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Antarctic bioprospecting: in pursuit of microorganisms producing new antimicrobials and enzymes

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Abstract

Intensive efforts are being made to find new compounds with antimicrobial activity. The search for these novel bio-products in sparsely explored environments may be the key to providing solutions for many emerging problems. Antarctic environments are valuable locations for bioprospecting. In this study, 63 cold-adapted bacterial strains of 6100 different colony morphotypes were isolated from Antarctic seawater samples around South Shetland and Deception islands. Strains were selected based on cold-active antimicrobial production and were grouped into 11 operational taxonomic units by internal spacer region-PCR and amplified ribosomal DNA restriction analysis. Isolates arbitrarily named 2D, 5D, and 6D were closely related to *Halomonas titanicae*, with 99.8, 98.9, and 96.7% identity according to 16S rDNA sequencing, and 99.7, 99.3, and 98.3% according to *gyrB* region sequence analysis, respectively. The isolate 18SH was closely related to *Candida sake* (99.2%) based on sequence analysis of the ITS1–5.8S rDNA–ITS2 and D1/D2 domain of 26S rDNA. Antimicrobials produced by isolates 2D, 5D, and 6D exhibited a low-molecular weight (< 6000 Da) and stability in wide pH and temperature ranges. When tested against foodborne and phytopathogenic bacteria, selected isolates exhibited a wide spectrum of activity. This work reports the isolation and identification of cold-adapted microorganisms with the ability to produce antimicrobial compounds with potential application in the pharmaceutical or in cold-chain management in the food industry. The current results highlight the potential of the Antarctic environment as a valuable and underexploited source of new antimicrobial molecules with exceptional properties for different biotechnological applications.

Keywords Bioprospection · Antarctica · Psychrophile · Antimicrobial · Enzyme

Introduction

Research and development of novel antimicrobial compounds are a worldwide priority owing to factors such as increasing resistance in bacterial pathogens caused by inappropriate and abusive use of antibiotics, the emergence of new pathogens, and a shortage of pharmaceutical companies involved in drug discovery (Davies and Webb 1998; O´Brien et al. 2004; Wratschko 2009; Lodato and Kaplan 2013). At present, pathogen resistance causes high mortality rates in hospitals as well as placing a major financial burden on healthcare institutions (Reddy et al. 2015). Despite advances in the therapy of bacterial infections, the frequency of bacterial resistance to common antibiotics continues to climb. Common examples of such pathogens include methicillin-resistant *Staphylococcus aureus* (Reynolds et al. 2004), penicillin-resistant *Streptococcus pneumoniae* (Karchmer 2004), and beta-lactam-resistant *Pseudomonas aeruginosa* (Paterson 2006). Therefore, the pursuit of novel antimicrobial compounds that are effective against resistant bacteria has become an urgent priority globally (Wenzel 2004; Spížek et al. 2010; Alves et al. 2012).

Microbes have been revealed to be an excellent source of new natural drugs. Efforts to discover novel drugs have focused on unexplored microbial ecosystems (Fischbach and Walsh 2006; Dionisi et al. 2012a), since most antibiotics

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or scaffolds for chemical modification have been derived from natural products (Singh and Barrett 2006; Newman and Cragg 2007).

Antarctica is the largest of the poorly explored pristine areas on Earth, and bioprospecting from this region represents one of the most valuable strategies for discovering new biologically active substances or new producer microorganisms (Kennedy et al. 2008; Rojas et al. 2009; Liu et al. 2013; Chávez et al. 2015), since its microbial diversity has been poorly explored (Teixeira et al. 2010; Loperena 2012; Pearce et al. 2012). In this context, given their size, complex topography, water circulation patterns, as well as high biological activity and biodiversity, Argentinian marine environments contain numerous niches that are particularly valuable for the bioprospection of microbial potential. In support of this, different biotechnologically relevant bioactive compounds have already been isolated from microorganisms belonging to these natural pristine ecosystems (Reed 2004; Arnau et al. 2016).

