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EXPERIMENTAL ARTICLES

The Extracellular Protein of *Luteococcus japonicus* subsp. *casei* Reactivates Cells Inactivated by UV Irradiation or Heat Shock

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Abstract—The culture liquid of *Luteococcus japonicus* subsp. *casei* was found to be able to reactivate cells of this bacterium inactivated by UV irradiation or heat shock. The antistress activity of the culture liquid was due to the presence of an extracellular exometabolite of a protein nature with a molecular mass of more than 10 kDa. When the bacterium was grown in a nutrient broth or glucose-containing mineral medium, the antistress protein was secreted by cells in the logarithmic growth phase. The reactivating effect of the antistress protein was inversely proportional to the survival rate of stressed cells.

Key words: *Luteococcus japonicus* subsp. *casei*, stress, reactivation, UV irradiation, thermoinactivation, extracellular protein, culture liquid.

Due to their simple organization, bacteria are convenient objects for studying stress responses, which are generally the same in pro- and eukaryotes. On the other hand, some researchers propose to use minor differences in the adaptive stress responses of bacteria for their intraspecies differentiation [1]. The study of stresses in bacteria may help solve some biotechnological and medical problems. The stress proteins of starter cultures can be used as molecular indicators of their physiological state, since the presence of stress proteins in a culture indicates that it has recently suffered stress [2]. This implies that such a culture either cannot function properly (since the induction of stress proteins is frequently accompanied by a suppression of the synthesis of many other proteins) or, conversely, is well adapted to stresses due to adaptive response to the first aggression of a stressor. The finding that bacteria with a weak stress response is negligibly virulent is of great interest from the standpoint of microbial pathogenesis.

The mechanisms of bacterial adaptation to stresses are fairly well studied at a subcellular level, however, little is known about the role of chemical cell-to-cell communications and the exometabolites of stressed and unstressed bacterial cells in this adaptation. The few recent studies on this problem were carried out with the bacterium *Escherichia coli* [3–7].

In contrast, this investigation deals with the bacterium *Luteococcus japonicus* subsp. *casei*, which was isolated from fresh cheese. The bacterium is gram-positive, non-spore-forming, and halotolerant. On the basis of physiological and biochemical properties, G+C content, and high DNA–DNA homology to *Propionibacterium jensenii*, the bacterium was first described as a new species of the genus *Propionibacterium*, *P. coccoides* [8]. Recently, however, based on the data of ribotyping, DNA–DNA hybridization, 16S rRNA gene sequencing, PCR–RFLP, and phylogenetic analysis, *P. coccoides* was found to be very close to the species *L. japonicus* [10] and was reevaluated as *L. japonicus* subsp. *casei* [9]. The similar species *L. japonicus* and *P. coccoides* form a cluster within the family *Propionibacteriaceae*.

L. japonicus subsp. *casei* is used for the ripening of curd cheese, during which the bacterium suffers stresses from heat and cold shock, high acidity and osmolarity, dehydration, starvation, and combinations of these stressors. This poses the problem of the stress adaptation of this bacterium.

The aim of the present work was to study the reactivating effect of the culture liquid and the protein exometabolite of *L. japonicus* subsp. *casei* on cells of this bacterium exposed to UV radiation and heat shock.

MATERIALS AND METHODS

Microorganism and cultivation conditions. Experiments were carried out with the bacterium *Luteococcus japonicus* subsp. *casei*, which was obtained from the culture collection at the Department of Microbiology, Faculty of Biology, Moscow State University.

The bacterium was grown under stationary conditions at 30°C in an Oxoid N2 nutrient broth (Oxoid, United Kingdom) supplemented with 1% glucose or in a liquid medium containing (g/l) $(NH_4)_2SO_4$, 0.3; KH_2PO_4, 0.2; $CaCl_2$, 0.002; $MgSO_4$, 0.002; NaCl, 0.002; $CoCl_2 \cdot 6H_2O$, 0.001; Difco tryptone, 0.1; Difco

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yeast extract, 0.05; and glucose, 1.5 in distilled water. The initial pH of the medium was 6.8–7.0. In the process of bacterial growth, the pH of the medium was maintained at the specified level by adding 10% NaOH.

