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EXPERIMENTAL ARTICLES

Characteristics of Psychrotolerant Pseudomonads Isolated from Organogenic Clay Deposits of the Mramornaya Cave (Primorskii Krai)

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Abstract—The community of cultured pseudomonads isolated from clayey organogenic deposits of the Mramornaya Cave (Primorskii krai) was investigated. The bacterial strains isolated in this work were eurythermal and psychrotolerant. Their taxonomic position was determined by high-throughput sequencing of the 16S rRNA gene fragments. Members of the genus *Pseudomonas* are known to inhabit all the ecological niches on Earth and, accordingly, have a wide range of adaptive functions. Microscopic techniques were used to establish the changes in the character of motility and the cell size stability with changes in the cultivation temperature. The studied strains are of scientific and applied interest due to their enzymatic activity against several substrates simultaneously at different temperatures (25 and 4°C), as well as to the ability to secrete cold-active pectinase, protease, and lipase. However, phosphate-solubilizing activity both at 4 and at 25°C became preferable for the strains. The Mramornaya Cave is of karst origin and is characterized by carbonate karst, which explains the preference for calcium phosphate in the studied strains. Analysis of the obtained data showed that the collection of cultivated bacteria obtained in the present work included both typical psychrotolerant ones, which exhibited enzymatic activity at optimal growth temperature, and unique ones, capable of synthesizing a wide range of enzymes under the temperature different from their growth optimum.

Keywords: Mramornaya Cave, psychrotolerant and eurythermal bacteria of the genus *Pseudomonas*, enzymatic activity

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Cave ecosystems are highly unusual: they are characterized by unique microclimate due to the lack of light, permanently low temperatures (3–4°C), high humidity (up to 100%), and deficiency of organic matter. In order to overcome growth-limiting factors, microorganisms are forced to adapt by forming complex networks of mutualistic or competitive relationships. For this reason, cave microorganisms produce peculiar secondary metabolites, which can be potentially used in various spheres of human life (Lopes da Silva et al., 2019). It was shown using both cultural and molecular methods that *Proteobacteria* make up a substantial part of total microbial diversity in caves. At the genus level, the most abundant groups are *Pseudomonas*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Brevundimonas*, *Caulobacter*, and *Bosea*. However, their abundance and biodiversity depend on the evolutionary maturity of a given microbial community, as well as on the current inflow of foreign organic substances and allochthonous microflora (Kuzmina et al., 2012; Jaroszewicz et al., 2021; Zhu et al., 2021; Kosznik-Kwaanicka et al., 2022)

In Primorskii Krai, there are about 90 known accessible caves more than 20 m long. The most numerous are solutional (karst) caves. Their formation, as it follows from the name, is associated with karst, i.e., water-mediated dissolution and leaching of rocks. In the Khasanskii District of Primorskii Krai, there are more than 10 currently known caves of the Barabash Formation confined to the Upper Permian limestone outcrops. One of them is a little-studied Mramornaya Cave, which is of interest as a cave ecosystem not exposed to anthropogenic load (Bersenev, 1990, 2017).

Much of the recent research has been concerned with cave microbiomes, because microorganisms isolated from such habitats possess interesting properties that can be used in biotechnology, medicine, and ecology (Ghosh et al., 2017; Jaroszewicz et al., 2021). For example, *Pseudomonas frederiksbergensis* RRC23 and *Rhodococcus* sp. RRC75 isolated from the Rasp-

berry Rising Cave (Canada) exhibited antimicrobial activity against *Escherichia coli* 15-318 with multiple drug resistance (Ghosh et al., 2020). The strain *Bacillus subtilis* CV16 isolated from a cave in the eastern part of the Amazon River (Brazil) has a potential to be used in cementing materials due to its ability to precipitate $CaCO_3$ as calcite crystals (Nicole et al., 2022). *Pseudomonas* sp. IB-K 13-1A isolated from the Kinderlinskaya cave (Russia) has a high capacity of dissolving various phosphorus compounds and producing growth-stimulating compounds, auxins (Kuzmina et al., 2015).

In view of the above, the goal of this work was to study the physiological and biochemical properties of bacteria of the genus *Pseudomonas* isolated from the samples of clayey organogenic sediments of the karst cave Mramornaya (Primorskii Krai).

MATERIALS AND METHODS

Objects. The objects of the study were bacterial strains of the genus *Pseudomonas* isolated from the samples of clayey organogenic sediments of the karst cave Mramornaya (Primorskii Krai). The Mramornaya Cave (Khasanskii District, Primorskii Krai) is situated in the upper reaches of the Amba River, 30 km to the northwest from the village of Zanadvorovka (43.34456° N, 131.39599° E). It is located at the foot of a hill close to the Amba River, has a horizontal entrance and a vertical exit, and is abundantly filled with rocks such as limestone and marble. The cave is flooded due to its closeness to the river and vertical holes; this fact is confirmed by frog bone remains and a sandy layer in the soil.

Samples were collected in spring (April 27, 2021) into a sterile hermetically sealed container. Soil samples were taken with sterile instruments and placed into sterile glassware. Spot samples were taken on a test plot from one layer by the "envelope" method, so that each sample represented a part of soil. The combined sample was composed by mixing five spot samples taken on the same test plot, 200 to 250 g each, from the depth of 5–10 cm. Before the experiments, the samples were stored in a refrigerator at a temperature from 4 to 5°C.

