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S. Hnatush [1, *], S. Komplikevych [1], O. Maslovska [1], O. Moroz [1], T. Peretyatko [1], A. Dzhulai [2], T. Krasnozhon [1]

[1] Ivan Franko National University of Lviv, Lviv, 79005, Ukraine
[2] State Institution National Antarctic Scientific Center, Ministry of Education and Science of Ukraine, Kyiv, 01601, Ukraine

*** Corresponding author:** shnatush1965@gmail.com

Bacteria of the genus *Pseudomonas* isolated from Antarctic substrates

Abstract. The study's primary purposes were establishing the number of microorganisms that exhibit hydrolytic activity in Antarctic soil and mosses samples, isolation of metal-resistant strains of bacteria, and description of their physiological and biochemical properties. Samples collected during the XXIII Ukrainian Antarctic Expedition in 2019 were used. The number of colony-forming units of microorganisms exhibiting proteolytic, amylolytic, cellulase, lipolytic activity was studied. Pure bacterial cultures were isolated using standard microbiological methods. Determination of resistance of isolates to heavy metals was estimated after their cultivation during ten days on agar plates with different concentrations of $CdCl_2 \cdot 2.5H_2O$, $CoCl_2 \cdot 6H_2O$, $K_2Cr_2O_7$, $FeSO_4 \cdot 7H_2O$, $CuCl_2 \cdot 2H_2O$. Identification of strains was based on the sequencing of the 16S rRNA gene, morphological, physiological, and biochemical properties. Among the 23 isolates, nine metal-resistant strains were selected, four of which were identified as *Pseudomonas yamanorum* IMV B-7916 and 79_102, and as *P. arsenicoxidans* 5A_1N_24, and 89_1T_89. Among the selected strains, the most resistant to heavy metals was *P. yamanorum* 79_102. All studied strains synthesize lipases during growth on medium with tween-20, which contains 0.5–1 mM of ferrous sulfate and copper (II) chloride. The studied strains produce exopolysaccharides during growth at 6 and 22 °C. The most effective among these strains exopolysaccharides are synthesized by *P. arsenicoxidans* 5A_1N_24 — 768 mg/g of dry weight. Our results expand the knowledge about the diversity of microorganisms of extreme biotopes, their properties, resistance to heavy metal compounds.

Keywords: enzymatic activity, exopolysaccharides, metal resistance, phylogenetic reconstruction, *Pseudomonas arsenicoxidans, Pseudomonas yamanorum*

1 Introduction

Antarctic biotopes have unique environmental conditions, such as low temperatures, high levels of UV radiation, and high concentrations of sodium chloride (Correa & Abreu, 2020). Moreover, the substrates are relatively rich in certain chemical elements. It was shown that the content of organogenic elements in the samples from the Argentine Islands was higher than in soils of mineral origin (Abakumov et al., 2016). In particular, the nitrogen content in the samples from the Petermann Island, Berthelot Island, Cape Rasmussen, and Galindez Island exceeded its content in the soils of Ukraine 9.65–16.17 times; phosphorus content — 5.37–54.69 times; carbon — 1.50–5.43 times (Abakumov et al., 2016; Parnikoza et al., 2017). The soils and plant shoots of the Argentine Islands contain variable and often high content of heavy metals (Parnikoza et al., 2017; Bedernichek et al., 2020). In different types of soils of the Galindez Island and the Argentine Islands, copper concentration ranged from 0.2 to 1856.3 mg/kg, zinc — from 0.4 to 667.1 mg/kg, lead — from 3.1 to 1760.0 mg/kg, cadmium — from 0.3 to 29.8 mg/kg, nickel — from 1.5

to 12.2 mg/kg, manganese — from 0.6 to 495.0 mg/kg, iron — from 3.8 to 18623.8 mg/kg (Parnikoza et al., 2007). In recent years, the concentration of heavy metals in the substrates of Antarctica has increased. Fabri-Jr. R. et al. (2018) have found an increased content of Cr, Cu, Ni in soil, lichens, and mosses from Fildes Peninsula, Antarctica, compared to previous studies (Ribeiro et al., 2011). This is associated with anthropogenic influence, which is indicated by the value of the enrichment factor of individual metals over ten, and a positive correlation with air masses movement (Liu et al., 2021). The composition of substrates and environmental factors determine the diversity of microorganisms that inhabit these areas and their adaptations. The efficiency of using Antarctic metal-resistant and metal-tolerant psychrophilic microorganisms in the destruction of oil hydrocarbons in seawater has been proved (Zakaria et al., 2020). Microorganisms susceptible to cadmium are promising for developing biosensors for detecting metal ions in samples (Salwan & Sharma, 2020). Exopolysaccharides of Antarctic microorganisms can be used as biosurfactants in detoxifying soils contaminated with petroleum products (Poli et al., 2010; Papa et al., 2013; Asencio et al., 2014). However, the enzymatic activity of Antarctic strains under the influence of heavy metal compounds has not been studied enough. It is important to search for strains that are characterized by hydrolytic activities. Screening for the ability to form hydrolytic enzymes was performed among microorganisms of seawater, marine sediment, algae, and different marine animal from King George Island (Tropeano et al., 2012), soil samples from Deception Island and Galindez Island (Tomova et al., 2014a) and Windmill Islands region, Wilkes Land, East Antarctica (Tomova et al., 2014b). In these habitats, microorganisms with protease, α-amylase, lipase, RNAse or DNAse, cellulase, urease, phytase, β-glucosidase, polygalactunorase, endocellulase, xylanase, chitosanase, β-galactosidase activities were detected (Tropeano et al., 2012; Tomova et al., 2014a; Tomova et al., 2014b). Extreme environmental conditions allow adapted microorganisms to survive with a wide range of enzymatic activities required for hydrolysis and redox conversion of existing substrates and for protection against adverse factors. Therefore, the search for strains with enzymatic activities capable of synthesizing exopolysaccharides and other biologically active substances is important to carry out in extreme habitats that have not been exposed to anthropogenic impact. Due to the increased resistance to environmental factors, the enzymes of Antarctic microorganisms can be used in agriculture, energy, food, pharmaceutical, and other industries (Kour et al., 2019).

The study aimed to determine the number of microorganisms with hydrolytic (protease, amylase, cellulase, and lipase) activity in Antarctic samples; isolation and characterisation of morphological, physiological, and biochemical properties of metal-resistant bacteria.

2 Materials and methods

2.1 Samples

We used samples of moss, soil, underground parts of *Deschampsia antarctica* E. Desv., 1854, which were taken during the XXIII Ukrainian Antarctic expedition in February–March 2019 (Fig. 1). Samples were taken: № 1 — from Berthelot Island (soil, *D. antarctica*, moss); № 2 — from Cape Rasmussen (Antarctic Peninsula) (soil, moss, mushrooms); № 3 — from Petermann Island (soil, *D. antarctica*, moss); № 4 — from Galindez Island (*D. antarctica*).

