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EXPERIMENTAL ARTICLES

Effect of Alkylresorcinols on Thermal Denaturation and Refolding of Bacterial Luciferase and Synthesis of Heat Shock Proteins Revealed in the Luminescent Molecular and Cellular Test Systems

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Abstract—Molecular and cellular luminescent biotests were used to reveal the effects of five alkylresorcinol homologues (C_7-, C_9-, C_{11}-, C_{12}-, and C_{18}-AR) on the thermally-induced denaturation and refolding of bacterial luciferases, as well as on the synthesis of heat shock proteins. The ARs activities were found to depend on their fine structure and concentration. The direct heat-protective effect of short-chain C_7- and C_9-AR on the chromatographically pure *Photobactrium leiognathii* luciferase/oxidoreductase was shown within a broad range of concentrations (10^{-6}–10^{-3} M). The long-chain ARs homologues exhibited a similar heat-protective effect at micromolar concentrations only, while their millimolar concentrations increased the sensitivity of the model proteins to thermal treatment. The recombinant strain *Escherichia coli* K12 MG1655, bearing constitutively expressed *Vibrio fischeri luxAB* genes was used to investigate the ARs effect on the intracellular chaperone-independent refolding of bacterial luciferase. The functional activity of heat-inactivated enzyme was restored by micromolar concentrations of short-chain ARs, while long-chain homologues inhibited refolding over the wide concentration range. The recombinant luminescent *E. coli* strain bearing the inducible *ibpA'::luxCDABE* genetic construction was used to determine the effect of ARs on the synthesis of heat shock proteins (HSP). The preincubation mode of bacterial cells with long-chain alkylresorcinols led to the dose-dependent stimulation of HSP synthesis (2.7 to 4 times), which confirmed that ARs function as "alarmones." Subsequent thermal treatment resulted in a 5- to 15-fold decrease of the following HSP induction compared to the control, while the number of viable cells opposite increased by 1.5- to 4-fold. Thus, pretreatment of the bacterial cells with long-chain ARs resulted in their preadaptation to subsequent thermally induced stress. Short-chain ARs caused less pronounced HSP suppression, although this was still was accompanied by increased heat resistance of the AR-pretreated bacterial cells.

Keywords: alkylresorcinols, protein heat denaturation, refolding, heat shock proteins, *Escherichia coli*, *ibpA* gene, *lux* genes, bioluminescence, luciferase

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In natural ecosystems, temperature is one of the most important factors controlling many aspects of prokaryotic physiology [1]. At temperatures beyond the tolerance limits of a species, the microorganism enters the stress state. The standard events of heat shock include impairment of the tertiary structure of enzymes, a sharp decrease in their catalytic activity, blockade of metabolic pathways, and the consequently growth arrest or death of a bacterial cell [2]. A number of ancient AR adaptation systems—unprogrammed by their development cycle—therefore exist to provide resistance of microorganisms to temperature stresses of various intensity [3].

Traditionally, an important role in cell protection against temperature stress is ascribed to a wide group of heat shock proteins (HSPs), non-enzymatic proteinst that act as molecular chaperones and chaperonins [4]. The major effects of HSPs are prevention of the aggregation and restoration of the native tertiary structure of proteins, while they are involved in folding of de novo synthesized proteins and control of protein translocation across the cytoplasmic membrane in the absence of the temperature factor, as well as in some other processes [5]. Thus, in *E. coli* cells, the system of molecular chaperones switches on the IbpA and IbpB proteins; the former generates a system of intracellular filaments in the absence of temperature stress, which is blocked upon interaction with a thermally aggregated substrate or co-chaperone IbpB [6].

Another mechanism of heat resistance is associated with the ability of bacteria to synthesize the so-called chemical chaperones, or low molecular weight com-

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Table 1. Molecular and cellular luminescence test systems used to study the protein-modifying activity of ARs

Luminescence est system	Composition or specific genetic features	Studied processes
Bioluminescence analytical reagent kit	Mixture of chromatographically pure enzymes luciferase and NAD(P)H : FMNB oxidoreductase of *P. leiognathi*, myristyl (C_{14}) aldehyde, flavin mononucleotide, and reduced nicotinamide dinucleotide	Denaturation
Eschericia coli K12 MG1655 (pF2) [*lux AB*]	The pF2 plasmid with inserted *luxAB* genes of *Vibrop fischeri* constitutively transcribed under the *lac* promoter	Chaperone-independent refolding
Escherichia coli pibpA' ::luxCDABE-Amp^R	The pibpA'::luxCDABE-Amp^R plasmid with complete cassette of *Photorhabdus luminescence* ZM 1 lux genes cloned under an inducible promoter of the IbpA chaperone protein	Synthesis of heat shock proteins

pounds that form complexes with biomolecules by means of weak interactions (hydrophobic, van der Waals, and hydrogen bonds), which are nonspecific for the biopolymer structure, stabilize the spatial structure of proteins, and promote the dissipation of the damage energy [7]. Along with glycerol, betaine, trehalose, and polyatomic alcohols, the properties of chemical chaperones have been found among alkylhydroxybenzenes (ARs) which are the low molecular weight autoregulatory factors of bacterial and yeast cells controlling their transition into a hypometabolic and anabiotic (quiescent) state with the simultaneous acquisition of high stability to extreme effects [8]. The documented protective effects of AHBs are mediated by both direct interaction with the protein globules being stabilized [9] and control of expression of the stress regulons [10].

