POLISH POLAR RESEARCH
vol. 35, no. 4, pp. 593–607, 2014 doi: 10.2478/popore-2014-0028

Characterization of heavy metals resistant heterotrophic bacteria from soils in the Windmill Islands region, Wilkes Land, East Antarctica

Iva TOMOVA, Margarita STOILOVA-DISHEVA
and Evgenia VASILEVA-TONKOVA*

Institute of Microbiology, Bulgarian Academy of Sciences, Acad. Georgi Bonchev Str., Bl. 26, 1113 Sofia, Bulgaria
** corresponding author <evaston@yahoo.com>*

This article is dedicated to the memory of Dr. Victoria Gesheva gratefully

Abstract: In this study, selected heavy metals resistant heterotrophic bacteria isolated from soil samples at the Windmill Islands region, Wilkes Land (East Antarctica), were characterized. Phylogenetic analysis revealed affiliation of isolates to genera *Bacillus*, *Lysinibacillus*, *Micrococcus* and *Stenotrophomonas*. The strains were found to be psychrotolerant and halotolerant, able to tolerate up to 10% NaCl in the growth medium. The Minimum Inhibitory Concentration of the seven heavy metals Cr, Cu, Ni, Co, Cd, Zn, and Pb was determined in solid media for each bacterial strain. Gram-positive Vi-2 strain and Gram-negative Vi-4 strain showed highest multiply heavy metals resistance, and Vi-3 and Vi-4 strains showed multi-antibiotic resistance to more than a half of the 13 used antibiotics. Plasmids were detected only in Gram-negative Vi-4 strain. The bacteria were able to produce different hydrolytic enzymes including industrially important proteases, xylanases, cellulases, and β-glucosidases. High heavy metals resistance of the Antarctic bacteria suggests their potential application for wastewater treatment in cold and temperate climates. Highly sensitive to Cd and Co ions Vi-1, Vi-5 and Vi-7 strains would be promising for developing biosensors to detect these most toxic heavy metals in environmental samples.

Key words: Antarctica, soil bacteria, hydrolytic enzymes, antibiotic resistance, metal resistance.

Introduction

Although the extreme environmental conditions in Antarctica such as low temperature, high salinity, osmotic stress, and high doses of UV-radiation, various microorganisms have colonized diverse habitats such as lakes, rivers, streams, rocks and

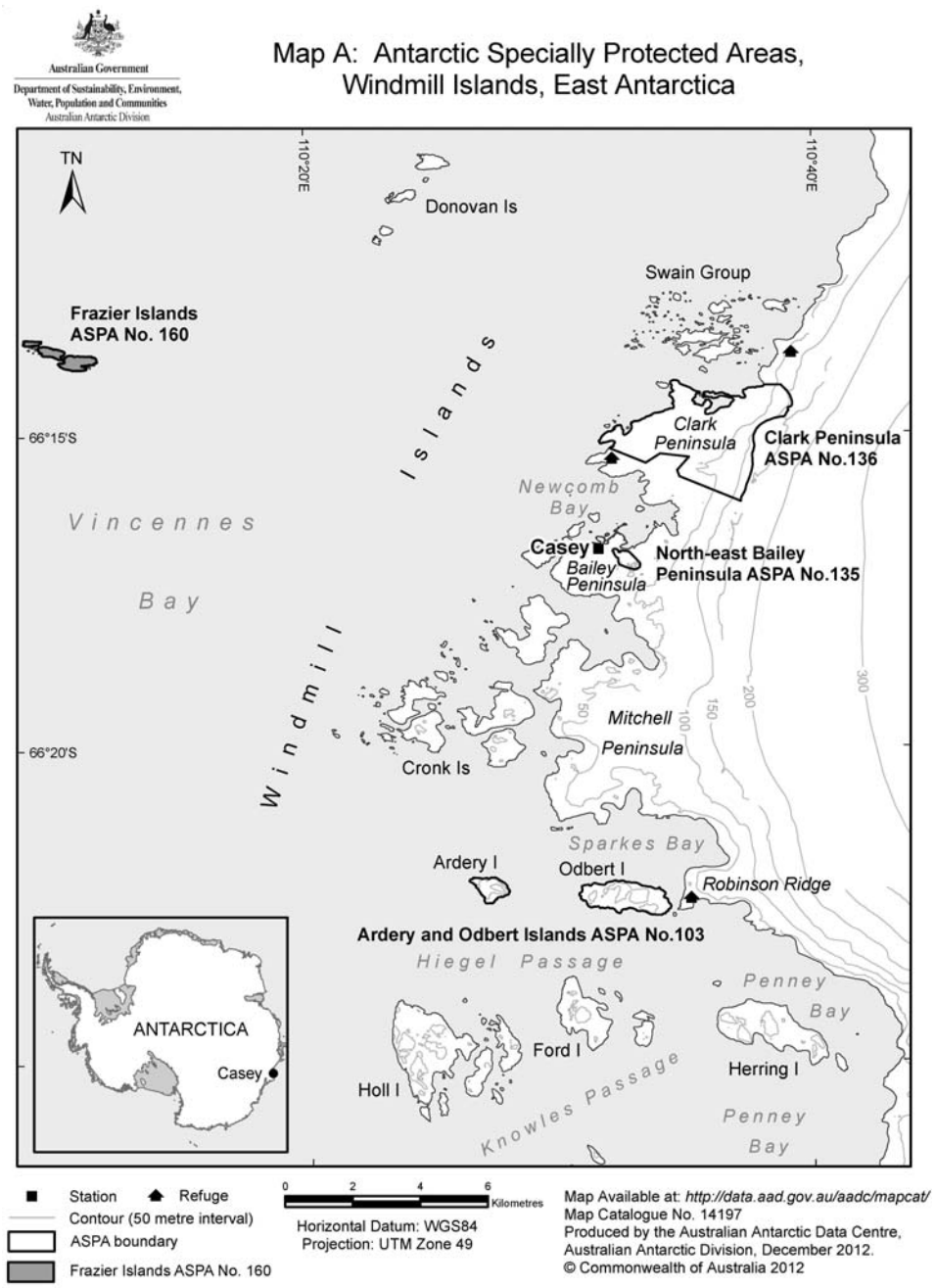

Fig. 1. Map showing the two studied sites of sampling in the Windmill Islands region, Wilkes Land, Antarctica.

soils (Cavicchioli *et al*. 2002; Cowan and Tow 2004; Margesin and Miteva 2011; Gesheva and Negoita 2012; Vasileva-Tonkova *et al*. 2014). Among them, bacteria

dominate in many terrestrial Antarctic ecosystems and play essential role in food chains, biogeochemical cycles and mineralization of pollutants (Nedwell and Walker 1993, 1995; Aislabie *et al*. 2006, 2009; Franzmann *et al*. 1997; Tindall 2004; Walker 2005; Tomova *et al*. 2014; Tytgat *et al*. 2014; Vasileva-Tonkova and Gesheva 2004).