Logarithmic-phase cells were harvested by centrifugation at 10000 *g* for 5 min, washed with a 50 mM Na-phosphate buffer (pH 7.0), and suspended in the same buffer to be used in experiments. The first supernatant (culture liquid) was used as the source of reactivating factors. To obtain these factors, the culture liquid was passed through a 0.22-μm filter (Nuclepore, United States), and the extracellular proteins adsorbed on the filter were desorbed with a 3% solution of NaCl. The saline solution was centrifuged to remove bacterial cells. The clear supernatant containing extracellular proteins was used in experiments as the antistress agent. The presence of contaminating bacterial cells in this preparation was controlled by plating it onto nutrient agar.

Stress factors. The stress factors (stressors) used in experiments were UV irradiation and cell heating at 53°C. The indicated temperature was chosen in preliminary experiments. The source of UV radiation was a pair of BUV-15 lamps (30 W), whose major spectral line has a wavelength of 253.7 nm. The bacterial suspension exposed to UV light or heating contained (100–300) × 10^6 cells/ml. To determine the survival rate of bacteria, an unexposed (control 1) or exposed to UV radiation or heat shock (control 2) bacterial suspension was plated onto nutrient agar and incubated at 30°C for 72 h.

Investigation of reactivating effect. A stressed bacterial suspension was mixed with the culture liquid or the preparation of antistress proteins in certain proportions and incubated at 30°C for 15 min (preliminary experiments showed that the reactivating effect of antistress factors was maximum under such incubation conditions). Then the serial tenfold dilutions of the bacterial suspension were plated, in triplicate, onto nutrient agar. The reactivating effect was evaluated by comparing the number of colonies grown in control 1 (unstressed bacterial suspension), control 2 (stressed bacterial suspension), and in the experiment (bacterial suspension exposed to stress and then incubated with antistress factors). The survival rate of bacteria was calculated as the relative number (expressed as a percent) of colony-producing cells with respect to the total number of cells in control 1. The reactivation index was calculated as the ratio of the number of colonies grown in the experiment to the number of colonies grown in control 2. The data presented in the tables are the means of triplicate experiments.

Protein was quantified by the method of Lowry *et al.* [11].

RESULTS

The reactivating effect of the culture liquid on UV-inactivated cells. Figure 1 shows the effect of UV radiation on the survival rate of bacterial cells. The data presented in Table 1 show that the culture liquid of *L. japonicus* subsp. *casei* exerted a reactivating effect (within 0.007–10%) on the UV-inactivated cells of this bacterium, the effect being the more pronounced, the lower the survival rate of the UV-exposed cells. Further experiments were carried out with the UV irradiation dose equal to 113 J/m^2, at which the reactivating effect of the culture liquid was maximum.

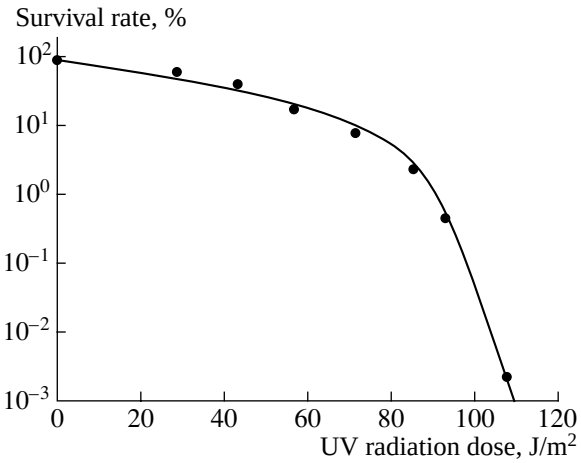

Fig. 1. The dose dependence of the effect of UV radiation on the survival rate of *L. japonicus* subsp. *casei* cells.

