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MICROBIOLOGY

Potential for resistance to freezing by non-virulent bacteria isolated from Antarctica

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Abstract: Industrial sectors are searching for new compounds to improve the preservation of food and blood, human tissues, and fuels used at low temperatures. Antarctic microorganisms have mechanisms to overcome injuries caused by low temperatures, making them sources of compounds with antifreeze activity. However, it is mandatory that such compounds do not pose a risk to human health. The present study evaluated the potential of Antarctic bacteria to resist freezing, produce virulence factors, their tolerance to physiological pHs/temperature and resistance to antibiotics. Sixty-five isolates were tested for antifreeze compound production, among which, 31 grew after the test. Of these, 3 strains of *Arthrobacter* sp. (356, 358 and 443), one *Psychromonas arctica* (ESH238) and one unidentified strain (363) showed positive results for hemolytic activity. *Psychrobacter* sp. 456 showed proteinase activity. None of the isolates showed resistance to the antibiotics. All isolates were able to grow in one of the three pHs (4, 7 and 8) and/or temperature (36, 38 and 40 ºC). Antarctic bacterial present potential for the production of antifreeze compounds and may be considered safe in industrial processes. The characterization of the genes responsible for virulence factors should be carried out to reinforce the potential applicability of such bacteria.

Key words: extremophilic bacteria, low temperature, virulence factors, hydrolytic enzymes, proteinase, hemolysis.

INTRODUCTION

Psychrophilic microorganisms that inhabit cold environments such as Antarctica have adopted a strategy to circumvent the effects of low temperatures, prevent freezing and keep their liquid cell fluids even below the freezing point (Muñoz et al. 2017). Antifreeze proteins (AFPs) (cryoprotectant agents) are a structurally diverse group of ice-binding proteins produced by microbial cells with unique properties, which may include thermal hysteresis (TH - decrease of the freezing point in the intracellular fluid) (Kristiansen et al. 2005, Muñoz et al. 2017, Białkowska et al. 2020), inhibition of ice recrystallization (IRI) and interaction with membranes and/or membrane proteins (Singh et al. 2014, Kim et al. 2017). AFPs can act at low concentration and is reported that they are 200–300 times more effective in the IRI than other intracellular solutes (Devries 1971, Muñoz et al. 2017). Freezing can be fatal to bacteria, as ice crystals have the ability to destroy cellular components and/or rupture the plasma membrane causing cell death (Provesi & Amante 2015).

There are three forms of action of AFPs on ice crystals: *i)* interference in the ice nucleation process; *ii)* decrease of the freezing temperature of cellular fluids without significantly altering the melting point; *iii)* inhibition of the recrystallization process (Provesi & Amante

2015). Through interference in the nucleation process, AFPs bind to nucleating substances and prevent crystallization from starting (Griffith & Ewart 1995, Hassas & Goff 2012). The decrease of the freezing temperature of body fluids occurs through the process known as thermal hysteresis, where there is an increase in the concentration of antifreeze proteins, reducing the freezing point by up to 3 °C (Pudney et al. 2003, Qiu et al. 2013). On the other hand, inhibition of recrystallization is mediated via protein absorption in various parts of the ice surface through interactions such as Van der Waals forces and hydrogen bridge (Cruz et al. 2009). The use of AFPs in food preservation can decrease up to 500 times the concentration of the use of other cryoprotective substances such as sucrose and sorbitol (Provesi & Amante 2015).

There are several compositions of synthetic agents used to assist in the cryopreservation of materials including cells, human tissues, and fuels. However, these agents are complex to prepare, costly and potential pollutants, thus arousing the need to find more appropriate compounds (Castro et al. 2011). There are some factors that interfere with the functional efficiency of synthetic cryoprotective agents, such as type, concentration, application time and cell differences (animal and plant), making their use more expensive (Castro et al. 2011). The use of synthetic cryoprotective agents to control the freezing of fuels and water from radiators in cold environments is a well-used technique, however it requires a large number of products and appropriate concentrations to fulfill its role, making these processes industrially costly (Jintao et al. 2011). Thus, the use of AFPs from microbial cells may be a sustainable strategy to extend the viability of food, the integrity of diverse types of cells and biological tissues as well as to meet the need to preserve seeds and plant species by the reduction of freezing damage, maintaining the original characteristics of the product (Provesi & Amante 2015).