Psychrophilic and psychrotolerant microorganisms have developed various adaptations to extreme environments through physiological and ecological mechanisms (Russell 1998; De Maayer *et al*. 2014). Enzymes are an essential target for adaptation of microorganisms to extreme environments with considerable potential for industrial application (Feller and Gerday 2003; Cavicchioli *et al*. 2011; Kumar *et al*. 2011; Gesheva and Vasileva-Tonkova 2012; Loperena *et al*. 2012; Tropeano *et al*. 2012). Moreover, Antarctic bacteria may develop ecologically important capabilities in response to the impact of different stresses such as halotolerance, metal tolerance and antibiotic resistance, which provide them with selective advantages. Reduced growth rates of organisms in Antarctica promote high concentrations of potential contaminants in Antarctic biota (Mangano *et al*. 2014). Bacteria are generally the first organisms affected by the increased presence of toxic compounds in the Antarctic environment including antibiotics and heavy metals (Lo Giudice *et al*. 2013). Investigation of microbial ecophysiology represents an essential tool for assessing functional diversity and adaptive strategies of microbial communities (Yergeau and Kowalchuk 2008; Vasileva-Tonkova *et al*. 2014).

Although numerous studies on microbial communities colonizing Antarctica, our knowledge on biodiversity, ecology, physiology, genetics, and biosynthetic potential of microbial groups in this extreme environment is still limited. Data about the resistance of the Antarctic bacteria towards heavy metals and antibiotics are still scarce and refer mainly to bacteria in Antarctic seawater (De Souza *et al*. 2006). A better understanding of prokaryotic diversity in Antarctica will contribute to our knowledge of biogeochemical processes in this extreme region and is a prerequisite for possible biotechnological exploitation of the resident microbiota. In the present study, selected bacterial isolates from the Windmill Islands region (East Antarctica) were characterized in more detail for their psychro- and halotolerance, antibiotic and heavy metals resistance, and hydrolytic enzymes production. Highly resistant to heavy metals bacterial strains producers of promising cold tolerant enzymes aims to be detected that could be valuable resource for ecological and biotechnological applications.

Materials and methods

Microorganisms.—Bacterial strains used in the present study were isolated from soil samples taken in the Windmill Islands region, East Antarctica (Gesheva 2010): fellfield (raw mineral soil) near Casey Station, Wilkes Land, and ornitho-

genic soil on Dewart Island, Frazier Islands (Fig. 1). The soil samples were collected from the surface to a depth of 3 cm, in sterile bags, and kept at 4°C during their transport and storage at the Institute of Microbiology in Sofia (Bulgaria). Bacterial strains were isolated from the soil samples on Nutrient agar (NA) and Kuster agar (KA) media. Suspensions of pure cultures mixed with 30% (v/v) glycerol were stored at -80°C.

Molecular identification.—Phylogenetic affiliation of the Antarctic bacteria was determined using 16S rDNA gene sequence analysis. Bacterial genomic DNA was extracted from the bacterial cells using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) in accordance with the manufacturer's instructions. The 16S rRNA gene was amplified from the extracted DNA using universal bacterial primers 8F and 1492R (Weisburg *et al*. 1991). PCR was performed as described in our previous work (Tomova *et al*. 2013). The obtained PCR products were purified and sequenced in Macrogen Inc. (South Korea). The DNA sequences were analyzed for similarities using BLAST program. They were not submitted to a database due to the lack of novelty (they showed less than 3% divergence with the closest phylogenetic neighbor). Multiple sequence alignment and phylogenetic tree construction were done using MEGA software version 4.0 (Tamura *et al*. 2007).

The effect of temperature and NaCl.—The growth of the Antarctic bacteria was monitored in nutrient broth (NB) medium at a temperature range 0–37°C. After inoculation, the strains were cultivated for 2 days at 10, 15, 20, 25, 30 and 37°C, and 5 days at 4°C. Salt tolerance of bacterial strains was tested in NB containing 5% and 10% (w/v) NaCl. Growth of the strains was determined by measuring the optical density at 570 nm (OD_{570}).

Screening for production of hydrolytic enzymes.—The Antarctic bacterial isolates were screened for production of hydrolytic enzymes by the agar diffusion method using specific substrates. The basal mineral agar medium (pH 7.0) contained (%): KH_2PO_4 0.1, $(NH_4)_2SO_4$ 0.5, $MgSO_4$.$7H_2O$ 0.01, NaCl 0.01, and agar 2.0. Inoculated plates were incubated for 3-10 days at 18 ± 2°C. The growth of cultures, zones of clearing around the colonies or color-diffusion zones on respective specific media were used as an indication of the presence of the relevant enzyme activity. Protease (PRO) was determined using 30% (v/v) skim-milk (sm) or 1% (w/v) gelatin (gel); the a-amylase (AMY) – 1% (w/v) insoluble starch; lipase (LipA) – 1% (v/v) Tween 80; ribonuclease (RNAse) or desoxiribonuclease (DNAse) – 0.5% (w/v) RNA or DNA, respectively; cellulase (CEL) – 2% (w/v) carboxymethyl cellulose (cmc), urease (Ure) – 2% (w/v) urea; phytase (PHY) – 0.5% (w/v) sodium phytate; β-glucosidase (β-GLU) – 0.4% (w/v) arbutin, and polygalacturonase (PGAse) – polygalacturonic acid. Endo-cellulase, xylanase (XYL) and chitosanase (CSN) activities were detected in peptone-yeast extract agar (PYA) supplemented with 0.05% (w/v) of each insoluble azurine cross-linked (AZCL) substrates: hydroxyethyl-cellulose (AZCL-HE-cell), AZCL-Xylan and AZCL-Chitosan (Megazyme,

Bray, Ireland). AZCL-substrate degradation appeared as a blue zone around the colony as a result of substrate hydrolysis when the chromophore compounds are released in the agar medium. The β-galactosidase (β-GAL) activity was determined by the appearance and intensity of blue-colored colonies during growth on LB agar with X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) as an inducer (Karasová *et al*. 2002).

