ISSN 0006-2979, Biochemistry (Moscow), 2025, Vol. 90, No. 7, pp. 911-920 © Pleiades Publishing, Ltd., 2025.
Russian Text © The Author(s), 2025, published in Biokhimiya, 2025, Vol. 90, No. 7, pp. 993-1003.

Effect of Cultivation Conditions on the Expression of the *Exiguobacterium sibiricum* Proteorhodopsin Gene

Lada E. Petrovskaya[1,2,a]*, Elena V. Spirina[3], Artemiy Yu. Sukhanov[4], Elena A. Kryukova[1], Evgeniy P. Lukashev[5], Rustam H. Ziganshin[1], Elizaveta M. Rivkina[3], Dmitrii A. Dolgikh[1,5], and Mikhail P. Kirpichnikov[1,5]

[1]*Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117997 Moscow, Russia*

[2]*Moscow Institute of Physics and Technology, 141701 Dolgoprudny, Moscow Region, Russia*

[3]*Institute of Physicochemical and Biological Problems in Soil Science, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia*

[4]*FRC Kazan Scientific Center, Russian Academy of Sciences, 420111 Kazan, Russia*

[5]*Faculty of Biology, Lomonosov Moscow State University, 119991 Moscow, Russia*

[a]*e-mail: lpetr65@yahoo.com*

Received March 28, 2025
Revised May 25, 2025
Accepted May 28, 2025

Abstract—Recombinant proteorhodopsin ESR of the gram-positive bacterium *Exiguobacterium sibiricum* isolated from permafrost deposits in northeastern Siberia binds retinal and acts as a light-dependent proton pump, but not much is known about its expression under natural conditions. In this work, expression of the *esr* gene in *E. sibiricum* cultures grown under various conditions was studied by quantitative PCR. It has been discovered that cultivation on poor media at low temperatures contributes to a significant increase in the content of the corresponding mRNA. The data obtained are confirmed by the results of the analysis of the membrane fraction of cells using label-free quantitative chromatography-mass spectrometry. Also, at 10°C, increased content of phytoene desaturases, which are involved in the biosynthesis of carotenoids, is observed. However, we were unable to detect the presence of a functional retinal-containing protein in the cells, presumably due to the lack of an enzymatic retinal synthesis system in *E. sibiricum*. The possible functions of ESR in *E. sibiricum* cells are discussed in connection with the characteristics of the extreme habitat of the bacterium. The results of this study contribute to expanding the understanding of the molecular mechanisms of microbial adaptation to environmental conditions and the potential role of microbial rhodopsins in these processes.

DOI: 10.1134/S0006297925600917

Keywords: *Exiguobacterium sibiricum*, permafrost deposits, microbial rhodopsins, retinal, carotenoids

INTRODUCTION

One of the primary types of energy on our planet is the energy of sunlight, which various green plants and photosynthetic microorganisms use to maintain metabolic processes. Recently, a lot of attention has been attracted by the contribution of microorganisms containing rhodopsin-like proteins to the accumulation and transformation of solar energy [1, 2]. Microbial rhodopsins are integral membrane proteins consisting of seven alpha-helical segments and containing a retinal cofactor in the all-*trans* configuration [3, 4]. Absorption of a quantum of light results in retinal molecule isomerization from the all-*trans* to the 13-*cis* state. Subsequent conformational changes in the protein are accompanied by various functional

* To whom correspondence should be addressed.

events, such as ion transport or sensory signal transmission [5]. As a result of transmembrane proton transfer, proton-motive force is generated and ATP is synthesized, which is then used by cells to meet their energy needs [6].

Coding sequences of potential retinal proteins have been found in the genomes of microorganisms inhabiting various ecological niches, including salt and fresh water bodies, ice, plant leaves, etc. [7-10]. It has been established that the presence of rhodopsins allows microbial cells to survive in unfavorable environmental conditions, particularly in the case of a lack of nutrients [11-13]. Thus, the genomes of almost half of the marine microorganism species contain genes of potential microbial rhodopsins [14, 15]. Genes of rhodopsin-like proteins have been found in many psychrotrophic and psychrophilic microorganisms [16, 17], as well as in the analysis of metagenomic DNA isolated from natural samples of cold habitats, mainly of aquatic origin [18-20]. In rare cases, the presence of rhodopsins has been described in representatives of soil microbial communities, for example, in the dry valleys of Antarctica [20].

Previously, we have obtained and studied the retinal protein of the gram-positive soil bacterium *Exiguobacterium sibiricum* (ESR), isolated from permafrost deposits of northeastern Siberia (age – 3 million years) [21]. ESR belongs to the proteorhodopsin family, carries out light-dependent proton transport [22] and has a number of structural and functional features that distinguish it from homologous proteins and make it an interesting study object [23-28]. It should be noted that the abovementioned studies were carried out using a recombinant protein, therefore, the functions and regulation of ESR expression in *E. sibiricum* cells remain unexplored.

In this work, we aimed to investigate the expression of the *esr* gene in the culture of *E. sibiricum* cells grown under various conditions and identify the factors contributing to an increased level of proteorhodopsin synthesis in cells.

MATERIALS AND METHODS

The study utilized reagents from Bio-Rad (USA), Merck (USA), Panreac (Spain); components of bacterial culture media (Difco, USA); organic solvents from Khimmed (Russia). The solutions were prepared using MilliQ deionized water. The *E. sibiricum* 255-15 strain was provided by Dr. T. A. Vishnivetskaya.

Cultivation of *E. sibiricum* cells. Cells of *E. sibiricum* strain 255-15 were stored at –70°C in 1/2 TSB medium (trypticase soy broth diluted 2-fold with distilled water) supplemented with 20% glycerol. Frozen cells were reconstituted on 1/2 TSA (trypticase soy agar) solid nutrient medium at 30°C. The grown colonies were transferred to 50 ml tubes (Corning, USA) and incubated in 1/2 TSB nutrient medium at 30°C for 16 h to OD600 ≈ 4. The resulting inoculum (10 ml) was added to 1 liter shaker flasks containing 200 ml of sterile tap water or liquid TSB medium diluted 2 and 10 times with sterile tap water. The final dilution of the medium was thus 1/2, 1/10, or 1/40 TSB. Cultivation was carried out under an illumination intensity of 30 μmol photons $m^{-2}\cdot sec^{-1}$ (natural light and fluorescent lamps) under forced aeration (200 rpm) on an Innova shaker (Brunswick, USA) at 10 or 25°C. For incubation in the dark, the flasks were covered with foil.