The described effects of ARs determine the interest in elucidation of relationships between the mechanisms for the protection of protein molecules from heat stress, which occurs with the potential involvement of both chemical (AHB) and molecular (IbpA) chaperones. Bacterial luciferases, exhibition in vivo and in vitro catalytic activity of which can be assessed directly by the intensity of developing glow (bioluminescence), these seems a convenient tool to solve the problem [11]. Design of temperature-induced genetic constructs [12] provides for the possibility of bioluminescence assessment of the activity of the relevant stress regulons, thus allowing for a better understanding of the mechanisms of the biological effects of ARs.

Therefore the goal of the present work was, to carry out a comprehensive study of the effects of alkylhydroxybenzenes on bacterial protective mechanisms against heat shock (the processes of thermal denaturing and further refolding of enzymes, as well as the synthesis of heat shock proteins), performed using molecular and cellular luminescent test systems.

MATERIALS AND METHODS

In this study, we used synthetic analogues of microbial AHBs, including the commercially available agents C_7-AR, C_{11}-AR, and C_{12}-AR (Sigma, United States) and C_9-AR and C_{18}-AR synthesized by the company Enamine (Ukraine), all 99.9% pure [13].

To study AR effects on the processes of thermal denaturing, refolding, and heat shock protein synthesis, the following molecular and cellular test systems were used (Table 1).

Effect of ARs on the process of thermal denaturation of enzymes was determined using the Reagent Kit for Bioluminescence Analysis (Institute of Biophysics, Siberian Branch, Russian Academy of Sciences) containing a mixture of chromatographically pure luciferase and NAD(P)H:FMN oxidoreductase of *Photobacterium leiognathi* [14]. Solutions of AR homologues (100 μL) were added to the solution of luciferase/oxidoreductase to the final concentration of 10^{-6}–10^{-3} M, and after 60 min the mixture was heated in a Termit (DNA-Technology, Russia) solid-state temperature controlled chamber in the temperature range from 30 to 44°C with an increment of 2°C for 5 min. In control samples, equivalent volumes of solvents were added to the enzyme solution instead of ARs. Then, the control and experimental samples were transferred into cuvettes containing the mixture of substrates for the luminescence reaction including 5.4×10^{-4} M flavin mononucleotide (Sigma), 4×10^{-4} M reduced nicotinamide dinucleotide (AppliChem, Germany), and 0.002% myristyl (C_{14}) aldehyde (Merck, Germany) in 0.02 M phosphate buffer (pH 6.8). Luminescence dynamics in the experimental and control samples were measured in parallel on a BLM 8802M2K (Nauka, Russia) double-beam bioluminometer. Based on the data obtained, the relative index of bioluminescence was calculated using the formula $I_{\text{norm}} = (I_n^{\text{exp}} \times I_0^{\text{contr}}) / (I_0^{\text{exp}} \times I_n^{\text{contr}})$, where I_0^{exp} and I_n^{exp} are the levels of luminescence of the experimental sample (enzymes in the presence of ARs) at the starting time point at minute n and I_0^{contr} and I_n^{contr} are the levels of luminescence of the control samples (enzymes without ARs) at the starting time point and at minute n.

AR effects on the process of chaperone-independent refolding of bacterial luciferase were studied using a

recombinant *E. coli* K12 MG1655 strain carrying a hybrid pF2 plasmid with constitutively transcribed *luxAB* genes of *Vibrio fischeri* [15]. The bacteria were grown at 28°C with forced aeration for 3 h in 15-mL flasks containing 5 mL LB broth (Sigma) in the presence of 100 μg/mL ampicillin to the early exponential growth phase (OD = 0.2, λ = 640 nm; Specord, Germany). Heat denaturation of intracellular luciferase was performed as described above. To suppress de novo protein synthesis, the antibiotic chloramphenicol (167 μg/mL) was introduced into the experimental and control samples prior to thermal denaturation. Immediately after the completion of thermal denaturing, AR homologues were introduced into experimental samples to final concentrations of 10^{-3}–10^{-6} M (an equivalent volume of solvent was introduced into the control samples), and the mixture was additionally incubated at 20–22°C for 60 min. Then, after the putative refolding, 20 μL long-chain decanal (C_{10}) aldehyde was added to the experimental and control samples and luciferase activity was measured using an LM 01T (Immunotech, Czech Republic) microplate bioluminometer. The effect of AR homologues on the model enzyme refolding was judged by the ratio of intensities of bioluminescence in the experimental and control samples (I_o/I_k).

To study the effects of AR on heat shock protein (molecular chaperone) synthesis, recombinant *E. coli pibpA'::luxCDABE*-AmpR strain, in which a complete *lux* gene cassette of *P. luminescence* had been cloned under the *ibpA* gene promoter, was used [16]. Such gene arrangement allows for a relatively low background luminescence level, which increases multifold upon heat treatment of a bacterial cell as a result of synchronous induction of both the ibpA chaperone gene and the bioluminescence genes under its control. The strain was grown for 16–18 h at 37°C in LB broth in the presence of 20 μg/mL ampicillin. Immediately before the start of the experiment, the culture was diluted (1 : 20) with fresh medium and incubated for 3–5 h to reach the early exponential growth phase (OD = 0.2, λ = 640 nm). Then, AR homologues were introduced into the experimental samples to final concentrations of 10^{-3}–10^{-6} M (equivalent volumes of solvent were introduced to the control samples), and the mixture was incubated for 60 min. Heat treatment of bacterial cells was performed by incubation of 300-μL aliquots of the cultures in a Termit solid-state temperature-controlled chamber in the temperature range from 25 to 60°C for 0–10 min. Aliquots of analyzed samples were collected at every 2 min and their bioluminescence intensity was measured using the LM 01T bioluminometer. In parallel, the number of viable cells (as CFU number) was determined in the samples by plating of 10-μL aliquots on LB agar with subsequent incubation for 24 h at 37°C. Quantitative assessment of heat shock protein induction was performed using the formula $F_i = (luxA_i/luxA_0)/N_B$, where $luxA_0$ is light emission by the cell suspension immediately after heat treatment, $luxA_i$ is light emission induced after 60 min of incubation in growth medium after heat treatment, and N_B is the relative number of cells in the sample (in fractions of unit to the initial number) retaining capacity for growth (judging by CFU number) after heat treatment.