Antibiotic susceptibility test.—The Antarctic bacteria were checked for antibiotic resistance with the following antibiotics: ciprofloxacin (Cp, 5 µg), gentamicin (G, 10 µg), amikacin (Am, 30 µg), tobramycin (Tb, 10 µg), novobiocin (Nb, 5µg), lincomycin (L, 15 µg), tetracycline (T, 30 µg), ampicillin (A, 10 µg), chloramphenicol (C, 30 µg), vancomycin (V, 30 mg), erythromycin (E, 15 µg), kanamycin (K, 30 µg), and cefazolin (Cfz, 30 µg).

Antibiotic susceptibility of the strains was assayed following the Kirby-Bauer disc diffusion method (Bauer *et al*. 1969) on peptone-yeast extract agar (PYA) medium. Aliquots of each bacterial suspension (grown exponentially in NB) were spread on the surface of PYA plates. Antibiotic discs (BUL BIO, NCIPD, Ltd, Sofia, Bulgaria) impregnated with known amounts of antibiotics were placed aseptically on the surface of the inoculated plates and incubated at 18 ± 2°C for 24 h. After incubation, the organisms were classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zones (including the disc) given in standard antibiotic disc chart.

Heavy metals resistance tests.—The Antarctic bacteria were screened for their heavy metals resistance patterns using the agar well diffusion method (Hassen *et al*. 1998). Seven heavy metals: Cr, Cu, Ni, Co, Cd, Zn, and Pb were used as salts: $K_2Cr_2O_7$, $CuSO_4{\cdot}5H_2O$, $NiCl_2$, $CoCl_2{\cdot}6H_2O$, $CdSO_4{\cdot}8H_2O$, $ZnSO_4{\cdot}7H_2O$ and $Pb(CH_3COO)_2{\cdot}3H_2O$, respectively. In a preliminary test, 0.05% (w/v) metal salt solutions were prepared in distilled water and sterilized in boiling water bath for 20 min. Sterile PYA plates were prepared and wells (7 mm in diameter) were punched by sterile borer. After inoculation of the plates with the indicator cultures (exponentially grown on NA), 100 µl of each metal salts solution were added into the wells. After incubation of the plates at 18–20°C for 48 h, the inhibition (sterile) zones were measured as an indicator of resistance/sensitivity. Zones were recorded as the distance from the edge of the zone to the edge of the well. Isolates showing clearance zone of one mm or less were considered as resistant strains (Rani *et al*. 2010).

The Minimum Inhibitory Concentration (MIC) of the Antarctic bacteria was determined by gradually increasing or decreasing the heavy metals concentration at the following cationic concentrations (mM): Cu, 0.2 to 32; Cd, 0.14 to 14; Ni, 3.8 to 38; Cr, 1.4 to 17; Zn, 0.2 to 17; Co, 0.2 to 21; Pb, 1.3 to 26. The MIC value was defined as the lowest concentration of the metal ion at which a visible clear zone of one-two mm around the well was observed after incubation of the plates at 18–20°C for 48 h (Sabdono *et al*. 2012).

Plasmid profiling.—Plasmid DNA was extracted from the bacterial cultures grown in NB using the commercial GenElute Plasmid Midiprep Kit (Sigma-Aldrich) in accordance with manufacturer's instructions. Agarose gel electrophoresis was performed in horizontal slab gel of 0.7% agarose submerged in Tris acetate running buffer for at 70 V for 2 h. Plasmid DNA bands were stained with ethidium bromide (0.5 µg/ml) for 15 min and visualized by UV transilluminator. Approximate sizes of plasmids were calculated from logarithmic plots against reference plasmids of DNA loader supercoiled (SIGMA D-5292).

Results

Molecular identification.—BLAST analysis showed affiliation of the sequences of the Antarctic strains with the sequences from the database with high percent of similarity (99–100%). Phylogenetic analysis based on 16S rRNA gene sequencing revealed affiliation of the bacterial isolates to the phyla *Gammaproteobacteria* (genus *Stenotrophomonas*), *Firmicutes* (genera *Bacillus* and *Lysinibacillus*) and *Actinobacteria* (genus *Micrococcus*) (Table 1). The constructed neighbor-joining tree confirmed the phylogenetic position of the strains among their closest neighbors (Fig. 2).

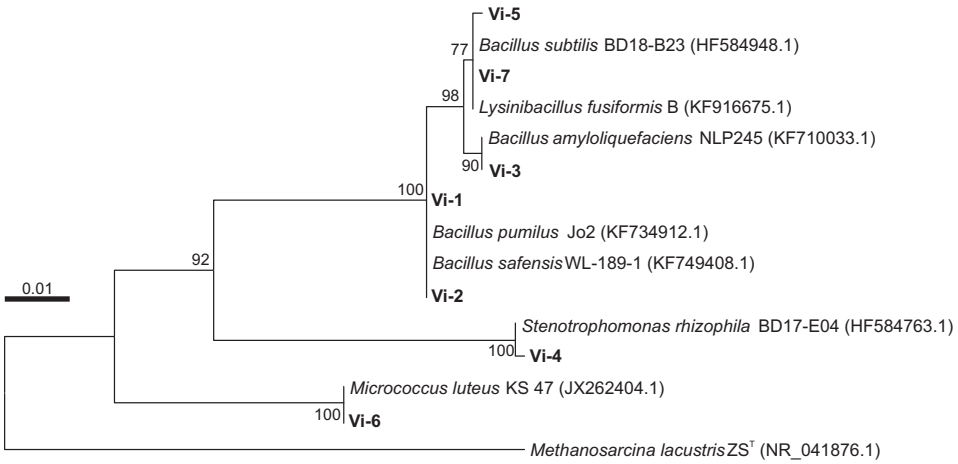

Fig. 2. Neighbor-joining tree showing the phylogenetic position of the Antarctic isolates among their closest neighbors. Bar, 1 % substitutions in nucleotide sequence. Bootstrap values greater than 70% confidences are shown at branching points. The archaeon *Methanosarcina lacustris* was used as an out-group. Accession numbers are given in parentheses.