Among the extremophiles, psychrophile and psychrotolerant microorganisms, which thrive at low temperatures, are a valuable and interesting source of novel bio-products, including antimicrobials (Horikoshi 1995; Brizzio et al. 2007; Sánchez et al. 2009; Margesin and Feller 2010; Vollú et al. 2014; Hamid and Benazir 2015; Tomova et al. 2015). Such antimicrobial metabolites exhibit the peculiar feature of being active at low temperatures and thus have a range of potential applications in medicine, food preservation, and the agronomic and cosmetic industries (Sánchez et al. 2009).

In this study, soil, sediment, and water samples from around the Antarctic Peninsula were collected and used for isolating and screening microbial specimens with outstanding antimicrobial and enzyme-producing abilities at the laboratory scale. The produced antimicrobial compounds were preliminarily characterized based on their spectrum of activity against drug-resistant human pathogens as well as phytopathogens of current concern. Further tests, such as of net charge, sensitivity to enzymatic treatment, relative molecular weight, and pH stability, were used to investigate the nature of these inhibitory compounds. This work emphasizes the potential of cold-adapted microorganisms as successful producers of cold-active antimicrobial compounds with potential application in the pharmaceutical, cosmetic, and chilled-food preservation industries. As a supplementary outcome, the results support the assertion that the Antarctic is a valuable natural reservoir of potentially new antimicrobials that warrants attention and environmental surveillance.

Materials and methods

Sampling

Sampling was carried out during an Antarctic campaign aboard the oceanographic ship ARA-Puerto Deseado during summer 2011. This Antarctic campaign covered the Antarctic Peninsula and South Shetland Islands area. Samples of sea water and marine sediment were collected from different locations. The geographic coordinates of each sampling site were fed into a Geographic Information System (Quantum GIS 2.18.10), which allowed the creation of a detailed map with accurate delineation of the sampling area (Fig. 1, Table 1). Sea water was collected at different depths using 5-L Niskin bottles coupled to conductivity–temperature–pressure probes for in situ determination of salinity, temperature, and depth. Water was immediately filtered through a set of three filters with different pore sizes (0.8, 0.45, and 0.22 µm). Filters were conserved in 5 mL of sterile sea water and stored at − 20 °C. In addition, sediment portions were collected using a snapper, a sediment-collecting device that collects mud from the ocean floor. Samples were kept at − 20 °C until processing.

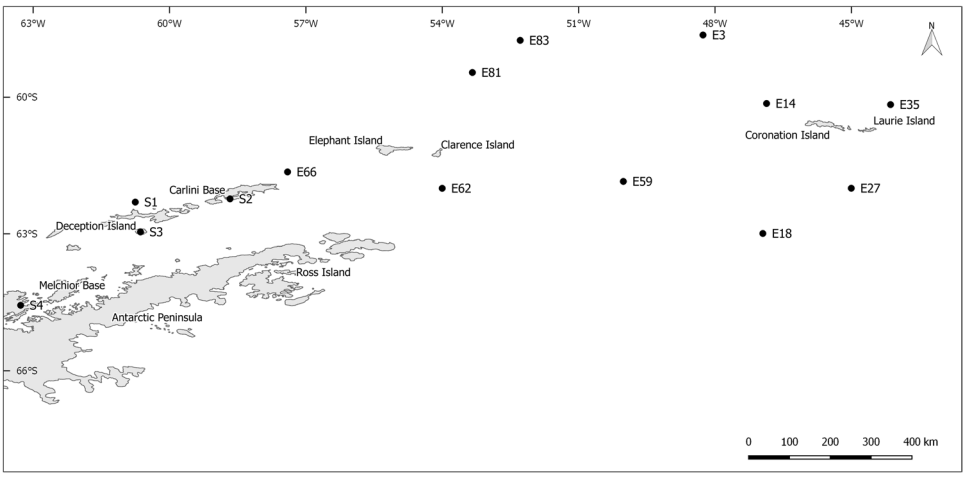

Fig. 1 Sampling sites during Antarctic campaigns aboard the oceanographic ship ARA-Puerto Deseado in summer 2011 and 2014