Antarctica is considered the most isolated continent on Earth, however, it has not escaped the negative impacts of anthropogenic activity. Antarctic marine ecosystems and their endemic faunas are affected on local and regional scales by pollution, and the introduction of exotic species (Aronso et al. 2011). Currently, pollutant compounds of different classes, including PAHs, polychlorinated biphenyls, drugs, and plastics, are arriving in Antarctica through air and sea currents and also through human activities, such as tourist visitation. With a large number of tourists visiting the polar ecosystem, the majority of them making repeated landings, there is great potential for negative environmental impacts on both terrestrial and marine habitats (Aronso et al. 2011, Lacerda et al. 2019). In addition, human-associated microorganisms containing antibiotic resistance genes have reached Antarctica, possibly through military bases, fishing boats, scientific expeditions, and/or ship-borne tourism (Hernández & González-Acuña 2016). According et al. (2020), the human activity has an impact on the local Antarctica microbiota, and strains recovered from regions under anthropic influence were considerably more resistant than those collected from regions that do not suffer human action.

Several microbial virulence factors have been discovered in Antarctica, such as extracellular hydrolytic enzymes, including phospholipases (enzymes capable of degrading epithelial membranes and destroying physiological substrates important for health) and proteinases (enzymes with proteolytic activity on proteins of the host's immune system, including immunoglobulins and cytokine) (D'Eça Júnior et al. 2011, Andreola et al. 2016). On the other hand, other biosynthetic pathways showing hemolytic activities (promoting lysis of

red blood cells), growth at different physiological pHs, as well as at different temperatures during a peak of infection, may facilitate the spread of these organisms in several tissues of the human organism and, consequently, increase the damage caused by these microbial cells (Guerra et al. 2007, Andreola et al. 2016, Nithya et al. 2010).

In this sense, the confirmation of the absence of virulence factors in bacteria potentially applicable to the production of compounds intended to be used in health and/or food sectors represents an important strategy to prevent the spread of these strains. Thus, the aims of the present work were to evaluate the biotechnological potential of bacteria isolated from Antarctica to potentially produce antifreeze compounds, in addition to confirm the lack of virulence factors as well as resistance to antibiotics of commercial use.

MATERIALS AND METHODS

Strains and standardization of cell growth

Sixty-five bacteria previously isolated by Silva et al. (2018) from marine invertebrates and waterlogged soil samples from Antarctica were evaluated. These samples were collected during an expedition to Antarctica in the austral summer of 2013/2014 by the MycoAntar - Brazilian Antarctic Program team. Purified bacteria preserved at -80 °C in 20% glycerol were obtained from Biological and Agricultural Pluridisciplinary Research Center (CPQBA) at the Campinas State University (UNICAMP) (Supplementary Material – Table SI).

Bacterial strains used in this work were grown on R2A agar (0.05 g L^{-1} yeast extract, 0.05 g L^{-1} peptone, 0.05 g L^{-1} Casamino acid, 0.05 g L^{-1} glucose, 0.05 g L^{-1} starch, 0.03 g L^{-1} sodium pyruvate, 0.03 g L^{-1} K_2HPO_4, 0.005 g L^{-1} $MgSO_4$, 1.5 g L^{-1} agar in pH 7) (Margesin et al. 2012). After growth in solid medium, each isolate was transferred to 5 mL of R2B (R2A without agar), in triplicate and samples were incubated at 15 ºC for 15 days. After growth, all strains had the optical density (OD) standardized at 0.08 in a spectrophotometer at 600 nm. All other experiments were carried out in triplicate and using the same OD of 0.08.

Potential microorganisms showing antifreeze activity

Bacterial isolates under study were exposed to the protocol described by Wilson et al. (2012), with modifications, to evaluate their freeze resistance. After standardization of cell growth, as previously described, cell suspensions (3 mL) were centrifuged at 4000 rpm for 5 minutes. Supernatants were discarded and cell pellets were added of sterile 0.9% saline solution and 20% glycerol, separately, both in triplicate. After 30 days at -80 °C, cells were thawed and cultivated in R2B culture medium in triplicate. Microbial growth after freeze of strains without 20% glycerol was considered as a positive result for the potential to antifreeze activity. The strains *Escherichia coli* ETEC 5041-1 and *Cryobacterium* sp. JCM 19503 (which showed AFP activity, according to Singh et al. 2014) were used as negative and positive controls, respectively.