Isolation and cultivation of bacteria were performed on the GRM agar (State Research Center for Applied Microbiology and Biotechnology, Russia). Soils were treated by the standard technique of inoculating aliquots from serial dilutions of suspension onto the medium surface. The plates with the inocula were incubated at 4°C, which is close to the climatic conditions of the cave.

Microscopy. Bacterial cell morphology was studied in Gram-stained smears with a Carl Zeiss Axioskop 40 microscope (Germany) with 40× and 100× objective lenses, using an immersion system in the phase contrast mode. Bacterial motility was observed in the "crushed drop" preparation (Netrusov et al., 2005).

Determining the temperature optimum. The optimal growth temperatures for the isolated strains were determined by culturing them at 4, 10, 15, 25, 30, and 37°C on the medium with GRM broth (State Research Center for Applied Microbiology and Biotechnology). The concentration of bacterial mass was measured by direct cell counting in a Goryaev chamber; then the changes in concentration were recorded on days 3, 6, 8 and 10 with an APEL AP-101 digital photoelectrocolorimeter (Japan). Biomass concentration was calculated by the formula for plotting growth curves:

$$C\left[\frac{\text{CFU}}{\text{mL}}\right] = \left(a\frac{4000b}{c}\right)\times 1000, \quad (1)$$

where a is the sum of cells calculated in 5 (or 10) big squares of the grid; b is the dilution of the initial substrate; and c is the number of small squares where the count was performed.

Specific growth rates of the strains were determined using the formula:

$$\mu\left[\text{h}^{-1}\right] = 2.3\left(\ln\frac{x}{x^0}\right)\Big/ t, \quad (2)$$

where μ is the specific growth rate of microorganisms (h^{-1}), x^0 and x are the initial and final concentrations of microbial cells (CFU/cm^3); and t is the time of cultivation (h) (Firsova, 2019).

Identification of enzymatic properties. The disc diffusion method was used to characterize in detail the features of bacterial metabolism, to identify extracellular enzymes (proteases, pectinases, and lipases), and to determine the ability to dissolve low-soluble phosphorus compounds. Paper disks, 5 mm in diameter, were impregnated with bacterial suspension and applied on the surface of agar media containing one of the substrates as a nutrient source. Enzymatic activity was estimated by the halo around bacterial colonies (the zone of hydrolysis for proteolytic activity, the zone of turbidity for lipolytic activity, and the zone of clarification for pectinase activity).The results were recorded in mm. The screening of enzymatic activity was performed at incubation temperatures of 4 and 25°C.

Proteolytic activity was determined on Eijkman's milk agar (g/L): $CaCO_3$, 1.0; K_2HPO_4, 0.2; peptone, 5.0; yeast extract, 5.0; agar, 30.0; skimmed milk, 5.0 (Netrusov et al., 2005).

Pectinase activity was determined on the pectin-containing growth medium (g/L): pectin, 5.0; tryptone, 5.0; yeast extract, 5.0; NaCl, 5.0; agar, 15.0 (Roy et al., 2018).

Lipase activity was determined on the medium containing (g/L): tryptone, 10.0; yeast extract, 5.0; agar, 20.0; sterile lipid homogenate (warm distilled water, 400 mL; olive oil, 100 mL; Tween 80, 1 mL) was

added separately in a volume of 30 mL per 1 L of the medium (Netrusov et al., 2005).

The test for the ability to mobilize inorganic phosphate was performed on Pikovskaya's agar (g/L): glucose, 20.0; NaCl, 0.2; $CaPO_4$, 5.0; $MgSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot 7H_2O$, traces; $FeSO_4 \cdot 7H_2O$, traces; agar, 20.0 (Kadyrova et al., 2022).

Glycolytic activity was determined on differential Hiss's media (State Research Center for Applied Microbiology and Biotechnology). Glucose, lactose, sucrose, mannitol, sorbitol, dulcitol, maltose, arabinose, inositol, xylose, rhamnose, and fructose were used as carbohydrate substrates. The results were evaluated by the ability of bacteria to utilize sugars with the production of acid, leading to a change in the medium color.

Molecular genetic identification of bacteria and phylogenetic analysis. Genomic DNA from bacterial cultures was isolated with the NK-sorbent Base kit (Litech, Russia) according to the manufacturer's instructions. The 16S rRNA gene fragment was analyzed using a BioMaster HS-*Taq*PCR-Color (2×) kit of reagents (Biolabmix, Russsia), as well as the universal bacterial primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1350R (5'-GACGGGCGGTGTGTACAAG-3'). Amplification was performed with a T100 Thermal Cycler (BioRad, United States) in the following mode: 4 min at 94°C (1 cycle); 60 s at 94°C, 60 s at 48°C, and 90 s at 72°C (5 cycles); 60 s at 92°C, 110 s at 50°C, and 90 s at 72°C (10 cycles); 60 s at 92°C, 60 s at 52°C, and 60 s at 72°C (10 cycles); 60 s at 92°C, 60 s at 54°C, and 110 s at 72°C (10 cycles); 10 min at 72°C (1 cycle). PCR products were separated by electrophoresis (at ~2 V/cm) in a 1% agarose gel with addition of ethidium bromide; the results were recorded on a transilluminator under UV light. Amplification products were purified from the remainder of the reaction mixture with an ExoSAP-IT Express kit (Thermo FS, United States).

