ISSN 0026-2617, Microbiology, 2019, Vol. 88, No. 2, pp. 132–136. © Pleiades Publishing, Ltd., 2019.
Russian Text © E.A. Rogozhin, L.I. Vorob'eva, E.Yu. Khodzhaev, E.S. Gerasimov, 2019, published in Mikrobiologiya, 2019, Vol. 88, No. 2, pp. 137–143.

EXPERIMENTAL ARTICLES

Optimized Fractioning and Structure Analysis of the Reactivating Factor from *Luteococcus japonicus* subsp. *casei*

E. A. Rogozhin[a, b], L. I. Vorob'eva[c, *], E. Yu. Khodzhaev[c], and E. S. Gerasimov[c]

[a] *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, 117997 Russia*
[b] *Gause Institute of New Antibiotics, Moscow, 119435 Russia*
[c] *Biological Faculty, Moscow State University, Moscow, 119192 Russia*
**e-mail: livorobjeva@mail.ru*

Received September 25, 2018; revised November 20, 2018; accepted November 30, 2018

Abstract—The chemical structure of the extracellular reactivating factor (RF) from *Luteococcus japonicus* subsp. *casei* was determined; this factor promotes survival of a small subpopulation of the producer cells under lethal stress impact. For the isolation and purification of this RF, the previously developed method for RF isolation from *Saccharomyces cerevisiae* was optimized. A total of 15 fractions were obtained from the culture liquid of *Luteococcus casei*, two of which (I and IV) exhibited reactivation activity against the cells subjected to a lethal stress impact (UV irradiation). The method included solid-phase extraction of the peptides on a hydrophobic sorbent with the C_8 phase and subsequent multistage separation using RP-HPLC. Mass spectral analysis (MALDI-TOF) was used to determine the molecular characteristics of fraction IV. Efficient ionization was not achieved for fraction I. Mass charges for fraction IV were 773.394 and 788.102 Da. Edman automatic sequencing was used to identify these components as peptides: Ala-Pro-Asn-Glu-Asn-Gln-Gly and Ala-Pro-Asn-Glu-Glu-Gln-Gly. No similarity to any known full-size functional peptide molecules in the databases on polypeptide primary structures was revealed. Formation of biologically active peptides by *L. casei* may be associated with non-template synthesis and probably involves proteolysis of a large protein.

Keywords: Luteococcus japonicus subsp. *casei*, reactivating factor, stress, structure, peptide, RP-HPLC
DOI: 10.1134/S0026261719020097

Investigation of stress responses in microorganisms and elucidation of the mechanisms of their formation are among the most important aspects of fundamental biology. The evolutionarily developed resistance of microorganisms to stressors of various nature is considered to be the primary cause of the increasing inefficiency of antimicrobial therapy. Under natural conditions, the ability of microorganisms to resist stresses due to production of extracellular compounds plays a key role in the formation and maintenance of homeostasis of microbial communities. Our previous studies have demonstrated the ability of bacteria, archaea and lower eukaryotes (yeasts) to produce extracellular compounds of peptide nature, which exert a protective and reactivating effect on the cells of both producers and members of other domains after exposure to various stressors, including UV radiation, heating, oxidants, and bile salts (Vorobjeva et al., 2013, 2015).

The test objects exposed to lethal impacts were gram-positive and gram-negative bacteria (Vorob'yova et al., 2010; Vorob'eva et al., 2013), yeasts of different physiological and ecological groups (Vorob'eva et al., 2011), and archaea (Vorob'eva et al., 2013). The basic model organism for studying the patterns of reactivating factor (RF) formation, its physicochemical characteristics, and conditions of manifestation of its biological activity was the bacterium *Luteococcus japonicus* subsp. *casei* (*L. casei*). Object selection was determined by a broad range of its adaptive properties, i.e., the ability to utilize various substrates (Vorobjeva, 1999; Thorenoor et al., 2009) and by occurrence of representatives of the genus *Luteococcus* in a wide environmental range. *Luteococcus* sp. strains were isolated from soil and water (Tamura et al., 1994), dairy products and cheese (Vorob'eva et al., 1983), blood samples (Collins et al., 2003), deep-sea sediments of the Pacific Ocean (Fan et al., 2014), and activated sludge of wastewater treatment plants at cellulose factories (Thorenoor et al., 2009).