Assessment of virulence potential

The strains considered positive in the antifreeze-producing assay were evaluated for virulence potential. Isolates were subjected to phospholipase, proteinase, protease and hemolytic activity assays. In addition, bacterial growth at physiological temperature and different pH and resistance to commercial antibiotics were assessed. The strains *E. coli* ETEC 5041-1 and *Staphylococcus aureus* CBMAI 485 were used as positive controls. All assays were performed in triplicate.

Phospholipase activity was determined according to Kantarcioğlu & Yücel (2002), with modifications. Egg yolk agar (EYA) culture medium containing 0.5% meat extract, 1% peptone, 1.5% bacteriological agar and 10% egg yolk emulsion was used. The egg yolk emulsion was prepared with 50% saline (0.9%) and 50% egg yolk. The bacterial isolate was inoculated onto the Petri dish and incubated at 15 °C for 15 days.

Proteinase activity was determined according to Andreola et al. (2016), with modifications. R2A culture medium supplemented with 0.2% bovine serum albumin (BSA) (Sigma – Aldrich, St Louis, USA) was used. The bacterial isolate was inoculated onto the Petri dish and incubated at 15 °C for 15 days.

Hemolytic activity was determined as described by Suhet et al. (2011) with adaptations. Blood agar medium (Kasvi) plus 5% human blood was used. The isolate was inoculated in the Petri dish and incubated at 15 °C for up to 15 days.

The halo formation around the colony was scored as positive for phospholipase, proteinase and hemolytic activities.

For all isolates, the enzyme activity index was measured. The diameter of the bacterial colonies and the halos formed were measured. The extracellular enzymatic activity index (EI) was calculated by the relation between the average diameter of the enzymatic halo (EH) and the average diameter of the colonies (DC) (EI = EH/DC). The level of enzyme production for each bacterial isolate was established by the following score: *non-producer* - those colonies that did not show visible halo; *moderate producer* - those colonies with a halo between 1.0-2.0 mm in diameter; *high producer* - those colonies with a halo size greater than 2.0 mm in diameter (Andreola et al. 2016).

High temperature growth assay was performed as described by Xavier et al. (2007), with adaptations. All isolates were grown in R2A culture medium at 15 ºC (as a control), and at 36 ºC, 38 ºC and 40 ºC, during 15 days of incubation. Growth at different pHs was performed as described by Hoyos et al. (2019) with adaptations. R2A culture medium was prepared using KH_2PO_4 buffer (0.2 M) prepared at pHs 4.0 (vaginal), 7.0 (blood) and 8.0 (urine) (Proksch 2018). Strains were inoculated onto Petri dish in triplicate and incubated at 15 ºC for 15 days. The isolates that were able to grow under different conditions (temperature and pH) were considered tolerant and possibly capable of infecting the human body.

Commercial antibiotic resistance assay

The strains considered positive in the antifreeze activity assay were evaluated for commercial antibiotic resistance. Bacteria were grown in R2B culture medium until OD 0.008, then 1.0 μL aliquots were transferred to 1.5 mL tubes for centrifugation at 4.000 rpm for 5 minutes. Supernatants were discarded and 1.0 mL of sterile distilled water was added to each tube to suspend pelleted cells. A sterile swab was soaked in the bacterial suspension and seeded in a Petri dish with Mueller Hinton (MH) agar culture medium. Sterile filter paper discs (2 mm of diameter) were immersed in commercial antibiotic solutions, including amoxicillin + potassium clavulanate 500 + 125 mg L^{-1}, azithromycin 500 mg mL^{-1} and chloramphenicol 250 mg L^{-1} and were arranged in triplicates in the MH. The plates were incubated at 15 ºC for 15 days. Microbial growth around the disk with antibiotic (without the formation of an inhibition halo) was indicative of resistance to the antibacterial agent. On the other hand, formation of halo around the bacterial colonies was indicative of antibiotic sensitivity (BRCAST 2017).

