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EXPERIMENTAL ARTICLES

Stress-Protective and Cross Action of the Extracellular Reactivating Factor of the Microorganisms of the Domains *Bacteria*, *Archaea*, and *Eukaryota*

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Abstract—Cross protection of members of the domains *Bacteria, Archaea*, and lower *Eukaryota* from stress factors due to the action of extracellular low-molecular metabolites with adaptogenic functions was shown. The adaptogen produced by *Luteococcus japonicus* subsp. *casei* and described previously as a reactivating factor (RF) was shown to protect the yeasts *Saccharomyces cerevisiae,* archaea *Haloarcula marismorti*, and the cells of higher eukaryotes (HeLa) against weak stressor impacts. Production of an archaeal extracellular metabolite with a weak adaptogenic effect of the producer cells and capable of a threefold increase in survival of heat-inactivated yeast cells was discovered. Our results confirm the similarity of the compensatory adaptive reactions in prokaryotes (bacteria and archaea) and eukaryotes.

Keywords: stress protection, cross effect, RF peptide reactivating factor, bacteria, archaea, yeasts, HeLa cells
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The protective mechanisms of microorganisms and multicellular organisms against stress are remarkably similar [1]. Microorganisms may be an adequate model for the studies of stress response, which at the population level is implemented by dose-dependent involvement of low-molecular metabolites in physicochemical, biochemical, and genetic reactions of a cell. Stress response also stimulates the synthesis of these metabolites, which in bacteria and lower eukaryotes are involved in signal transmission and may have a function of the environment probes [2], stress protection molecules [3, 4] and adaptogens [5–7].

These molecules diffuse readily into the environment and, being organism-unspecific, may protect not only the producing population, but also other stress-affected populations (cross effect) [5]. This is important for the maintenance of the homeostasis in microbial communities, and, at the level of heterologous systems, demonstrates the role of the microbiota in formation of the protective reactions in host organisms.

The goal of the present work was to study the cross effect of extracellular reactivating factors of *Luteococcus casei*, *Haloarcula marismortui*, and *Saccharomyces cerevisiae* in protection of bacteria, archaea, yeasts, and HeLa mammalian cell culture from various stress factors.

MATERIALS AND METHODS

Research subjects and cultivation. Gram-positive bacteria *Luteococcus japonicus* subsp. *casei*, isolated from cheese and described previously [8], yeasts *Saccharomyces cerevisiae* VKPM Y-1200, haloarchaea *Haloarcula marismortui* B-1809 (ATCC 43049), originally isolated from the Dead Sea and received from the All-Russian Collection of Microorganisms (VKM), and HeLa, a cell line derived from human cervical tumor, were used in this work.

L. casei was cultivated under static conditions in 100-mL flasks at 30°C in glucose-mineral medium containing the following (%): glucose, 1.5; $(NH_4)_2SO_4$, 0.3; KH_2PO_4, 0.1; NaH_2PO_4, 0.2; $MgSO_4$, 0.002; $CaCl_2$, 0.002; NaCl, 0.002; and yeast extract, 0.1; pH 7.0 was maintained with a 5% NaOH solution.

S. cerevisiae was cultivated in 500-mL flasks containing 100 mL of medium on a shaker (180 rpm) at 28°C in 4B malt wort at pH 6.5. The optical density was controlled by nephelometry (FEC 56 PM, Russia, 2 mL cells, filter no. 6). For the experiments with HeLa cells, the yeast were cultivated on the starvation

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medium containing the following (%): glucose, 1.0; $(NH_4)_2SO_4$, 0.3; $MnSO_4 \cdot 7H_2O$, 0.02; KH_2PO_4, 0.1; NaCl, 0.002; $ZnSO_4 \cdot 7H_2O$, 0.01; $FeCl_3 \cdot 6H_2O$, 0.001; Ca pantothenate, 1000 μg/L; vitamin B_1, 200 μg/L; biotin, 1 μg/L; pH 7.0.