The PCR products were sequenced according to Sanger with a Big Dye Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, United States) to prepare nucleotide sequences for reading with a Nanofor 05 genetic analyzer (Syntol, Russia). Sequencing was performed at the Laboratory of Marine Microbiology, Institute of the World Ocean, Far Eastern Federal University. Phylogenetic analysis was performed by searching for homologous sequences in the GenBank database using the BLAST software (Altschul et al., 1997) (http://www.ncbi.nlm.nih.gov/blast).The sequences were edited with BioEdit; the CLUSTAL W algorithm was used for sequence alignment (http://www.genebee.msu.su/clustal). The phylogenetic tree was constructed by the neighbor-joining method based on the Kimura two-parameters algorithm in the MEGA 11 software (Kumar et al., 2016). The branching order confidence index was determined by the bootstrap analysis of 100 alternative trees.

The work was carried out with research equipment of the Core Facility "Biotechnology and Genetic Engineering" (Federal Research Center for Biodiversity, the Far Eastern Branch of the Russian Academy of Sciences).

Statistical analysis. No less than three independent experiments were conducted in at least two replicates. Statistical data processing was performed by the analysis of variance (ANOVA) (at significance level $p \leq 0.05$); statistics of the center (the median) and statistics of the range (the quartile) were calculated. The selected values were depicted as a box plot using Statistica 13 and Microsoft Excel 2007.

RESULTS AND DISCUSSION

Bacteria of the genus *Pseudomonas* were described long ago (Palleroni et al., 1973). As a rule, they occur ubiquitously in soil (Lujan et al., 2015), in water (Majorina et al., 2022), and in the rhizosphere of some plants (Molina et al., 2020). They have also been described from solutional caves in different regions of the world: Lesu (Romania) (Bogdan et al., 2023), Kapovaya (Russia) (Galimzyanova et al., 2020), Majorca (Spain) (Busquets et al., 2021), and unexploited caves of the Kuankuoshui Nature Reserve, Zunyi, Guizhou Province (China) (Zhu et al., 2021). It is known that *Pseudomonas* bacteria have a broad range of industrial applications for antibiotic production, extraction of residual oil from wells, environmental pollution control, and as models for numerous theoretical studies; among them, there are psychrophilic forms (Shcherbakov et al., 2017; Glushakova et al., 2021; Sidorenko and Rusakova, 2022).

Phylogenetic analysis. The cultivation of clayey organogenic sediments of the karst cave Mramornaya on GRM agar resulted in isolation of 13 bacterial strains. To determine their taxonomic affiliation, the 16S rRNA gene sequences 1100–1310 bp long were obtained for each strain and deposited in the NCBI database (Table 1). Phylogenetic analysis of the sequences showed that all of them belonged to the genus *Pseudomonas* (Fig. 1).

The comparative analysis of nucleotide sequences showed that the genes of the strains MP5 and MP25 had 99.77 and 98.52% homology to the sequences of *Pseudomonas* sp. MDT1-85 and *Pseudomonas* sp. PAMC 27331, respectively. *Pseudomonas* sp. MDT1-85 was previously isolated from a glacier, and *Pseudomonas* sp. PAMC 27331 was isolated from Antarctic soil.

The closest relative of the strain MP3 is *P. frederiksbergensis* MRCER1 49 (98.70% similarity) isolated from the rhizosphere of an olive tree. The enzymatic activity of this bacterial species is well studied. It is known that *P. frederiksbergensis* is an effective bioin-

Table 1. Phylogenetic affiliation of bacteria of the genus *Pseudomonas* isolated from the samples of clayey organogenic sediments of the Mramornaya Cave based on the GenBank search for closest relatives of the obtained 16S rRNA gene sequences

Strain	NCBI number	Fragment length, bp	Homology percentage	Homolog
MP10	OR352475	1160	99.48	*Pseudomonas brassicacearum* ICMP 14356 (MK356421)
MP16	OR352477	1120	99.88	*Pseudomonas arsenicoxydans* Y24-2 (MH817850)
MP20.1	OR352478	1270	99.35	*Pseudomonas gessardii* YL-179 (OK135846)
MP11	OR352479	1190	98.73	*Pseudomonas gessardii* YL-179 (OK135846)
MP24.2	OR352480	1260	99.21	*Pseudomonas fragi* 8d-S10 (MN062067)
MP2	OR352481	1195	99.75	*Pseudomonas lini* KNUC164 (DQ424866)
MP25	OR352482	1310	98.52	*Pseudomonas* sp. PAMC 27331 (MT555369)
MP17	OR352483	1300	99.69	*Pseudomonas fluorescens* CP DB12 (MH304227)
MP1	OR352484	1100	99.64	*Pseudomonas lini* KNUC164 (DQ424866)
MP5	OR352485	1280	99.77	*Pseudomonas sp.* MDT1-85 (JX949570)
MP3	OR352486	1280	98.70	*Pseudomonas frederiksbergensis* MRC ER1 49 (OK605778)
MP24.1	OR352487	1300	99.70	*Pseudomonas fragi* 8d-S10 (MN062067)
MP4	OR352476	1300	99.61	*Pseudomonas mandelii* SY03134 (KT369882)

oculant for increasing plant resistance to cold stress (Chatterjee et al., 2017) and can grow at temperatures from 4 to 30°C, but not at 37°C (Andersen et al., 2000).