Statistical analysis

Statistical analysis was performed according to Ottoni et al. (2020), by using Variance analysis (ANOVA) and Tukey (at 5% probability) in software PAST version 2.17c.

RESULTS AND DISCUSSION

For the freeze resistance assay, 31 (47.7%) out of the 65 bacterial isolates showed the ability to resist freezing at -80 °C in the absence of the cryoprotectant (glycerol 20%), demonstrating the potential as producers of antifreeze compounds (Table I).

The strains potentially producing AFPs were affiliated to nine distinct genera recovered from soil biofilm, marine invertebrate, waterlogged soil, and sea sponge (Table I). Members of the genus *Leifsonia* (isolated from sediment) were tested for AFP activity in the work performed by Singh et al. (2014), however, no AFP activity in the culture medium was found. Regarding the *Arthrobacter* genus, AFP encoding gene was found in strains isolated in China (Sun et al. 2016) and Antarctic soil (See-Too et al. 2017), yet no protein expression test was performed to confirm their activities. *Arthrobacter* (Muñoz et al. 2017), *Psychrobacter* (isolated from permafrost), *Rhodococcus, Pseudoalteromonas* (Lorv et al. 2014) and *Psychromonas* (Kim et al. 2017) have already been reported as AFP producers and/or resistant to freeze-thaw stress. In the same way, *Cellulophaga* and *Carnobacterium* genera have been reported as cold-adapted bacteria in cold environments (Leisner et al. 2007, Bowman 2017). However, as far as we know, this is the first report on the potential AFP production by bacteria associated with marine invertebrates in cold environments, as well as the first report of anti-freezing potential *Cellulophaga* genus.

The extracellular cryoprotective agents induce water to escape from the intracellular medium to the extracellular medium, preventing the formation of ice inside the cell during freezing by the increase in osmolarity (Hubálek 2003). The medical industry also shows interest in AFPs for preserving organs and blood (Felle & Gerday 2003). In agriculture, there is a need to preserve seeds and plant species. The possibility of introducing the gene responsible for the production of AFP into vegetables grown in cold regions has also been discussed to produce transgenic organisms that are able to withstand low temperatures (Aragão 2003, Provesi & Amante 2015). However, studies still need to be deepened in relation to the toxicity of this substance in the human body, despite the fact that consumption already exists through natural fish and vegetables from cold environments (Wen-Li et al. 2005, Crevel et al. 2002).

Raymond et al. (2008) found a protein produced by *Psychromonas ingrahami* isolated from sea ice that showed 71% similarity with ice-binding protein, a protein similar to AFPs, however, this protein was not characterized. A study conducted by Duman & Olsen (1993) was the first one to discover AFPs produced by *Rhodococcus erythropolis*. According to Singh et al. (2014), *Pseudoalteromonas* species were able to produce enhancer molecules such as polysaccharides (trehalose), amino acids (glycine and betaine), and salts (NaCl), compounds which may increase the TH of AFPs. Yim et al. (2008), , filed a patent for an exopolysaccharide derived from *Pseudoalteromonas* sp. strain CY01 (KCTC 12867BP) from polar regions. This exopolysaccharide showed ability to cryoprotect cells without presenting cytotoxicity. The inventors claimed that this new compound can replace conventional cryoprotective agents that exhibit cytotoxicity when used in high concentrations.

Table I. Bacterial isolates responses for freezing resistance, antibiotics resistance, virulence factors and growth under different values of temperature and pH.