Table 1 Sampling sites surrounding areas of Antarctic Peninsula

Sample	Position		Depth (m)	Salinity (PSU)
	Latitude (S)	Longitude (W)		
E3	58° 38,077	48°15,732	50	34,50
E14	60°8335	46°51,811	30	34,17
E18	62°59,569	46°56,741	50	34,34
E27	61°59,984	44°59,995	50	34,35
E35	60°9779	44°8129	50	34,26
E45	57°59,422	45°27,440	100	34,33
E59	61°51,006	50°00,874	80	34,69
E62	62°00,073	54°00,020	50	34,29
E66	61°38,483	57°24,143	50	34,14
E81	59°27,468	53°20,155	100	34,26
E83	58°45,109	52°17,046	50	33,50
Shetland Islands	62°18,235	60°45,164	100	ND
Carlini Base	62°13,971	58°40,121	30	ND
Melchior Base	64°33,779	63°16,554	4	ND
Deception Island	62°57,549	60°38,293′	4	ND

PSU g Kg^{-1}, *ND* not determined

Isolation of psychrophilic/psychrotolerant bacteria

During this study, 15 points for sampling sea water were selected (Fig. 1). Sediment samples were taken from South Shetland and Deception islands and soil samples from Carlini and Melchior Argentinian Antarctic bases. For the isolation of both psychrophilic and psychrotolerant bacteria, filters were gently mixed in sea water and kept for 24 h under conditions with continuous mixing. Subsequently, 100 µL of each sample was serially diluted and poured onto plates containing LB-SW medium (5 g L^{-1} pancreatic casein hydrolysate, 5 g L^{-1} yeast extract, 24.6 g L^{-1} $MgSO_4 \cdot 7H_2O$, 23.4 g L^{-1} NaCl, 2.9 g L^{-1} $CaCl_2 \cdot 2H_2O$, and 1.5 g L^{-1} KCl, pH 7.3) and R_2A (0.25 g L^{-1} pancreatic casein hydrolysate, 0.5 g L^{-1} acid casein hydrolysate, 5 g L^{-1} yeast extract, 0.25 g L^{-1} peptone from meat, 0.5 g L^{-1} glucose, 0.5 g L^{-1} starch, 0.3 g L^{-1} K_2HPO_4, 0.3 g L^{-1} sodium pyruvate, 24.6 g L^{-1} $MgSO_4 \cdot 7H_2O$, 23.4 g L^{-1} NaCl, 2.5 g L^{-1} $CaCl_2 \cdot 2H_2O$, and 1.5 g L^{-1} KCl, pH 7.2). Solid media were prepared by adding 15 g L^{-1} bacteriological-grade agar.

Sediment and soil samples (1 g) were resuspended in 20 mL of sterile marine water with continuous mixing for 24 h, serially diluted, and plated as described above. Plates were incubated at 5 and 15 °C for 7–14 days and bacterial colonies were counted by macroscopic examination. Those colonies showing a distinctive morphotype were selected and transferred by picking them up and placing them onto separate replica plates.

Detection of antimicrobial producers

To identify antimicrobial producers, the antagonism method described by Gratia (1946) and Fredericq (1948) was used, in combination with a modified version of the agar-well diffusion method described by Portrait et al. (1999). Specifically, colonies were picked up from duplicate plates containing either LB-SW or R_2A medium and incubated for 7–14 days at both 5 and 15 °C. Subsequently, plates were overlaid with soft LB medium (0.8% w/v agar) previously inoculated with 10^7–10^9 CFU mL^{-1} *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 25923 as indicator strains. Clear zones (halos) observed around the bacterial colonies after incubation at 37 °C for 24 h were taken to indicate the presence of an inhibitory compound. Once producers had been detected, they were picked out and their antimicrobial production ability was evaluated and compared with that of other indicator bacteria.

Internal transcribed spacer (ITS) amplification by PCR

Isolates were incubated for 7 days in 100-mL flasks containing 20 mL of LB-SW medium at 15 °C in an orbital shaker, with agitation at 200 rpm. DNA extraction was performed in accordance with the work of Sambrook et al. (1989). DNA preparations were visualized to assess their integrity by electrophoresis in 0.8% (w/v) agarose gel in TAE buffer. DNA was stored at 4 °C until PCR amplification.