The strains MP1 and MP2 are close to *Pseudomonaslini* KNUC164 isolated from the rhizoplane of wild-growing cereals. This species is little studied; there are several works concerning its ability to dissolve phosphate compounds and to extract growth-stimulating substances (Sandhya et al., 2017). *P. lini* can grow at temperatures from 4 to 36°C, but not at 41°C (Delorme et al., 2002).

The nucleotide sequence of the 16S rRNA gene of the strain MP16 was by 99.88% homologous to the sequence of the strain *P. arsenicoxydans* Y24-2 isolated from the samples of bottom deposits from the Camarones valley in the Atacama Desert, which showed the ability to oxidize arsenite. The growth temperature range of *P. arsenicoxydans* is is 4–37°C (Campos et al., 2010).

The strain MP4 is close to *P. mandelii* SY03134 (99.61% similarity), which was isolated from soil samples of the Qilian Mountains in China. It should be noted that *P. mandelii* is characterized in literature as a psychrophile with cold-adapted glucose-6-phosphate dehydrogenases involved in the pentose phosphate pathway (DangThu et al., 2020).

The strains MP24.1 and MP24.2 were clustered into the same group, being close to *P. fragi* 8d-S10 with a similarity of 99.70 and 99.21%, respectively. Noteworthy, the strain *P. fragi* 8d-S10 is facultatively psychrophilic and can grow within a temperature range from 0 to 30°C (Bao et al., 2023).

The sequence analysis of the 16S rRNA gene of the strains MP11 and MP20.1 demonstrated their similarity with *P. gessardii* YL-179 at a level of 98.73 and 99.35%, respectively. This bacterial species is known for its thermostable proteolytic activity. A thermostable protease is also produced by bacteria of the species *P. fluorescens*. The optimal growth temperature for these species is above 20°C; however, they can grow within a temperature range of 4–42°C (Menget al., 2017). One of the strains of this species, namely *P. fluorescens* CP DB12, had 99.48% similarity to the strain MP17 isolated from organogenic sediments of the Mramornaya Cave.

The strain MP10 is the closest relative of *P. brassicacearum* ICMP 14356 (99.48% similarity) isolated from a necrotic spot on a tomato stem. Bacteria of the species *P. brassicacearum*, which possess 1-aminocyclopropane-1-carboxylate deaminase, can exhibit both pathogenic and growth-stimulating properties when interacting with tomato plants (Belimov et al., 2007). The growth range for this species is from 5 to 37°C; at the same time, some weak growth was observed at 40°C on a complex Luria–Bertani medium (Zachow et al., 2017).

Thus, representatives of the genus *Pseudomonas* are found in large amounts in all major natural environments (terrestrial, freshwater, and marine) and develop close relationships with plants and animals. This universal occurrence implies a high degree of physiological and genetic adaptivity, which may underlie the manifestation of a wide range of biochemical properties.

The detected similarity of the 16S rRNA gene sequences and the comparison with the type strains