H. marismortui was cultivated on a shaker (180 rpm) at 37°C in 250-mL flasks with 100 mL of the medium containing the following (%): NaCl, 21.4; $MgSO_4 \cdot 7H_2O$, 1.7; sodium citrate, 0.3; KCl, 0.17, $CaCl_2 \cdot 2H_2O$, 0.023; yeast extract, 0.43; peptone, 0.85; glucose, 0.34; Tris-HCl, 0.52. After adjusting pH to 7.3, trace element solution, containing 0.0218 g of $MnCl_2 \cdot 4H_2O$ and 0.486 g of $FeCl_2 \cdot 6H_2O$ in 100 mL water, was added (1.7 mL/L). Cell-free filtrate (CF), obtained after centrifugation of the exponential growth phase culture at 10500 *g* for 15 min, was used as the protector factor from the archaea.

Test systems were designed as follows. Late exponential microbial cultures were centrifuged at 10000 *g* for 20 min, and the bacterial and yeast cells were washed and resuspended in 0.05 M Na phosphate buffer (pH 7.4). The archaeal cells were washed and resuspended in 25% NaCl solution. The resulting cell suspensions were the test objects for stress effects. The culture liquids (CL) separated from bacterial and yeast cells were used for extraction of the reactivating factors (RF) according to the procedure previously described [6, 7].

HeLa cell culture was used as a test object for determination of the protective activity of microbial RF on animal cells. Cells were grown in the medium containing the following (%): F12 medium, 45; DMEM medium (Paneco, Russia), 45; and fetal bovine serum (Biolot, Russia), 10, supplemented with gentamycin, in plastic culture vials under standard conditions at 37°C and 5% CO_2 in the gas phase. The day before the experiment, HeLa cells were placed on glass cover slips.

The numbers of viable cells of *L. casei* and *S. cerevisiae* were determined by the number of the colony-forming units (CFU/mL) after plating of 5 μL of sequential tenfold dilutions of the suspensions on respective 1.5% agar media in six replicates for each dilution and incubation at 32°C for 48–72 h. Apart from CFU monitoring, the number of viable and nonviable cells was determined by fluorescence microscopy of 5 μL of the suspension stained with the Live/Dead Baclight™ kit (Molecular Probes Inc., United States). Cells with green and yellow fluorescence were considered viable, while cells with red fluorescence were considered nonviable. The cell count was carried out in at least 20 microscopic fields for each preparation.

Phase contrast and epifluorescence observations were performed with an Axioplan microscope (Carl Zeiss, Germany).

Determination of cross activity of bacterial and yeast RF. Bacterial or yeast cells' suspensions were adjusted to OD_{540} = 0.4–0.5 (1 cm cells, an SF-26 spectrophotometer, Russia), and 2-mL samples were subjected to UV irradiation using two parallel-mounted 30-W BUV-15 lamps (Russia), with emission at 253.7 nm and the radiation dose of 1.9 kJ/m^2. The irradiated preparations were supplemented with RF (experiment) or fresh culture medium (control) in a standard ratio (1 : 1 vol/vol) and incubated at 32°C for 10 min. The protective effect of RF added to the suspensions prior to irradiation was studied under the same conditions. The efficiency of the reactivating protective effect was evaluated by the ratio of CFU in the experimental and control samples.