The *L. japonicus* subsp. *casei* strain was isolated from Sovetskii cheese. Molecular biological studies, ribotyping and restriction analysis of the 16S and 23S rDNA fragments have shown that the isolated *Luteococcus* strain should be assigned to the family *Propionibacteriaceae* (Van Niewholtz, 1998; Vorobjeva, 1999). The closest relative of the strain is *Propioniferax innocuum*. Previously, from the culture liquid of *L. casei* we have isolated a reactivating factor exerting protective and reactivating effects on the cells of its producer and other organisms: bacteria, archaea, and yeasts. It has

been shown that RF is a peptide and a complex of two active fractions (Vorob'eva et al., 2015). RF is synthesized and excreted by undamaged cells under normal growth conditions at extremely low concentrations (Vorob'eva et al., 2013). An inverse dependence was found between stressor intensity and RF efficiency (the survival rate of microbial cells) (Vorob'eva et al., 2011; Vorob'eva et al., 2013). Thus, the function of RF is of particular importance under stresses of lethal intensity. RF has no strict species specificity as was demonstrated by the cross-domain effect of the preparation (Vorob'eva et al., 2013). The type of concentration dependence, the high rate of reactivating effect and stress resistance suggest the signaling mechanism of action of the peptide RF (Vorobjeva et al., 2009). Using the constructed tester strain *Escherichia coli* bearing the *umuD-lacZ* operon it was established that the protective effect of RF was not associated with the induction of SOS response (Loiko et al., 2013) and seemed to be provided by the signaling-type RF effect on the cytoplasmic membrane.

The goal of the present work was to identify the structure of the peptide reactivating factor from *L. casei*.

MATERIALS AND METHODS

Research subjects and cultivation conditions. The research subject was a gram-positive bacterium *Luteococcus japonicus* subsp. *casei* originally isolated from cheese (Vorob'eva et al., 1983). The strain was cultivated under static conditions at 32°C in flasks with 100 mL of glucose-mineral medium containing the following (%): glucose, 1.0; $(NH_4)_2SO_4$, 0.3; KH_2PO_4, 0.1; NaH_2PO_4, 0.2; $MgSO_4$, 0.002; $CaCl_2$, 0.002; NaCl, 0.002; yeast extract, 0.1; pH 7.0. The *L. casei* culture in the linear growth phase was centrifuged (10000 *g*, 20 min) and the culture liquid (CL) was used for RF isolation. *L. casei* cells were washed twice with 0.05 M Na-phosphate buffer solution, pH 7.4, and resuspended in the same buffer to the cell density of OD_{540} = 0.4–0.6. The cell suspension was used to determine the biological activity of RF.

RF activity assay. CL was run through a nitrocellulose membrane filter with 0.22-μm pore diameter (Millipore, United States); the components adsorbed on the filter were eluted with 3% NaCl solution and then the solution was run through a low protein affinity membrane filter (Pall, United States) to completely remove the cells.

The cell number in the suspensions of *L. casei* and in the strains of yeasts, lactic acid bacteria, and propionic acid bacteria was determined by the titer of colony-forming units (CFU/mL) after plating of 10^N-fold diluted suspensions on the relevant solid (2% agar). Micro inoculation with 5-μL aliquots of suspensions was performed with six replicate samples per dilution.