Antimicrobial producers were grouped according to amplification profiles of the 16S–23S rDNA intergenic spacer region. Internal sequences located between the nuclear ribosomal sequences, especially ITS1 and ITS2, are well known for exhibiting differential rates of nucleotide changes, defining a hypervariable region between preserved sequences (Jensen et al. 1993). Universal primers SM (5′-AAG TCG GGT AGC TAA CAA C-3′) and BR3 (5′-GTC GTA ACA AGG TAG CCG TA-3′) were used to amplify this region, as described by Willems et al. (2001).

Amplified ribosomal DNA restriction analysis

In addition to obtaining the ITS profiles of selected isolates, amplified ribosomal DNA restriction analysis (ARDRA) was also used for isolate differentiation based on enzyme-restriction profiles of the 16S rDNA gene previously amplified by PCR (Malik et al. 2008). *Hae*III and *Pst*I restriction enzymes were used for digesting the amplified 16S rDNA products. To do this, 300 ng of amplicons were diluted to 17.5 µL with sterile distilled water and 2 µL of 10 × buffer was added for each enzyme reaction prior to the incorporation of 0.5 µL of the appropriate enzyme (5–10 U). Tubes were incubated overnight at 37 °C. The digestion reaction was stopped by heating the mixture at 70 °C for 10 min. Finally, restriction products were visualized in 2% (w/v) agarose gels and the isolates were grouped into operational taxonomic units (OTUs) according to their differential restriction profiles.

PCR amplification and phylogenetic analysis

PCR amplification of the 16S rRNA gene was performed using the universal primers 8-27 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492-1509 (5′-GTT TAC CTT GTT ACG ACT T-3′), as previously reported by Sánchez et al. (2009). Amplification was carried out in a Thermocycler Gene Amp PCR System 9700 (Applied Biosystems). Sequencing of amplified DNA fragments was outsourced to Macrogen Inc. (Seoul, South Korea). Sequences were edited with Chromas Lite software (version 1.4) and aligned using Mega 5.2 (Tamura et al. 2004). The 16S rDNA sequences were compared to those previously deposited in Ez-taxon server V 2.1 and NCBI GenBank databases and either belonging to the same genus or representative closely related species were aligned and a similarity matrix was calculated (Maidak et al. 2000). A dendrogram was constructed based on 16S rRNA gene sequences by the neighbor-joining approach and using the Jukes–Cantor correction method (Saitou and Nei 1987) with a bootstrap level of 1000. Owing to limitations in the sequence analysis and databases of 16S rDNA, bacterial isolates were subjected to partial sequencing of the *gyr*B gene (Fukushima et al. 2002), which was amplified by PCR in accordance with the work of Kazunori et al. (2003).

Yeast strain was identified by sequence analysis of the 18S rRNA gene, the ITS1–5.8S–ITS2 chromosomal region, and the D1/D2 domain of the 26S rRNA gene sequence.

The primers used were ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (White et al. 1990; O'Donnell 1993). Almost complete bacterial sequences were obtained and deposited in the NCBI GenBank database under the Accession Numbers KU221086 to KU221088, while yeast isolate was deposited under the accession number MF801627.

Isolate characterization

Isolates were initially characterized by microscopic observation and Gram staining. Cell morphology was examined with the aid of a Nikon Eclipse 80i phase contrast microscope at 1000 × magnification and biochemical profiling was performed using API® systems (bioMérieux, France). The sugar profile was evaluated by API® 50CHB and other physiological features were tested by API® 20Strep and API® Coryne, in accordance with a modified version of the procedure reported by Logan and Berkeley (1984). For the isolate 18SH, the physiological profile was obtained with API® 20AUX. The incubation time in all cases was 7 days at 15 °C, in accordance with the manufacturer's instructions. Growth assays at different temperatures, pH values, and increasing concentrations of NaCl were performed as described by Labrenz et al. (1998).

The susceptibilities of selected antimicrobial producers (isolates 2D, 5D, and 6D) to commonly used antibiotics were tested by the disk diffusion method, in accordance with the instructions of the manufacturer of each test. Tests included the Gram-negative 1, 2, and 3, and Gram-positive A and B series (Laboratorios Britania, CABA, Argentina).