2.2 CFU number in Antarctic substrates

The number of microorganisms was quantified as the number of CFU in 1 g of the sample. 1 g of the sample was placed into 9 ml of 0.9% NaCl solution and then shaken for ten minutes. Serial dilutions of these suspensions were plated on tryptic soy agar (TSA) (Merck, USA) to determine the number of microorganisms that metabolize nitrogen of organic compounds, tryptic soy broth (Merck, USA), containing 12% gelatin (TSB-gelatin) — to determine the number of microorganisms that exhibit proteolytic activity, starch-ammonia agar (SAA) (g/l: starch – 10.0; $(NH_4)_2SO_4$ – 2.0; K_2HPO_4 – 1.0; $MgSO_4$ – 1.0; $CaCO_3$ – 3.0; agar – 20; distilled water; pH 7.0) — for determining the number of microorganisms exhibiting amylolytic activity, Hutchinson's medium

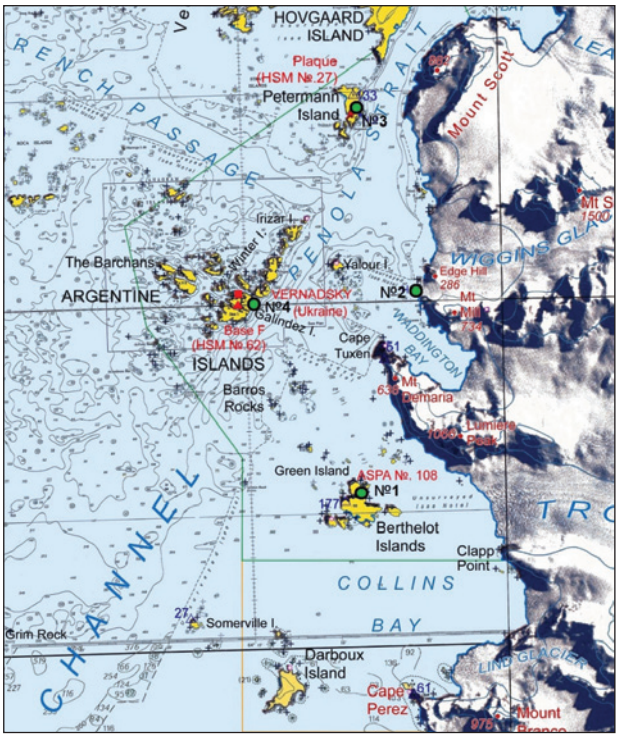

Figure 1. Map of sampling sites used in the study (cartographic materials provided by P. Malov)

(g/l: cellulose – 10.0; $NaNO_3$ – 2.5; K_2HPO_4 – 1; $MgSO_4 \cdot 7H_2O$ – 0.3; NaCl – 0.1; $CaCl_2 \cdot 4H_2O$ – 0.1; $FeCl_3 \cdot 6H_2O$ – 0.01; distilled water; pH 7.0) — for determining the number of microorganisms that show cellulase activity, medium with Tween-20 (g/l: peptone – 10.0; NaCl – 5; $CaCl_2 \cdot 6H_2O$ – 0.1; agar – 20; distilled water; pH 7.0; Tween-20 – 10 g/l was sterilized separately) — to determine the number of microorganisms that exhibit lipolytic activity. Bacteria were grown at a temperature of 20 °C for ten days.

2.3 Isolation of microorganisms and cultivation conditions

Morphologically different colonies grown on TSA, TSB-gelatin, SAA, Hutchinson's medium, or Tween-20 medium that had proteolytic, amylolytic, cellulase, or lipolytic activity, were selected as separate isolates. Isolation of pure cultures of microorganisms from Antarctic samples was performed using standard microbiological cultivation methods on agar and liquid nutrient media. Isolates characterized by several activities were plated on TSA media with heavy metal salts for primary screening of metal resistance. To do it 0.1 mg/l $CdCl_2 \cdot 2.5H_2O$, 10 mg/l $MnCl_2 \cdot 4H_2O$, 10 mg/l $CoCl_2 \cdot 6H_2O$, 25 mg/l $K_2Cr_2O_7$, 30 mg/l $FeSO_4 \cdot 7H_2O$, 100 mg/l $CuCl_2 \cdot 2H_2O$ were added into TSA. Used concentrations of metal salts were 100 times higher than the maximum permissible concentrations (MPC) for these metals for water (Ministry of Health of Ukraine, 2019). To study the resistance of isolates of *Pseudomonas* to the higher concentrations of corresponding salts of heavy metals 2, 5, 10, 50, 100, 250, 500 μM $CdCl_2 \cdot 2.5H_2O$; 1, 5, 10, 15, 20 mM $CoCl_2 \cdot 6H_2O$; 0.1, 0.25, 0.5, 2, 5, 10 mM $K_2Cr_2O_7$; 0.5, 1, 5, 10, 15, 20 mM $FeSO_4 \cdot 7H_2O$; 1, 2, 3, 4, 5, 6 mM $CuCl_2 \cdot 2H_2O$ were added into TSA. To exclude known pathogenic microorganisms isolates that for several inoculations were resistant to all test salts, were additionally inoculated onto differential diagnostic media (Chromocult Listeria agar OTTAVIANI and AGOSTI (Merck, USA), ENDO agar (Merck, USA), bile-esculin agar (Merck, USA), Baird-Parker Agar (Oxoid, UK), Brilliance *Bacillus cereus* agar (Oxoid, UK)). Isolates that did not form characteristic colonies on the diagnostic media were selected for further studies.

2.4 PCR amplification of 16 S rRNA gene and phylogenetic analysis

Chromosomal DNA was isolated using the soft lysis method (Green & Sambrook, 2012). Purification from proteins was carried out by salting with potassium acetate. DNA was precipitated with isopropanol and washed with 70% ethanol. DNA was dissolved in deionized water.

The 16S rRNA gene was amplified using universal primers: 27F AGAGTTTGATCCTGGCTCAG and 1492R GGTTACCTTGTTACGACTT (Turner et al., 1999). PCR reaction was performed in a volume of 50 μl using Taq polymerase (NEB M0273X) on a Mastercycler pro thermal cycler (Eppendorf, Germany). Genomic DNA of strains was used as a template for the PCR reaction. The reaction mixture typically contained 1.0 U of Taq Polymerase and 1OX

PCR buffer (ThermoFischer Scientific, USA), 0.04 mM of each deoxynucleotide, 600 nM of each amplification primer, ca. 50 ng of genomic template DNA and purified water to volume. Temperature and cycling conditions were as follows: one 95 °C denaturation cycle for 3 min, followed by 30 cycles of 95 °C denaturation for 30 s, primer annealing at 49 °C for 30 s, and elongation at 72 °C for 90 s. The PCR reaction products were analyzed by electrophoresis of DNA in agarose gel and visualized by staining with ethidium bromide. PCR products about 1.5 kbp were purified from the gel using silica columns "QiaQuick" ("Qiagen", USA), analyzed for DNA concentration and purification quality using DeNovix DS-11 microvolume spectrophotometer. The products were sequenced from primers 27F and 1492R using BigDye terminators mix, and fragments were analyzed on ABI Prism 3130 xl. The resulting nucleotide sequences (two for each sample corresponding to DNA readings from 27F and 1492R primers) were quality checked, assembled, trimmed, and compared with the sequences in the GenBank database by BLAST search.