Strain	Identification (16S rRNA)	Antifreeze-producing (-80 °C)	Antibiotic resistance (halo in mm/±SD)			Virulence potential (Index/±SD)								
			Amo+Clav	Azi	Clo	Proteinase	Phospholipase	Hemolytic activity	Temperature (mm/±SD)			pH (mm/±SD)		
			F = 0.3573	F = 0.04658	F = 1.681	F = 0.6777 / *p* = 0.5227 *p* = 0.5623 *p* = 0.7829			F = 0.5832	F = 0.2462	F = 0.0472	F = 6.511	F = 4,862	F = 5.8342
			p = 0.7017	*p* = 0.9545	*p* = 0.1985				*p* = 0.9601	*p* = 0.00268	*p* = 0.00937	*p* = 0.006424		
									36 °C	**38 °C**	**40 °C**	**4.0**	**7.0**	**8.0**
Controls	*Escherichia coli* ETEC 5041-1	-	n.d.			-	-	1.1±0.0	7.6±1.2	13±0.8	9.3±0.9	n.d.		
	Staphylococcus aureus (CBMAI 485)							1.1±0.0	7.6±0.5	11.3±0.9	7.6±0.9	n.d.		
	Cryobacterium sp. (JCM 19503)	+	n.d.			n.d.	n.d.	n.d.	n.d.			n.d.		
268	*Leifsonia antarctica*	+				-	-	*	*	*	*	5.0±1.4	5.0±0.5	7.3±0.5
356	*Arthrobacter* sp.		**			1.7±0.4	2.6±0.5			6.0±0.0	6.0±0.8	6.3±0.5		
358	*Arthrobacter psychrochitiniphilus*					*	*			7.0±0.8	7.0±0.5	7.3±0.5		
359	NI		43.3±2.3	29.0±2.9	41.0±2.9	*	23±1.2	1.2±0.0	5.0±0.8	1.0±0.0	1.0±0.0	7.6±0.5	7.7±0.8	7.6±0.5
363						1.9±0.0	*	1.3±0.0	3.1±0.5	1.0±0.5	1.0±0.0	8.6±0.5	8.7±0.9	7.3±0.5
366	*Arthrobacter* sp.		**				4.3±0.5	16±3.2	19.6±2.3	7.0±0.8	7.0±0.8	7.6±0.5		
367	NI						*	2.0±0.0	1.0±0.0	*	7.0±0.0	7.0±0.8		
369			36.6±2.3	33.3±2.0	27.3±1.2		2.6±0.5			*		*	6.6±0.5	7.3±0.5
372							*			-		9.0±0.0	9.0±0.5	8.0±1.4
380							4.6±1.2		2.0±0.8	8.0±0.8	8.6±1.2	8.6±0.5		
381			**				6.6±0.5			7.6±0.5	8.6±0.4	8.3±0.5		
382	*Arthrobacter stackebrandtii*								*		9.0±0.0	7.0±0.8		
383	NI		40.0±0.0	30.0±0.0	40.0±0.0				6.3±1.2	2.3±0.5	1.0±0.0	6.6±0.5	7.3±0.5	6.6±0.5
386			35.0±0.0	35.0±0.0	35.0±0.0				*	*	*	7.3±0.5	3.3±0.9	7.0±0.0
408	*Arthrobacter* sp.		38.3±2.3	34.0±0.8	40.0±0.0				*	1.0±0.5	1.0±0.0	7.3±0.5	9.0±0.8	6.0±0.8
409	*Psychrobacter* sp.		36.0±2.3	36.0±2.1	35.6±1.6				5.3±1.2	2.6±0.5	1.0±0.0	8.3±1.2	8.6±0.5	8.3±0.5
411	*Arthrobacter* sp.		41.3±1.2	35.3±2.9	31.0±0.4				*	*	*	9.0±0.8	8.6±1.2	7.6±0.5
422	*Psychrobacter* sp.		36.3±2.6	31.6±2.3	43.6±0.9				5.0±0.0	1.0±0.5	1.0±0.0	9.3±0.5	9.0±0.2	9.0±0.8

Table I. Continuation.