Determination of the stress-protective activity of bacterial and yeast RF to HeLa cells. HeLa cell cultures were subjected to the oxidation stress, which was caused by introduction of Na arsenite to the final concentration of 50–100 μM. The stress development was monitored by the number of cells with the stress granules (SG), which are formed in the cytoplasm of eukaryotic cells under various extreme factors [9, 10]. In the course of this process, the total translation level in the cells is significantly reduced, and the translation apparatus elements are redistributed into the SG, which contain the small ribosome subunit, the translation initiation factors (eIF3, eIF4E, eIF4G), RNA-binding proteins (PABP, TIA, TIAR), and mRNA [11]. In our experiments, the SG in the cytoplasm of eukaryotic cells were detected by indirect immunofluorescence with the antibodies to eIF3 protein, an SG-specific translation initiation factor. The cells were fixed with methanol at –20°C for 1 min, postfixed freshly prepared 3% paraformaldehyde for 10 min, treated with the antibodies to eIF3a [11] conjugated with the fluorochrome tetramethylrhodamine isothiocyanate (TRITC), then mounted on glass slides and examined with an Axiovert 200 M microscope (Carl Zeiss, Germany) equipped with an ORCA-IIERG2 digital camera (Hamamatsu, Japan). The development of oxidative stress was monitored by counting the number of cells with stress granules after exposure to different concentrations of Na arsenite.

When studying the stress-protective activity of the yeast and bacterial RF, the HeLa cells suspension was incubated for 30 minutes in the mixture of Hanks and RF solutions(1 : 1 vol/vol) for the experiment, and in pure Hanks solution for the control. Oxidative stress was induced by 30-min incubation with Na arsenite at 50 and 100 μM concentration, after which the number of cells with stress granules was counted.

Determination of the stress-protective activity of archaea cell-free filtrate (CF). Protective functions of CF with respect to archaea. A 6-mL sample of *H. marismortui* cell suspension in 25% NaCl solution was supplemented with 3 mL of CF or of 25% NaCl solution for the experiment and the control, respectively, and pre-incubated for 10 min at 25°C. To induce the acid stress, citrate buffer (2 mL) was added to both control and experimental samples to reach the

Table 1. Cross-effect against of *L. casei* and *S. cerevisiae* RF on *S. cerevisiae* subjected to UV irradiation (1.9 kJ/m^2)

Experimental conditions	Number of viable cells, $\times 10^4$ mL^{-1}	Efficiency of protection*
S. cerevisiae culture before UV irradiation	210 ± 1.14	
Cell suspensions in buffer after UV irradiation	0.020 ± 0.0014	1.0
Cell suspensions incubated with *S. cerevisiae* RF after UV irradiation	0.038 ± 0.0020	1.9
Cell suspensions incubated with *L. casei* RF after UV irradiation	0.054 ± 0.0034	2.7
Cell suspensions simultaneously incubated with *S. cerevisiae* and *L. casei* RF after UV irradiation	0.040 ± 0.0028	2.0
Cell suspensions sequentially incubated with *S. cerevisiae* and *L. casei* RF after UV irradiation	0.040 ± 0.0011	2.0
Cell suspensions sequentially incubated with *L. casei* and *S. cerevisiae* RF after UV-irradiation	0.058 ± 0.0019	2.9

* The CFU ratio for the experimental (irradiated, with RF addition) and control (irradiated, without RF) variants.

final pH of 5.5, 5.0, 3.5, and 2.6 and the samples were incubated for 1 h. To determine the effectiveness of CF, the cells were separated by centrifugation, washed with 25% NaCl solution, inoculated in 10 mL of the culture medium, and incubated for two days on a shaker (120 rpm) at 37°C. In the second control, the acid stress was not induced. Cell growth was monitored by optical density at 540 nm. The CF protective activity of was evaluated by the difference of cell growth in the experimental and control flasks.

To determine the protective effect of archaeal CF on yeast, cell suspension of *S. cerevisiae* was pre-incubated for 10 min at 32°C with CF, 1 : 1 vol/vol, for the experiment and with fresh archaeal growth medium for the control. The control and experimental samples (2 mL) were subjected to heat stress by 10 min incubation at 55°C. The protective effect was evaluated by the number of colony-forming units in the experimental and control samples (CFU/mL).

Statistics. The paper presents the mean arithmetic values and their standard deviations from three independent experiments.