Reactivating factor isolation and structure determination. In this study we used the previously developed scheme of fractionation and purification of polypeptide components secreted by *L. casei* into the culture liquid (Vorob'eva et al., 2015). The culture liquid was desalted, concentrated, and fractionated by affine chromatography in a Heparin HiTrap Sepharose column (GE HealhCare, Sweden). The anionic total fraction not bound to the sorbent was rechromatographed by the analytical reversed-phase (RP) HPLC in a Vydac C_{18} Protein & Peptide 4.6 × 250 mm column (GraceVydac, United States). Chromatography was carried out with the linear gradient of solvent B (80% CH_3CN, 0.1% TFA) relative to solvent A (0.1% TFA) from 0 to 50% for 50 min at a flow rate of the mobile phase of 1 mL/min. The peptides were detected by absorption at 214 nm. Molecular masses of the active components were measured on an Ultraflex MALDI-mass spectrometer (Bruker Daltonics, Germany) equipped with an UV laser (337 nm) in the positive ion mode. The matrix used was 2,5-dihydroxybenzoic acid. Equal volumes (1 μL) of the samples and the matrix (20 mg matrix/mL in 80% aqueous CH_3CN, 0.1% TFA in MQ deionized water) were mixed on the target and the resultant mixture was air-dried. The mass spectra were analyzed using FlexAnalysis for TOF. The error of measurement was 2 ppm. The primary structure of the biologically active peptide was determined by the Edman degradation with a PPSQ-33A automated protein and peptide sequencer (Shimadzu Corp., Japan) according to the manufacturer's protocol. The experimental data were interpreted using the LabSolutions software package (Shimadzu Corp., Japan). The homologies of amino acid sequences were searched in the UniProt/SwissProt and trEMBL databases using the BLASTP algorithm; the translated sequences were searched on the basis of the *L. casei* annotated genome kindly provided by V. Loux (Collective Genomes Gbk l7320. contigs final gbk).

Determination of the RF biological activity. The stress factors used were UV radiation, bile salts, and oxidative stress. The source of UV radiation consisted of two 30-W BUV-15 lamps (Russia) mounted in parallel, with the major part of emission at 253.7 nm. Preliminary experiments showed the "dose–response" dependence. At a radiation dose of 81 J/m^2, the survival rate of irradiated cells was 0.01–0.03%. The target activity was determined in the cell suspension incubated with RF for 10 and 15 min before and after stress exposure, respectively. The reactivating effect was assessed by comparing viable cell numbers (CFU) emerging on a solid medium (2% agar) in experimental and control variants; in the latter there was no reactivating factor. The bacterial survival rates in experimental and control variants were expressed in percentage relative to non-irradiated control. The test objects were the cells of RF producer *Luteococcus casei*, as well

Table 1. Reactivating effect of the RF from *L. casei* on stress-exposed microbial cells

Test object, stress factor	Cell number, CFU/mL		
	before stress (K1) (%)	after stress (K2) (%)	after stress and incubation with RF (%)
L. casei exponential phase, UV radiation*	175×10^5 (100)	0.12×10^5 (0.07)	0.47×10^5 (0.27)
L. casei stationary phase, UV radiation*	200×10^5 (100)	0.012×10^5 (0.06)	0.22×10^5 (0.11)
L. casei, H_2O_2**	212×10^6 (100)	25×10^6 (11.8)	182×10^6 (85.8)
S. cerevisiae, UV radiation*	130×10^5 (100)	0.012×10^5 (0.009)	0.050×10^5 (0.038)
Lb. casei, bile salts***	50×10^6 (100)	0.042×10^6 (0.084)	0.554×10^6 (1.11)
Lb. acidophilus, bile salts***	160×10^6 (100)	0.441×10^6 (0.275)	1.880×10^6 (1.175)
P. freudenreichii, bile salts***	130×10^6 (100)	0.014×10^6 (0.011)	0.032×10^6 (0.025)
P. acidipropionici, bile salts***	100×10^6 (100)	0.031×10^6 (0.031)	0.064×10^6 (0.064)

* Dose of UV radiation was 108 J/m^2 for *L. casei* and 81 J/m^2 for *S. cerevisiae*.
** H_2O_2 concentration was 600 mM.
*** Final concentration of the mixture of cholate and deoxycholate Na salts (1 : 1) was 2 g/L.

as the cells of lactic acid bacteria from the collection of the Department of Microbiology (Moscow State University, the Faculty of Biology): *Lactobacillus acidophilus* (*Lb. acidophilus*), *Lactobacillus casei* (*Lb. casei*). The propionic acid bacteria *Propionibacterium freudenreichii* (no. 1857) and *P. acidipropionici* (no. 1859) were obtained from the Czech Collection of Microorganisms (CCM). The yeast *Saccharomyces cerevisiae* VKPM Y-1200 was obtained from the All-Russian Collection of Industrial Microorganisms. The cell suspensions of tester strains were obtained as described above.