Multiple alignment was performed using the program ClustalW (Thompson et al., 1994). For aligned sequences, the search for the optimal model of nucleotide substitution (Nei & Kumar, 2000) and phylogenetic reconstruction in the program MEGA X (Kumar et al., 2018) was performed. Phylogenetic reconstruction by the method of maximum likelihood after 1000 bootstrap replications was performed using the Jukes-Cantor model (Jukes & Cantor, 1969).

2.5 Characteristics of isolates

The morphology of cells (cell shape, size, ability to form spores, determination of the composition and structural organization of the cell wall after Gram staining) was investigated using light (Carl Zeiss Axio Lab.A1 binocular microscope, an Olympus IX73 inverted microscope with a DP-74 digital camera) and transmission electron microscopy (Reynolds, 1963). Gram staining was performed using a dye kit (Merck, USA). Bacterial ability to spore formation was determined microscopically (Peshkov-Trujillo method) and by culturing a pre-pasteurized cell suspension (Hudz' et al., 2014). Catalase activity was detected by the apparent release of O_2 after applying a few drops of 10% H_2O_2 (Arenas et al., 2014; Hudz' et al., 2014). Oxidase activity was detected using strips with N,N-dimethyl-p-phenylenediamine oxalate, and α-naphthol (Millipore, USA). Relation to oxygen was determined by the nature of growth in fluid thioglycollate medium (Merck, USA). Bacterial motility was detected by the nature of growth in the TSA column with 0.2% agar. The optimum growth temperature was determined after five days of growing at 2, 6, 16, 20, 28, or 37 °C. The optimal pH for the cultivation of isolates was determined after five days of cultivation in TSB with pH 4–9. Halotolerance of isolates was established after five days of cultivation in TSB with 0.5–15% NaCl. ID 32 GN kit (bioMérieux, France) was used to detect the ability of isolates to metabolize different carbon sources. Different carbon sources fermentation was detected during growth in Hiss media with arabinose, glucose, dulcite, inositol, xylose, lactose, maltose, mannitol, mannose, rhamnose, sucrose, sorbitol. Peculiarities of metabolism of nitrogen-containing compounds were determined after growth in TSB with cysteine (0.01%) by a color change of litmus (ammonia release) and lead acetate (hydrogen sulfide, mercaptan release) indicator papers (Hudz' et al., 2014). To detect the ability to fix nitrogen, bacteria were grown on Ashby medium (Hudz' et al., 2014). Bacterial ability to reduce nitrate ions was detected using qualitative reactions for nitrate (with sulfuric acid and diphenylamine) and nitrite ions (with Griess reagent — a mixture of sulfanilic acid and 2-naphthylamine in acidic medium), and the formation of N_2 bubbles in the Bubble Durham tube after seven days of growth in the TSB with 0.2% KNO_3 (Hudz' et al., 2014). The proteolytic activity of the investigated isolates was evaluated by their ability to liquefaction of gelatin after growth in a column of TSB-gelatin (Loperena et al., 2012; Hudz' et al., 2014). Amylase activity was evaluated by the growth on SAA and the formation of visible zones of starch hydrolysis around the colonies after applying Lugol's solution on the colony (Sushma et al., 2012; Hudz' et al., 2014). Lipase activity was evaluated by the ability of isolates to form crystals of calcium salts of fatty acids around

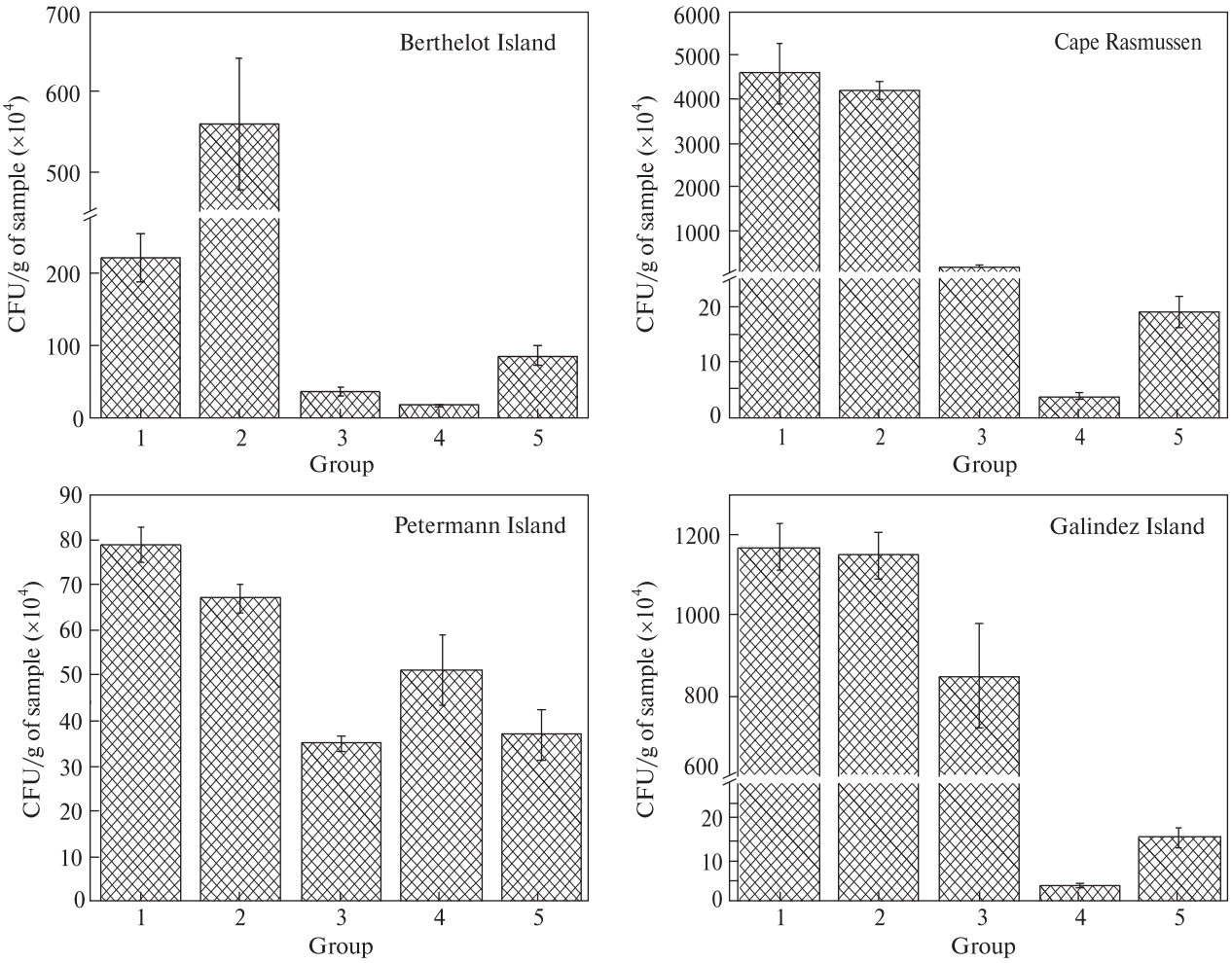

Figure 2. The number of microorganisms that metabolize nitrogen of organic compounds (1), show proteolytic (2), amylolytic (3), cellulolytic (4), lipolytic (5) activities in samples taken from Antarctic substrates