RESULTS AND DISCUSSION

The reactivating effect of the extracellular metabolites of *S. cerevisiae* culture liquid on UV-irradiated *L. casei* cells was demonstrated earlier, which suggested certain homology of the active components of bacterial an yeast culture liquid and/or of their cellular targets [12]. The anti-stress activity of yeast and bacterial reactivating factors (RF) was found to be determined by metabolites of proteinaceous nature with MW less than 10 kDa. The study of the extracellular RF cross-action was continued in the present work.

Bacteria and yeast. The RF of bacteria and yeast were introduced into the test system (a suspension of yeast cells after UV irradiation, which caused the death of 99.99% of the cells). The preparations were added in various ways: separately, of either bacterial or yeast origin and together, or in a varying sequence. The RF of the peptide nature, isolated from *L. casei* and *S. cerevisiae* culture liquids by the same method [6, 7] demonstrated a competition for the yeast cellular targets (Table 1). The strongest effect on survival of yeast was observed after sequential introduction of *L. casei* RF for 10 min and of *S. cerevisiae* for further 10 min, as well as after introduction of *L. casei* RF alone into the UV-irradiated yeast suspension. Preincubation of yeast cells with *S. cerevisiae* RF alone, or first with the yeast RF and then with *L. casei* RF resulted in a weaker effect. These results indicate a similarity in the action of *L. casei* and *S. cerevisiae* extracellular reactivating factors and higher efficiency of the bacterial RF.

The extracellular microbial autoregulators of other nature, as alkylhydroxybenzenes, homoserine lactones, and butyrolactones, also did not demonstrate strict species specificity [3, 13, 14].

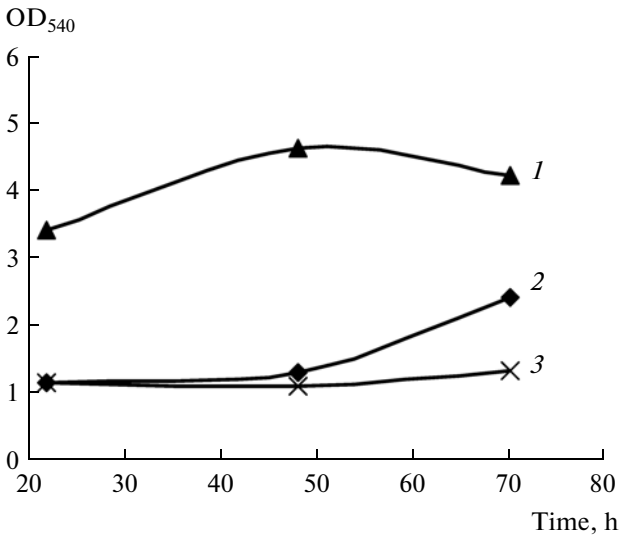
Archaea and yeast. Due to high resistance of archaea to various stress impacts, the choice of the factor with lethal intensity was a special task, which was even more complicated due to the inapplicability of quantitative enumeration of archaeal cells by CFU counts. Plating of diluted suspensions on solid media did not result in colony formation. Thus, the effect of the protection factor was evaluated by comparing the growth rates in the experimental and control variants inoculated with the samples subjected to stress. The culture growth rate depended on the number of cells remaining viable after the exposure. The same method was used previously in osmosensitivity studies of *Halobacterium salinarium* [15]. UV irradiation of the archaeal cell suspension in 25% NaCl solution for 20 min with a dose of 1.9 kJ/m^2 had almost no effect on their viability (data not shown).

Heat shock as a stress factor did not give consistent results due to a very narrow temperature range, 10 min at 68–70°C, at which the effect on the survival rates

would have reach at least 60–70%. Thus, when the archaea suspension was heated to 70°C for 10 min, the number of viable cells decreased so critically, that when they were used as inoculum, the growth rate decreased by 75% after 48 h. Importantly, preincubation of the archaea with their own extracellular protective factor prior to heat shock resulted in reduction of their growth rate by only 29%. However, with the increase of the heating temperature by two degrees, the loss of viability of all the cells was observed.