RESULTS AND DISCUSSION

Biological characteristics of RF. The bacterium *L. casei* is a facultative anaerobe represented by large, nonmotile non-spore-forming spherical cells producing orange colonies on agar surface. The major fermentation products are propionic and acetic acids; caprylic, isocaproic, butyric, hexanoic (caproic), and formic acids are produced in minor amounts (Vorob'eva et al., 2014). It was previously shown that the number of surviving bacteria exposed to UV radiation and heating increased in the case of short-term (10–15 min) pre- or postincubation of *L. casei* cells with the fraction of extracellular peptide metabolites of different degrees of purity (Vorob'eva et al., 2003).

The extracellular peptide fraction referred to as RF (reactivating factor) isolated from *L. casei* CL had a protective effect on stress-exposed cells of not only the producer but also of other prokaryotic and eukaryotic objects.

Peptide exometabolites with antistressor properties are formed by the cells and excreted into the medium under the conditions favorable for normal growth of microorganisms and are accumulated in the medium by the beginning of the stationary growth phase (Table 1).

RF exhibited a universal protective effect on the cells exposed to unrelated stresses: UV radiation, heating, oxidants and bile salts (Table 1).

There is an inverse dependence between the efficiency of protective and reactivating effects of RF and the survival rate of stress-exposed cells. The maximum antistress effect of the exometabolite was observed at an extremely low number of survivor cells after lethal impact (0.01–0.1%). The protective effect (preincubation with RF) was several times more pronounced than the reactivating effect (postincubation with RF) exhibited after lethal impact (Vorob'eva et al., 2003). It has also been noted that the efficiency of reactivation of *L. casei* cells in the stationary phase was 2 times less compared to the cells in the logarithmic growth phase (Vorob'eva et al., 2009).

RF showed a universal biological effect against microorganisms from different taxa (including probiotic) exposed to stress factors of diverse nature (Table 1).

The above-mentioned patterns in manifestation of the biological activity of RF suggest that its effects are associated with formation of a small subpopulation of stress-resistant persister cells. RF probably induces the phenotypic transition of regular vegetative cells into persisters, which accounts for its much (up to 10-fold) greater effect in the variants with culture preincubation before the stress compared to the variant with postincubation. However, the possible effect of RF at the stage of induction of reversed transition of persisters to vegetative growth cannot be ruled out either. In this case, the higher efficiency of RF during preincubation may be explained by an increase in its biological activity due to UV radiation. This explanation is not contrary to the data on enhanced efficiency of RF under exposure to radiation (as part of the puri-

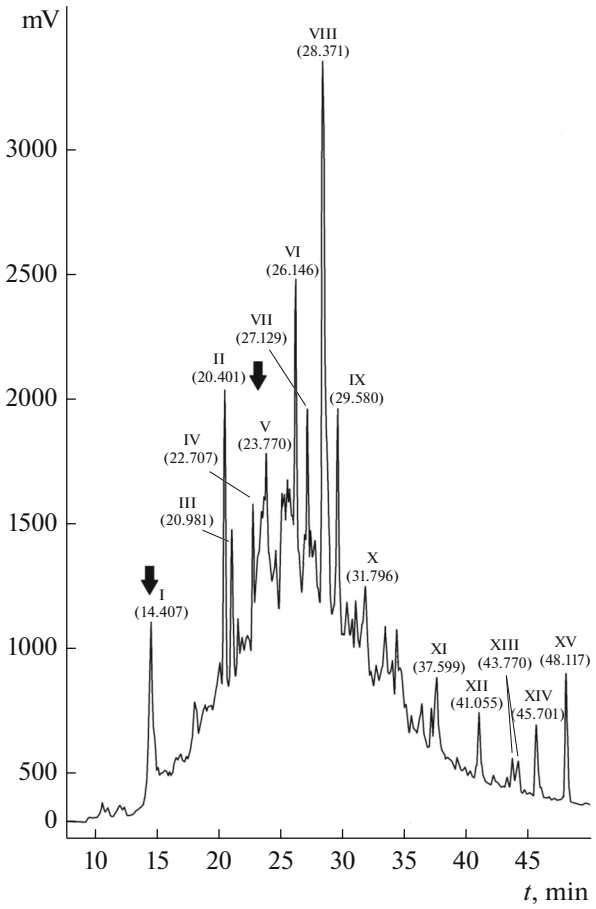

Fig. 1. The RP-HPLC profile of fractionation of the unbound fraction after separation of desalted concentrated extracellular liquid of *L. japonicus* by affine chromatography with a stepwise gradient. The collected fractions are denoted by Roman numerals (I–XV); their retention times on the column are shown. Black arrows indicate the peaks showing the presence of a reactivating effect.

fied fraction) before the effect on the cells in the post-incubation variant (Vorob'eva and Khodzhaev, 2010).