All experiments were performed in triplicate. The results were processed using the Statistica software package.

RESULTS

Effect of alkylhydroxybenzenes on luciferase thermal stability. In the first stage of the research, the pattern of thermal denaturation of the luciferase/NAD(P)H : FMN oxidoreductase enzyme complex of *P. leiognathi* was established (Fig. 1). The luminescence intensity of the enzyme complex, which characterized its activity, remained stable upon heating of the control samples within the range from 30 to 34°C, while a further stepwise increase in the intensity of heat treatment was accompanied by progressive quenching of bioluminescence until its complete inhibition was achieved at 44°C and above. Heating at 40°C for 5 min was therefore chosen as the treatment damaging the individual enzyme complex with pronounced denaturation of the enzymes and maintenance of up to 20% initial activity (Fig. 1a).

Pre-incubation of the enzyme complex with AR homologues during 60 min followed by thermal treatment as described above influenced the processes of thermal denaturation, while the direction of the effect and its intensity depended on the chemical structure of ARs and their concentrations (Fig. 1b). Preincubation of the enzyme complex with short-chain C_7-AR and C_9-AR in a wide range of concentrations (10^{-6}–10^{-3} M) caused a proportional increase of thermal stability of the complex. For example, C_7-AR at the lowest concentration applied (10^{-6} M) caused a 1.9-fold increase in residual activity of luciferase/oxidoreductase compared to thermally denatured control samples, and the increase in its concentration up to 10^{-3} M led to a 4.9-fold increase in the residual activity.

Long-chain AR homologues with five or more carbon atoms in the alkyl chain produced a different effect on thermal stability of the enzymes (Fig. 1b). In the range of concentrations from 10^{-6} to 10^{-5} M they increased the thermal stability of luciferase, which resulted in a 3.4-fold increase in the relative bioluminescence index for C_{18}-AR and a 3.7-fold increase for C_{12}-AR, while higher concentrations of these homologues, up to 10^{-4}–10^{-3} M, on the contrary, promoted the thermal denaturation processes of the model enzyme complex and caused a decrease in the bioluminescence indices to the values below unit, 0.15 for C_{12}-AR and 0.13 for C_{18}-AR.

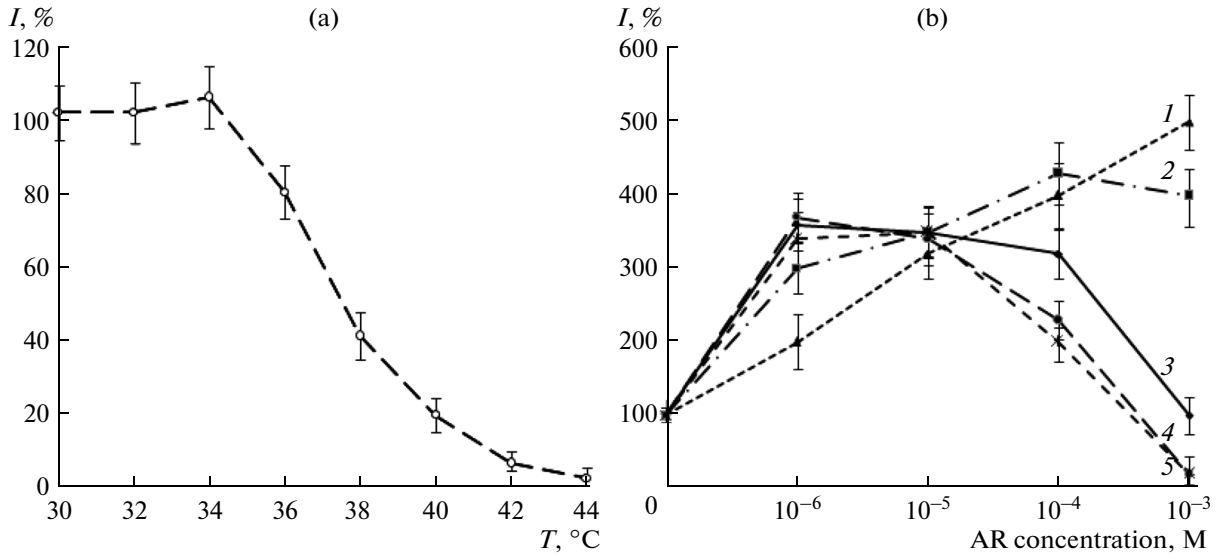

Fig. 1. Residual activity of *P. leiognathi* luciferase after thermal denaturation for 5 min (a) and relative values of luminescence of luciferase preincubated with C_7-AR (*1*), C_9-AR (*2*), C_{11}-AR (*3*), C_{12}-AR (*4*), and C_{18}-AR (*5*) during 60 min and then subjected to thermal treatment at 40°C for 5 min (b). Designations: temperature, °C (a) and AR concentration, M (b) are plotted against the X axis and bioluminescence intensity in % to the control, against the Y axis.