Table 1. Substrate samples and isolates used in the study

Sample	Substrate	Sampling location	Coordinates	Isolates
5 A 2019	soil, *D. antarctica*, moss	Berthelot Island	65°19.705, 064°08.060	5A_101, 5A_102, 5A_103, 5A_104, 5A_106, 5A_1N_24
9.9 A 2019	soil, moss, mushrooms	Cape Rasmussen, Antarctic Peninsula	65°14.848, 064°05.080	9.9_101, 9.9_102, 9.9_103, 9.9_104
79 A 2019	soil, *D. antarctica*, moss	Petermann Island	65°10.342, 064°08.317	79_101, 79_102
89 A 2019	*D. antarctica*	Galindez Island	65°14.783, 064°14.788	89A_105, 89A_106, 89A_107, 89A_108, 89A_110, 89A_111, 89_1T_89, 89A_2T_20, 89A_2N_20

the colonies after growth on medium with Tween-20 (Lo Giudice et al., 2006). Exopolysaccharides were extracted from EDTA (2%) for 3 hours at 4 °C, then centrifuged at 15000 g for 20 min at 4 °C (Pan et al., 2010). The content of exopolysaccharides in the obtained supernatant was determined using anthrone (Frølund et al., 1996). All other features were detected using Remel RapID™ ONE system.

3 Results

Microorganisms that metabolize nitrogen of organic compounds were found in the samples taken from Berthelot Island, Petermann Island, Galindez Island, and Cape Rasmussen (Antarctic Peninsula). The CFU count of microorganisms that exhibit proteolytic, amylolytic, cellulase, and lipolytic activity in all samples was 10^4–10^7. The largest number of microorganisms that metabolize nitrogen of organic compounds was in a sample taken from Cape Rasmussen (Antarctic Peninsula) ($4.6 \cdot 10^7$ CFU/g of the sample) (Fig. 2). Also, the largest number of microorganisms exhibiting proteolytic activity was detected in this sample. Microorganisms that showed amylolytic activity were most abundant in the sample from Galindez Island and which showed cellulolytic activity — in the sample from Petermann Island. Microorganisms that exhibited lipolytic activity in the four samples ranged from 1.6 to $8.5 \cdot 10^5$ CFU/g of the sample.

From these samples, 21 bacterial isolates were isolated (Table 1). Because bacteria were isolated on different media, proteolytic, amylolytic, cellulase, li-

Table 2. Enzymatic activity of isolates

Isolates	Proteolytic	Amylolytic	Cellulase	Lipase
5A_101	–	+	–	+
5A_102	+	–	–	+
5A_103	–	±	–	–
5A_104	–	±	–	+
5A_106	+	±	–	+
5A_1N_24	+	–	–	+
9.9_101	+	±	–	+
9.9_102	+	±	–	+
9.9_103	–	±	–	+
9.9_104	–	±	–	–
79_101	–	±	–	+
79_102	+	±	–	+
89A_105	–	±	–	+
89A_106	–	±	–	–
89A_107	–	±	–	+
89A_108	–	±	–	–
89A_110	–	±	–	+
89A_111	±	±	–	+
89_1T_89	+	–	–	+
89A_2T_20	–	+	–	+
89A_2N_20	+	–	–	+

Note: «+» — intensive activity, «±» — less intensive activity, «–» — no activity.

pase activities, and resistance to metal salts were screened.

None of the isolates was characterized by cellulase activity (Table 2). The 16S rRNA genes of isolates resistant to all test metal salts at concentrations higher by 100 times the MPC of these metal ions for water (Table 3) and characterized by a combination of at least two of the enzymatic activities were sequenced.

Isolates 5A_1N_24, 9.9_102, 79_102, and 89_1T_89 that were resistant to various concentrations of copper (II) chloride, ferrous sulfate, cobalt (II) chloride, cadmium (II) chloride, and potassium dichromate according to the results of sequencing were identified as members of the genus *Pseudomonas*.

Pairwise alignment of the 16S rRNA gene sequence of these isolates to the GenBank database allowed us to establish high values of the identity of *Pseudomonas yamanorum* LBUM636 and *Pseudomonas yamanorum* 8H1 (100%) to the sequence of isolates 9.9_102 and 79_102. *Pseudomonas arsenicoxydans* Y24-2 has the highest degree of identity in terms of the nucleotide sequence of the 16S rRNA gene of isolate 5A_1N_24 — 99.93%. The nucleotide sequence of the 16S rRNA gene of isolate 89_1T_89 is 100% identical to the 16S rRNA gene sequence of *P. arsenicoxydans* Y24-2. Accordingly, isolate 5A_1N_24 was identified as *Pseudomonas arsenicoxidans* 5A_1N_24; isolate 9.9_102 as *Pseudomonas yamanorum* IMV B-7916; isolate 79_102 as *Pseudomonas yamanorum* 79_102; isolate 89_1T_89 as *Pseudomonas arsenicoxidans* 89_1T_89. Gene sequences of 16S rRNA of isolates are available at GenBank: *P. yamanorum* IMV B-7916 as MW362268; *P. yamanorum* 79_102 as MW362274; *P. arsenicoxidans* 5A_1N_24 as MW362276; *P. arsenicoxidans* 89_1T_89 as MW362282. After reconstructing the 16S rRNA gene, the high bootstrap

Table 3. Growth of isolates under the influence of heavy metal compounds

Isolates	0.1 mg/l $CdCl_2 \cdot 2.5H_2O$	10 mg/l $MnCl_2 \cdot 4H_2O$	10 mg/l $CoCl_2 \cdot 6H_2O$	25 mg/l $K_2Cr_2O_7$	30 mg/l $FeSO_4 \cdot 7H_2O$	100 mg/l $CuCl_2 \cdot 2H_2O$
5A_101	+	+	+	–	+	+
5A_102	+	+	+	–	+	+
5A_103	+	+	+	+	–	+
5A_104	+	+	+	+	–	+
5A_106	+	+	+	+	+	+
5A_1N_24	+	+	+	+	+	+
9.9_101	–	+	+	–	+	+
9.9_102	+	+	+	+	+	+
9.9_103	+	+	+	–	+	+
9.9_104	+	+	–	+	+	+
79_101	+	+	+	+	+	+
79_102	+	+	+	+	+	+
89A_105	+	+	+	+	+	+
89A_106	+	+	–	–	+	+
89A_107	+	+	+	+	+	+
89A_108	+	+	+	–	+	–
89A_110	+	+	+	–	–	–
89A_111	+	+	+	+	+	+
89_1T_89	+	+	+	+	+	+
89A_2T_20	+	+	–	+	+	–
89A_2N_20	+	+	–	–	+	–

Note: «+» — growth, «–» — no growth.