Membrane fraction isolation. The cells were centrifuged at 7000*g* for 10 min at 5°C, after which the pellet was resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 20% sucrose and lysozyme (0.4 mg/ml), incubated for 1 h and disrupted by ultrasonication. The suspension was centrifuged for 30 min (6000*g*, 5°C). The resulting supernatant was centrifuged for 1 h (100,000*g*, 5°C) and the membrane fraction pellet was resuspended in 50 mM Tris-HCl (pH 8.0).

To remove cytoplasmic protein impurities, 300 μl of a cooled 100 mM Na_2CO_3 solution was added to 100 μl of the suspension, incubated for 1 h with stirring in an ice bath, after which centrifugation at 100,000*g* and 5°C was repeated. The pellet was resuspended in 50 mM Tris-HCl (pH 8.0) and used for subsequent proteomic analysis.

Membrane fraction spectroscopy was performed after solubilization of the fraction by adding a solution of n-dodecyl-β-D-maltopyranoside (Anatrace, USA) to 0.5% and NaCl to 100 mM, incubation at room temperature for 3 h and centrifugation (20,000*g*, 10 min). The absorption spectrum of the supernatant was obtained using a Hitachi-557 spectrophotometer (Hitachi, Japan). The Origin 8.1 program was used for multiexponential approximation of the curves.

Changes in absorption per single flash [532 nm; 7 ns duration, 10 mJ pulse energy; YAG-Nd LS-2131M laser (LOTIS TII, Belarus)] were recorded using a flash photolysis setup with double monochromatization of the measuring light. Using an Octopus CompuScope 8327 analog-to-digital converter (GaGe, Canada), 100 single signals were accumulated.

Total RNA isolation. Total RNA was obtained using the ExtractRNA reagent (Evrogen, Russia). *E. sibiricum* cells in an amount corresponding to 1 OD unit at 600 nm were sedimented by centrifugation in a tabletop centrifuge for 2 min at 10,000*g* and suspended in 1 ml of the reagent with the addition of 200 μl of a mixture of 150-300 μm and 400-600 μm glass beads (Sigma-Aldrich, USA). The suspension was shaken in a Mini Bead Mill (VWR, USA) at the maximum speed for 1 min, then heated for 10 min at 70°C.

This treatment was repeated 3 times, ensuring effective cell destruction and maximum RNA yield.

The subsequent steps were carried out according to the protocol of the ExtractRNA manufacturer. The resulting lysate was centrifuged for 10 min at 15,000*g* at room temperature, 0.2 ml of chloroform was added to the supernatant, after which the mixture was incubated for 5 min at room temperature with periodic shaking. After 15 min centrifugation (12,000*g*, 4°C), the upper aqueous phase was collected and 0.5 ml of 100% isopropanol was added to it, the mixture was incubated for 10 min at room temperature, then centrifuged for 10 min at 12,000*g*. The sediment was washed with 1 ml of 75% ethanol and centrifuged at room temperature (20,000*g* for 5 min), then air-dried and dissolved in 30 µl of RNase-free water.

To remove genomic DNA, the samples were treated with DNase I (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations, then the preparation was purified using the CleanRNA Standard kit (Evrogen). If DNA impurities were detected in the total RNA preparation, additional purification was performed using the TURBO DNA-free Kit (Thermo Fisher Scientific). The concentration of the obtained RNA was determined using Qubit 2.0 (Thermo Fisher Scientific).

cDNA synthesis and qPCR. RevertAid reverse transcriptase (Thermo Fisher Scientific) was used for cDNA synthesis. The reaction mixture (20 µl) included 4 µl of 5× buffer, 1 µl of random hexamer primer mix (Invitrogen, USA), 0.5 µl of RiboLock RNase inhibitor (Thermo Fisher Scientific), 2 µl of dNTP mix, 1 µl of enzyme, and 1 µg of isolated RNA. The sample was incubated for 10 min at room temperature and then for 60 min at 42°C. The reaction was stopped by heating for 10 min at 70°C. The concentration of the obtained cDNA was determined by using Qubit 2.0.

Quantitative PCR (qPCR) was performed using a LightCycler 96 PCR analyzer (Roche, Switzerland). The relative quantitative analysis mode was selected for measurements; the *esr* gene fragment was amplified in the presence of ESR_F1 and ESR_R1 primers. Fragments of the genes of DNA gyrase, *gyrA*, and of the β-subunit of RNA polymerase, *rpoB*, were amplified as references using primers gA2F and gA2R, rB1F and rB1R, respectively. The primers (synthesized by Evrogen) were selected using the Primer3Plus program. The sequences of all the primers used in this study are given in Table 1.

The stability of reference gene expression was confirmed using the geNorm test. The reaction mixture (20 µl) contained 4 µl of the ready-made qPCRmix-HS SYBR mixture (Evrogen), 8 pM of each primer, and 100 ng of cDNA. The reaction conditions were as follows: denaturation at 95°C for 5 min; 45 cycles (95°C for 20 sec; 60°C for 20 sec; 72°C for 20 sec); melting at 95°C for 10 sec, then at 65°C for 60 sec. All measurements were performed in three independent replicates with a negative control (template-free mixture). The absence of genomic DNA impurities in RNA samples was checked by adding the appropriate amount of the RNA preparation that had not been reverse transcribed to the reaction mixture instead of cDNA. The data were processed using LightCycler Software 1.1.

Table 1. The sequences of the primers used in the study

Primer name	5′→3′ primer sequence
ESR_F1	AATCGACGGTTTTCCAACAG
ESR_R1	TAGATCCAGGCGAAACATCC
gA2F	GACGATGATTCCGGTCAACT
gA2R	ATTGATTCGGGCATAAGCAG
rB1F	GACGTTTCACCGAAACAGGT
rB1R	AGGATTTCACGTGCCGTTAC

Protein hydrolysis with trypsin in solution. An aliquot of the suspension containing 20 µg of protein was dried thoroughly in a SpeedVac centrifugal vacuum concentrator (Savant, France) and dissolved in 20 µl of a buffer solution containing 100 mM Tris-HCl (pH 8.5), 1% sodium deoxycholate, 10 mM TCEP (Tris(2-carboxyethyl)phosphine) and 20 mM 2-CAA (2-chloroacetamide); heated for 10 min at 85°C and cooled to room temperature. Trypsin (0.4 µg) in 10 µl of 100 mM Tris-HCl (pH 8.5) was added to the protein solution and the mixture was incubated at 37°C overnight. After incubation, an equal volume of 2% TFA was added to the reaction mixture and the peptides were desalted on a laboratory-made SDB-RPS StageTip microcolumn constructed as described by Rappsilber et al. [29]. The peptide solution was applied to the microcolumn by centrifugation at 300*g*, washed twice with a solvent mixture (50 µl of 1% TFA, 50 µl ethyl acetate), then with 50 µl of 0.2% TFA and eluted with 60 µl of a solution containing 5% ammonium hydroxide and 60% acetonitrile in water. The eluate was dried and stored at −80°C. Before analysis, the peptides were dissolved in 40 µl of a solution containing 0.1% TFA and 2% acetonitrile in water.