These data enrich our knowledge on ARs as chemical chaperones [17], i.e. low molecular weight compounds that modify the enzyme structure and functional stability upon interaction [18, 19].

Effect of ARs on luciferase refolding. Enhancing the thermal stability of protein molecules by ARs may be achieved via their participation in the refolding process after thermal denaturation, which is traditionally considered one of the mechanisms of action of both molecular [4] and chemical [7] chaperones, but has never been demonstrated for ARs. In our experiments, we used a recombinant *E. coli* strain K12 MG1655 previously proposed for evaluation of the functional activity of Hsp70 (DnaK-DnaJ) heat shock proteins [15] and characterized by the presence of a constitutively transcribed thermolabile luciferase of marine luminescent bacteria *Vibrio fischeri*. The following requirements for the experimental series were met: (1) complete denaturation of intracellular luciferase was achieved by heating of the cell suspension at 42°C for 5 min, and (2) luciferase and molecular chaperone *de novo* synthesis was suppressed by the introduction of a translation blocker chloramphenicol into the test system prior to heating (Fig. 2a). During the experiments, in the 60 min after the heating and complete denaturation of the enzyme, *E. coli* K12 MG1655 luminescence was restored by 34%, which corresponded to the normal values of chaperone-independent renaturation (I_k) of luciferase (Fig. 2a).

Introduction of AR homologues into the test system significantly modified the efficiency of refolding, the parameters of which depended on the chemical structure and concentration of ARs (Fig. 2b).

At low concentrations (10^{-6} M), short-chain homologues, especially C_7-AR, promoted refolding, reliably increasing the restoration efficiency of luciferase functional activity up to 1.35 times when compared to the relevant control (Figs. 2a and 2b). At the same time, an increase in the concentrations of C_7- and C_9-AR above 10^{-5} M led to inversion of the effect, and the efficiency of refolding decreased ($I_o/I_k < 1$); that is, high concentrations of C_7- and C_9-AR prevented refolding (Fig. 2b). Another important feature of the effect of short-chain ARs was an increase in the rate of restoration of luciferase native structure: already after 30 min activity of the enzyme preincubated with 10^{-6} M C_7-AR exceeded the control activity level 2.5 times and reached its maximum, while the maximum activity was observed only after 60 min in the control.

On the contrary, introduction of long-chain homologues C_{11}-, C_{12}-, and C_{18}-ARs into the test system consistently blocked the restoration of functional activity of thermally inactivated luciferase; at all AR concentrations the I_o/I_k ratio values were below 1 (Fig. 2b). The degree of the inhibitory effect increased in the order C_{11}-AR → C_{12}-AR → C_{18}-AR with their increasing hydrophobicity, and with an increase in concentration of each of the homologues. The effect of C_{18}-AR at the lowest concentration used (10^{-6} M) caused a decrease in the value of restored activity of bacterial luciferase (I_o/I_k) down to 0.44 against the control ratio ($P < 0.05$), and an increase in the homologue concentration to 10^{-3} M resulted in the I_o/I_k value of 0.06 ($P < 0.01$).

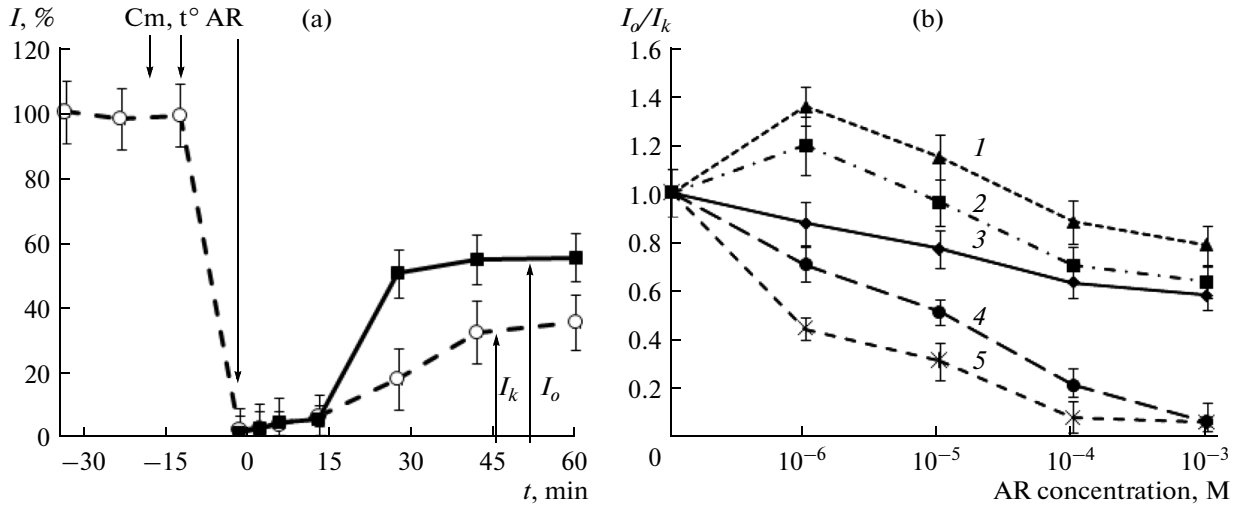

Fig. 2. AR effect on restoration of luciferase activity after heat denaturation (a) and the characteristic I_o/I_k values in the presence of C_7-AR (*1*), C_9-AR (*2*), C_{11}-AR (*3*), C_{12}-AR (*4*), and C_{18}-AR (*5*) after 60 min of refolding (b). Designations: time, min (a) and AR concentration, M (b) are plotted against the X axis and bioluminescence intensity expressed as (a) % to control and (b) I_o/I_k value, against the Y axis. Arrows indicate the moments of heating (t°), introduction of chloramphenicol (Cm), and alkylhydroxybenzenes (ARs).