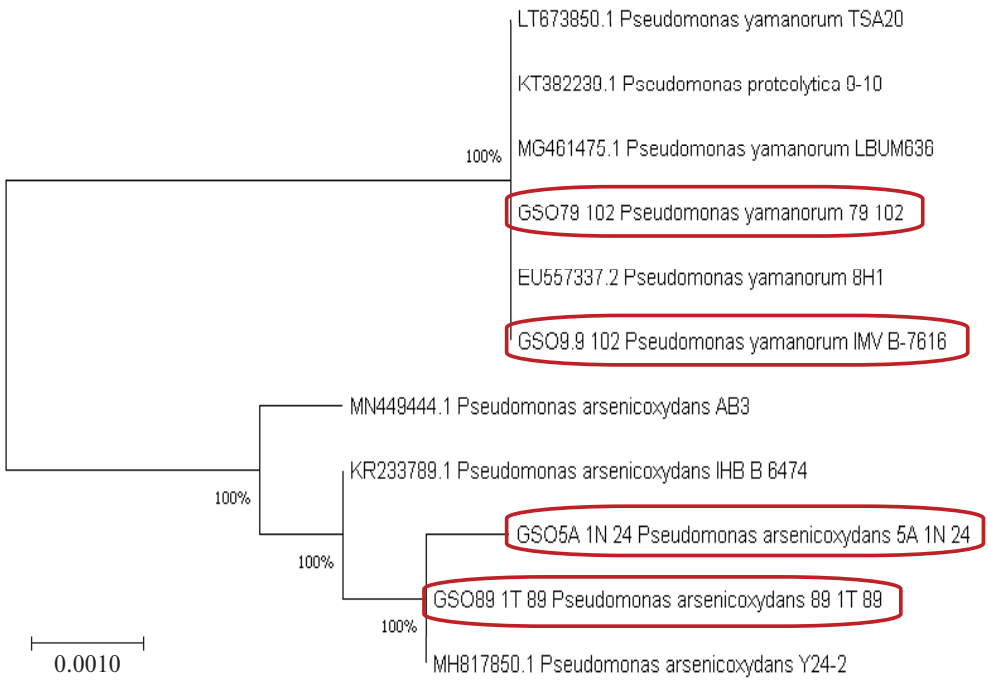

Figure 3. Phylogenetic reconstruction of the 16S rRNA gene of strains *Pseudomonas yamanorum* 79_102, *Pseudomonas yamanorum* IMV B-7916, *Pseudomonas arsenicoxidans* 5A_1N_24 and *Pseudomonas arsenicoxidans* 89_1T_89 with the highest log likelihood (-1962.90) by the maximum likelihood method according to the Jukes-Cantor model with 1000 bootstrap replications. Near the branches are the values of the percentage of trees in which these sequences are placed side by side

value confirms the affiliation of isolates to one or another species (Fig. 3).

Cells of all four strains are Gram-negative rods (Fig. 4). The cell sizes of *P. arsenicoxidans* 5A_1N_24 – 1.9 × 0.6 μm, *P. yamanorum* IMV B-7916 – 1.8 × 0.7 μm, *P. yamanorum* 79_102 – 2.3 × 1.1 μm, *P. arsenicoxidans* 89_1T_89 – 2.1 × 0.8 μm. The studied bacteria are aerobes, do not form spores, grow at +8 ... + 28 °C (*P. arsenicoxidans* 89_1T_89 – 2–28 °C) with an optimum of 16–20 °C. For all four strains, the optimum pH is 7.0. Bacteria of *P. arsenicoxidans* 5A_1N_24 grow in the pH range 6–8, *P. yamanorum* IMV B-7916 and *P. yamanorum* 79_102 — in the pH range 4–9, *P. arsenicoxidans* 89_1T_89 — in the pH range 4–8. All strains can grow with 0.5–5% NaCl in the cultivation medium, and *P. yamanorum* IMV B-7916 can withstand up to 7.5% of this salt in the medium. The studied bacteria are catalase- and oxidase-positive. The results of studies of the ability of bacteria to hydrolyze starch, Tween-20, gelatin, degrade certain carbohydrates, alcohols, carboxylic acids, amino acids, hydrolyze aliphatic thiols, to reduce NO_3^-, features of amino acid metabolism, urease, decarboxylase, glycosidase, amidase activity of *P. arsenicoxidans* 5A_1N_24, *P. arsenicoxidans* 89_1T_89, *P. yamanorum* IMV B-7916, and *P. yamanorum* 79_102 are presented in Table 4.

P. yamanorum strain 79_102 and *P. yamanorum* strain IMV B-7916 grow on starch-ammonia agar but do not form zones of hydrolysis of starch. All strains produce exopolysaccharides at 6 and 22 °C, synthesize lipases during growth on a medium with Tween-20 that contains 0.5–1 mM of ferrous sulfate and copper (II) chloride. At 6 °C *P. arsenicoxidans* 89_1T_89 forms 37 mg of exopolysaccharides/g of dry cell mass, *P. yamanorum* IMV B-7916 – 105.16, *P. yamanorum* 79_102 – 144.97, *P. arsenicoxidans* 5A_1N_24 – 768.05. For comparison: *Pseudoalteromonas* CAM025 at growth at –2 and 10 °C form about 100 mg of exopolysaccharides/g dry weight, which is 30 times