Chromatography-mass spectrometry analysis. The samples were loaded onto a laboratory-made precolumn (50 × 0.1 mm) packed with Reprosil-Pur 200 C18-AQ 5 µm sorbent (Dr. Maisch, Germany) in a solution containing 2% acetonitrile, 98% H_2O, 0.1% TFA at a flow rate of 4.2 µl/min and separated at room temperature on a fused silica column (300 × 0.1 mm) with an emitter manufactured on a P2000 Laser Puller

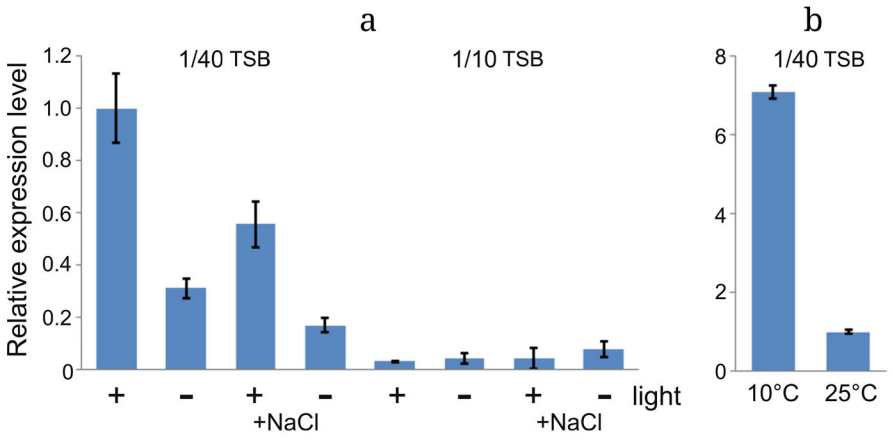

Fig. 1. The effects of medium composition, illumination (a) and temperature (b) on the *esr* gene expression level; +NaCl – with the addition of NaCl up to 30 g/liter. Normalization was performed for the expression level under illumination during growth on 1/40 TSB at 25°C.

(Sutter, USA) and packed with Reprosil-Pur 200 C18-AQ 1.9 μm sorbent (Dr. Maisch) in the laboratory. Reversed-phase chromatography was performed on an Ultimate 3000 Nano LC System chromatograph (Thermo Fisher Scientific) coupled to an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) via a nanoelectrospray source (Thermo Fisher Scientific). For chromatographic separation of peptides, solvent systems A (99.9% water; 0.1% formic acid) and B (19.9% water; 0.1% formic acid; 80% acetonitrile) were used. The peptides were eluted from the column using a linear gradient: A → 3% B for 3 min; 3 → 6% B for 2 min; 6 → 32% B for 100 min; 32 → 50% B for 18 min; 50 → 99% B for 0.1 min; 99% B for 3 min; 99 → 3% B for 0.1 min at a flow rate of 500 nl/min.

Analysis of mass spectrometric data was carried out by using the MaxQuant 2.4.2.0 [30] and Perseus 2.0.10.0 [31] programs. The Uniprot protein sequence database (version date 06.2023) was used to correlate tandem mass spectra with amino acid protein sequences.

RESULTS

The effect of cultivation conditions on *esr* gene expression. To determine the optimal cultivation mode providing the maximum level of ESR proteorhodopsin gene expression in *E. sibiricum* cells, the bacterial culture was grown while varying the following parameters: 1) medium composition (TSB nutrient medium diluted 2, 10, and 40 times with sterile tap water); 2) presence/absence of light; 3) presence/absence of NaCl; 4) temperature (10 and 25°C). It should be noted that *E. sibiricum* cells turned out to be resistant to standard treatments used in RNA isolation, which required the development of a special technique for their destruction. The *esr* gene expression level was determined by qPCR; normalization was performed according to the expression level of the reference genes *gyrA* and *rpoB*.

The experiments demonstrated that the *esr* gene expression level was 4 times higher when grown on 1/10 TSB than on 1/2 TSB (data not shown). Cultivation of *E. sibiricum* cells on 1/40 TSB showed that limiting nutrient resources further increases the content of the corresponding mRNA. Thus, the *esr* gene expression level in the culture grown on 1/40 TSB under natural light was 30 times higher than in the culture grown on 1/10 TSB, but when grown in the dark, it decreased by about 3 times (Fig. 1a). Addition of NaCl to the nutrient medium at a concentration of 30 g/liter resulted in a decrease in the expression level by 1.8 times, regardless of the presence or absence of a light source.

It is known that *E. sibiricum* is a psychrotrophic organism with the ability to grow at low ambient temperatures [21, 32]. In the course of this study, it was established that *E. sibiricum* cells retain their viability during cultivation on various media for a week at 10°C (Fig. 2). We studied the expression of the *esr* gene

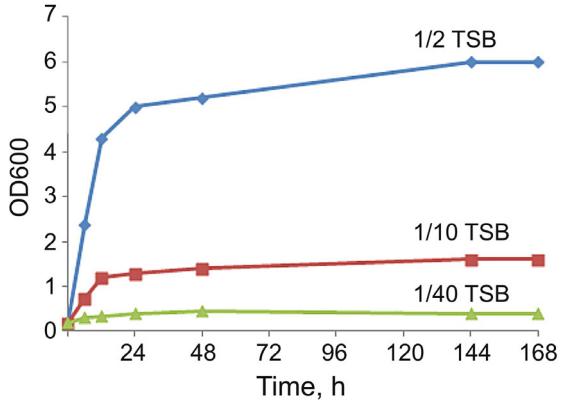

Fig. 2. Growth dynamics of *E. sibiricum* on various media at 10°C.