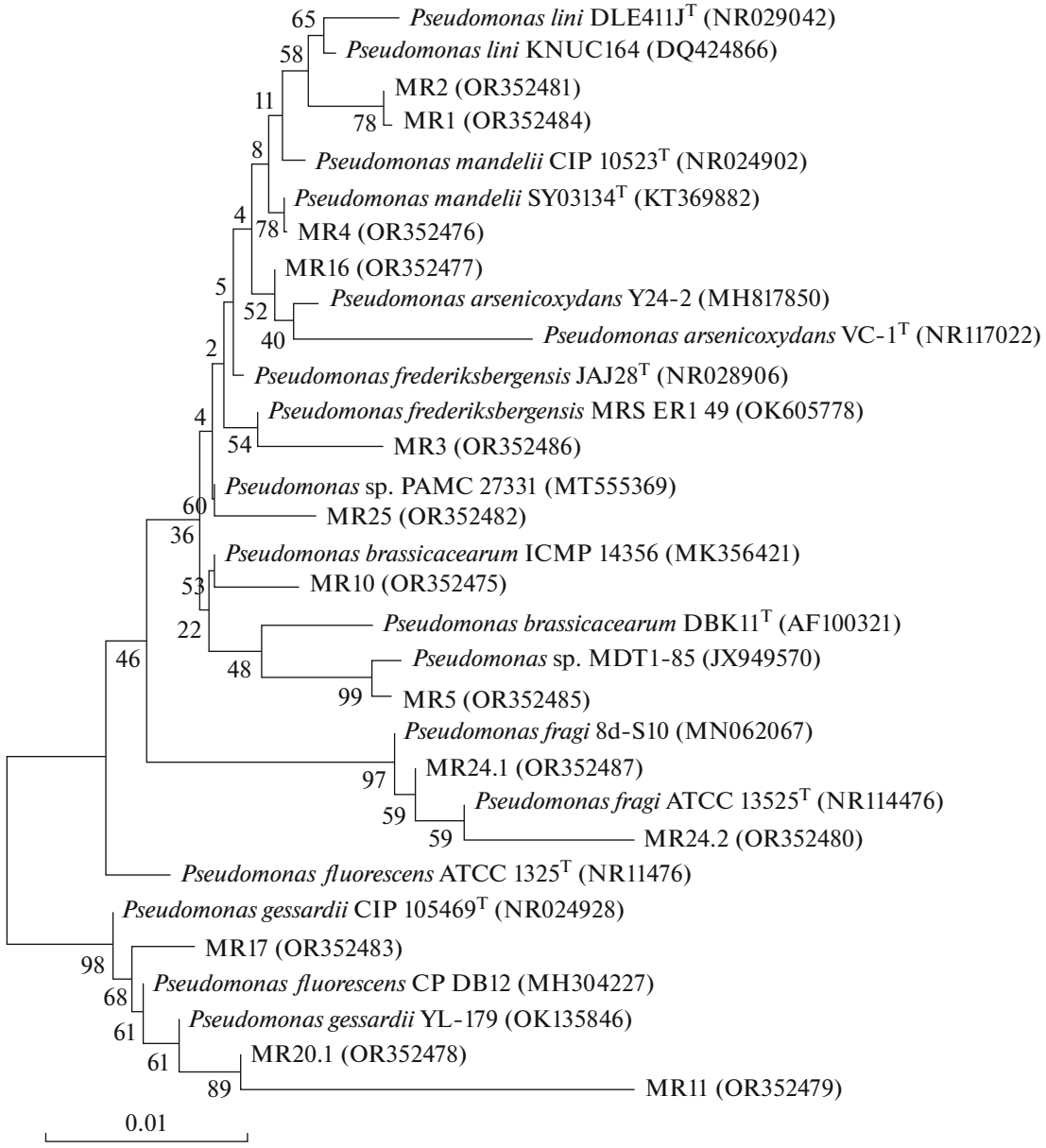

Fig. 1. Phylogenetic tree constructed based on sequence analysis of the 16S rRNA gene fragments of bacterial strains isolated from organogenic sediments of the Mramornaya Cave and showing their positions among representatives of the genus *Pseudomonas*. The dendrogram is based on the algorithm of the neighbor joining (NJ) method. The scale bar corresponds to two nucleotide substitutions per 100 b.p. Bootstrap support values above 50% are presented.

P. brassicacearum DBK11, *P. arsenicoxydans* VC-1, *P. gessardii* CIP 105-469, *P. fragi* ATCC 13525, *P. lini* DLE411J, *P. fluorescens* ATCC 1325, *P. frederiksbergensis* JAJ28 and *P. mandelii* CIP 10523 suggest that the studied bacteria belong to the genus *Pseudomonas*.

Carbon sources. Pseudomonades are known to be capable of nitrogen fixation, phosphate dissolution (Sandhya et al., 2017), and the synthesis of proteases, lipases, and other enzymes (Meng et al., 2017; Pabai et al., 1995; Ramani et al., 2010). The genus *Pseudomonas* is characterized by the following saccharolytic properties: most of the species use sucrose, arabinose, mannose and mannitol as a carbohydrate source (*Bergey's Manual...*, 1986). In the present work, the glycolytic activity against a range of carbohydrates (extended Hiss series) was studied in two cultivation modes: at 4 and 25°C. It was found that all strains under study exhibited glycolytic activity against sorbitol, sucrose, lactose, mannitol, maltose, glucose, and xylose at 25°C. At 4°C, the range of substrates utilized for oxidation was limited to sorbitol, sucrose, lactose, mannitol, maltose, and rhamnose. The absence of response was observed in all tested strains at 25°C in the presence of dulcitol and at 4°C in the presence of

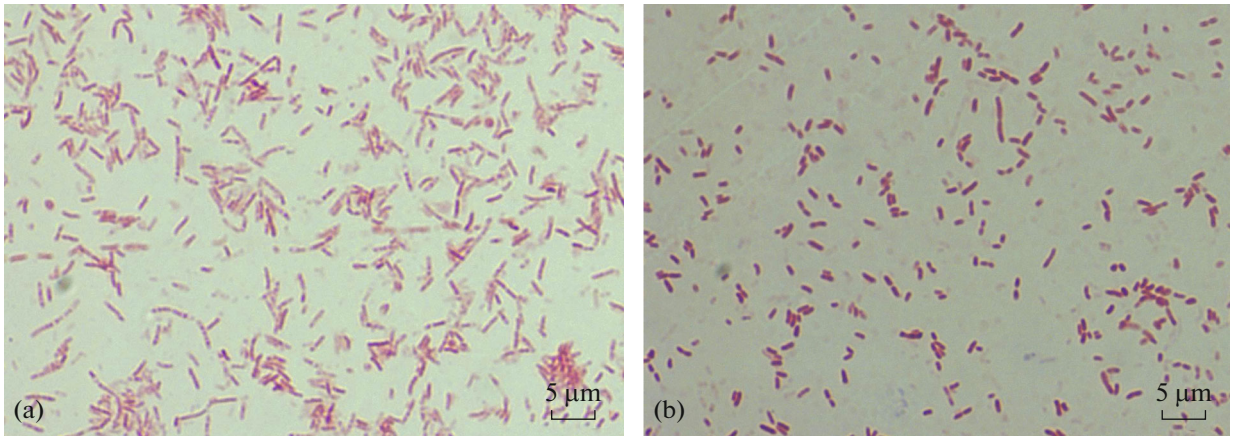

Fig. 2. Cell morphology of the strain MP4 at 25 (a) and 4°C (b); scale bar, 5 μm.