Determination of the structure of the extracellular RF from *L. casei*. Extracellular RF was isolated from the culture liquid of *L. casei* by the optimized protocol including solid-phase extraction (desalting) of concentrated CL on a hydrophobic sorbent with the C_8 phase, followed by evaporation, lyophilization and separation by affine chromatography with a stepwise gradient of increasing NaCl concentration from 0 to 1 M. The total unbound fraction, without the stage of separation by analytical gradient anion-exchange chromatography, was separated by RP-HPLC; as a result, 15 predominant fractions were collected and designated as I–XV (Fig. 1). These fractions were lyophilized to remove residual amounts of TFA. The results of testing the fractions for the sought-for reactivating effect showed its presence only in two components (I and IV). For determining the structural characteristics, both fractions were analyzed by MALDI-TOF mass spectrometry. We failed to obtain effective ionization and, accordingly, mass spectrum signals for fraction I, while the mass-to-charge ratios for component IV were 773.394 and 788.102 Da. Further identification of this active fraction was performed by the Edman's method of stepwise automated sequencing; as a result, the complete amino acid sequences of two peptides within component IV were established: ^{1}Ala-Pro-Asn-Glu-Asn-Gln-Gly^{8} (peptide IV-1) and ^{1}Ala-Pro-Asn-Glu-Glu-Gln-Gly^{8} (peptide IV-2). Consequently, both peptides were highly homologous, differing in only one amino acid substitution: Asn5Glu. Their formation apparently was not associated with template synthesis, because such sequences have not been found in the genome of *L. casei* (V. Loux, Collective Genomes Gbk). The search of homologies in the databases of the primary structures of polypeptides revealed no similarity with any of the known full-size functional peptide molecules. However, the data obtained make it possible to state with certain probability that this peptide is a fragment of partial proteolysis of a larger protein. It is characteristic that, according to the genomic data, this protein may contain a unique repeating motif of the **APNENQC**APNENQ-C**APNENQC** type, which later on will undergo limited proteolysis with formation of short sequences with the signaling function. Moreover, an additional argument in favor of proteolytic origin of RF may be partial coincidence of the given short sequence with certain parts of some bacterial proteins according to the data (GenBank reference sequence IDs: WP_012104305.1, WP_007615369.1, WP_020569836.1). The identified peptides have no homology with the previously determined RF possessing the functions of a stress "reactivator" from baker's yeast (*S. cerevisiae*) (Vorob'eva et al., 2017); however, the similar physicochemical properties, as well as the similar predicted elements of the random coil-type secondary structure, are evidence in favor of the similar mechanisms of signal peptide generation in response to stresses in phylogenetically distant living organisms belonging to different kingdoms.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

REFERENCES

Collins, M.D., Hitson, R.A., Nikolaitchouk, N., Nyberg, A., and Folsen, E. *Luteococcus sanguinis* sp. nov., isolated from human blood, *Int. J. Syst. Evol. Microbiol.*, 2003, vol. 53, pp. 1889–1891.

Fan, X., Zhang, Z., and Zhang, X.H., *Luteococcus sediminum* sp. nov., isolated from subseafloor sediment of the

South Pacific Gyre, *Int. J. Evol. Microbiol.*, 2014, vol. 64, pp. 2522–2527.

Loiko, N.G., Vorob'eva, L.I., Khodzhaev, E.Yu., Kozlova, A.N., Gal'chenko, V.F., and El' Registan, G.I., Effect of the reactivating factor of *Luteococcus japonicus* subsp. *casei* on the expression of SOS response genes, *Microbiology* (Moscow), 2013, vol. 82, pp. 126–132.

Tamura, T., Takaechi, M., and Yokota, A., *Luteococcus japonicus* gen. nov., sp., nov., a new gram-positive coccus with *LL*-diaminopimelic acid in the cell wall, *Int. J. Syst. Bacteriol.*, vol. 44, pp. 348–356.