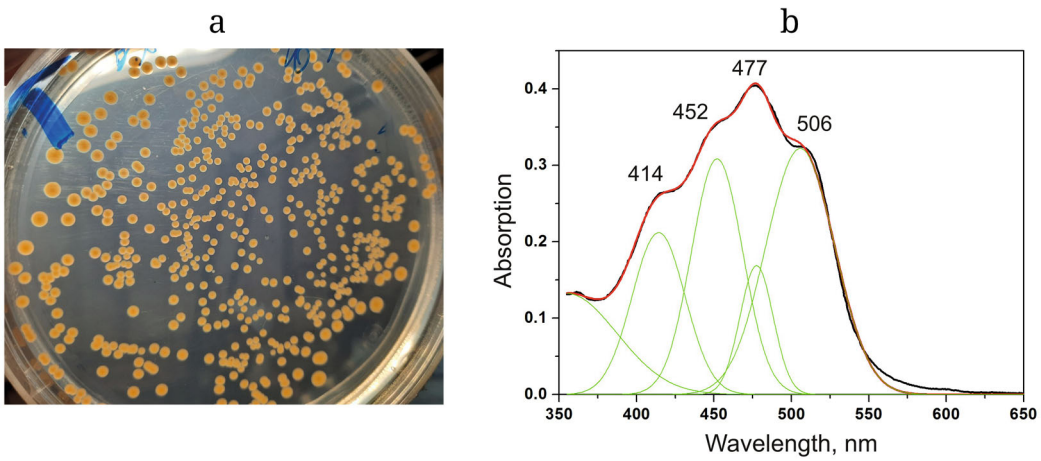

Fig. 3. Carotenoid synthesis in *E. sibiricum* cells. Colonies grown on 1/2 TSA plates (a) and the absorption spectrum of the membrane fraction of cells cultured at 10°C (b). The black line is the experimental curve; the red line is the result of approximation using Gaussian functions; the corresponding curves are shown in green. The calculated positions of the absorption maxima are indicated.

in cells cultured at 10°C for seven days on 1/40 TSB under illumination. Determination of the amount of mRNA by qPCR showed that the expression level under these conditions increases significantly. After a week of cultivation at 10°C it is 7 times higher than the expression level achieved at room temperature (Fig. 1b).

Spectroscopic analysis of the membrane fraction of *E. sibiricum* cells. *E. sibiricum* cells exhibit a yellow-orange color during cultivation in liquid and solid media [21] (Fig. 3a). We isolated the membrane fraction from cells grown for a week at 10°C and analyzed it using spectroscopic methods. The absorption spectrum of the membrane fraction solubilized in the presence of n-dodecyl-β-D-maltopyranoside detergent contains characteristic peaks indicating the synthesis of carotenoids (Fig. 3b). Approximation of the curves showed the presence of absorption maxima at 414, 452, 477, and 506 nm. Previously, the presence of carotenoids, including lycopene, β-carotene and zeaxanthin was demonstrated in extracts of *Exiguobacterium acetylicum*, *Exiguobacterium auranticum*, *Exiguobacterium profundum* [33, 34] and other representatives of this genus. It should be noted that the maximum corresponding to proteorhodopsin of *E. sibiricum* (532 nm) was not detected in the absorption spectrum. Also, no light-induced changes in absorption (photocycle) were detected in the studied sample at the wavelengths characteristic of ESR mediated by the functional activity of the retinal protein (data not shown).

Proteomic analysis of the membrane fraction of *E. sibiricum* cells. To confirm the obtained data, we performed a comparative analysis of the membrane fractions of cells grown at 25 and 10°C using label-free quantitative chromatography-mass spectrometry (high performance liquid chromatography with tandem mass-spectroscopy, HPLC-MS/MS). A total of 1604 polypeptides were identified, including enzymes, transporters, components of the secretion system, sensory and other proteins. In particular, the presence of peptides corresponding to *E. sibiricum* rhodopsin was detected, and the ESR content in the sample obtained as a result of cultivation at 10°C was 1.57 times higher than its content in the sample from cells grown at room temperature. Also noteworthy is the increased level of synthesis of phytoene desaturases, which are involved in the biosynthesis of carotenoids (Exig_0517, Exig_0735, Exig_0736, and Exig_0738). The content of these enzymes turned out to be 1.54, 1.81, 2.47, and 2.93 times higher, respectively, in samples of membrane fractions of cells grown at a lower temperature.

DISCUSSION

Permafrost deposits are characterized by a set of conditions that hinder survival of living organisms (decreased temperature, low nutrient concentration, low water activity) [35, 36]. A number of studies have shown that growth of microorganisms (*Pelagibacter ubique*, *Vibrio* sp., etc.) on minimal or highly diluted media promotes an increase in the expression of rhodopsin genes [12, 37, 38]. Similar results were obtained in our study as well. Growing *E. sibiricum* cultures under resource-limited conditions (on 1/40 TSB) led to a 30-fold increase in *esr* gene expression, with illumination promoting a higher expression level, as was shown, for example, for *Dokdonia* sp. MED134 [38].

The optimum temperature for *E. sibiricum* is 36°C, however, this bacterium is capable of growing in the temperature range from −2.5 to +40°C [21]. It has been established that at low temperatures, the bacterial cells

Fig. 4. Fragments of the *E. sibiricum* genomic sequence containing the *esr* (Exig_1419; a) and phytoene desaturase (Exig_0735–Exig_0738; b) genes. The data were obtained from the KEGG website, https://www.kegg.jp/genome/T00687. The numbers indicate the locations of genes on the *E. sibiricum* chromosome.

undergo a significant changes of their gene expression profile, with many of their genes encoding proteins synthesized only under certain conditions [32]. In this study, we found that a decrease in temperature to 10°C promotes a significant (7-fold) increase in the *esr* gene expression. Oligotrophic environment and decreased temperature correspond to the conditions observed in the seasonally thawed permafrost layer in summer, and access to solar energy on the soil surface is mainly preserved during this season due to poor vegetation cover. Previously, similar regulation of synthesis was described for xanthorhodopsin in *Sphingomonas glaciales* AAP5 from an alpine lake, which begins to accumulate in cells only at temperatures below 16°C [39].

ESR-specific peptides were detected in the membrane fraction of cells using HPLC-MS/MS analysis. The protein content was significantly higher in the sample obtained from the culture grown at a lower temperature. The lower degree of increase in protein content compared to the degree of increase in mRNA content (1.57 and 7 times, respectively) may indicate differences in the regulation of ESR expression at the level of transcription and translation.

Despite the obtained data demonstrating the presence of ESR in *E. sibiricum* cells, the absence of a maximum corresponding to the retinal protein in the absorption spectrum of the membrane fraction as well as light-induced absorption (photocycle) changes suggests that under the studied conditions the cells do not synthesize retinal, which is necessary for the formation of functional rhodopsin. The absence of retinal in cells and an increased level of proteorhodopsin gene synthesis with a low level of *blh* gene expression were previously described for a number of *Flavobacterium* strains isolated from glaciers [40].

The coding sequences of rhodopsin-like proteins are often included in operons that contain genes of enzymes of the β-carotene and retinal biosynthesis pathway [41]. Thus, the coding sequence of proteorhodopsin (PR) is located before a cluster of six genes (*idi*, *crtEIBY* and *blh*) responsible for the synthesis of such enzymes [6]. In the *E. sibiricum* genome, the proteorhodopsin gene is not a part of such a cluster (Fig. 4a). At a significant distance from the *esr* gene are the genes of phytoene synthases and phytoene desaturases (Fig. 4b), which could potentially carry out the synthesis of carotenoids [32, 41]. However, the gene for β-carotene-15,15′-dioxygenase (*blh*), which produces all-*trans*-retinal from β-carotene, is not detected. It should be noted that for approximately 28% of *E. sibiricum* proteins, no possible functions could be predicted due to a lack of homologous sequences in the databases [32].