Enzymatic activity

Because of their potential biotechnological application, isolates were also tested according to their enzymatic activity profile. An actively growing culture (10 µL) of each selected isolate containing 10^7 CFU mL^{-1} was spotted onto a specific agar medium as described below. The results were determined based on the observation of either a clear zone or a dark precipitation around the grown colony, depending on the tested activity (see below).

Starch degradation—Nutrient agar medium for analyzing starch degradation was prepared as follows (in g L^{-1}): peptone, 5; yeast extract, 5; NaCl, 5; agar, 15; and soluble starch, 10. Starch hydrolysis was detected by the presence of clear halos around colonies after covering with Lugol iodine solution.

Lipase activity—Nutrient agar medium for analyzing lipase activity was prepared as follows (in g L^{-1}): peptone, 5; yeast extract, 5; NaCl, 5; agar, 15; gum arabic, 20; rhodamine B, 1; and 3% (v/v) olive oil. Lipase activity was detected by exposing the plates to UV light, where an orange–pink fluorescence zone around the colonies indicated a positive result.

Gelatin hydrolysis activity—Nutrient agar medium was supplemented with 12% (w/v) pure gelatin to analyze gelatin hydrolysis activity. Colonies were picked up, placed onto the solid medium, and incubated for 7 days at 15 °C for growth. Gelatin liquefaction indicated a positive reaction.

Chitinase activity—Nutrient agar medium was supplemented with 15 g L^{-1} colloidal chitin to analyze chitinase activity. After 7 days of incubation at 15 °C, strains showing a cleared zone around the colony were taken as positive.

Esterase activity—Medium containing (in g L^{-1}) peptone, 10; NaCl, 5; $CaCl_2$, 4; Tween 80, 1; and agar, 15 was used for testing esterase activity. A positive reaction was considered when an opaque precipitate formed around the colony after growth for 7 days.

Cellulase and xylanase activities—To measure cellulase and xylanase activities, nutrient agar medium was amended with 1% (w/v) carboxymethyl cellulose or 1% (w/v) xylan, respectively. To detect activity on plates, they were overlaid with 1% (w/v) Congo red solution for 30 min and then washed twice with 1% (w/v) NaCl solution. Positive results were considered when there was a clear halo around the colonies.

Influence of growth medium and incubation temperature on antimicrobial production

To check the influence of culture medium on antimicrobial production, individual Erlenmeyer flasks containing different media (LB, LB-SW, M9, or M63) were inoculated (10% v/v) with ~ 10^7 cells mL^{-1} of an overnight culture of each antimicrobial producer strain. Flasks were incubated at 15 °C for 7 days in an orbital shaker, with agitation at 200 rpm. Cells were removed by centrifugation at 8000 × *g*. The influence of medium composition on antimicrobial production was assessed according to the antimicrobial titer from cell-free supernatants. To this end, the serial twofold dilution method described by Mayr-Harting et al. (1972) was applied and results are expressed as arbitrary units per milliliter (AU mL^{-1}), considering the last dilution giving an inhibition zone against *Salmonella enterica* ser. Newport used as the indicator strain (Sánchez et al. 2009).

To evaluate the influence of incubation temperature on antimicrobial production, Erlenmeyer flasks containing 10 mL of LB-SW medium were inoculated with 10% (v/v) of an overnight culture containing ~ 10^7 cells mL^{-1} of each isolate and incubated at different temperatures (5, 15, 20, 25, 30, and 40 °C) for 7 days. In a similar way, the influence of pH on antimicrobial production was tested by adjusting the LB-SW medium's initial pH to different set values, namely, 4.0, 6.0, 9.0, and 10.0, and incubating it at 15 °C for 7 days. The antimicrobial activity in each experiment was determined by the twofold dilution method, as described above.

Characterization of antimicrobial activity

Inhibition spectrum

The antimicrobial spectra of the selected isolates were evaluated against different enteropathogenic and phytopathogenic bacteria by means of antimicrobial diffusion tests on solid (1.5% w/v agar) and semisolid (0.8% w/v agar) culture media. Producer strains were picked up, placed onto LB-SW medium, and incubated at 15 °C for 7 days. After incubation, antimicrobial-producing strains were killed by exposing them to chloroform vapors before inoculating the sensitive strains. Antimicrobial activity was evaluated by the deferred antagonism method, as described above.