Psychro- and halotolerance.—We found that the tested Antarctic bacteria are psychrotolerant (psychrotrophs) able to grow at both +4°C and above 20°C (Table 1). Four strains showed highest growth rate at 25–30°C, and three strains – at

Table 1

Some characteristics of the Antarctic soil bacteria

Isolate code	Isolation medium	Location	Temperature (°C) for:		Closest related species / Accession number / Similarity (%)
			Isolation	Maximal growth rate	
Vi-1	NA	Casey Station, Wilkes Land	12	30	*Bacillus pumilus* Jo2 / KF734912.1 / 100
Vi-2	NA		12	30	*Bacillus safensis* WL-189-1 / KF749408.1 / 99
Vi-3	NA		12	25–30	*Bacillus amyloliquefaciens* NLP245 / KF710033.1 / 99
Vi-4	NA	Dewart Island, Wilkes Land	12	25–30	*Stenotrophomonas rhizophila* BD17-E04 / HF584763.1 / 99
Vi-5	KA		25	37	*Bacillus subtilis* BD18-B23 / HF584948.1 / 99
Vi-6	KA		25	37	*Micrococcus luteus* KS 47 / JX262404.1 / 99
Vi-7	KA		25	37	*Lysinibacillus fusiformis* B / KF916675.1 / 99

Table 2

Hydrolytic enzymes activities of the Antarctic soil bacteria

Isolate code	PRO (sm)	PRO (gel)	AMY	RNAse	CEL (cmc)	CEL HE-cell	XYL	PGAse	PHY	β-GLU	β-GAL
Vi-1	+ +	+	–	+ +	–	+ +	+ + + +	+	–	–	–
Vi-2	+ + +	–	–	–	±	+ +	+ + + +	–	–	–	–
Vi-3	+ + +	+ + +	+	+	–	–	+ + + +	–	+	+ + +	–
Vi-4	+ + +	+ + +	–	–	+	–	–	–	+	+ + +	–
Vi-5	+ + +	–	+	–	±	+ + + +	–	–	–	–	+
Vi-6	–	–	–	–	–	–	–	–	–	–	–
Vi-7	–	+ + +	–	+	–	+ + + +	+ + + +	–	–	–	–

Symbols: ±, zone <1 mm; +, zone 1–2 mm; + +, zone 3–4 mm; + + +, zone 5–9 mm; + + + +, zone ≥ 10 mm; –, no zone observed. The β-GAL activity was measured by the intensity of blue-colored colonies. Lipase, urease, DNAse and chitosanase activities were negative for all isolates and are not included in the table. Abbreviations of the enzymes are indicated in Materials and methods section.

37°C. All strains were able to tolerate up to 10% NaCl in the growth medium and can be considered moderately halotolerant.

Detection of hydrolytic enzymes production.—The Antarctic strains were screened for hydrolytic enzymes activities on solid media supplemented with specific substrates (Table 2). The results revealed that the tested strains, except for Vi-6 strain, were able to produce at given conditions wide range of hydrolytic enzymes. Strain Vi-3 showed broadest spectrum of hydrolytic enzymes activities (seven enzyme activities of the 15 tested) followed by strains Vi-1 (six enzyme activities), Vi-4 and Vi-5 (five enzyme activities), Vi-2 and Vi-7 (four enzyme activities). No production of any of the tested enzymes was observed by strain Vi-6. None of the studied isolates showed lipase, urease, DNAse and chitosanase activities.

Antibiotic resistance.—The susceptibility of the Antarctic bacteria was tested towards 13 antibiotics and the results are shown in Table 3. Two strains were

Table 3

Antibiotic susceptibility of the Antarctic soil bacteria expressed by inhibition zones (mm in diameter)

Strains	Antibiotic												
	Cp	G	Am	Tb	Nb	L	T	A	C	V	E	K	Cfz
Vi-1	S (34)	S (28)	S (37)	S (30)	I (18)	R (–)	S (26)	S (30)	S (22)	S (29)	S (30)	S (29)	S (45)
Vi-2	S (45)	S (30)	S (38)	S (29)	S (27)	S (40)	R (–)	S (40)	S (25)	S (29)	I (18)	S (27)	S(35)
Vi-3	S (34)	S (38)	S (45)	S (36)	S (30)	R (15)	R (9)	R (–)	R (–)	R (11)	R (10)	R (–)	R (–)
Vi-4	S (39)	I (18)	S (25)	R (12)	R (–)	R (–)	I (20)	R (–)	I (20)	R (–)	S (24)	S (22)	R (–)
Vi-5	S (44)	S (35)	S (46)	S (38)	S (27)	R (12)	S (35)	R (–)	S (30)	S (35)	S (33)	S (35)	S (45)
Vi-6	S (28)	S (30)	S (40)	S (25)	S (35)	S (38)	S (30)	S (45)	S (35)	I (20)	S (40)	S (30	S (35)
Vi-7	S (42)	S (35)	S (44)	S (30)	S (26)	I (16)	S (30)	S (30)	S (30)	S (30)	S (28)	S (40)	S (55)

Degree of susceptibility: S, sensitive (≥21 mm); I, intermediate (16–20 mm); R, resistant (≤15 mm).

found to be multi-antibiotic resistant: strain Vi-3 – to eight antibiotics, and strain Vi-4 showing resistance to six antibiotics and intermediate susceptibility to three antibiotics. Strain Vi-5 was found to be resistant to lincomycin and ampicillin, and Vi-1 and Vi-2 strains were single antibiotic resistant to lincomycin and tetracycline, respectively. Strains Vi-6 and Vi-7 were sensitive to all used antibiotics.

Heavy metals resistance.—The Antarctic bacteria were tested for resistance towards seven heavy metals, and MICs for each metal ion were determined. As can be seen in Fig. 3, bacterial strains showed a varying response to the tested heavy metals. MICs of the metal ions were determined in the range: for Cu 1.6–8.0 mM; for Zn 0.35–10.4 mM; for Ni 1.7–6.9 mM; for Cd 0.085–0.85 mM; for Co 0.42–8.5 mM; for Cr 1.4–3.4 mM, and for Pb 5.3–15.8 mM. Highest MIC values of each metal ion were determined as follows: up to 15.8 mM for Pb (strain Vi-1),