Thus, at present, no bioinformatic or experimental evidence has been obtained proving that *E. sibiricum* is capable of synthesizing retinal as a cofactor for proteorhodopsin. The ESR molecule contains a lysine residue, necessary for the formation of a Schiff base, in a position corresponding to that of homologous proteins. Heterologous expression of the *esr* gene in *Escherichia coli* cells upon the addition of retinal is accompanied by its binding with the formation of a functional holo-form of the protein [22]. It can be assumed that retinal synthesis by *E. sibiricum* cells occurs under conditions that could not be reproduced in our experiment and with the participation of enzymes that have no homologues among the known proteins. There is also a hypothetical possibility of using bacterial retinal from the environment, as is probably the case with the rhodopsins of Saccharibacteria [42] or *Rhodoluna lacicola* [43]. However, given the low concentration of microbial cells in permafrost sediments, this option seems unlikely.

An alternative explanation may be the presence of additional functions of ESR that do not depend on the presence of retinal and facilitate cell survival under extreme environmental conditions. For example, the presence of scramblase activity, which improves biophysical properties of a cell membrane (its fluidity and membrane potential), was established for proteorhodopsin of the psychrophilic bacterium *Psychroflexus torquis* [44]. *Escherichia coli* cells that express this protein demonstrate increased resistance to stress even in the absence of light and without the addition of retinal. Increased mobility of membrane lipids could contribute to maintaining the viability of *E. sibiricum* cells at low temperatures. It is known that increased synthesis of desaturases is one of the adaptation mechanisms of psychrophilic microorganisms [45, 46].

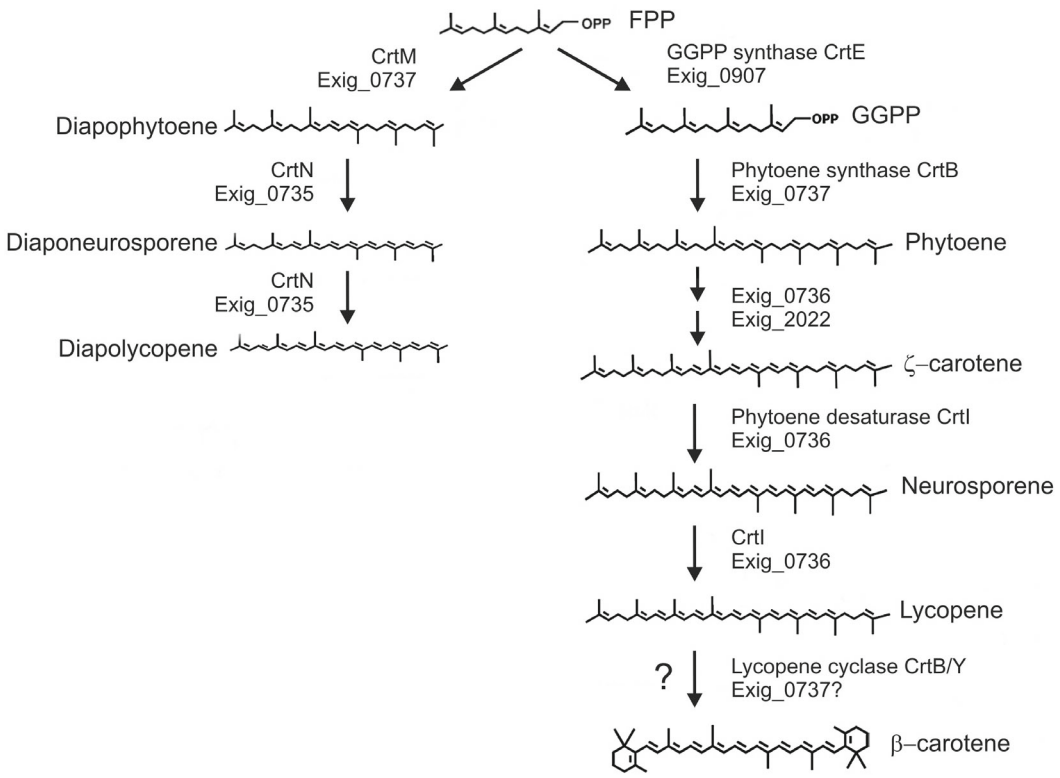

Fig. 5. A proposed scheme of carotenoid biosynthesis pathways in *E. sibiricum* cells according to KEGG data, https://www.kegg.jp/pathway/esi00906. FPP – farnesyl pyrophosphate; GGPP – geranylgeranyl pyrophosphate. The enzymes whose genes have been identified in the *E. sibiricum* genome are shown. The lycopene cyclase gene *crtY* was not found in the genome; the putative enzyme catalyzing this stage is indicated.

To confirm this hypothesis, further analysis of the presence of scramblase activity in ESR is proposed.

Along with the increased expression of the *esr* gene in bacterial cells at 10°C, we also found an increase in the content of some enzymes involved in carotenoid synthesis at this temperature. At present, it has not been experimentally established which carotenoids are contained in *E. sibiricum* cells; however, based on the presence of coding sequences for the corresponding enzymes in the genome, it can be positively stated that lycopene and/or its derivatives are synthesized (Fig. 5). The enzyme that synthesizes β-carotene (CrtY lycopene cyclase) has not been identified in the genome of *E. sibiricum* and other representatives of the *Exiguobacterium* genus; however, it is known that many of them contain this carotenoid [33, 34]. It can be assumed that the function of CrtY in *Exiguobacterium* cells is performed by phytoene synthase Exig_0737 or some other enzyme of this metabolic pathway. Indeed, enzymes with both activities have been found in some microorganisms [47]. Carotenoids are of particular importance for the survival of cold-adapted bacterial species, as they possess antioxidant and photoprotective properties, and also have a cryoprotective effect on membranes, protecting them during repeated freeze-thaw cycles [48-52]. Increased carotenoid synthesis at low temperatures has been noted in many species of psychrophilic and psychrotolerant bacteria, including *Staphylococcus xylosus* [51], *Sphingobacterium antarcticus* [52], etc. [49].

CONCLUSION

In this work, the expression of the proteorhodopsin gene of the soil bacterium *E. sibiricum* under various conditions was studied for the first time. It was found that cultivation on a poor medium at a low temperature promotes a significant increase in the expression level of ESR and the enzymes of the carotenoid biosynthesis pathways, which may reflect the corresponding changes in cell phenotypes under seasonal thawing of permafrost deposits. However, we failed to detect the presence of a functional retinal-containing protein in the cells, presumably due to the absence of an enzymatic system for retinal synthesis in *E. sibiricum*. The results of the study facilitate the development of ideas concerning the molecular mechanisms of microorganism adaptation to extreme environmental conditions and the possible role of microbial rhodopsins in these processes.