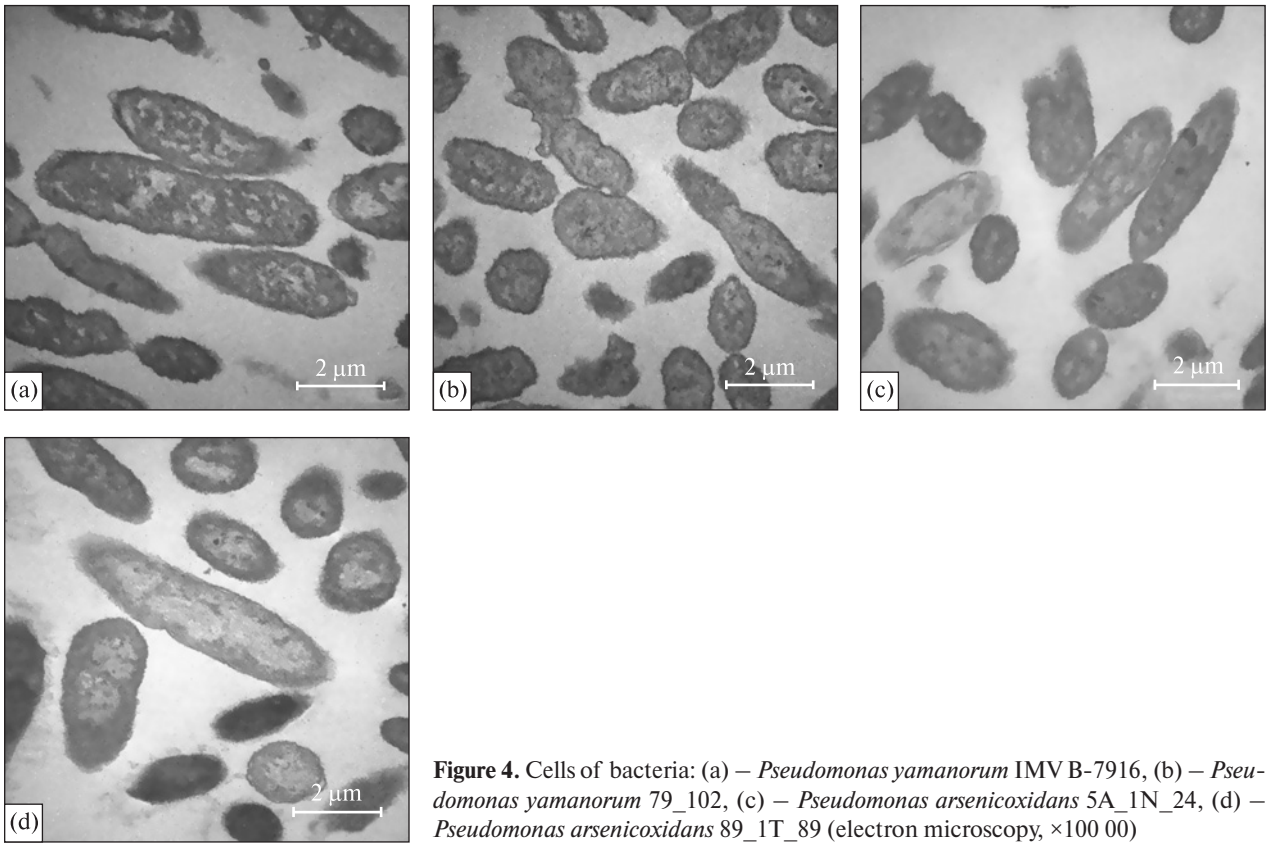

Figure 4. Cells of bacteria: (a) – *Pseudomonas yamanorum* IMV B-7916, (b) – *Pseudomonas yamanorum* 79_102, (c) – *Pseudomonas arsenicoxidans* 5A_1N_24, (d) – *Pseudomonas arsenicoxidans* 89_1T_89 (electron microscopy, ×100 00)

higher than the amount of exopolysaccharides produced by this strain at 25 °C (Mancuso Nichols et al., 2004). All strains are characterized by arginine decarboxylase, ornithine decarboxylase activities, and the ability to cleave proline-β-naphthylamide.

Bacteria of *P. arsenicoxidans* 5A_1N_24 were resistant to 5 mM copper (II) chloride, 20 mM ferrous sulfate, 5 mM cobalt (II) chloride, 0.05 mM cadmium (II) chloride, and 0.25 mM potassium dichromate (Table 5).

P. yamanorum 79_102 was the most resistant to the influence of heavy metal compounds among the studied strains. It grew on TSA with 5 mM copper (II) chloride, 20 mM ferrous sulfate, 1 mM cobalt (II) chloride, 0.05 mM cadmium (II) chloride, 2 mM potassium dichromate. *P. arsenicoxidans* 89_1T_89 was resistant to 4 mM copper (II) chloride, 15 mM ferrous sulfate, 5 mM cobalt (II) chloride, 0.01 mM cadmium (II) chloride, 0.25 mM potassium dichromate. Bacteria *P. yamanorum* IMV B-7916 were resistant to 5 mM copper (II) chloride, 20 mM ferrous sulfate, 1 mM cobalt (II) chloride, 0.05 mM cadmium (II) chloride, 2 mM potassium dichromate. The slight growth of *P. yamanorum* IMV B-7916 was observed on TSA with 5 mM potassium dichromate.

4 Discussion

Enzymes of psychrophilic microorganisms, in particular, lipases, proteases, amylases, and cellulases, have significant biotechnological value (Yarzábal, 2016). In each studied sample (Fig. 2), the most microorganisms with proteolytic activity were detected. Proteolytic activity was found in 42.9% of isolates (Table 2). The obtained results correlate with the results of studies of isolates from Deception Island and Galindez Island (Tomova et al., 2014a), where 58.3% of isolates showed protease activity, and from King

Table 4. Physiological and biochemical properties of *Pseudomonas arsenicoxidans* 5A_1N_24, *Pseudomonas arsenicoxidans* 89_1T_89, *Pseudomonas yamanorum* IMV B-7916, and *Pseudomonas yamanorum* 79_102

Feature	*Pseudomonas arsenicoxidans* 5A_1N_24	*Pseudomonas arsenicoxidans* 89_1T_89	*Pseudomonas yamanorum* IMV B-7916	*Pseudomonas yamanorum* 79_102
Starch hydrolysis	–	–	±	±
Hydrolysis of Tween-20	+	+	+	+
Hydrolysis of gelatin	+	+	+	+
Metabolism of:				
L-rhamnose	–	–	–	+
N-acetylglucosamine	–	–	+	+
D-ribose	–	–	+	–
inositol	–	–	+	+
D-sucrose	–	–	–	–
D-maltose	–	–	–	–
itaconic acid	–	–	+	+
suberic acid	–	–	–	–
sodium malonate	–	–	+	+
sodium acetate	–	+	+	+
lactic acid	+	+	+	+
L-alanine	+	+	+	+
potassium 5-ketogluconate	–	+	–	+
glycogen	–	–	–	–
3-hydroxybenzoic acid	–	–	–	–
L-serine	+	+	+	+
D-mannitol	+	+	+	+
D-glucose	+	+	+	+
salicin	–	–	–	–
D-melibiose	–	–	–	–
L-fucose	–	–	+	+
D-sorbitol	–	–	+	+
L-arabinose	–	+	+	+
propionic acid	–	–	–	–
capric acid	+	+	+	+
valeric acid	–	+	–	–
sodium citrate	+	+	+	+
L-histidine	–	–	+	+
potassium 2-ketogluconate	+	+	+	+

End of Table 4

Feature	*Pseudomonas arsenicoxidans* 5A_1N_24	*Pseudomonas arsenicoxidans* 89_1T_89	*Pseudomonas yamanorum* IMV B-7916	*Pseudomonas yamanorum* 79_102
3-hydroxybutyrate	–	+	+	+
4-hydroxybenzoic acid	–	+	–	+
L-proline	+	+	+	+
Reduction of NO_3^-	–	–	–	+
Amino acid metabolism:				
H_2S formation	–	–	–	–
NH_3 formation	+	+	+	+
indol formation	–	–	+	–
Enzymatic activity:				
urease	–	–	–	–
arginine decarboxylase	+	+	+	+
ornithine decarboxylase	+	+	+	+
lysine decarboxylase	–	+	+	+
hydrolysis of aliphatic thiols	+	–	–	–
Cleavage:				
p-nitrophenyl-β, D-glucuronide	–	–	–	–
γ-nitrophenyl-β, D-galactoside	–	–	–	–
p-nitrophenyl-β, D-glucoside	–	–	–	–
p-nitrophenyl-β, D-xyloside	–	–	–	–
p-nitrophenyl-n-acetyl-β, D-glucosaminide	–	–	–	–
proline-β-naphthylamide	+	+	+	+
γ-glutamyl-β-naphthylamide	–	–	+	–
pyrrolidonyl-β-naphthylamide	–	–	+	–

Note: «+» — activity, «–» — no activity.