Molecular weight estimation

The molecular weight of the antimicrobial compounds was estimated as described by Asensio et al. (1976). To do this, small sterile pieces of dialysis membranes with different molecular weight cutoffs (6000–12,000 MWCO) were deposited on the top of plates containing LB-SW agar medium. Colonies were deposited over dialysis membranes using a blunt toothpick. Plates were incubated for 7–15 days at 15 °C. After incubation, the dialysis membranes containing cells were taken out of the plates and the antimicrobial activity was determined according to the deferred antagonism technique, as described above.

Alternatively, 2 mL of cell-free supernatants were filtered through Centricon (Amicon®) devices with cellulose filtration membranes using different cut-off limits (6000, 10,000, and 30,000 MWCO). Loaded devices were centrifuged at 3000×*g* for 25 min at room temperature. After centrifugation, retentate and flow through portions were subjected to antimicrobial activity determination against sensitive strains as described above.

Net charge determination

The net charge of antimicrobials was tested by electrophoresis in agarose gel by a modified version of the method for the in situ electrophoresis of colicins described by Davies and Reeves (1975). Accordingly, 50 µL of cell-free supernatant with antimicrobial activity (400 AU mL^{-1}) was deposited in the center of a 1% (w/v) agarose gel. Once drops had been absorbed, the gel was placed in a horizontal electrophoresis

device and filled with Tris–acetate buffer (pH 8.0). Electrophoresis was performed for 6 h at 35 V, after which the gel was covered with a thin layer of soft LB medium inoculated with 10^7 CFU mL^{-1} of *Salmonella enterica* ser. Enteritidis and incubated overnight at 37 °C. The net charge was determined by observing the movement of the inhibition zone after the electrophoresis.

Sensitivity to enzymatic treatment

To obtain a preliminary insight into the chemical nature of the produced antimicrobials, cell-free supernatants from selected isolates were treated with different proteases: trypsin (EC.3.4.21.4), type II chymotrypsin from bovine pancreas (EC 3.4.21.1), pronase E (EC 3.4.24.31), and proteinase K (EC 3.4.21.64), to assess their proteinaceous nature. Catalase (EC 1.11.1.6) was also tested to rule out inhibition due to hydrogen peroxide. Lipase (EC 3.1.1.3) and α-amylase (EC 3.2.1.1) treatments were used to determine the presence of lipid or carbohydrate moieties in the antimicrobial compounds. The enzymes were used at a concentration of 25 mg mL^{-1} and prepared in accordance with the manufacturer's instructions. Deferred antagonism procedures were performed in accordance with the work of O'Brien et al. (2004), as depicted above.

Stability to pH and temperature

To estimate the antimicrobial pH stability, the pH of cell-free supernatants was adjusted within the range of 2.0–12.0 by adding either HCl or NaOH, held for 1 h at room temperature, and then readjusted to pH 7.0 to examine the residual inhibitory activity as described above. In the same way, thermostability was determined after freezing/heating cell-free supernatants from − 20 to 85 °C for 1 h, and at 105 °C for 15, 30, 45, or 60 min. After these pH and temperature treatments, activity was measured by the twofold dilution method as described above against *S. enterica* ser. Newport and expressed as AU mL^{-1}. In addition, inhibition of the psychrotolerant *Pseudomonas yamanorum* $8H1^T$ strain (Arnau et al. 2015) was evaluated to determine the antimicrobial activity at a low temperature (8 °C).

Hemolytic, bio-emulsifying, and bio-surfactant activities

Antimicrobial hemolytic activity of cell-free supernatants was evaluated on LB soft-agar medium supplemented with 2% (v/v) of a fresh erythrocyte suspension (Banat 1993; Morán et al. 2002). Ten microliters of a cell-free supernatant with antimicrobial activity (≥ 400 AU mL^{-1}) was deposited over LB-blood agar plates and incubated at 37 °C for 3 days. Cell-free supernatant of *S. aureus* ATCC 29213 was used as a positive hemolytic control (Cooper et al. 1964).