Abbreviations. ESR, *Exiguobacterium sibiricum* proteorhodopsin; TSB, trypticase soy broth.

Contributions. L. E. Petrovskaya, E. M. Rivkina, D. A. Dolgikh, and M. P. Kirpichnikov – concept and supervision of the work; E. V. Spirina, E. A. Kryukova, A. Yu. Sukhanov, E. P. Lukashev, R. H. Ziganshin, and L. E. Petrovskaya – experiments; L. E. Petrovskaya, E. P. Lukashev, R. H. Ziganshin, E. M. Rivkina, and D. A. Dolgikh – discussion of the results of the work; L. E. Petrovskaya, A. Yu. Sukhanov, E. V. Spirina, and E. M. Rivkina – writing the text of the paper.

Funding. This work was financially supported by the Russian Science Foundation (project no. 23-14-00160; qPCR, spectroscopic studies and proteomic analysis) and by the State Assignment no. 12204500038-3 “Permafrost deposits and frozen soils: temperature regime, biota, biogeochemical processes, and prospects for ecosystem functioning under global warming” (bacterial cultures acquisition, RNA isolation).

Ethics approval and consent to participate. This work does not contain any studies involving human and animal subjects performed by any of the authors.

Conflict of interest. The authors of this work declare that they have no conflicts of interest.

REFERENCES

1. Rozenberg, A., Inoue, K., Kandori, H., and Béjà, O. (2021) Microbial rhodopsins: the last two decades, *Annu. Rev. Microbiol.*, **75**, 427-447, https://doi.org/10.1146/annurev-micro-031721-020452.
2. Pinhassi, J., DeLong, E. F., Béjà, O., González, J. M., and Pedrós-Alió, C. (2016) Marine bacterial and archaeal ion-pumping rhodopsins: genetic diversity, physiology, and ecology, *Microbiol. Mol. Biol. Rev.*, **80**, 929-954, https://doi.org/10.1128/mmbr.00003-16.
3. Govorunova, E. G., Sineshchekov, O. A., Li, H., and Spudich, J. L. (2017) Microbial rhodopsins: diversity, mechanisms, and optogenetic applications, *Annu. Rev. Biochem.*, **86**, 845-872, https://doi.org/10.1146/annurev-biochem-101910-144233.
4. Gordeliy, V., Kovalev, K., Bamberg, E., Rodriguez-Valera, F., Zinovev, E., Zabelskii, D., Alekseev, A., Rosselli, R., Gushchin, I., and Okhrimenko, I. (2022) Microbial rhodopsins, in *Rhodopsin: Methods and Protocols* (Gordeliy, V. ed) Springer US, New York, NY, pp. 1-52, https://doi.org/10.1007/978-1-0716-2329-9_1.
5. Brown, L. S. (2022) Light-driven proton transfers and proton transport by microbial rhodopsins – A biophysical perspective, *Biochim. Biophys. Acta*, **1864**, 183867, https://doi.org/10.1016/j.bbamem.2022.183867.
6. Martinez, A., Bradley, A. S., Waldbauer, J. R., Summons, R. E., and DeLong, E. F. (2007) Proteorhodopsin photosystem gene expression enables photophosphorylation in a heterologous host, *Proc. Nat. Acad. Sci. USA*, **104**, 5590-5595, https://doi.org/10.1073/pnas.0611470104.
7. Atamna-Ismaeel, N., Finkel, O. M., Glaser, F., Sharon, I., Schneider, R., Post, A. F., Spudich, J. L., von Mering, C., Vorholt, J. A., Iluz, D., Béjà, O., and Belkin, S. (2012) Microbial rhodopsins on leaf surfaces of terrestrial plants, *Environ. Microbiol.*, **14**, 140-146, https://doi.org/10.1111/j.1462-2920.2011.02554.x.
8. Béjà, O., Spudich, E. N., Spudich, J. L., Leclerc, M., and DeLong, E. F. (2001) Proteorhodopsin phototrophy in the ocean, *Nature*, **411**, 786-789, https://doi.org/10.1038/35081051.
9. Atamna-Ismaeel, N., Sabehi, G., Sharon, I., Witzel, K.-P., Labrenz, M., Jürgens, K., Barkay, T., Stomp, M., Huisman, J., and Beja, O. (2008) Widespread distribution of proteorhodopsins in freshwater and brackish ecosystems, *ISME J.*, **2**, 656-662, https://doi.org/10.1038/ismej.2008.27.
10. Koh Eileen, Y., Atamna-Ismaeel, N., Martin, A., Cowie Rebecca, O. M., Beja, O., Davy Simon, K., Maas Elizabeth, W., and Ryan Ken, G. (2010) Proteorhodopsin-bearing bacteria in antarctic sea ice, *Appl. Environ. Microbiol.*, **76**, 5918-5925, https://doi.org/10.1128/AEM.00562-10.
11. Gómez-Consarnau, L., Akram, N., Lindell, K., Pedersen, A., Neutze, R., Milton, D. L., González, J. M., and Pinhassi, J. (2010) Proteorhodopsin phototrophy promotes survival of marine bacteria during starvation, *PLOS Biol.*, **8**, e1000358, https://doi.org/10.1371/journal.pbio.1000358.
12. Steindler, L., Schwalbach, M. S., Smith, D. P., Chan, F., and Giovannoni, S. J. (2011) Energy starved candidatus *Pelagibacter ubique* substitutes light-mediated ATP production for endogenous carbon respiration, *PLoS One*, **6**, e19725, https://doi.org/10.1371/journal.pone.0019725.
13. DeLong, E. F., and Beja, O. (2010) The light-driven proton pump proteorhodopsin enhances bacterial survival during tough times, *PLoS Biol.*, **8**, e1000359, https://doi.org/10.1371/journal.pbio.1000359.
14. Gómez-Consarnau, L., Raven, J. A., Levine, N. M., Cutter, L. S., Wang, D., Seegers, B., Arístegui, J., Fuhrman, J. A., Gasol, J. M., and Sañudo-Wilhelmy, S. A. (2019) Microbial rhodopsins are major contributors to the solar energy captured in the sea, *Sci. Adv.*, **5**, eaaw8855, https://doi.org/10.1126/sciadv.aaw8855.
15. Finkel, O. M., Béjà, O., and Belkin, S. (2013) Global abundance of microbial rhodopsins, *ISME J.*, **7**, 448-451, https://doi.org/10.1038/ismej.2012.112.
16. Feng, S., Powell, S. M., Wilson, R., and Bowman, J. P. (2013) Light-stimulated growth of proteorhodopsin-bearing sea-ice psychrophile *Psychroflexus torquis* is salinity dependent, *ISME J.*, **7**, 2206-2213, https://doi.org/10.1038/ismej.2013.97.
17. Kondo, K., Ohtake, R., Nakano, S., Terashima, M., Kojima, H., Fukui, M., Demura, M., Kikukawa, T., and

Tsukamoto, T. (2024) Contribution of proteorhodopsin to light-dependent biological responses in *Hymenobacter nivis* P3T isolated from red snow in antarctica, *Biochemistry*, **63**, 2257-2265, https://doi.org/10.1021/acs.biochem.4c00286.