George Island (Tropeano et al., 2012), where 44.4% were microorganisms characterized by proteolytic activity. Amylase activity was detected in 80.9% of the studied isolates; however, only two isolates (9.5%) formed a zone of starch hydrolysis. Lipase activity was detected in 76.2% of isolates.

No isolate had cellulase activity. It is known that bacteria can form a biofilm on the hyphae of fungi (Cai et al., 2019; Kjeldgaard et al., 2019). Such an association is possible in the natural habitat of these isolates because all colonies that were grown on Hutchinson's medium (Fig. 2) and were characterized by cellulase activity had the morphology of microscopic fungi. Among the isolates, bacteria with cellulase activity were not detected.

Among the microorganisms isolated from Antarctic habitats, the genera *Pseudomonas*, *Psychrobacter*, *Arthrobacter*, and *Flavobacterium* predominate (Romaniuk et al., 2018). Bacteria of the genus *Pseudomonas*, common in various habitats, are components of the rhizosphere and phyllosphere of plants. Among the representatives of this genus are plant growth-promoting bacteria (Glick, 2012; Afegbua & Batty, 2019; Qessaoui et al., 2019). The study (Cid et al., 2017)

found that members of the genus *Pseudomonas* are associated with the phyllosphere of *D. antarctica*. They are also quantitatively predominant, contain genes whose products promote plant growth and mitigate the effects of stressors, and are isolated in pure culture because five of the six sequenced isolates from the phylosphere of *D. antarctica* belong to this genus (Cid et al., 2018). Nine of the 12 bacterial cultures of

Table 5. Resistance to heavy metals salts

Salt	mM	*P. arsenicoxidans* 5A_1N_24	*P. arsenicoxidans* 89_1T_89	*P. yamanorum* IMV B-7916	*P. yamanorum* 79_102
	0	+	+	+	+
$CuCl_2 \cdot 2H_2O$	1	+	+	+	+
	2	+	+	+	+
	3	+	+	+	+
	4	+	+	+	+
	5	±	–	+	+
	6	±	–	–	±
$FeSO_4 \cdot 7H_2O$	0.5	+	+	+	+
	1	+	+	+	+
	5	+	+	+	+
	10	+	+	+	+
	15	+	+	±	+
	20	±	–	–	+
$CoCl_2 \cdot 6H_2O$	1	+	+	+	+
	5	±	±	–	–
	10	–	–	–	–
	15	–	–	–	–
	20	–	–	–	–
$CdCl_2 \cdot 2.5H_2O$	0.002	+	+	+	+
	0.005	+	+	+	+
	0.010	±	±	+	+
	0.050	±	–	–	±
	0.100	–	–	–	–
	0.250	–	–	–	–
	0.500	–	–	–	–
$K_2Cr_2O_7$	0.1	+	±	+	+
	0.25	+	±	+	+
	0.5	–	–	±	±
	2	–	–	±	±
	5	–	–	±	–
	10	–	–	–	–

Note: «+» — growth, «±» — slight growth, «–» — no growth

endophytes of *D. antarctica* roots are members of the genus *Pseudomonas* (Podolich et al., 2021). The prevalence of bacteria of the genus *Pseudomonas* in different habitats is due to their ability to use many organic compounds as carbon and energy sources to produce biologically active compounds (Higuera-Llanten et al., 2018) and to be resistant to various environmental factors. Bacteria of the genus *Pseudomonas* include producers of exopolysaccharides (Vásquez-Ponce et al., 2017; Zhao et al., 2018), polyhydroxyalkanoates (Mozejko-Ciesielska et al., 2019), bacterial lipases (Rios et al., 2018; Salwoom et al., 2019). Bacteria of this genus can degrade aromatic hydrocarbons (benzene and toluene) (Skvortsov et al., 2018) and also exhibit multiple resistance to antibiotics and heavy metal ions (Higuera-Llanten et al., 2018). From the biotopes of Antarctica were isolated *Pseudomonas* sp. LSK25 — producer of bacterial lipases (Salwoom et al., 2019), *Pseudomonas extremaustralis* — producer of poly (3-hydroxybutyrate) (López et al., 2009), *Pseudomonas* sp. MPC6 — producer of polyhydroxyalkanoates (Orellana-Saez et al., 2019), *Pseudomonas mandelii* — producer of alginates (Vásquez-Ponce et al., 2017). *Pseudomonas antarctica* PAMC 27494 synthesize bacteriocins (Lee et al., 2017).

From the Antarctic and subantarctic habitats, bacteria of the genus *Pseudomonas* were isolated mostly from soil (Bozal et al., 2007; Kosina et al., 2013; Tomova et al., 2014a; Arnau et al., 2015; See-Too et al., 2017), but also cyanobacterial mat from ponds (Reddy et al., 2004), algal culture (Hwang et al., 2009), marine sediment sample (Carrión et al., 2011), sedimentary rock (Kosina et al., 2016), seawater or freshwater (López et al., 2009; Vásquez-Ponce et al., 2017; Higuera-Llanten et al., 2018).

Very few culturable strains of *P. yamanorum* are known (8H1 (Arnau et al., 2015), LP2 (Komesli et al., 2020), LBUM636 (Morrison et al., 2016)), and *P. arsenicoxidans* (VC-1 (Campos et al., 2010)), however, only *P. arsenicoxydans* ACM1 (GenBank: ASM413599v1) was isolated from the moss rhizosphere in Antarctica.

The isolates we isolated are different strains of *P. yamanorum* and *P. arsenicoxidans*. The bacteria *P. yamanorum* IMV B-7916 and *P. yamanorum* 79_102 we isolated compared with *P. yamanorum* 8H1 (Arnau et al., 2015) differ slightly in the temperature range for growth, are characterized by a narrower range of optimal pH. Unlike *P. yamanorum* 8H1, both isolated strains of *P. yamanorum* can grow on SAA, indicating the presence of amylolytic activity. Also, these strains differ in their ability to assimilate certain organic compounds and individual enzymatic activities. Both strains of *P. yamanorum* isolated by us assimilate lactate, D-mannitol, show ornithine decarboxylase and lysine decarboxylase activities, which is not typical for *P. yamanorum* 8H1. Also, some characteristics of *P. yamanorum* 8H1 differ in one of the studied strains. In particular, the ability to assimilate D-ribose is present in the *P. yamanorum* IMV B-7916. *P. yamanorum* 79_102 can reduce nitrates. In contrast to *P. yamanorum* IMV B-7916, *P. yamanorum* 79_102 can assimilate L-rhamnose, potassium 5-ketogluconate, and 4-hydroxybenzoic acid. Bacteria *P. yamanorum* 79_102 cannot break down pyrrolidonyl-β-naphthylamide. Bacteria *P. yamanorum* IMV B-7916 form indole from tryptophan.