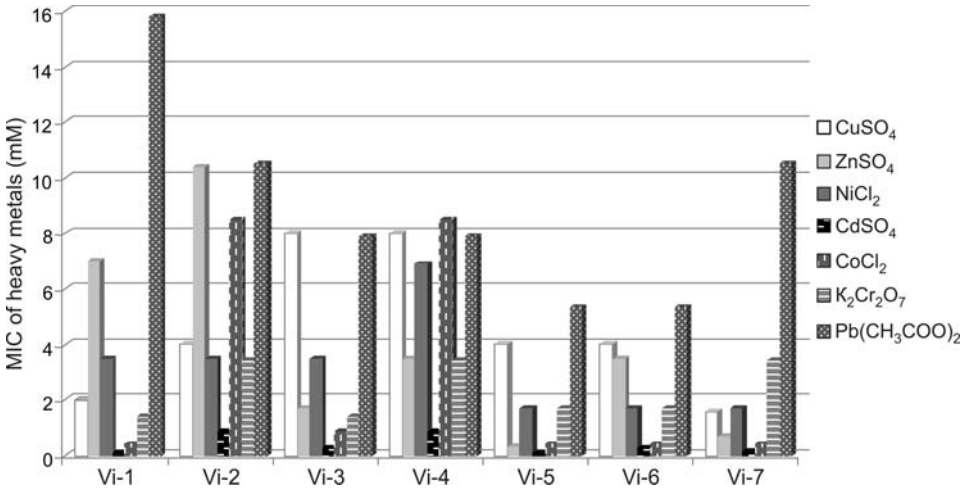

Fig. 3. Minimum Inhibitory Concentration (MIC) of heavy metal ions against the Antarctic bacteria.

10.4 mM for Zn (strain Vi-2), 8.5 mM for Co (strains Vi-2 and Vi-4), 8.0 mM for Cu (strains Vi-3 and Vi-4), 6.9 mM for Ni (strain Vi-4), and 3.4 mM for Cr (strains Vi-2, Vi-4 and Vi-7). All tested strains were found to be resistant (MICs above 1.0 mM) to Pb, Cu, Ni and Cr ions, five strains were resistant to Zn ions, and two strains – to Co ions. All strains were sensitive to Cd ions with MICs determined below 1.0 mM (in the range 0.08–0.85 mM). Strains Vi-2 and Vi-4 showed multiple resistance to six metal ions followed by Vi-1, Vi-3 and Vi-6 strains with resistance to five metal ions, and Vi-5 and Vi-7 strains – to four metal ions.

Plasmid testing.—The Antarctic bacteria were tested to determine if metal/ antibiotic resistance genes are plasmid encoded. Plasmid DNA, probably encoded metal/antibiotic resistance genes, was detected and isolated only in Vi-4 strain: four plasmid bands with molecular size 2.4, 3.2, 5.6 and 6.35 kb in Vi-4 strain were observed (Fig. 4). The other tested strains carried no detectable plasmids although their resistance to some antibiotics and heavy metals, suggesting location of the resistance genes on chromosomes.

Discussion

In this study, we characterized selected heavy metal resistant bacterial isolates from soils in the Windmill Islands region, Wilkes Land, East Antarctica. After performing phylogenetic analysis based on 16S rRNA gene, the belonging of the strains to the phyla *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* was established. These three phyla are among the most often encountered bacterial groups in Antarctica (Aislabie *et al*. 2006; Peeters *et al*. 2011). The abundance of *Firmicutes* phylum is favored by greater availability of nutrients such as ornithogenically-influenced soils (Aislabie *et al*. 2009).

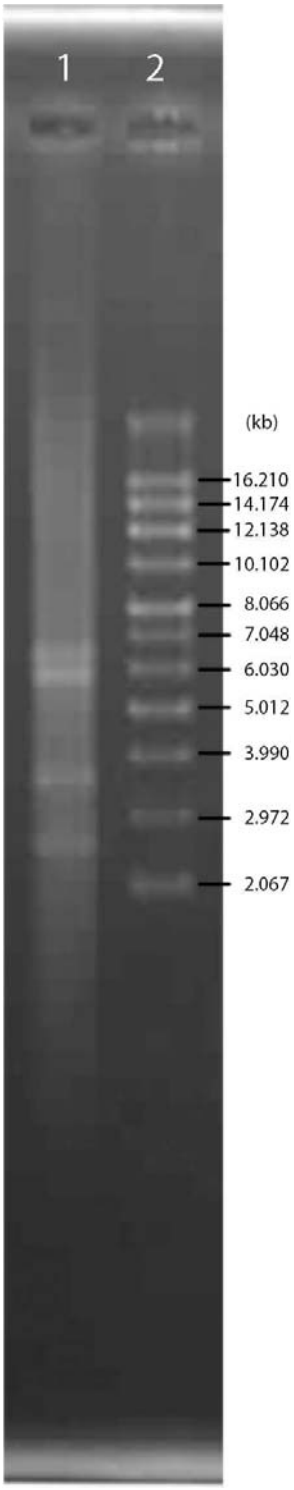

Fig. 4. Agarose gel electrophoresis of plasmid DNA isolated from Vi-4 strain. Line 1: plasmids from Vi-4 strain; Line 2: DNA ladder (11 supercoiled fragments).

Finding that the tested Antarctic bacteria are predominantly psychrotolerant was expected as they have been reported as more abundant in cold ecosystems than obligate psychrophiles (Helmke and Weyland 2004), that occur probably in response to the wide temperature fluctuations in the Antarctic ecosystems. All tested strains were able to tolerate up to 10% NaCl in the growth medium and can be considered moderately halotolerant. It could suggest that the halotolerance of microbial strains is due to permanent impact of high salinity sea water during formation of soil surface microcenoses in maritime Antarctica. It has been reported that salinity may be the major environmental determinant of microbial community structure rather than extremes of temperatures, pH, or other physical and chemical factors (Tamames *et al*. 2010).

Enzyme activities pattern is a useful tool for assessing functional diversity of soil microbial communities, responsible for soil organic matter turnover in their natural habitats (Ferrer *et al*. 2007). Antarctic bacteria have been reported to produce a range of polymer degrading enzymes (Kumar *et al*. 2011; Loperena *et al*. 2012; Tropeano *et al*. 2012), but few studies on ribonuclease and phytase production by Antarctic bacteria have been reported (Reddy *et al*. 1994; Park and Cho 2011). Detection of a variety of biopolymer-degrading enzymes produced by the tested Antarctic bacteria suggested their key role in the degradation of the organic matter in their natural environments. Our study revealed promising bacterial strains that could be a valuable source of industrially important cold active proteases, cellulases, xylanases, β-gucosidases, phytases. As the complete degradation of cellulose requires the activity of diverse enzymes, isolation of cellulotic enzymes active over a wide range of temperatures is highly desirable (Soares *et al*. 2012). In this regard, cellulase and xylanase producing strains Vi-1, Vi-2, Vi-3, Vi-5 and Vi-7 would be of particular interest.