18. Kim, S.-H., Jung, B., Hong, S. G., and Jung, K.-H. (2020) Temperature dependency of proton pumping activity for marine microbial rhodopsin from antartic ocean, *Sci. Rep.*, **10**, 1356, https://doi.org/10.1038/s41598-020-58023-5.
19. Lamm, G. H. U., Marin, E., Alekseev, A., Schellbach, A. V., Stetsenko, A., Bourenkov, G., Borshchevskiy, V., Asido, M., Agthe, M., Engilberge, S., Rose, S. L., Caramello, N., Royant, A., Schneider, T. R., Bateman, A., Mager, T., Moser, T., Wachtveitl, J., Guskov, A., and Kovalev, K. (2024) CryoRhodopsins: a comprehensive characterization of a group of microbial rhodopsins from cold environments, *bioRxiv*, https://doi.org/10.1101/2024.01.15.575777.
20. Guerrero, L. D., Vikram, S., Makhalanyane, T. P., and Cowan, D. A. (2017) Evidence of microbial rhodopsins in Antarctic Dry Valley edaphic systems, *Environ. Microbiol.*, **19**, 3755-3767, https://doi.org/10.1111/1462-2920.13877.
21. Rodrigues, D. F., Goris, J., Vishnivetskaya, T., Gilichinsky, D., Thomashow, M. F., and Tiedje, J. M. (2006) Characterization of *Exiguobacterium* isolates from the Siberian permafrost. Description of *Exiguobacterium sibiricum* sp. nov, *Extremophiles*, **10**, 285-294, https://doi.org/10.1007/s00792-005-0497-5.
22. Petrovskaya, L. E., Lukashev, E. P., Chupin, V. V., Sychev, S. V., Lyukmanova, E. N., Kryukova, E. A., Ziganshin, R. H., Spirina, E. V., Rivkina, E. M., Khatypov, R. A., Erokhina, L. G., Gilichinsky, D. A., Shuvalov, V. A., and Kirpichnikov, M. P. (2010) Predicted bacteriorhodopsin from *Exiguobacterium sibiricum* is a functional proton pump, *FEBS Lett.*, **584**, 4193-4196, https://doi.org/10.1016/j.febslet.2010.09.005.
23. Balashov, S. P., Petrovskaya, L. E., Lukashev, E. P., Imasheva, E. S., Dioumaev, A. K., Wang, J. M., Sychev, S. V., Dolgikh, D. A., Rubin, A. B., Kirpichnikov, M. P., and Lanyi, J. K. (2012) Aspartate-histidine interaction in the retinal Schiff base counterion of the light-driven proton pump of *Exiguobacterium sibiricum*, *Biochemistry*, **51**, 5748-5762, https://doi.org/10.1021/bi300409m.
24. Balashov, S. P., Petrovskaya, L. E., Imasheva, E. S., Lukashev, E. P., Dioumaev, A. K., Wang, J. M., Sychev, S. V., Dolgikh, D. A., Rubin, A. B., Kirpichnikov, M. P., and Lanyi, J. K. (2013) Breaking the carboxyl rule: lysine 96 facilitates reprotonation of the Schiff base in the photocycle of a retinal protein from *Exiguobacterium sibiricum*, *J. Biol. Chem.*, **288**, 21254-21265, https://doi.org/10.1074/jbc.M113.465138.
25. Petrovskaya, L., Balashov, S., Lukashev, E., Imasheva, E., Gushchin, I. Y., Dioumaev, A., Rubin, A., Dolgikh, D., Gordeliy, V., Lanyi, J., and Kirpichnikov, M. (2015) ESR – a retinal protein with unusual properties from *Exiguobacterium sibiricum*, *Biochemistry (Moscow)*, **80**, 688-700, https://doi.org/10.1134/S000629791506005X.
26. Siletsky, S. A., Mamedov, M. D., Lukashev, E. P., Balashov, S. P., Dolgikh, D. A., Rubin, A. B., Kirpichnikov, M. P., and Petrovskaya, L. E. (2016) Electrogenic steps of light-driven proton transport in ESR, a retinal protein from *Exiguobacterium sibiricum*, *Biochim. Biophys. Acta*, **1857**, 1741-1750, https://doi.org/10.1016/j.bbabio.2016.08.004.
27. Petrovskaya, L. E., Siletsky, S. A., Mamedov, M. D., Lukashev, E. P., Balashov, S. P., Dolgikh, D. A., and Kirpichnikov, M. P. (2023) Features of the mechanism of proton transport in ESR, retinal protein from *Exiguobacterium sibiricum*, *Biochemistry (Moscow)*, **88**, 1544-1554, https://doi.org/10.1134/s0006297923100103.
28. Gushchin, I., Chervakov, P., Kuzmichev, P., Popov, A. N., Round, E., Borshchevskiy, V., Ishchenko, A., Petrovskaya, L., Chupin, V., Dolgikh, D. A., Arseniev, A. S., Kirpichnikov, M., and Gordeliy, V. (2013) Structural insights into the proton pumping by unusual proteorhodopsin from nonmarine bacteria, *Proc. Nat. Acad. Sci. USA*, **110**, 12631-12636, https://doi.org/10.1073/pnas.1221629110.
29. Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips, *Nat. Protocols*, **2**, 1896-1906, https://doi.org/10.1038/nprot.2007.261.
30. Tyanova, S., Temu, T., and Cox, J. (2016) The MaxQuant computational platform for mass spectrometry-based shotgun proteomics, *Nat. Protocols*, **11**, 2301-2319, https://doi.org/10.1038/nprot.2016.136.
31. Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data, *Nat. Meth.*, **13**, 731-740, https://doi.org/10.1038/nmeth.3901.
32. Rodrigues, D. F., Ivanova, N., He, Z., Huebner, M., Zhou, J., and Tiedje, J. M. (2008) Architecture of thermal adaptation in an *Exiguobacterium sibiricum* strain isolated from 3 million year old permafrost: a genome and transcriptome approach, *BMC Genomics*, **9**, 547, https://doi.org/10.1186/1471-2164-9-547.
33. Jinendiran, S., Dileep Kumar, B. S., Dahms, H.-U., Arulanandam, C. D., and Sivakumar, N. (2019) Optimization of submerged fermentation process for improved production of β-carotene by *Exiguobacterium acetylicum* S01, *Heliyon*, **5**, e01730, https://doi.org/10.1016/j.heliyon.2019.e01730.
34. Patel, V. K., Srivastava, R., Sharma, A., Srivastava, A. K., Singh, S., Srivastava, A. K., Kashyap, P. L., Chakdar, H., Pandiyan, K., Kalra, A., and Saxena, A. K. (2018)

Halotolerant *Exiguobacterium profundum* PHM11 tolerate salinity by accumulating L-proline and fine-tuning gene expression profiles of related metabolic pathways, *Front. Microbiol.*, **9**, 423, https://doi.org/10.3389/fmicb.2018.00423.