Another test for the presence of archaeal stress-protective metabolites in the culture liquid was the cells defense from the acid stress. Haloarchaea do not form acids and their growth is suppressed at low pH values [16]. The conditions for the acid stress (using citrate buffer solutions at different pH values) were chosen based on the previously established fact that the extracellular RF exhibited the highest protective activity when the growth of a test organism was suppressed by 99–99.9% [6, 7]. Such a strong decrease of the haloarchaeal growth was not achieved with the medium acidification in the pH range of 5.5–2.6, which resulted in a 30% decrease in survival compared to the control. In further experiments, the citrate buffer of pH 2.6 was used as the stress factor.

In the control variants, where the exponential-phase cells not exposed to acid stress were used as the inoculum, the cultures were growing without lag phase with μ of 0.04 h^{-1}, the maximum cell accumulation was observed after 48 h, was followed by the stationary phase and cell lysis by 72 h (figure). In the culture inoculated with the cells subjected to stress by incubation for 1 h in citrate buffer, pH 2.6, a long lag phase lasting up to 48 h and subsequent low rate growth (μ = 0.015 h^{-1}) were observed. The lag phase of the culture of the cells preincubated with archaeal RF was shorter than the exponential growth phase, which commenced with almost the same growth rate as that in the control (μ = 0.03 h^{-1}). In this case, the protective effect of RF was not associated with the effect of the growth medium components' pre-incubation, with which had almost no effect on growth. These results demonstrated that haloarchaea, like eubacteria and yeast, synthesize extracellular metabolites with the stress-protective function, and that the protective



Growth of the control and experimental cultures inoculated with the suspension of acid-stressed *H. marismortui* cells. Effect of the pre-incubation with the archaeal protective factor on the growth rate. Designations: inoculum not subjected to acid stress (control) (*1*); inoculum subjected to acid stress after 10 min preincubation with RF (*2*); and inoculum subjected to acid stress after 10 min preincubation in 25% NaCl solution (*3*).

effect of these metabolites is expressed in the preservation of a higher number of viable producer cells than in unprotected conditions, which was evident from a significant shortening of the rehabilitation period of the protected cell suspensions.

Extracellular stress-protecting metabolites found in archaeal RF exhibited a protective effect on *S. cerevisiae* cells subjected to heating at 55°C for 10 min, increasing their survival threefold in the variants with preincubation of the yeast suspension mixed with archaeal RF in the ratio 1 : 1 (Table 2). The growth medium for haloarchaea, which was used as the control, had no protective effect. Incubation of *H. marismortui* RF with proteinase K did not remove its protective effect, which is an indirect evidence of non-protein nature of the protective metabolite or metabolites. Several factors with an adaptogenic effect, including alkylhydroxybenzenes (AHB), were found in the cells

Table 2. Protective effect of the cell-free filtrate (CF) of *H. marismortui* cells on *S. cerevisiae* subjected to heat shock at 55°C for 10 min

Experimental conditions	Number of viable cells, $\times 10^5$ CFU/mL	Protection efficiency
Control	90 ± 4.8	–
Cells incubated:		
with growth media, then heated	0.009 ± 0.0009	1.0
with RF, then heated	0.027 ± 0.0023	3.0
with RF, previously incubated with proteinase K, the heated	0.025 ± 0.0027	2.8

Table 3. Protective effect of *H. marismortui* RF according to the ratio of *S. cerevisiae* intact (viable) and damaged (nonviable) cells before and after heat shock

S. cerevisiae suspension	Total cell number, $\times 10^5$ CFU/mL	The proportion of cells,%	
		"live"	"dead"
Before heating	2.0 ± 0.11	88	12
After heating (55°C, 10 min)	0.02 ± 0.003	0.9	99
After preincubation with RF and heating	0.06 ± 0.005	38	57