Of the tested strains, Vi-3 and Vi-4 were found to be multi-antibiotic resistant, and Vi-6 and Vi-7 – sensitive to all used antibiotics. Antibiotic-resistant bacteria are widespread in the environment, especially in aquatic habitats (Kümmerer 2009), and even in habitats that seem unlikely to have been exposed to anthropogenic antibiotics impact (Séveno *et al*. 2002). Antibiotic resistance genes are often located on plasmids that are able to be horizontally transferred into diverse bacterial populations contributing to the widespread dissemination of antibiotic resistance in the environment (Davison 1999; Michaud *et al*. 2004). Antibiotic-resistant bacteria have also detected in cold environments such as sandstones in McMurdo valley, Antarctica (Siebert *et al*. 1996); the Arctic permafrost subsoil in Siberia (Mindlin *et al*. 2008); near Palmer Station, Antarctica (Miller *et al*. 2009); West Antarctica King George Island (Wong *et al*. 2011); glacier environments in the Arctic, Antarctic, Central Asia, North and South America (Segawa *et al*. 2013).

Although there are no currently acceptable concentrations of metal ions, which can be used for distinguishing metal-resistant and metal-sensitive bacteria, strains that were able to grow above 1.0 mM concentration of metal ions were considered

resistant (Malik and Jaiswal 2000). We found that all tested Antarctic strains are resistant towards Pb, Cu, Ni and Cr ions, five strains – to Zn ions, and two strains – to Co ions. Microorganisms have developed a variety of mechanisms to tolerate the presence of heavy metals in their habitats (Silver 1996; Bruins *et al*. 2000; Nies 2003; Spain and Alm 2003; Haferburg and Kothe 2007). As suggested by Shruti *et al*. (2012), varying response of the tested bacteria to the heavy metal ions might be due to the difference in their cell wall composition or to variations in the resistance mechanisms. Different resistance mechanisms developed in bacteria could serve as a basis for their use in bioremediation approaches (Filali *et al*. 2000; Malik 2004). In this regard, Vi-2 and Vi-4 strains showing high resistance to most of the tested heavy metals are especially promising. High heavy metals resistance of these strains may suggest that they overproduce some multidrug resistance efflux pumps that are known to be involved in bacterial resistance to a wide range of toxic compounds (Pages *et al*. 2008). Metal resistance of the studied Antarctic bacteria associated with their ability to produce hydrolytic enzymes makes them promising for application in bioremediation of heavy metals polluted sites. A model of mixed strains culture could be developed with potential for wastewater treatment in cold and temperate climates. Highly sensitive to Cd and Co ions Vi-1, Vi-5 and Vi-7 strains would be promising in developing biosensors to detect these most toxic heavy metals in environmental samples.

We detected plasmid DNA (four plasmid bands) only in Vi-4 strain. The resistance to heavy metals in bacteria is usually associated with plasmids, which also encode resistance to antibiotics, although a direct correlation between antibiotic resistance and heavy metals resistance of the strains tested in this study cannot be established. Clustered resistance genes are more likely to simultaneously pass to other bacteria in the environment (Filali *et al*. 2000; Lawrence 2000; Spain and Alm 2003). Thus, in an environment with multiple stresses, for example antibiotics and heavy metals, it would be more beneficial to the survival of bacteria to acquire resistance to both stresses. Horizontal transfer of resistance genes between bacteria of different species and genera occurs easily and frequently in nature (Silver 1996; Coral *et al*. 2005). The presence of bacterial plasmids has also been reported for natural microbial communities of the marine ecosystem in Antarctica (Kobori *et al*. 1984; De Souza *et al*. 2006).

In conclusion, the present work reveals promising psychrotolerant and halotolerant Antarctic bacteria with high multiple heavy metals resistance that could be used as producers of industrially important enzymes and in biological treatment of heavy metals contaminated wastewaters in cold and temperate climates. Further studies are underway to assess the potential of the isolated bacterial strains for their biotechnological applications.

Acknowledgements.—The authors thank the National Fund for Scientific Research for financial support of this work (Contract DNTS / Ukraine 01/1-2012). We would like to thank also the reviewers: Prof. Tony Walker and Prof. Andrzej Tatur for carefully examining our paper.

References

AISLABIE J., JORDAN S., AYTON J., KLASSEN J.L., BARKER G.M. and TURNER S. 2009. Bacterial diversity associated with ornithogenic soil of the Ross Sea region, Antarctica. *Canadian Journal of Microbiology* 55: 21–36.

AISLABIE J.M., CHHOUR K.-L., SAUL D.J., MIYAUCHI S., AYTON J., PAETZOLD R.F. and BALKS M.R. 2006. Dominant bacteria in soils of Marble Point and Wright Valley, Victoria Land, Antarctica. *Soil Biology and Biochemistry* 38: 3041–3056.

BAUER A.W., KIRBY W.M.M., SHERRIS J.C. and TURCK M. 1966. Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology* 45: 493–496.

BRUINS M.R., KAPLL S. and OETME F.W. 2000. Microbial resistance in the environment. *Ecotoxicology and Environmental Safety* 45: 198–207.

CAVICCHIOLI R., SIDDIQUI K.S., ANDREWS D. and SOWERS K.R. 2002. Low-temperature extremophiles and their applications. *Current Opinion in Biotechnology* 13: 253–261.

CAVICCHIOLI R., CHARLTON T., ERTAN H., MOHD OMAR S., SIDDIQUI K.S. and WILLIAMS T.J. 2011. Biotechnological uses of enzymes from psychrophiles. *Microbial Biotechnology* 4: 449–460.

CORAL M.N.Ü., KORKMAZ H., ARIKAN B. and CORAL G. 2005. Plasmid mediated heavy metal resistances in *Enterobacter* spp. isolated from Sofulu landfill, in Adana, Turkey. *Annals of Microbiology* 55: 175–179.

COWAN D.A. and TOW L.A. 2004. Endangered Antarctic environments. *Annual Review of Microbiology* 58: 649–690.

DAVISON J. 1999. Genetic exchange between bacteria in the environment. *Plasmid* 42: 73–91.