Strain	Identification (16S rRNA)	Antifreeze-producing (-80 °C)	Antibiotic resistance (halo in mm/±SD)			Virulence potential (Index/±SD)								
			Amo+Clav	Azi	Clo	Proteinase	Phospholipase	Hemolytic activity	Temperature (mm/±SD)			pH (mm/±SD)		
423	*Arthrobacter* sp.			**					4.3±0.5	*	*	9.0±0.0	9.3±0.9	8.0±1.6
425	*Cellulophaga fucicola*		21.6±2.3	35.0±4.0	39.0±1.4				*	3.3±0.5	6.3±0.4	7.3±0.5	9.0±1.4	6.6±0.0
426	*Rhodococcus* sp.		35.0±2.3	35.0±0.0	35.0±0.0				5.3±1.2	0.6±0.5		6.3±0.9	8.0±0.0	7.0±0.0
427	*Carnobacterium* sp.								3.6±0.9	*		9.0±0.8	9.3±0.4	9.0±0.0
428	NI			**						*		6.6±0.5		
443	*Arthrobacter* sp.							1.0±0.0	1.0±0.0	7.6±0.5	8.0±0.8	7.0±0.0		
444	*Sporosarcina* sp.	+	32.0±5.8	32.3±3.2	41.6±1.2			*	*	5.3±1.2	*	*	10±0.0	9.0±0.0
445	*Psychrobacter* sp.			**				7.3±0.5	*	*		*	7.3±0.4	7.3±0.5
449	*Carnobacterium* sp.		40.0±0.0	40.0±0.0	40.0±0.0							*	7.6±0.4	7.0±0.8
450	*Arthrobacter* sp.			**					5.0±1.4			*	8.3±0.9	12±2.1
456	*Psychrobacter fozii*			1.2±0.1					1.0±0.0	6.0±1.6	2.3±0.0	7.6±0.5		
ES26	*Pseudoalteromonas* sp.		12.3±5.8	18.6±0.9	11.0±0.0	-			9.3±0.5	4.5±0.8	1.0±0.0	4.0±2.1	6.6±0.4	2.3±0.5
ESH238	*Psychromonas arctica*			**				1.5±0.1	*	*	1.0±0.0	*	*	6.6±0.5

n.d.: not determinated.
***: lack of growth.**
****: total inhibition (the strain was completely inhibited by the antibiotic (there was no growth on the plate).**
CLO: chloramphenicol ≥17 mm sensitive / <17 mm resistant.
AMO + CLAV: amoxicillin + potassium clavulanate ≥19 mm sensitive / <19 mm resistant.
AZI: azithromycin ≥21 mm sensitive / <21 mm resistant.
The values for the effect of pH, temperature and virulence index, are the average of three replicated measurements. One-way ANOVA with Dunnet post hoc (F and *p*) was used to determine if there were statistically significant differences (p <0.05) over time in response between assays.
NI: not identified.

Extracellular enzymes such as proteases, that are able to degrade host cell proteins including those related to the immune system such as cytosine and immunoglobulins, as well as phospholipases, which can degrade epithelial membranes and destroy important physiological components, are studied as virulence factors (Kokkayil & Dhawan 2015). Some organisms have other infection strategies such as hemolytic activity and growth favored under different physiological pHs and temperatures, facilitating the dissemination of these organisms in different tissues of the human body and consequently increasing the damage caused by them (Pereira et al. 2016). Some microorganisms may have hemolytic activity, disrupting the host red blood cells and causing serious health problems *in vivo* (Rörig et al. 2009).

The 31 isolates potentially resistant to freezing were subjected to virulence potential assessment, including phospholipase, proteinase, protease and hemolytic activities, in addition to the evaluation of bacterial growth under physiological temperatures, different pH values and resistance to commercial antibiotics. Six out of 31 isolates (19%) showed positive results for virulence potential (Table I). The strains *Arthrobacter* sp. 356 and 443, *Psychromonas arctica* ESH238 (Figure 1a) and the unidentified (NI) strains 359 and 363 showed hemolytic activity in medium supplied with red cells, with virulence index ranging from 1.2 to 1.9, thus being considered moderate producers of virulence factor. The strain safety is one of the main criteria when selecting strains for biotechnological use (Leuschner et al. 2010). The hemolytic activity of *Arthrobacter* species isolated from cold environments has been evaluated by a few studies which found no positive results (Gangwar et al. 2011, Nam et al. 2011, Mogrovejo-Aruas et al. 2020), however, none of them assessed microorganisms from the Antarctic continent. Concerning *Psychromonas arctica,* no research has been done so far to determine if these bacteria have hemolytic activity.