Thorenoor, N., Kim, Y., Lee, C., Yu, M.H., and Engesser, K.H., A previously uncultured papermill *Propionibacteria* is able to degrade O-aryl alkyl ethers and various aromatic hydrocarbons, *Chemosphere*, 2009, vol. 75, pp. 1287–1293.

Van Niewholtz, J.A., A taxonomic re-evaluation of *Propionibacterium coccoides, Ph.D. Thesis*, Dep. Microbiol. Biochem. Univ. Orange Free State, Bloemfontein, South Africa, 1998.

Vorob'eva, L.I., Turova, T.P., Kraeva, N.I., and Alekseeva, A.A., Propionic acid cocci and their systematic position, *Microbiology* (Moscow), 1983, vol. 52, pp. 368–374.

Vorobjeva, L.I., *Propionibacteria*, Kluwer Academic, 1999.

Vorob'eva, L.I., Khodjaev, E.Yu., and Ponomareva, G.M., The extracellular protein of *Luteococcus japonicus* subsp. reactivates cells inactivated by UV-irradiation or heat shock, *Microbiology* (Moscow), 2003, vol. 72, pp. 428–433.

Vorob'eva, L.I., Khodzhaev, E.Yu., Mulyukin, A.L., and Toropygin, I.Yu., The mechanism of action of reactivating factor from *Luteococcus japonicus* subsp. *casei, Appl. Biochem. Microbiol.*, 2009, vol. 45, pp. 489–493.

Vorob'eva, L.I. and Khodzhaev, E.Yu., Protective and reactivating effect of the protein exometabolite on yeast cells inactivated by the ultraviolet irradiation, *Appl. Biochem. Microbiol.*, 2010, vol. 46, pp. 177–183.

Vorob'yova, L.I., Fedotova, A.V., and Khodzhaev, E.Yu., Protective action of reactivating factor of *Luteococcus japonicus* subsp. *casei* toward cells of *Escherichia coli* reparation mutants inactivated with UV-light, *Appl. Biochem. Microbiol.*, 2010, vol. 46, pp. 567–573.

Vorob'eva, L.I., Khodzhaev, E.Yu., and Vustin, M.M., Extracellular protein metabolite of *Luteococcus japonicus* subsp. *casei* reactivates cells subjected to oxidative stress, *Appl. Biochem. Microbiol.*, 2011, vol. 47, pp. 264–269.

Vorob'eva, L.I., Khodzhaev, E.Yu., Novikova, T.M., Mulyukin, A.L., Chudinova, E.M., Kozlova, A.N., and El'-Registan, G.I., Stress-protective and cross action of the extracellular reactivating factor of the microorganisms of the domains bacteria, archaea, and eukaryota, *Microbiology* (Moscow), 2013, vol. 82, pp. 594–599.

Vorob'eva, L.I., Khodzhaev, E.Yu., Rogozhin, E.A., Samoilenko, V.A., and Kharchenko, N.V., Structural characterization of the extracellular peptide metabolites of *Luteococcus japonicus* subsp. *casei* and their protective effect on probiotic bacteria, *Microbiology* (Moscow), 2015, vol. 84, pp. 502–511.

Vorob'eva, L.I., Rogozhin, E.A., Khodzhaev, E.Yu., Nikolaev, I.V., and Turova T.P., Reactivating factor of *Luteococcus japonicus* subsp. *casei*: isolation and characterization, *Appl. Biochem. Microbiol.*, 2015, vol. 51, pp. 44–51.

Vorob'eva, L.I., Khodzhaev, E.Yu., Rogozhin, E.A., Cherdyntseva, T.A., and Netrusov, A.I., Characterization of extracellular yeast peptide factors and their stress-protective effect on probiotic lactic acid bacteria, *Microbiology* (Moscow), 2016, vol. 85, pp. 411–419.

Vorob'eva, L.I., Rogozhin, E.A., Khodzhaev, E.Yu., Volodyazhkin, R.A., and Samoilenko, V.A., Characterization and stress-protective action of *Saccharomyces cerevisiae* extracellular peptide factors on propionic acid bacteria, *Microbiology* (Moscow), 2017, vol. 86, pp. 698–707.

Translated by E. Makeeva