35. Gilichinsky, D. A., and Rivkina, E. M. (2011) Permafrost microbiology, in *Encyclopedia of Geobiology*, Springer, pp. 726-732, https://doi.org/10.1007/978-1-4020-9212-1_162.
36. Jansson, J. K., and Tas, N. (2014) The microbial ecology of permafrost, *Nat. Rev. Microbiol.*, **12**, 414-425, https://doi.org/10.1038/nrmicro3262.
37. Akram, N., Palovaara, J., Forsberg, J., Lindh, M. V., Milton, D. L., Luo, H., González, J. M., and Pinhassi, J. (2013) Regulation of proteorhodopsin gene expression by nutrient limitation in the marine bacterium *Vibrio* sp. AND4, *Environ. Microbiol.*, **15**, 1400-1415, https://doi.org/10.1111/1462-2920.12085.
38. Kimura, H., Young, C. R., Martinez, A., and DeLong, E. F. (2011) Light-induced transcriptional responses associated with proteorhodopsin-enhanced growth in a marine flavobacterium, *ISME J.*, **5**, 1641-1651, https://doi.org/10.1038/ismej.2011.36.
39. Kopejtka, K., Tomasch, J., Kaftan, D., Gardiner, A. T., Bína, D., Gardian, Z., Bellas, C., Dröge, A., Geffers, R., Sommaruga, R., and Koblížek, M. (2022) A bacterium from a mountain lake harvests light using both proton-pumping xanthorhodopsins and bacteriochlorophyll-based photosystems, *Proc. Nat. Acad. Sci. USA*, **119**, e2211018119, https://doi.org/10.1073/pnas.2211018119.
40. Liu, Q., Li, W., Liu, D., Li, L., Li, J., Lv, N., Liu, F., Zhu, B., Zhou, Y., Xin, Y., and Dong, X. (2021) Light stimulates anoxic and oligotrophic growth of glacial *Flavobacterium* strains that produce zeaxanthin, *ISME J.*, **15**, 1844-1857, https://doi.org/10.1038/s41396-020-00891-w.
41. Klassen, J. L. (2010) Phylogenetic and evolutionary patterns in microbial carotenoid biosynthesis are revealed by comparative genomics, *PLoS One*, **5**, e11257, https://doi.org/10.1371/journal.pone.0011257.
42. Jaffe, A. L., Konno, M., Kawasaki, Y., Kataoka, C., Béjà, O., Kandori, H., Inoue, K., and Banfield, J. F. (2022) Saccharibacteria harness light energy using type-1 rhodopsins that may rely on retinal sourced from microbial hosts, *ISME J.*, **16**, 2056-2059, https://doi.org/10.1038/s41396-022-01231-w.
43. Keffer, J. L., Hahn, M. W., and Maresca, J. A. (2015) Characterization of an unconventional rhodopsin from the freshwater actinobacterium *Rhodoluna lacicola*, *J. Bacteriol.*, **197**, 2704-2712, https://doi.org/10.1128/jb.00386-15.
44. Fang, J., Zhang, Y., Zhu, T., and Li, Y. (2023) Scramblase activity of proteorhodopsin confers physiological advantages to *Escherichia coli* in the absence of light, *iScience*, **26**, 108551, https://doi.org/10.1016/j.isci.2023.108551.
45. Raymond-Bouchard, I., and Whyte, L. G. (2017) From transcriptomes to metatranscriptomes: cold adaptation and active metabolisms of psychrophiles from cold environments, in *Psychrophiles: From Biodiversity to Biotechnology* (Margesin, R. ed), Springer International Publishing, Cham, pp. 437-457, https://doi.org/10.1007/978-3-319-57057-0_18.
46. Wu, G., Baumeister, R., and Heimbucher, T. (2023) Molecular mechanisms of lipid-based metabolic adaptation strategies in response to cold, *Cells*, **12**, 1353, https://doi.org/10.3390/cells12101353.
47. Zhao, Z., Liu, Z., and Mao, X. (2020) Biotechnological advances in lycopene β-cyclases, *J. Agric. Food Chem.*, **68**, 11895-11907, https://doi.org/10.1021/acs.jafc.0c04814.
48. Sajjad, W., Din, G., Rafiq, M., Iqbal, A., Khan, S., Zada, S., Ali, B., and Kang, S. (2020) Pigment production by cold-adapted bacteria and fungi: colorful tale of cryosphere with wide range applications, *Extremophiles*, **24**, 447-473, https://doi.org/10.1007/s00792-020-01180-2.
49. Collins, T., and Margesin, R. (2019) Psychrophilic lifestyles: mechanisms of adaptation and biotechnological tools, *Appl. Microbiol. Biotechnol.*, **103**, 2857-2871, https://doi.org/10.1007/s00253-019-09659-5.
50. Singh, A., Krishnan, K. P., Prabaharan, D., and Sinha, R. K. (2017) Lipid membrane modulation and pigmentation: A cryoprotection mechanism in Arctic pigmented bacteria, *J. Basic Microbiol.*, **57**, 770-780, https://doi.org/10.1002/jobm.201700182.
51. Seel, W., Baust, D., Sons, D., Albers, M., Etzbach, L., Fuss, J., and Lipski, A. (2020) Carotenoids are used as regulators for membrane fluidity by *Staphylococcus xylosus*, *Sci. Rep.*, **10**, 330, https://doi.org/10.1038/s41598-019-57006-5.
52. Jagannadham, M. V., Chattopadhyay, M. K., Subbalakshmi, C., Vairamani, M., Narayanan, K., Rao, C. M., and Shivaji, S. (2000) Carotenoids of an Antarctic psychrotolerant bacterium, *Sphingobacterium antarcticus*, and a mesophilic bacterium, *Sphingobacterium multivorum*, *Arch. Microbiol.*, **173**, 418-424, https://doi.org/10.1007/s002030000163.