Thus, the ability of ARs to influence the processes of enzyme refolding as one of the mechanisms of spatial organization of protein tertiary structure was demonstrated for the first time. The effects of long-chain homologues may be explained by hydrophobic interactions with aromatic amino acid residues exposed in the process of thermal denaturation, which resulted in stabilization of the new conformation preventing protein renaturation into the initial functionally active form. On the other hand, low physiological concentrations of short-chain AR homologues, the effect of which has been demonstrated to result in weakening of intramolecular hydrogen bonds and increase in the interdomain mobility of the proteins [9, 20], promoted refolding processes presumably due to increased conformational lability of renatured enzymes.

Effect of ARs on induction of heat shock protein synthesis. Studies of AR functions in generation of bacterial stress response demonstrated that various extreme factors (for example, heat shock) stimulated AR biosynthesis [21], while elevated AR levels affected cell stability [8, 22, 23] by means of increased functional and operational stability of the enzymes [9, 20, 24] on the one hand, and activation of stress gene expression, on the other [10, 13]. It was therefore logical to assume that changes in AR concentration in a microbial culture can affect the induction of heat shock protein synthesis, in particular, syntesis of the IbpA chaperone [6]. Experiments of this part of the study were performed using a recombinant *E. coli* strain *pibpA'::luxCDABE*-AmpR which responded to heat treatment by the synchronous induction of both the *ibpA* chaperone gene and the bioluminescence genes under its promoter. Therefore, measurement of the luminescence intensity of the test strain allowed for real-time quantitative evaluation of the dynamics of heat shock protein synthesis, the rate of which was proportional to protein denaturation induced by heat treatment [16].

In preliminary experiments, we determined the optimal mode of heat treatment of the model system that preserved enough viable bacterial cells to generate a detectable bioluminescent response to heat stress (Table 2). Under the chosen temperature mode, the bacterial culture was heated at 55°C for 5 min, which resulted in the maximum absolute intensity of luminescence (~5 times higher than the background level) upon maintenance of up to 14.8 (±1.3)% of viable bacterial cells to the initial number of cells. The value of F_i calculated based on the obtained data was 128.7 (±13.4), evidencing an intense (over 100-fold against the background) induction of the synthesis of heat shock proteins (Table 2).

In the next series of experiments, the effects of ARs on the level of luminescence (HSP synthesis) and growth characteristics of *E. coli pibpA'::luxCDABE* cells were determined (Table 3). The results demonstrated a dose-dependent induction of bioluminescence: not exceeding 20% at the lowest AR concentration (10^{-6} M) and a decrease of cell luminescence upon AR dose increase. Analysis of AR effects on growth characteristics of the test strain revealed a decrease in CFU number, the degree of which progressively increased in the order C_9-AR → C_{18}-AR (C_7-AR stimulated cell growth at 10^{-6} M of and had no effect at higher doses). The F_i value relating the intensity of bioluminescence to the number of viable bacterial cells calculated based on our data evidenced the dose-dependent effect of ARs on cell luminescence

Table 2. Effect of heat treatment (T°C, 5 min) on absolute values of bioluminescence intensity (I), preservation of capacity for growth (CFU), and relative parameters of heat shock protein induction (F_i) in *E. coli ibpA'::luxCDABE* cells

Temperature, °C	I	CFU, $n \times 10^6$, cells/mL	F_i, r.u.
25	10767	1.65	1.00
35	16877	1.66	1.88
40	25434	1.59	2.11
45	38450	1.43	2.70
50	49900	1.67	13.49
55	**51000**	**0.24**	**128.70**
60	9768	0.16	167.30

The chosen optimal temperature of induction, with over 10% (14.7%) surviving cells providing for high absolute and relative levels of cell luminescence, is typed boldface.

and therefore on HSP synthesis (Table 3). The long-chain C_{11}-, C_{12}-, and C_{18}-AR caused a 2.7 to 4-fold increase in cell bioluminescence at a concentration of 10^{-4} M.

The results presented above were taken into account in the evaluation of viability and bioluminescence (I) (Fig. 3), as well as the heat shock protein induction value F_i derived from them (Table 4) in the cells of the *E. coli ibpA'::luxCDABE* test strain subjected to heat shock after preincubation (60 min) with ARs. After heat treatment, a statistically important, albeit minor (11–17% compared to the control) increase in viable cell numbers (determined as CFU) was observed only in the samples pretreated with C_7- and C_9-AR (10^{-6}–10^{-4} M), which was in good agreement with data on the heat-protective properties of short-chain AR homologues presented above and known from the literature [21, 22]. At the same time, the number of cells retaining viability decreased progressively in the order C_{11}-AR → C_{18}-AR, and for each of the long-chain homologues it decreased with an increase of their acting concentrations (Fig. 3). It should be noted that long-chain homologues C_{11}-AR → C_{18}-AR at concentrations above 10^{-5} M potentiated the stress factor effect.