The ability of antimicrobials to be emulsified was also evaluated as described by Morán et al. (2000). Specifically, 2 mL of a cell-free supernatant with antimicrobial activity (≥ 400 AU mL^{-1}) was mixed with 3 mL of kerosene in a test tube and vortexed for 2 min. After 24 h, the proportion of emulsified kerosene and the stability of this emulsion were evaluated. The emulsifying capacity is expressed as the emulsification (E_{24}) index. The E_{24} index is defined as the height of the emulsified layer (mm) divided by the total height of the liquid column (in mm) multiplied by 100 (Iqbal et al. 1995). Emulsification ability was considered stable if the E_{24} index remained ≥ 50% after 24 h (Bosch et al. 1988). In addition, bio-surfactant activity was evaluated by the drop-collapse test (Jain et al. 1991) using mineral and olive oil.

Results

Isolation of Antarctic bacteria

Two different culture media were used to enhance bacterial isolation: one for nutrient-deficient soils (R_2A) and the other for fastidious bacteria (LB-SW). Two different incubation temperatures were used to isolate either psychrophilic (5 °C) or psychrotolerant (15 °C) microorganisms.

In most cases, psychrotolerant specimens were more frequently isolated than psychrophilic ones for each isolation

Table 2 CFU mL^{-1} obtained from water and sediment samples using R_2A and LB-SW solid medium at both, 5 °C and 15 °C

Sample	CFU mL^{-1}			
	LB-SW		R_2A	
	5 °C	15 °C	5 °C	15 °C
E3	1351	4567	1245	4765
E14	672	819	503	949
E18	2568	7818	4722	1285
E27	2758	3442	5356	3255
E35	864	950	740	840
E45	527	1238	698	1322
E59	1145	1102	1029	2405
E62	867	2045	956	1435
E66	1056	3634	1564	2453
E81	1034	1454	1245	1845
E83	1058	2564	1034	1809
Melchior Base*	4056	7890	3049	5768
Shetland Islands*	2656	4678	2548	5960
Carlini Base*	4039	5069	3409	5084
Deception Island*	3043	4005	4301	6043
Total	27694	51275	32,399	45,218

*CFU g^{-1}

site (Table 2). However, no significant differences were evidenced in the number of isolates depending on the tested culture medium (R_2A or LB-SW). Water samples named E18 and E27 obtained from areas around the South Orkney Islands yielded more bacterial isolates than the other sampling sites, while sediment samples obtained around Carlini (permanent Argentinian base) and Melchior (temporary Argentinian base) yielded the highest counts for both psychrophilic and psychrotolerant microorganisms. This was probably due to the influence of humans and mammals in the areas with Antarctic bases.

Detection of inhibitory compound producers

For the isolation of inhibitor compound producers, 6100 different colony morphotypes were selected and tested for antimicrobial production. Initially, 63 isolates were detected as producers of inhibitory substances. South Shetland Islands showed the highest detection rate of antimicrobial producers with 30 inhibitor colonies, followed by the water sample E81 and the sediment from Deception Island with 22 and 11 colonies, respectively.

When antimicrobial activity was re-evaluated in the initial 63 selected isolates (after subculturing five times), only 27 of them were able to inhibit the tested sensitive strains. Twenty of them were isolated at 15 °C and the rest at 5 °C. Among the isolates obtained at 15 °C, eight (SH1–4, SH8, SH9, SH29, and SH30) were obtained from South Shetland Islands sediment, nine (E81/1–3, E81/5–7, E81/9, E81/13, and E81/15) from water sample E81, and three from Deception Island sediment (2D, 5D, and 6D). All isolates showed inhibitory activity against both *S. aureus* 25923 and *E. coli* ATCC 35218 sensitive strains.

Screening for antimicrobial-producing strains incubated at 5 °C yielded six selected isolates from the E81 water sample with antimicrobial activity against *E. coli* ATCC 35218 (5/10, 5/16, 5/20–22, and 5/28) out of 600 tested colonies. In addition, the colony named 18SH isolated from South Shetland Islands sediment at 5 °C showed yeast micro-morphology and antagonistic activity against *S. aureus* 25923.