DE MAAYER P., ANDERSON D., CARY C. and COWAN D.A. 2014. Some like it cold: understanding the survival strategies of psychrophiles. *EMBO Reports* 15: 508–517.

DE SOUZA M.J., NAIR S., LOKA BHARATHI P.A. and CHANDRAMOHAN D. 2006. Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic marine waters. *Ecotoxicology* 15: 379–384.

FELLER G. and GERDAY C. 2003. Psychrophilic enzymes: hot topics in cold adaptation. *Nature Reviews Microbiology* 1: 200–208.

FERRER M., GOLYSHINA O., BELOQUI A. and GOLYSHIN P.N. 2007. Mining enzymes from extreme environments. *Current Opinion in Microbiology* 10: 207–214.

FILALI B.K., TAOFIK J., ZEROUAL Y., DZAIRI F.Z., TALBI M. and BLAGHEN M. 2000. Waste water bacteria resistant to heavy metals and antibiotics. Current Microbiology 41: 151–156.

FRANZMANN P.D., DOBSON S.J., NICHOLS S.D. and MCMEEKIN T.A. 1997. Prokaryotic Antarctic biodiversity. *In*: B. Battaglia, J. Valencia and D.W.H. Walton (eds) *Antarctic communities species, structures and survival.* Cambridge University Press, Cambridge: 52–56.

GESHEVA V. 2010. Production of antibiotics and enzymes by soil microorganisms from the Windmill islands region, Wilkes Land, East Antarctica. *Polar Biology* 33: 1351–1357.

GESHEVA V. and NEGOITA T. 2012. Psychrotrophic microorganism communities in soils of Haswell Island, Antarctica, and their biosynthetic potential. *Polar Biology* 35: 291–297.

GESHEVA V. and VASILEVA-TONKOVA E. 2012. Production of enzymes and antimicrobial compounds by halophilic Antarctic *Nocardioides* sp. grown on different carbon sources. *World Journal of Microbiology and Biotechnology* 28: 2069–2076.

HAFERBURG G. and KOTHE E. 2007. Microbes and metals: interactions in the environment. *Journal of Basic Microbiology* 47: 453–467.

HASSEN A., SAIDI N., CHERIF M. and BOUDABOUS A. 1998. Resistance of environmental bacteria to heavy metal. *Bioresource Technology* 64: 7–15.

HELMKE E. and WEYLAND H. 2004. Psychrophilic versus psychrotolerant bacteria – occurrence and significance in polar and temperate marine habitats. *Cellular and Molecular Biology* 50: 553–561.

KARASOVÁ P., SPIWOK V., MALÁ Š., KRÁLOVA B. and RUSSEL N.J. 2002. Beta-galactosidase activity in psychrotrophic microorganisms and their potential use in food industry. *Czech Journal of Food Sciences* 20: 43–47.

KOBORI H., SULLIVAN C.W. and SHIZUYA H. 1984. Bacterial plasmids in Antarctic natural microbial assemblages. *Applied and Environmental Microbiology* 48: 515–518.

KUMAR L., AWASTHI G. and SINGH B. 2011. Extremophiles: A novel source of industrially important enzymes. *Biotechnology* 10: 121–135.

KUMMERER K. 2009. Antibiotics in the aquatic environment – a review. Part II. *Chemosphere* 75: 435–441.

LAWRENCE J.G. 2000. Clustering of antibiotic resistance genes: beyond the selfish operon. *ASM News* 66: 281–286.

LO GIUDICE A., CASTELLA P., BRUNI V. and MICHAUD L. 2013. Response of bacterial isolates from Antarctic shallow sediments towards heavy metals, antibiotics and polychlorinated biphenyls. *Ecotoxicology* 22: 240–250.

LOPERENA L., SORIA V., VARELA H., LUPO S., BERGALLI A., GUIGOU M., PELLEGRINO A., BERNARDO A., CALVINO A., RIVAS F. and BATISTA S. 2012. Extracellular enzymes produced by microorganisms isolated from maritime Antarctica. *World Journal of Microbiology and Biotechnology* 28: 2249–2256.

MALIK A. 2004. Metal bioremediation through growing cells. *Environment International* 30: 261–278.

MALIK A. and JAISWAL R. 2000. Metal resistance in *Pseudomonas* strains isolated from soil treated with industrial wastewater. *World Journal of Microbiology and Biotechnology* 16: 177–182.

MANGANO S., MICHAUD L., CARUSO C., LO GIUDICE A. 2014. Metal and antibiotic resistance in psychrotrophic bacteria associated with the Antarctic sponge *Hemigellius pilosus* (Kirkpatrick, 1907). *Polar Biology* 37: 227–235.

MARGESIN R. and MITEVA V. 2011. Diversity and ecology of psychrophilic microorganisms. *Research in Microbiology* 162: 346–361.

MICHAUD L., DI CELLO F., BRILLI M., FANI R., GIUDICE A.L. and BRUNI V. 2004. Biodiversity of cultivable psychrotrophic marine bacteria isolated from Terra Nova Bay (Ross Sea, Antarctica). *FEMS Microbiology Letters* 230: 63–71.

MILLER R.V., GAMMON K. and DAY M.J. 2009. Antibiotic resistance among bacteria isolated from seawater and penguin fecal samples collected near Palmer Station, Antarctica. *Canadian Journal of Microbiology* 55: 37–45.

MINDLIN S., SOINA V., PETROVA M. and GORLENKO Z. 2008. Isolation of antibiotic resistance bacterial strains from Eastern Siberia permafrost sediments. *Russian Journal of Genetics* 44: 27–34.

NEDWELL D.B., WALKER T.R., ELLIS-EVANS J.C. and CLARKE A. 1993. Measurements of seasonal rates and annual budgets of organic carbon fluxes in an Antarctic coastal environment at Signy Island, South Orkney Islands, suggest a broad balance between production and decomposition. *Applied and Environmental Microbiology* 59: 3989–3995.

NEDWELL D.B. and WALKER T.R. 1995. Sediment-water fluxes of nutrients in an Antarctic coastal sediment: influence of bioturbation. *Polar Biology* 15: 57–64.

NIES D.N. 2003. Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiology Reviews* 27: 313–339.

PAGES D., ROSE J., CONROD S., C_{UINE} S., CARRIER P., HEULIN T. and ACHOUAK W. 2008. Heavy metal tolerance in *Stenotrophomonas maltophilia*. *Plos One* 3: e1539.