The reactivating effect of the culture liquid of a 48-h-old culture of *L. japonicus* subsp. *casei* was twice as great as the reactivating effect of a 24-h-old culture. The incubation of the UV-inactivated cells with the culture liquids for 15 min was more efficient than the incubation for 30 min (data not presented). The agitation of bacterial suspensions during their exposure to UV light augmented the inactivating effect of the light. The number of colonies grown from suspensions incubated with the culture liquid at 30 and 37°C was almost the same. With account for these data, further experiments were carried out with a mild agitation of bacterial suspensions, the culture liquid was obtained from 48-h-old *L. japonicus* subsp. *casei* cultures, and stressed cells were incubated with the antistress factors at 30°C for 15 min.

The reactivating effects of the culture liquids of *L. japonicus* subsp. *casei* grown in nutrient broth and glucose-containing mineral medium were much the same (Table 2). Control experiments showed that fresh sterile growth media and phosphate buffer exerted no reactivating effect. With account for these data, further experiments were carried out with the culture liquid of the bacterium grown in a glucose-containing mineral medium in which nitrogen-containing organic compounds were present in negligible amounts as growth factors.

The reactivating effect of the extracellular proteins present in the culture liquid. After filtration

Table 1. The reactivating effect of the culture liquid of *L. japonicus* subsp. *casei* as a function of the survival rate of cells exposed to different UV radiation doses

Experimental conditions	Cell concentration, 10^6 cells/ml	Survival rate, %	Reactivation index
Unexposed cells (control 1)	254 ± 11.6	100	
Cells exposed to UV radiation, 68 J/m^2			
Postincubation with			
phosphate buffer (control 2)	24.4 ± 1.76	9.6	1.0
culture liquid	30.8 ± 2.11	12.1	1.3
Cells exposed to UV radiation, 98 J/m^2			
Postincubation with			
phosphate buffer (control 2)	0.5 ± 0.002	0.2	1.0
culture liquid	4.48 ± 0.23	1.77	8.9
Cells exposed to UV radiation, 113 J/m^2			
Postincubation with			
phosphate buffer (control 2)	0.018 ± 0.001	0.007	1.0
culture liquid	1.24 ± 0.07	0.49	70

Note: Culture liquid was mixed with cell suspensions at a volume ratio of 1 : 1.

Table 2. The reactivating effect of various antistress preparations on the UV-inactivated *L. japonicus* subsp. *casei* cells

Experimental conditions	Cell concentration, 10^6 cells/ml	Survival rate, %	Reactivation index
Unexposed cells (control 1)	184 ± 7.4	100	
Cells exposed to UV radiation, 113 J/m^2 Postincubation with			
phosphate buffer (control 2)	0.0092 ± 0.0006	0.005	1.0
glucose-containing mineral medium (GMM)	0.008 ± 0.0005	0.005	1.0
culture liquid of *L. japonicus* subsp. *casei* grown in GMM	0.768 ± 0.01	0.42	83.5
culture liquid passed through a Nuclepore filter	0.0078 ± 0.0003	0.004	1.0
nutrient broth	0.0072 ± 0.0003	0.004	1.0
culture liquid of *L. japonicus* subsp. *casei* grown in nutrient broth	0.739 ± 0.009	0.40	80.3

Note: Culture liquid was mixed with cell suspensions at a volume ratio of 1 : 1.

through a 0.22-μm Nuclepore filter, the culture liquid lost its reactivating activity almost completely, although the protein content in the filtrate decreased insignificantly (from 1.88 to 1.7 mg/ml). The proteins adsorbed on the filter were likely responsible for the antistress activity of the culture liquid. The treatment of the culture liquid with pronase nearly completely destroyed its reactivating activity (Table 3). The dialysis of the culture liquid in 10-kDa-cutoff cellulose sacks decreased its reactivating activity by only 30% (Table 3). Taken together, these data suggest that the reactivating effect of the culture liquid of *L. japonicus* subsp. *casei* is due to an exometabolite of a protein nature with a molecular mass of more than 10 kDa.

The protein (or proteins) desorbed from the filter with a 3% solution of NaCl was tested for reactivating activity. In preliminary experiments, 3% NaCl was shown not to inhibit the growth of the bacterium. The data presented in Fig. 2 show that the reactivating effect of the desorbed antistress protein was proportional to its concentration within a range from 1 to 50 μg/ml. At the latter concentration, the antistress protein increased the survival rate of severely inactivated cells by about 400 times.