dulcitol, inositol, and fructose. In some strains, the metabolic response was temperature-dependent. At 4°C, no fermentation was observed for fructose in the strains MP24, MP25, and MP17, for glucose and xylose in the strain MP16, for xylose and inositol in the strain MP10, and for inositol in MP20.1. At 25°C, the negative reaction was observed in the strain MP17 cultivated on rhamnose-containing media. Thus, the glycolytic activity of bacteria under study was temperature-dependent; however, cultivation temperature had no effect on the enzymatic machinery responsible for glycosis in the strain MP24.2.

Morphological, cultural and biochemical properties. The colonies of the *Pseudomonas* bacteria under study ranged from transparent to beige in color and did not produce pigment when grown on GRM agar. All strains were different in their cultural, morphological and tinctorial properties (Table 2).

It is known that temperature may have an effect on bacterial cell size (Shehata and Marr, 1975). Our studies showed that cultivation temperature had no effect on the size of bacterial cells of most strains: their length, regardless of temperature, varied from 0.54 to 2.14 μm in different isolates (Fig. 2). The exceptions were two strains, MP3 and MP4, with the cell size increasing from 1.2–1.8 to 5.6 μm with a temperature change from 4 to 25°C.

The ability to migrate to a favorable medium (chemotaxis) is of great significance for the survival of bacteria living in a challenging environment with nutrient deficiency (Vorotnikov, 2011). Our studies showed a temperature-dependent change in cell motility of the strains. For example, at 4°C, the cells of the strains MP4, MP5, MP3, and MP17 exhibited directional swimming motility; furthermore, the bacteria controlled their motion by changing the direction of rotation of the basal body. At 25°C, the activity noticeably decreased and the motility became twitching. The strain MP20.1, on the contrary, exhibited swimming motility at 25°C and twitching motility at 4°C. The MP10 bacteria showed twitching motility at 4°C and rotating motility at 25°C. Based on the findings and literature data analysis (McBride, 2001; Tsyganov et al., 2021), we believe that, under cold conditions, most of the studied strains activate defense mechanisms, which allow them to move actively searching for new energy sources. The defense mechanism may involve formation of unconventional locomotive organs: changes in the synthesis of receptor proteins responsible for taxis, flagellin proteins capable of self-organizing into filaments of bacterial flagella, transformation of the number of flagella and the shape of their basal body, and other phenomena.

The growth temperature range is one of the most important characteristics of microorganisms inhabiting caves. The temperature optimum for the growth of all bacteria under study was 4–25°C. At 30 and 37°C, most strains showed no increase in the concentration of bacterial cells. The exceptions were the strains MP16 and MP2, for which the growth rates were noticeably higher at 37°C throughout the entire period of cultivation (Fig. 3b). Figure 3a shows the growth curves for the strain MP17 illustrating the growth patterns of most strains at different temperatures.

Thus, both psychrotolerant and eurythermal bacteria of the genus *Pseudomonas* were present in the Mramornaya Cave. At the same time, psychrotolerant bacteria predominated among the strains under study. This fact is confirmed by their low specific growth rates at 37°C compared to the specific growth rates at 4 and 25°C (Fig. 4a): in the major pool of the strains, the mean values of specific growth rate ranged from 0.0023 to 0.0098 h^{-1} at 37°C and from 0.0065 to 0.0125 h^{-1} at 4 and 25°C. At the same time, the minimum–maximum range of specific growth rates at 4°C was wide: from 0.0050 to 0.0150 h^{-1}, probably due to the cold-adapted properties of the strains. As a result, for an overwhelming majority of strains under study, the increase in biomass had the lowest values at 37°C, with concentrations from 0.20 to 0.75 CFU/mL