PARK I. and CHO J. 2011. The phytase from antarctic bacterial isolate, *Pseudomonas* sp. JPK1 as a potential tool for animal agriculture to reduce manure phosphorus excretion. *African Journal of Agricultural Research* 6: 1398–1406.

PEETERS K., ERTZ D. and WILLEMS A. 2011. Culturable bacterial diversity at the Princess Elisabeth Station (Utsteinen, Sŕr Rondane Mountains, East Antarctica) harbours many new taxa. *Systematic and Applied Microbiology* 34: 360–367.

RANI M.J., HEMAMBIKA B., HEMAPRIYA J. and KANNAN V.R. 2010. Comparative assessment of heavy metal removal by immobilized and dead bacterial cells: A biosorption approach. *African Journal of Environmental Science and Technology* 4: 077–083.

REDDY G.S.N., RAJAGOPALAN G. and SHIVAJI S. 1994. Thermolabile ribonucleases from antarctic psychrotrophic bacteria: detection of the enzyme in various bacteria and purification from *Pseudomonas fluorescens*. *FEMS Microbiology Letters* 122: 211–216.

RUSSELL N.J. 1998. Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications. *Advances in Biochemical Engineering/Biotechnology* 61: 1–21.

SABDONO A., RADJASA O.K. and UTOMO H.S. 2012. Screening of multi-metal resistances in a bacterial population isolated from coral tissues of Central Java coastal waters, Indonesia. *International Journal of Oceanography and Marine Ecological System* 1: 11–23.

SEGAWA T., TAKEUCHI N., RIVERA A., YAMADA A., YOSHIMURA Y., BARCAZA G., SHINBORI K., MOTOYAMA H., KOHSHIMA S. and USHIDA K. 2013. Distribution of antibiotic resistance genes in glacier environments. *Environmental Microbiology Reports* 5: 127–134.

SEVENO N.A., KALLIFIDAS D., SMALLA K., VAN ELSAS J.D., COLLARD J.-M., KARAGOUNI A.D., WELLINGTON E.M.H. 2002. Occurrence and reservoirs of antibiotic resistance genes in the environment. *Reviews in Medical Microbiology* 13: 15–27.

SHRUTI M., BALI G. and SARANYA S.K. 2012. Lead biosorbtion by a bacterium isolated from industrial effluents. *International Journal of Microbiology Research* 4: 196–200.

SIEBERT J., HIRSCH P., HOFFMANN B., GLIESCHE C.G., PEISSL K. and JENDRACH M. 1996. Cryptoendolithic microorganisms from Antarctic sandstone of Linnaeus Terrace Asgard Range: diversity, properties and interactions. *Biodiversity and Conservation* 5: 1337–1363.

SILVER S. 1996. Bacterial resistances to toxic metal ions- a review. *Genetics* 179: 9–19.

SOARES F.L. Jr., MELO I.S., DIAS A.C. and ANDREOTE F.D. 2012. Cellulolytic bacteria from soils in harsh environments. *World Journal of Microbiology and Biotechnology* 28: 2195–2203.

SPAIN A. and ALM E. 2003. Implications of microbial heavy metal tolerance in the environment. *Reviews in Undergraduate Research* 2: 1–6.

TAMAMES J., ABELLAN J.J., PIGNATELLI M., CAMACHO A. and MOYA A. 2010. Environmental distribution of prokaryotic taxa. *BMC Microbiology* 10: 1–14.

TAMURA K., DUDLEY J., NEI M. and KUMAR S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24: 1596–1599.

TINDALL B.J. 2004. Prokaryotic diversity in the Antarctic: the tip of the iceberg. *Microbial Ecology* 47: 271–283.

TOMOVA I., GLADKA G., TASHYREV A. and VASILEVA-TONKOVA E. 2014. Isolation, identification and hydrolytic enzymes production of aerobic heterotrophic bacteria from two Antarctic islands. *International Journal of Environmental Sciences* 4: 614–625.

TOMOVA I., LAZARKEVICH I., TOMOVA A., KAMBOUROVA M. and VASILEVA-TONKOVA E. 2013. Diversity and biosynthetic potential of culturable aerobic heterotrophic bacteria isolated from Magura Cave, Bulgaria. *International Journal of Speleology* 42: 65–76.

TROPEANO M., CORIA S., TURJANSKI A., CICERO D., BERCOVICH A., CORMACK W.M. and VAZQUEZ S. 2012. Culturable heterotrophic bacteria from Potter Cove, Antarctica, and their hydrolytic enzymes production. *Polar Research* 31: 18507.

TYTGAT B., VERLEYEN E., OBBELS D., PEETERS K., DE WEVER A., D'HONDT S., DE MEYER T., VAN CRIEKINGE W., VYVERMAN W. and WILLEMS A. 2014. Bacterial diversity assessment in Antarctic terrestrial and aquatic microbial mats: a comparison between bidirectional pyrosequencing and cultivation. *PLoS ONE* 9: e97564.

VASILEVA-TONKOVA E. and GESHEVA V. 2004. Potential for biodegradation of hydrocarbons by microorganisms isolated from Antarctic soils. *Zeitschrift für Naturforschung* 59c: 140–145.

VASILEVA-TONKOVA E., ROMANOVSKAYA V., GLADKA G., GOULIAMOVA D., TOMOVA I., STOILOVA-DISHEVA M. and TASHYREV O. 2014. Ecophysiological properties of cultivable

heterotrophic bacteria and yeasts dominating in phytocenoses of Galindez Island, maritime Antarctica. *World Journal of Microbiology and Biotechnology* 30: 1387–1398.

WALKER T.R. 2005. Distribution of oxygen, sulfides and optimum temperature for sulfate reduction in maritime Antarctic sediments. *Polish Polar Research* 26: 215–230.

WEISBURG W.G., BARNS S.M., PELLETIER D.A. and LANE D.J. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173: 697–703.

WONG C., TAM H., ALIAS S., GONZALEZ M., GONZALEZ-ROCHA G. and DOMINGUEZ-YEVENES M. 2011. *Pseudomonas* and *Pedobacter* isolates from King George Island inhibited the growth of foodborne pathogens. *Polish Polar Research* 32: 3–14.

YERGEAU E. and KOWALCHUK G.A. 2008. Responses of Antarctic soil microbial communities and associated functions to temperature and freeze-thaw cycle frequency. *Environmental Microbiology* 10: 2223–2235.

Received 4 September 2014
Accepted 24 November 2014