The reactivating activity of the antistress protein, like that of the culture liquid, decreased as the cell survival increased (Table 4). Indeed, the reactivation index of the antistress protein was only 1.6 with the cell sur-

Table 3. The effect of dialysis, proteolysis, and heating on the reactivating activity of the culture liquid of *L. japonicus* subsp. *casei*

Experimental conditions	Cell concentration, 10^6 cells/ml	Survival rate, %	Reactivation index
Unexposed cells (control 1)	221 ± 13.2	100	
Cells exposed to UV radiation, 113 J/m^2			
Postincubation with			
phosphate buffer (control 2)	0.0096 ± 0.0006	0.0044	1.0
culture liquid of *L. japonicus* subsp. *casei* grown in GMM	0.956 ± 0.048	0.433	98.4
culture liquid treated with 1 mg/ml pronase	0.03 ± 0.0012	0.0136	3.1
1 mg/ml pronase solution in buffer	0.015 ± 0.002	0.005	1.2
dialyzed culture liquid	0.662 ± 0.035	0.300	69.0
culture liquid heated to 75°C	0.0238 ± 0.001	0.011	2.45
culture liquid heated to 92°C	0.024 ± 0.001	0.011	2.48

Note: Culture liquid was mixed with cell suspensions at a volume ratio of 1 : 1. GMM is glucose-containing mineral medium.

vival equal to 3.42%, while it exceeded 400 with the cell survival equal to 0.004%. The dialysis of a solution of the antistress protein in 10-kDa-cutoff cellulose sacks had little influence on the reactivation index (as a result of the dialysis, it decreased from 433 to 400). This observation confirms the above suggestion that the antistress protein has a molecular mass of more than 10 kDa.

The reactivating effect of the antistress protein on heat-inactivated cells. As can be seen from the data presented in Table 5, the culture liquid of *L. japonicus* subsp. *casei* augmented the survival rate of heat-inactivated cells by 26 times. The antistress protein at a concentration of 40 μg/ml enhanced the survival rate of such cells by 550 times. The filtrate of the culture liquid passed through a Nuclepore filter did not possess antistress activity. These data strongly suggested that the factor that reactivated the heat-inactivated cells of *L. japonicus* subsp. *casei* was identical to the protein factor that reactivated the UV-inactivated cells of this bacterium.

DISCUSSION

The data presented in this paper show that the unstressed logarithmic-phase cultures of *L. japonicus* subsp. *casei* grown in nutrient broth and a glucose-containing mineral medium produce exometabolite (or exometabolites) of a protein nature that possesses high antistress activity. This activity, like that of the intracellular antistress protein of *Propionibacterium shermanii* [12], is inversely proportional to the survival rate of stress-inactivated cells. With a total concentration equal to 40 μg/ml, the antistress protein of *L. japonicus* subsp. *casei* augmented the survival rate of the heat- or UV-inactivated cells of this bacterium by 400–500 times (from a level of 0.003–0.007%). With account for the fact that the antistress protein comprises only a small portion of the total extracellular proteins of the bacterium, these data suggest that chemical cell-to-cell communications play an important part in the stress phenomena under discussion.

The works concerned with the role of chemical cell-to-cell communications in stress phenomena are few in number and deal primarily with the study of the protective and sensory exometabolites of bacteria exposed or adapted to various stresses, such as heat and cold shocks, oxidative stress [4, 5], the action of tetracycline [3], and decreased pH [13–15]. In this paper, we described the antistress protein exometabolite of *L. japonicus* subsp. *casei* that is secreted by unstressed cells. For the first time, such a protective extracellular stress-sensing metabolite was detected in the unstressed culture of *E. coli* K12 grown at pH 7.0–9.0 [15]. The protein precursor secreted by this bacterium was activated by low pH, heat shock, and UV irradiation, transform-