Table 2. Cultural and morphological characteristics of strains under study

Strain	Temperature optimum, °C	Colony morphology						Microscopic characteristics (4°C)				Microscopic characteristics (25°C)			
		Color	Diameter, mm	Cross section	Surface	Edge	Pigment	Gram staining (+/–)	Shape	Size, μm	Motility	Gram staining (+/–)	Shape	Size, μm	Motility
MP1	25	White	1–3	Flat	Lustrous	Even	No	–	Rods	0.73–1.55	Weakly motile	–	Rods	0.73–1.55	Weakly motile
MP2	37	Transparent beige	3–5	Flat	Lustrous	Even		–	Rods	1.11–2.14	Weakly motile	–	Rods	1.12–2.13	Weakly motile
MP4	4	Transparent gray	10–15	Flat	Lustrous	Uneven		–	Rods	1.68–1.80	Motile	–	Rods	1.84–5.60	Weakly motile
MP5	4	Beige	1–3	Flat	Lustrous	Even		–	Rods	0.74–1.53	Motile	–	Rods	0.74–1.51	Weakly motile
MP25	25	Gray	3–5	Crater-shaped	Lustrous	Even		–	Rods	0.74–1.53	Motile	–	Rods	0.72–1.51	Motile
MP16	37	White	2–4	Flat	Lustrous	Even		–	Rods	0.68–1.76	Motile	–	Rods	0.70–1.75	Motile
MP10	4	Transparent white	3–4	Flat	Lustrous	Even		–	Rods	0.62–1.42	Motile	–	Rods	0.62–1.44	Motile
MP3	25	White	10–16	Flat	Opaque	Uneven		–	Rods	1.05–1.20	Motile	–	Rods	1.35–5.60	Motile
MP24.1	25	Light beige	14–16	Flat	Opaque	Uneven		–	Rods	0.54–0.78	Motile	–	Rods	0.54–0.76	Motile
MP24.2	25	Light beige	11–15	Flat	Opaque	Even		–	Rods	0.56–0.80	Motile	–	Rods	0.30–0.81	Motile
MP17	4	Gray	2–5	Convex	Lustrous	Even		–	Rods	0.68–1.76	Motile	–	Rods	0.66–1.77	Weakly motile
MP11	25	Transparent white	5–8	Convex	Lustrous	Even		–	Rods	0.64–1.43	Motile	–	Rods	0.66–1.44	Motile
MP20.1	25	White	10–13	Flat	Opaque	Even		–	Rods	0.58–0.86	Weakly motile	–	Rods	0.59–0.85	Motile

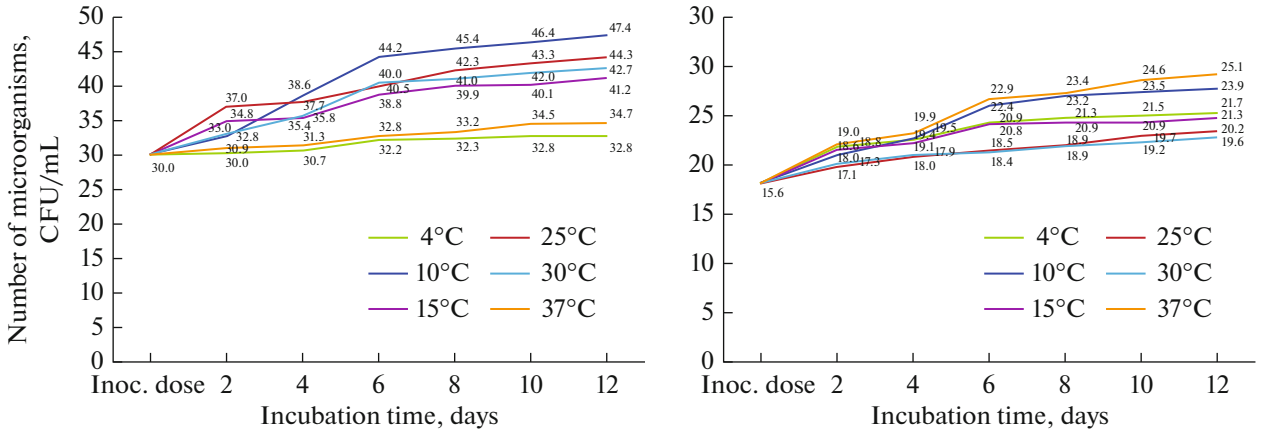

Fig. 3. Growth curves of the psychrotrophic culture of *Pseudomonas* sp. MP17 (a) and the eurythermic culture *Pseudomonas* sp. MP16 (b) at the tested temperatures.

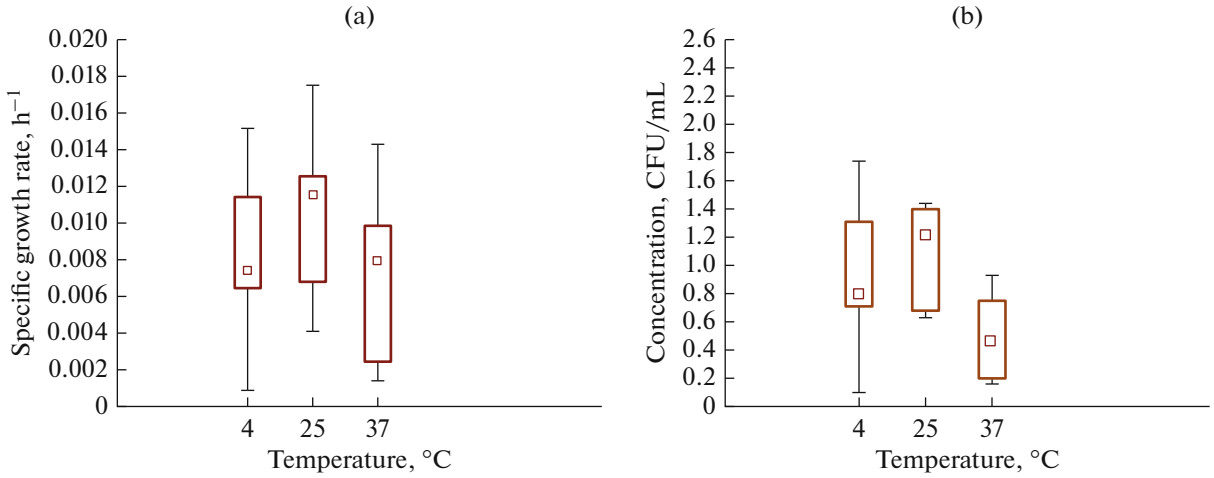

Fig. 4. Range of values of specific growth rate (a) and increase in cell biomass (b) for all strains under study at different temperatures.