Determination of another parameter, luminescence (I) of the cells preincubated with ARs and then subjected to heat shock evidenced that only the long-chain homologues C_{12}-AR and C_{18}-AR affected the bioluminescence response, and only at the lowest concentration used (10^{-6} M) (Fig. 3b). The algorithm for calculation of induction factor for the IbpA chaperone

Table 3. Effect of ARs on cell viability (CFU, % to the control) and heat shock protein induction parameters in *E. coli ibpA'::luxCDABE* cells (F_i) in the absence of thermal treatment

Compound under study, concentration, M	Residual number of CFU (% to the control)	IbpA chaperone induction factor (F_i)
C_7-AR, $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	108.98 ± 9.20	1.00 ± 0.10
	97.85 ± 8.30	1.30 ± 0.20
	98.87 ± 9.10	1.32 ± 0.10
	95.68 ± 8.00	1.42 ± 0.20*
C_9-AR, $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	104.03 ± 9.50	1.16 ± 0.10
	98.47 ± 8.60	1.19 ± 0.20
	90.96 ± 9.30	1.54 ± 0.20*
	66.70 ± 5.50	0.76 ± 0.08
C_{11}-AR, $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	100.50 ± 9.10	0.92 ± 0.08
	95.02 ± 8.10	0.87 ± 0.07
	51.32 ± 4.60*	2.73 ± 0.10**
	2.61 ± 1.70**	1.25 ± 0.09
C_{12}-AR, $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	98.27 ± 8.50	0.96 ± 0.08
	78.02 ± 8.20	1.15 ± 0.10
	26.59 ± 3.60**	2.75 ± 0.30**
	0.96 ± 0.10**	1.93 ± 0.20*
C_{18}-AR, $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	94.98 ± 7.60	1.09 ± 0.20
	79.18 ± 7.70	1.30 ± 0.10
	22.74 ± 3.00**	3.97 ± 0.40**
	0.77 ± 0.09**	1.09 ± 0.10
Control	100.00	1.00

Designations: * $P < 0.05$; ** $P < 0.01$.

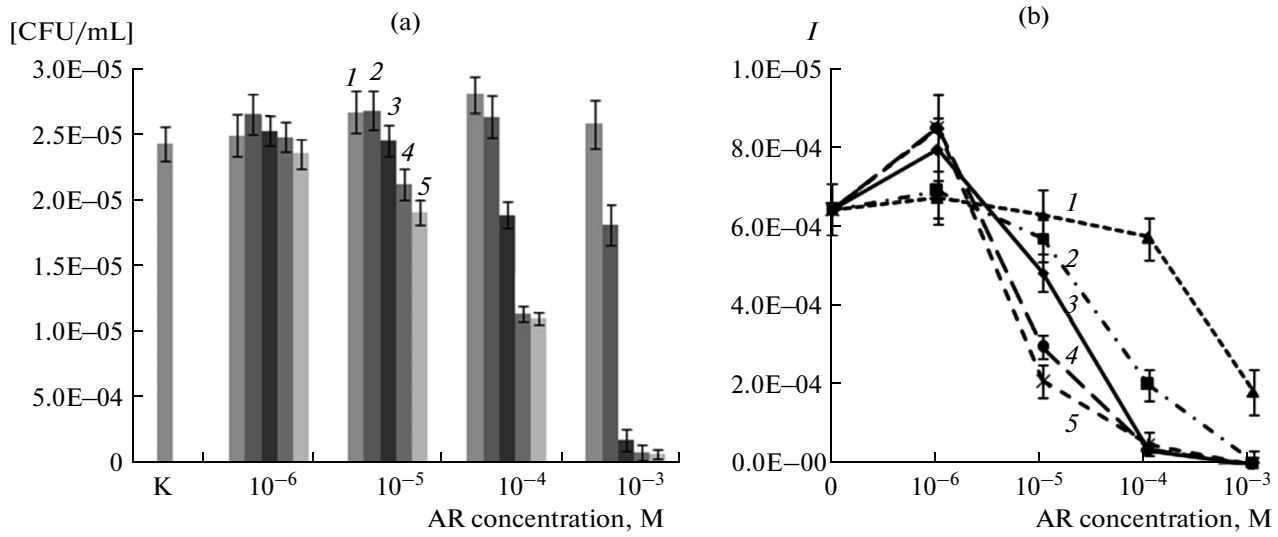

Fig. 3. Effect of heat shock (55°C, 5 min) on the viability (CFU/mL) (a) and bioluminescence level (*I*) (b) of *E. coli ibpA'::lux-CDABE* strain pretreated with C_7-AR (*1*), C_9-AR (*2*), C_{11}-AR (*3*), C_{12}-AR (*4*), and C_{18}-AR (*5*). Designations: AR concentration, M is plotted against the X axis and the number of viable cells, CFU/mL (a) and bioluminescence intensity, *I*, U (b), against the Y axis.