One strain, *Psychrobacter fozii* 456 showed proteinase activity (virulence index of 1.2) in medium supplied with albumin (Figure 1b). Species of this genus have been isolated from diverse sources, such as food, including fish, poultry, meat, (Ortiz-Alcántara et al. 2016) and environment, more frequently associated to cold and non-polar environments (Lasa & Romalde 2017). In addition, members of *Psychrobacter* are considered rare opportunistic pathogens in humans and have been found in diverse clinical samples such as urine, eyes, wounds, brain tissues, cerebrospinal fluid, and human blood (Ortiz-Alcántara et al. 2016). Nonetheless, the pathogenic species described so far does not include *Psychrobacter fozii*, whose virulence has been not tested either, which makes the results of the present study of great relevance. No isolate showed phospholipase activity since there was no halo formation in the culture medium.

Statistical analyses showed that there was a statistically significant difference for hemolytic activity of the *Arthrobacter* sp. 443, compared to other isolates that showed the same activity, due to a higher index related to its activity. However, this isolate was considered a moderate producer of virulence factor (Table I).

All isolates were able to tolerate and/or grow at different pH and temperature values, including isolates that showed hemolytic and proteinase activities (356, 359, 363, 443, ESH238 and 456) (Table I). The index of cell growth at temperatures of 36 °C, 38 °C and 40 ºC ranged from 2.6 to 23.0; 0.6 to 16.0; and 1.0 to 19.6, respectively. The index of cell growth at pH 4.0, 7.0 and 8.0 ranged from 4.0 to 9.3; 2.3 to 10.0; and 2.3 to 12.0, respectively. The results

demonstrate the pathogenic potential of these bacteria if an infection occurs. The results of the statistical analyses showed that there was a significant difference in the assays for the effect of temperature on microbial growth, due to the high growth of *Arthrobacter* sp. 366, the only strain that surpassed the growth rate of controls *E. coli* and *S. aureus* (Table I). However, we can say that it is an isolate able to grow at higher temperatures, in comparison to the Antarctic environmental temperature, but that did not show any virulence potential regarding proteinase, phospholipase and hemolytic activity.

Power et al. (2016) performed a study with *E. coli* strains recovered from untreated sewage in Antarctic environment where 11 genes found to be involved with virulence and antibiotic resistance factors were associated with mobile genetic elements. The authors demonstrated that sewage disposal in Antarctic marine environment is introducing non-indigenous microorganisms, specifically strains of *E. coli* with pathogenic characteristics. In the work carried out by Grillová et al. (2018), the authors identified four *Escherichia albertii* strains, isolated from feces of live animals on James Ross Island, Antarctica, and Isla Magdalena, Patagonia, encoding virulence factors. These included cytolethal and intimin distension toxins, bacteriocin determinants as well as were molecules able to cause cell cycle arrest in human cell. On the other hand, reports of studies that evaluated virulence factors such as proteinase, phospholipase and hemolytic activities from bacteria recovered from Antarctic samples were not found.

Microorganisms from environments with low temperatures have already been reported with the ability to tolerate a wide range of pH values, ranging from 2.0 to 14.0 (Dhakar & Pandey 2016). Ganzert et al. (2011) identified two novel bacterial strains of *Arthrobacter kerguelensis* and *Arthrobacter psychrophenolicus*, isolated from moss-covered soil from Livingston Island, Antarctica, near the Bulgarian station St Kliment Ohridski, that were tolerant to wide pH amplitude, ranging from 4 to 9.5. However, studies that have evaluated the tolerance of bacterial isolates from Antarctica to mesophilic temperatures, ranging from 36 °C to 40 °C, are scarce. Research generally assesses optimal bacterial growth, which varies between 15 °C to 30 °C (Humphry et al. 2001, Nevot et al. 2007, Rinnan et al. 2009), emphasizing the importance of the results presented in our study.

Although Antarctica has been considered one of the most pristine ecosystems in the world, human activities, especially in regions close to research stations, have increased significantly (Brasil- Ministério da Ciência e Tecnologia 2009). Anthropogenic activities at research stations have been identified as a source of environmental pollution that can cause public health problems (Lacerda et al. 2019). The virulence of microorganisms recovered from Antarctica with potential for use in biotechnology sectors, including products intended for humans, can pose a threat to health and, for this reason, it is crucial to carry out a prior investigation of the associated risks.