(Fig. 4b). At 4 and 25°C, the range of values for the bulk of the studied bacteria was from 0.70 to 1.40 CFU/mL. Comparable data were obtained for the karst caves of Central Siberia, because the isolates were unable to grow at 29°C (Vorobyeva et al., 2012). At the same time, other researchers found that, irrespective of the growth temperature optimum, cave bacterial isolates grew well at temperatures from 13 to 45°C (Laiz et al., 2003). These contradictory data probably reflect individual peculiarities of the cave systems, because each cave is unique in terms of biological, chemical and physical characteristics. The availability of solar light, water, nutrients, air flow, and interaction with organisms living outside the caves are different in each cave zone and influence the microflora capable of developing and surviving there (Ryan and Meiman, 2004).

All strains under study are Gram-negative, oxidase- and catalase-positive rods. Since it is known that oxidase is a catalyst of redox reactions and catalase is involved in cellular antioxidant defense of bacteria, decomposing hydrogen peroxide and performing electron transport (Ryazantseva, 2011), we believe that these strains can participate in redox processes and, at the same time, have protective properties against oxidative stress.

Enzymatic activity. The study showed that the experimental strains exhibited proteolytic, lipase, pectinase, and phosphate-solubilizing activities depending on the temperature of cultivation (Table 3). Most cultures demonstrated higher proteolytic activity at 25°C, though some strains (MP16, MP20.1, and MP24.1) demonstrated it at 4°C. The strains MP16 and MP24.1 acquired the ability to degrade protein-containing substrates at lower temperatures. For example, protease activity was detected when these strains were cultivated under cold conditions (4°C) but was not observed at 25°C. Proteolytic activity was

Table 3. Enzymatic activity of the bacterial strains of the genus *Pseudomonas* isolated from organogenic sediments of the Mramornaya Cave (Primorskii Krai)

Strain	Diameter of the zone of activity, mm							
	Proteolytic		Pectinase		Lipase		Phosphate-solubilizing	
	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C
MP10	20	26	0	17	0	17	12	21
MP16	13	0	17	0	17	0	14	20
MP2	0	0	0	0	0	0	0	0
MP11	28	28	0	0	0	0	22	30
MP1	0	18	15	28	15	28	18	18
MP20.1	25	20	0	20	0	20	14	19
MP24.2	16	21	0	35	0	35	25	15
MP4	0	0	30	20	30	20	14	19
MP25	17	21	31	0	31	0	15	16
MP17	27	28	24	0	24	0	19	20
MP3	0	14	20	10	20	10	15	25
MP24.1	13	0	18	0	18	0	20	0
MP5	0	21	0	0	0	0	15	0

found in the strains MP10, MP11, MP24.2, MP25, MP17, and MP20.1 at both 4 and 25°C; however, for most of these strains, the zone of hydrolysis observed at 25°C was larger than at 4°C. Probably, the proteases synthesized by these bacteria have elevated thermostability, because their activity was manifested in the temperature range from 4 to 25°C. The strains MP5, MP3, and MP1 exhibited proteolytic activity only at 25°C. At the same time, the optimal growth temperature was 25°C for the strains MP3 and MP1, but it was 4°C for MP5.

Most of the studied strains showed a highly specific pectinase activity dependent on cultivation conditions. For example, cold-active pectinase was found in four strains (MP16, MP25, MP17, and MP24.1) and the pectinase activity with the temperature optimum at 25°C was found in three strains (MP10, MP20.1, and MP24.2). Some strains had a thermotolerant pectinase: the enzyme activity was detected at both 4 and 25°C in the strains MP1, MP4, and MP3.

The strains MP10, MP11, MP20.1, MP25, MP17, and MP5 demonstrated lipolytic activity at 4 and 25°C. The cold-active lipolytic activity against olive oil and Tween 80 was shown for the strains MP16 and MP4. The strains MP1 and MP3 exhibited lipolytic activity at 25°C. The highest activity was observed for the strain MP25.

Nearly all strains under study exhibited phosphate-solubilizing activity at both 4 and 25°C. The exceptions were the strains MP2 (at 4°C), MP24.1, and MP5 (at 25°C), which did not demonstrate the ability to dissolve calcium phosphate. The largest clearance zone of 30 mm was observed for the strain MP11 at 25°C. The high ability of the tested strains to solubilize calcium phosphate is one more indicator of their adaptation to the cave environment, because one of the typical features of many solutional caves is the formation of calcium carbonate in soil.

Thus, the bacteria of the genus *Pseudomonas* isolated from clayey organogenic sediments of the Mramornaya Cave (Primorskii Krai) included psychrotolerant and eurythermic forms. Most of the studied strains did not change cell size with changing cultivation temperature but changed their motility mode. The screening of enzymatic activity at 4 and 25°C showed varying pectinase, protease, and lipase activities depending on the optimal growth temperature. The largest number of strains exhibited phosphate-solubilizing activity both at 4 and at 25°C.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

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

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