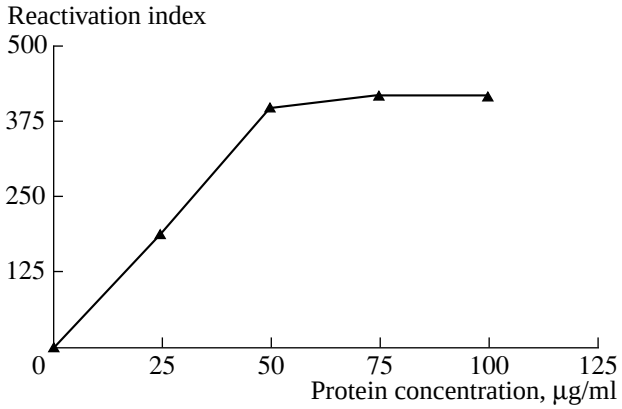

Fig. 2. The concentration dependence of the reactivating effect of the extracellular antistress protein of *L. japonicus* subsp. *casei* on the UV-inactivated bacterial cells. The survival rate of the UV-inactivated bacterial cells at zero concentration of the antistress protein was 0.017%.

Table 4. The reactivating effect of the extracellular antistress protein of *L. japonicus* subsp. *casei* as a function of the survival rate of cells exposed to different UV radiation doses

Experimental conditions	Cell concentration, 10^6 cells/ml	Survival rate, %	Reactivation index
Unexposed cells (control 1)	301 ± 5.5	100	
Cells exposed to UV radiation, 83 J/m^2			
Postincubation with			
phosphate buffer	6.54 ± 0.258	2.16	1.0
antistress protein solution	10.32 ± 0.378	3.42	1.6
Cells exposed to UV radiation, 98 J/m^2			
Postincubation with			
phosphate buffer	0.744 ± 0.034	0.246	1.0
antistress protein solution	5.64 ± 0.162	1.86	7.6
Cells exposed to UV radiation, 113 J/m^2			
Postincubation with			
phosphate buffer	0.011 ± 0.0007	0.004	1.0
antistress protein solution	4.68 ± 0.328	1.56	433

Note: The concentration of the antistress protein in the incubation medium was 40 μg/ml.

Table 5. The effect of various antistress preparations on the survival rate of *L. japonicus* subsp. *casei* cells inactivated by heating at 53°C for 40 min

Experimental conditions	Cell concentration, 10^6 cells/ml	Survival rate, %	Reactivation index
Intact cells (control 1)	252 ± 16.6	100	
Heat-inactivated cells incubated with			
phosphate buffer	0.0188 ± 0.001	0.0075	1.0
culture liquid	0.488 ± 0.022	0.194	26
40 μg/ml antistress protein	10.34 ± 0.31	4.1	550
culture liquid passed through a Nuclepore filter	0.017 ± 0.0008	0.007	0.92

Note: Culture liquid was mixed with cell suspensions at a volume ratio of 2 : 1.

ing into a factor that was able to induce acid tolerance in bacterial cells. In contrast, the protein exometabolite of *L. japonicus* subsp. *casei* does not need activation and is universal with respect to the type of stress (at least, UV radiation and heat shock). The antistress effect of the protein exometabolite of *L. japonicus* subsp. *casei* is most pronounced when the survival rate of stress-inactivated bacterial cells is very low, i.e., when the known repair bacterial systems are inefficient.

It can be suggested that the protein exometabolite of *L. japonicus* subsp. *casei* binds to the membrane receptor of bacterial cells, which transduces signal to the stress-specific σ-factor. In *Bacillus subtilis*, the general stress protein (GSP) genes, which are induced by heat shock, ethanol, salt, and starvation, refer to the α-B-regulon [16]. Kruger *et al.* [17] showed that the *B. subtilis clpC* operon working under the control of the σ-A-promoter encodes the DNA repair and competence proteins.

Work is now in progress in our laboratory aimed at isolating and purifying the *L. japonicus* subsp. *casei* exometabolite in order to investigate the mechanism of its antistress activity. The high antistress activity of this exometabolite shows the potential of propionibacteria as probiotics.

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