synthesis (F_i) included the following steps: (1) determination of F_{i1} for the cells pretreated with ARs and surviving after heat shock compared to the cells not treated with ARs (100%) (Table 4); and (2) determination of the F_{i2} value (%) for the cells pretreated with AR and surviving upon heating compared to the F_i of the cells pretreated with ARs and not heated (100%) (Table 4). Therefore, the calculation procedure (1) reflected the total stress response of the cells (CFU and IbpA induction) after two sequential effects, AR treatment as an alarm signal and the following heat shock, and procedure (2) revealed the protective effects of ARs in cell preadaptation to heat shock. Calculation of F_{i1} values and of the cell viability factor demonstrated activation (although weak) of IbpA chaperone induction (Table 4) upon the effect of long-chain ARs at the lowest concentration, which was probably the reason for the higher thermal stability of cells (CFU number) in these samples (Fig. 3 and Table 4). The increase in their concentration resulted in a pronounced repression of heat shock protein synthesis. For example, in the samples preincubated with C_{12}- and C_{18}-AR homologues at concentrations of 10^{-4} and 10^{-3} M with subsequent heat treatment, the F_{i1} value decreased 5–34 times, which correlated with the 2 to 29.6-fold decrease in the number of viable cells compared to the relevant controls. A decrease in the acting concentrations (to 10^{-5} M) of these AR homologues caused the partial induction of the synthesis of the IbpA chaperone with a proportional increase in the number of bacterial cells retaining their proliferative ability after extreme temperature stress. The increase in acting concentrations of short-chain homologues (to 10^{-4}–10^{-3} M), particularly of C_7-AR, led to repression of the synthesis IbpA chaperone (F_{i1} values of 23.53–46.38 against 128.70 in control), while it also increased the level of heat resistance of pretreated bacterial cells (Table 4). Such an effect of C_7- and C_9-AR may be ascribed to their adaptogenic and protective functions demonstrated previously [10, 21–23].

HSP synthesis induction factor F_{2i} and the residual number of CFU after heat shock of the cells pretreated with ARs were compared with the same values for non-heated cells, taken as 100%, at each AR concentration; this revealed the adaptogenic function of long-chain ARs (Table 4). In the cells pretreated with C_{11}-, C_{12}-, and C_{18}-AR (at concentrations of 10^{-4} and 10^{-3} M) the fraction of cells surviving heat treatment was the highest, 22.52–64.94%, compared with 14.08% in the absence of ARs. In the same samples, the increase in the HSP induction factor F_{2i} was much less than in the control (up to 348.62–2400.00% if compared to 12870.00% in the absence of AR) (Table 4). Therefore, cell treatment with long-chain ARs induced the development of stress responses, including HSP synthesis, which pre-adapted the cells to further stress effects.

DISCUSSION

A special group of phenolic lipids, termed alkylhydroxybenzenes (ARs) in the Russian literature [25] and resorcinols in the literature published in English [26], is present in a number of bacteria, lower fungi, and plants, where it performs important regulatory and adaptogenic functions. In particular, induction of cell transition into a hypometabolic quiescent state is a well-documented AR effect in microbial populations

Table 4. Effect of ARs on the preservation of cell viability and heat shock protein induction parameters in *E. coli ibpA'::luxCDABE* cells upon thermal treatment (+55°C, 5 min)

Compound under study, concentration, M	Residual number of cells (%) preserving the ability to grow after heat treatment with respect to the initial number of CFU (1) and the number of CFU after contact with AR (2)		Induction factor F_i calculated taking into account the number of CFU after heat treatment in a. u. (F_{i1}) and taking into account the induction upon contact with AR (%) (F_{i2})	
	1	2	F_{i1}	F_{i2}
C_7-AR $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	15.24 16.32 17.16 15.77	13.98 16.67 17.35 16.48	122.97 115.59 78.35* 46.38**	12297 8891.53 5935.60* 3266.20**
C_9-AR, $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	16.24 16.39 16.11 11.10*	15.61 16.64 17.71 16.64	124.26 110.10 63.86* 23.53**	10712.07 9252.10 4146.75** 3096.58**
C_{11}-AR, $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	15.45 14.97 11.56 1.12*	15.37 15.75 22.52 43.03**	129.84 109.33 37.54** 30.00**	14113.37 12566.67 1375.39** 2400.00**
C_{12}-AR, $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	15.15 12.95 6.98* 0.56**	15.41 16.59 26.25* 58.33**	132.99 79.37* 25.75** 4.00**	13853.13 6901.74* 936.37** 207.25**
C_{18}-AR, $10^{-6}/10^{-5}/10^{-4}/10^{-3}$	14.39 11.68 6.78* 0.50**	15.15 15.75 29.81* 64.94**	134.30 74.03* 21.60** 3.80**	12321.10 5694.62* 544.08** 348.62**
Control	14.80		128.70	12870.00

Designations: *$P < 0.05$; ** $P < 0.01$.

[27] accompanied by the formation of resistance to a wide range of environmental stress factors [8]. Many years of research into the nature of this phenomenon made it possible to associate the protective effects of ARs with two major mechanisms; the first is direct protection of bacterial cells from the damaging factors and the second is an alarm signal to mobilize resources for protection. While the alarm signal function of ARs is mediated through control of stress regulon expression [10, 13], their protective effect develops via structural modification of a wide range of cell biopolymers, of which enzymes are of particular importance [9].

The nonspecific multitargeted nature of AR binding with protein molecules governed by formation of hydrogen bonds, hydrophobic, and electrostatic interactions [24] is responsible for their regulatory functions not only in microbial, but also in heterologous living systems [28].

The current work is a continuation of the studies of mechanisms of protein modification by ARs, where bacterial luciferases are used as a tool, with their performance evaluated in vitro and in vivo by the developed (bio)luminescence intensity [11]. The design of versatile genetic constructs [12] provided the opportunity to assess both the preservation of enzymatic activity as such and the regulation of stress regulon functioning, including the system of heat shock proteins, which allowed for a comprehensive picture of AR biological effects under conditions of heat shock.

Realization of this approach resulted in obtaining new data on the direction and intensity of the effects of AR homologues of different structures, which affect the thermal stability of protein molecules in vitro and in vivo in processes of denaturation (Fig. 1) and the refolding of enzymes (by the example of luciferase) (Fig. 2), as well as HSP synthesis induction (Fig. 3, Tables 4 and 5).