Regarding the resistance to commercial antibiotics, results obtained herein demonstrated that all isolates were sensitive to the tested antibiotics, *i.e.* amoxicillin + potassium clavulanate, azithromycin and chloramphenicol (Table I, Figure 1c). It is important to note that for 15 strains evaluated (approximately 50%), the antibiotics tested were so effective that the bacteria did not even grow on the plate during the trial. Statistical analysis showed that there was a significant difference between microbial growth on the plates containing the antibiotics among the tested isolates (Table I).

Figure 1. Examples of strains evaluated in the virulence potential assessment. *Psychromonas arctica* ESH238 showed hemolytic activity (a). *Psychrobacter fozii* 456 showed proteinase activity (b). Formation of halo of microbial growth inhibition around the disks containing the antibiotics (c).

Even though our strains have been shown to be susceptible to the tested antibiotics, studies have already demonstrated the existence of antibiotic-resistant bacteria isolated from Antarctica, as well as of antibiotic resistance genes in soil, animal feces, seawater, sediment marine, among other samples from the Antarctic continent (Scott et al. 2020, Na et al. 2020, Jara et al. 2020). Power et al. (2016) conducted a study showing that the discharge of untreated sewage into Antarctic environments poses a risk of introducing allochthonous microorganisms into the icy ecosystem. The authors analyzed a variety of Antarctic samples including marine sediments, seawater, marine urchins, Antarctic soft-shelled clam, feces from southern elephant seals, penguins, and Weddell seals. The study concluded that sewage disposal introduces human derived *Escherichia coli* strains containing antibiotic resistance genes and virulence factors, which pose a risk to the diversity of Antarctic native microbial communities.

Anthropic activity in Antarctica has been increasing considerably in recent years, representing a major impact on the resident microbiota. Such increase in human presence, especially during summer, may lead to the appearance of pharmaceuticals on the continent (Hernández et al. 2019). Miller et al. (2009) estimated antibiotic resistance in microorganisms isolated from the Antarctic marine waters and a penguin rookery. The antibiotic resistance was higher among the presumed mesophiles (strains grown at 20 °C but not at 6 °C) than the presumed psychrophiles (stains grown at 6 °C) and increased with proximity to Palmer Station, suggesting that human presence has impacted the natural microbial community of the site. Rabbia et al. (2016) performed a study where *Escherichia coli* strains were isolated from Fildes Peninsula, a site with strong human influence in Antarctica. Strains were recovered from seawater, bird droppings, and water samples from inside a local wastewater treatment plant. Several isolates were resistant to β-lactams (the same antimicrobial class used in our work), aminoglycosides, tetracycline, and trimethoprim–sulfonamide. The authors concluded that the bacterial antibiotic resistance found may be associated with discharged treated wastewater originated from Fildes Peninsula treatment plants.

Na et al. (2020) analyzed the occurrence of Antarctic antibiotic-resistant bacteria using samples of animal feces, soil and sediments collected in Fildes Peninsula. The results showed

that bacteria resistant to antibiotics ciprofloxacin and sulfamethazin were the most abundant, and genera *Pseudomonas* and *Arthrobacter* (genus with virulence potential in our study) were the most dominant, for each of the antibiotics, respectively. In the study by Laganà et al. (2019), the authors found antibiotic resistance against cephalosporins, quinolones and amoxicillin + clavulanic acid in seven strains belonging to the genera *Alteromonas*, *Pseudomonas*, *Pseudoalteromonas* and *Halomonas*, isolated from a polystyrene macro-plastic piece stranded on the shores in King George Island. According to the study, the microbial resistance to some classes of antimicrobial compounds can be explained by the increasing presence of humans and the introduction of these compounds in the Antarctic region.

The results of our study showed that some of the strains potentially producing antifreeze compounds may, in fact, present virulence factors to humans. However, these isolates may be in lower abundance in the Antarctic environment (19% of the strains analyzed in this study) and may offer only moderate pathogenic potential. Thus, considering the increasing number of people visiting and performing research activities in the Antarctic continent, the assessment of the anthropogenic impact in this pristine environment should be taken into account more rigorously. Nonetheless, microorganisms recovered from Antarctica can still be safely explored for future bioprospecting studies of compounds of biotechnological interest.

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SUPPLEMENTARY MATERIAL

Table SI. Freeze-resistant Antarctic strains, respective sources of isolation and data from the sampling site.

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