Table 4. Protective effect of *L. casei* and *S. cerevisiae* RF on HeLa cells forming stress granules (SG) in response to Na arsenite-induced oxidative stress

Experimental conditions	Na arsenite concentration (μM)		
	0	50	100
Share of SG-containing cells, %			
In the intact culture (control)	0	6.0 ± 2.8	68.0 ± 8.0
Preincubated with RF from *S. cerevisiae*	0	1.0 ± 0.2	77.0 ± 5.0
Preincubated with RF from *L. casei*	0	2.5 ± 0.25	65.0 ± 3.0

and culture liquid of many microorganisms [13], including tyrosol of *S. cerevisiae* [17]. Such non-protein compounds may possibly serve as protective compounds in the case of archaea.

The numbers of viable and nonviable cells in yeast suspensions subjected to heat shock were also determined by direct microscopy after staining with the Live/Dead fluorescent dye (Table 3). The proportion of viable and nonviable cells was 88 and 12% in the initial suspension and 0.9 and 99%, respectively, after heat shock. Yeast pre-incubation for 15 min with the archaeal CF prior to heating at 55°C for 10 min resulted in an increase in the share of viable cells to 38%, a decrease of the share of nonviable cells to 57%, with 5% of the cells with yellow fluorescence, indicating a transition stage.

Thus, we discovered that haloarchaea, which due to their efficient repair systems and specific cellular organization [16] have a high degree of adaptation to extreme environmental conditions, also possess the ability to protect their cells and the cells of other microorganisms from unfavorable and damaging impacts by synthesizing the stress-protecting metabolites.

A number of fundamental molecular properties are known to be common for archaea and eukaryotes, such as the mechanisms of DNA replication, transcription, and translation, as well as the homology of certain heat shock proteins [19, 20]. The archaea and eukaryotes have proteosomes and exosomes, which are not present in bacteria [16, 19]. Recently, caspase activity of an enzyme similar to that of an enzyme involved in eukaryotic cells apoptosis was detected in haloarchaea [20]. The effect of *H. marismortui* exometabolites, protecting *S. cerevisiae* cells subjected to a heat shock, is an additional evidence of the similarity of molecular and biological properties of archaea and eukaryotes.

Yeast cells and HeLa. Lower eukaryotes, such as *S. cerevisiae*, are used as models for investigation of a number of fundamental processes [18]. Considerable amount of data demonstrates the functional activity of microbial metabolites in heterologous systems and the similarities between the metabolic processes of microorganisms and multicellular organisms. In particular, a homology of the enzyme specifically cleaving the human endorphin precursor, and the enzyme cleaving the yeast α-factor peptide hormone precursor, was reported [18]. Thus, it was possible to consider a protective effect of microbial peptide RF against mammalian cells. In the experiments, we used HeLa cells, which respond to unfavorable conditions by forming stress granules (SG), which may be detected by indirect immunofluorescence with the antibodies specific to the mammalian SG protein. The effect of yeast RF on the number of HeLa cells containing SG was studied at increasing arsenite concentrations, inducing the development of oxidative stress in the cells. The results presented in Table 4 demonstrate that RF of both bacterial and yeast origin reduced the SG formation only in the case of an insignificant stress exposure (50 μM Na arsenite). The RF derived from yeasts grown in poor medium and from *L. casei* reduced the SG proportion from 6% in the control to 1% and 2.5%, respectively, in the experiment.

In general, the results obtained in this work demonstrate a wide range of adaptogenic effects of extracellular microbial metabolites of both proteinaceous and non-proteinaceous nature on the living systems of various organization levels: both prokaryotes, including eubacteria and archaea, and eukaryotes, including yeasts and mammals. High level of evolutionary conservatism of the molecular mechanisms of stress response development in bacteria, archaea, yeasts, and higher eukaryotes makes it possible to reliably use prokaryotes and lower eukaryotes as models for studying the patterns of stress response and for developing efficient strategies and methods to prevent stress and distress, specifically in higher eukaryotes.

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