The study resulted in the discovery of new AR effects and in establishment of the dependence of the direction and intensity of these effects on the chemical structure of ARs. The effects controlled by ARs are principally different in short- and long-chain homologues and depend nonlinearly on the acting concentrations of ARs in model molecular and cell systems.

Table 5. Integral characteristics of the effect of alkylhydroxybenzenes on stress response in bacteria revealed in bioluminescence models in vitro and in vivo

Studied process	AR and their acting concentrations, M			
	short-chain (C_7- and C_9-AR)		long-chain (C_{11}-, C_{12}-, and C_{18}-AR)	
	10^{-6}–10^{-5}	10^{-4}–10^{-3}	10^{-6}–10^{-5}	10^{-5}–10^{-3}
Protein denaturation	Prevent		Prevent	Enhance
Protein refolding	Promote	Block	Block	
Heat shock protein synthesis	Produce no effect on HSP synthesis with improvement of cell resistance to heat stress		Activate HSP synthesis with improvement of cell resistance to heat stress	Inhibit HSP synthesis with decrease of cell resistance to heat stress

The data obtained confirm a pronounced heat-protective activity of short-chain AR homologues manifested through the protection of enzymes from thermal denaturation (Fig. 1) and potentiation of their subsequent refolding (Fig. 2) (within the range of concentrations from 10^{-5} to 10^{-6} M). The positive effect of the short-chain C_7-AR on refolding of luciferase is a newly discovered effect of AR. Confirmation of this new activity of short-chain ARs improves our understanding of their role in the stress response of organisms, where they are involved not only in the protection of cell structures from unfavorable conditions, but also in reactivation after damage. If the main mechanism of protective effects of short-chain ARs is their functioning as antioxidants, or as traps for reactive oxygen species, their involvement in the reactivation of proteins is associated with their functions as chemical chaperones aiding in the restoration of correct protein conformation. To understand the mechanisms of the effect of short-chain ARs in the processes of protein refolding, the previously obtained data on the influence of ARs on processes of intramolecular dynamics of protein seem extremely important. The data of Fourier analysis of diffuse X-ray scattering, elastic Rayleigh scattering of Mössbauer radiation, and computer modelling [20] evidence the direct interaction of ARs with the functional groups of enzymes, which leads to a decrease in denaturation temperature with a simultaneous increase in the amplitude of equilibrium fluctuations [19]. The latter fact increases the chance of correct orientation of amino acid sequences. Therefore, the complex of AR effects in thermal protection of proteins once again proves that the reference of this group of small regulatory molecules to chemical chaperones is valid; the result of AR interaction with protein globules is the acquisition of higher stability to a wide range of denaturing effects by the proteins.

In contrast to the effects of short-chain ARs, long-chain homologues improved luciferase heat resistance at low concentrations; however, when applied to a thermally denatured enzyme, they prevented its refolding (Fig. 2), which confirms the previously obtained data on the inhibition of renaturation of *Bacillus intermedias* RNase by the C_{12}-AR homologue [29]. The described effects of long-chain ARs can be described in frames of broader models of preferable hydration and preferable hydrophobic interactions of proteins with alkyl radicals of ARs [30].

Another newly discovered effect of ARs, and also a dose-dependent one, is moderate induction of HSP synthesis by long-chain homologues C_{11}-, C_{12}-, and C_{18}-AR, which resulted in a 2.7 to 4-fold stimulation of cell luminescence at concentrations of 10^{-4} M. These results agree with the previously demonstrated effect of long-chain ARs as alarm signals activating the expression of stress genes (SOS response and *rpoS* regulon) in *E. coli* [10, 13]. The effect of long-chain ARs on HSP synthesis has not been previously demonstrated; it adds to their effects as stress imitators. Application of the luminescent test system of *E. coli* strain *ibpA'::luxCDABE* allowed for documenting three groups of regulatory effects of ARs upon the generation of cell stress response to heat shock: (1) The long-chain C_{11}-, C_{12}-, and C_{18}-ARs at low concentrations cause moderate induction of HSP synthesis, which protects bacterial cells from subsequent heat impact (Table 4). (2) The effect of high concentrations of long-chain AR homologues, on the one hand, leads to a pronounced repression of HSP formation after heat shock, which correlates with a decrease in the residual CFU number (6% compared to 100% prior to thermal treatment), and on the other hand, increases cell resistance to the subsequent heat shock, considerably increasing the fraction of surviving cells if compared with the samples without AR pretreatment (Table 5). (3) Short-chain AR homologues, particularly C_7-AR, do not affect HSP synthesis over a wide range of concentrations, but promote the development of increased heat resistance in the treated bacterial cells. The latter effect is in agreement with the results obtained upon investigation of the effect of C_7- and C_9-AR on the processes of thermal denaturing and refolding of enzymes. This allows the authors to conclude, that in this case, protection of bacterial cells from the stress factor is ensured by (1) realization of the direct protective effects of ARs demonstrated pre-

viously for bacteria and yeasts [10, 21–23] and provided for by the chemical chaperone properties of ARs [9, 17–19] and (2) their functional interference with the system of heat shock proteins [23].

As for the applied aspect of the obtained results, the nonspecific and multitargeted nature of AR binding to protein molecules provides for its reproduction not only in microbial, but also in heterologous living systems. In particular, the change in catalytic activity, substrate specificity, and operational stability of enzymes achieved in the presence of AR confirms that ARs are promising for biotechnology production [20], the prolonged maintenance of functional characteristics of commercial protein preparations [31], and the development of candidate pharmaceutical substances for immune correction.

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