The selected strains of *P. arsenicoxidans* are characterized by a narrower temperature range and lower optimal pH value for growth than *P. arsenicoxidans* VC-1 (Campos et al., 2010). These isolates can withstand higher concentrations of NaCl (5%) compared to the reference strain (2%). Both strains of *P. arsenicoxidans* show ornithine decarboxylase activity, which is not typical for *P. arsenicoxidans* VC-1 (Campos et al., 2010). The studied strains are cannot assimilate N-acetylglucosamine and D-sorbitol, in contrast to the described *P. arsenicoxidans* VC-1 (Campos et al., 2010). Also, these strains do not cleave p-nitrophenyl-β, D-glucoside, p-nitrophenyl-n-acetyl-β, D-glucosaminide, γ-glutamyl-β-naphthylamide, pyrrolidonyl-β-naphthylamide. The ability to hydrolyze aliphatic thiols is in the strain *P. arsenicoxidans* 5A_1N_24; the ability to assimilate sodium acetate, L-arabinose, lysine decarboxylase activity is characteristic of *P. arsenicoxidans* 89_1T_89.

All isolates were resistant to at least three heavy metal salts. Nine of 21 isolates (42.9 %) were resistant to all tested salts in the concentration that 100 times exceeds MPC for these metals. *P. yamanorum* 79_102 were the most resistant to the heavy metal compounds

among the studied strains.The identified strains, in addition to resistance to heavy metals, were also characterized by hydrolase activity and the ability to synthesize exopolysaccharides. Resistance to heavy metals is primarily due to genetic elements (plasmids, transposons). The genome of *P. psychrotolerans* L19 contains genes and operons that determine resistance to copper, in particular, the *cus* operon, which encodes the RND-type efflux system, and multicopper oxidases encoding genes (Santo et al., 2012). Tn7-like transposon provides hyperresistance to copper phenotype for strains *P. syringae* pv. syringae (Aprile et al., 2021). Plasmid pMR68 *Pseudomonas* strain K-62 contains three clusters of *mer* gene, which determine the resistance to mercury compounds (Sone et al., 2013). In the genome of the Antarctic multimetal resistant *Pseudomonas putida* ATH-43 found genes and operons that provide resistance to Hg^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+}, CrO_4^{2-}, AsO_4^{3-}, SeO_3^{2-}, and TeO_3^{2-}, a wide range of antibiotics and medications, a number of stressors (Rodríguez-Rojas et al., 2016). However, metal resistance does not always correlate with metal content in the habitat of microorganisms, the presence of homologues of genes that provide resistance to a metal, by regulating transport and recovery of mercury, which was shown by principal component analysis of King George Island soil samples (Romaniuk et al., 2018). This property is probably related to the synthesis of exopolysaccharides by these strains, as exopolysaccharides are known to remove heavy metal ions from solutions (Muthu et al., 2017). This requires further research.

5 Conclusions

Isolated metal-resistant strains of *Pseudomonas yamanorum* 79_102, *Pseudomonas yamanorum* IMVB-7916, *Pseudomonas arsenicoxidans* 5A_1N_24, and *Pseudomonas arsenicoxidans* 89_1T_89 can be used for further research and development of technologies for environmental remediation or for obtaining of exopolysaccharides. The data expand the knowledge about the diversity of microorganisms of extreme biotopes, their properties, adaptation to the influence of heavy metal compounds.

Author contributions. SH supervised the research. SH, SK, OlM, TP, OM, TK did experiments, data analysis, text drafting. AD sample collection and processing. OlM, SK, OM edited the manuscript.

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*Conflict of Interest.*The authors declare that they have no conflict of interest.

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С. Гнатуш[1, *], С. Комплікевич[1], О. Масловська[1], О. Мороз[1], Т. Перетятко[1], А. Джулай[2], Т. Красножон[1]

[1] Львівський національний університет імені Івана Франка, м. Львів, 79005, Україна

[2] Державна установа Національний антарктичний науковий центр МОН України, м. Київ, 01601, Україна

* **Автор для кореспонденції**: shnatush1965@gmail.com

Бактерії роду *Pseudomonas*, виділені зі субстратів Антарктики

Реферат. Основною метою дослідження є встановлення чисельності в антарктичних зразках мікроорганізмів, які виявляють гідролітичну активність; виділення металорезистентних штамів бактерій та опис їхніх фізіолого-біохімічних властивостей. Для дослідження використали зразки грунту і моху, відібрані під час XXIII Української антарктичної експедиції у 2019 р. Досліджено кількість колонієутворювальних одиниць мікроорганізмів, що виявляють протеолітичну, амілолітичну, целюлазну, ліполітичну активність. Чисті культури бактерій виділяли із застосуванням стандартних мікробіологічних методів. Для дослідження стійкості ізолятів до солей важких металів у триптон-соєвий агар вносили різні концентрації $CdCl_2 \cdot 2{,}5H_2O$, $CoCl_2 \cdot 6H_2O$, $K_2Cr_2O_7$, $FeSO_4 \cdot 7H_2O$, $CuCl_2 \cdot 2H_2O$ та вирощували за 20 °C 10 діб. Ідентифікацію штамів проводили за результатами секвенування гена 16S рРНК, морфологічними та фізіолого-біохімічними властивостями. Серед 23 ізолятів відібрали дев'ять металорезистентних штамів, чотири з яких були ідентифіковані як *Pseudomonas yamanorum* IMB B-7916 та 79_102 і *P. arsenicoxidans* 5A_1N_24 та 89_1T_89. Серед виділених штамів найстійкішими до впливу сполук важких металів є *P. yamanorum* 79_102. Усі досліджувані штами синтезують ліпази за росту на середовищі з твіном-20 з 0,5—1 мМ ферум (II) сульфату та купрум (II) хлориду. Досліджувані штами утворюють екзополісахариди під час росту за 6 та 22 °C. Найбільше екзополісахаридів серед цих штамів синтезує *P. arsenicoxidans* 5A_1N_24 — 768 мг/г сухої маси. Отримані дані розширюють знання про різноманіття мікроорганізмів екстремальних біотопів, їхні властивості, стійкість до впливу сполук важких металів.

Ключові слова: *Pseudomonas arsenicoxidans*, *Pseudomonas yamanorum*, екзополісахариди, ензиматична активність, металорезистентність, філогенетична реконструкція