Isolate preliminary differentiation

Antimicrobial producers were initially grouped and differentiated by conventional and molecular methods. Internal spacer region profiling and ARDRA analysis were used. Based on molecular analysis together with macro- and micro-morphological features, the isolates could be grouped into 11 OTUs (Table 3).

Representative bacterial isolates from each OTU were first characterized based on their Gram staining, morphology, and oxidase reactions. Table 3 shows some physiological and

Table 3 OTUs obtained from ARDRA, ISR (16S-23S spacer), morphological characteristics and enzymatic profiles tested in isolates

Group	Isolates	Morphological characteristics (colonies)	Lipase	Cellulase	Xylanase	Protease	Gelatinase	Estearase	Chitinase	Amylase
G 1	E81/1 E81/2 E81/3 E81/5 E81/6 E81/7 E81/9 E81/13 5/10 5/16 5/21 5/22 5/28	Mucoid and yellow	+	+	–	–	–	–	–	–
G 2	E81/15	Mucoid and bright yellow	+	+	–	–	–	–	–	–
G 3	5/20, 6D	Mucoid and yellow	+	–	–	–	–	–	–	–
G 4	SH2, 5/15, SH1	Mucoid and pink	–	–	–	–	+	–	–	–
G 5	SH4	Mucoid and brown	–	+	–	–	–	–	–	+
G 6	SH30	Mucoid and orange	–	–	–	–	+	–	–	–
G 7	2D, 5D	Mucoid and yellow	+	–	–	–	–	–	–	–
G 8	SH3	Mucoid and white	+	–	–	–	+	–	–	–
G 9	SH29	Mucoid and pink	+	–	–	–	–	–	–	–
G 10	SH9	Mucoid and pink	+	–	–	–	+	–	–	–
G11	18SH	Creamy white	–	–	–	–	–	–	–	–

+ Indicates positive reaction after incubation time

morphological features of the isolates grouped into OTUs. In addition, the enzymatic profile of one representative isolate of each OTU was characterized following the previously described procedures. The results showed seven isolates with lipase activity, three with cellulase activity, and four with gelatinase activity (Table 3). Protease activity could not be evidenced in these isolates under the conditions tested, and only one isolate showed amylase activity according to the method used.

Based on the size and clearance of inhibition halos against sensitive strains and the growth rate, four isolates were selected for further studies. Bacterial isolates named 2D, 5D, and 6D, and the isolated yeast 18SH showed the clearest inhibition halos and their antagonistic activity was evidenced after 3 days of incubation at 15 °C.

Sugar fermentation profiles, catalase activity, and physiological features of selected isolates are shown in Table 4. Physiological tests showed some similarities between clustered strains within the same group (Table 4). Sensitivity/resistance to commonly used antibiotics is also listed for each selected isolate in Table 5.

Isolate identification

Bacterial isolates 2D, 5D, and 6D were identified by amplifying and sequence analysis of the 16S rRNA gene. Molecular characterization showed that isolates 2D (1261 bp), 5D (1264 bp), and 6D (1257 bp) were phylogenetically related to members of the genus *Halomonas* (Dobson and Franzmann 1996), belonging to the Halomonadaceae family (Franzmann et al. 1988), γ-proteobacteria group. The 16S rDNA sequence analysis of isolates 2D and 5D showed 99.8 and 98.9% identity with *Halomonas titanicae* BH1^T (Sánchez-Porro et al. 2010), while isolate 6D also showed proximity to the same species but with a lower similarity value (96.7%). It was also shown to be closely related to *Halomonas alkaliantarctica* strain CRSS^T (Poli et al. 2007) and *Halomonas neptunia* strain Eplume1^T (Kaye et al. 2004) (Fig. 2, Table 6).

A 1200-bp nucleotide sequence of the *gyrB* gene was additionally obtained for these bacterial isolates. The *gyrB* sequence analysis allowed us to confirm isolates' filiation: 2D, 5D, and 6D sequences showed ~ 96.0–99.7% identities with *Halomonas* members, with *H. titanicae* being the most closely related species (Table 6). The isolate named 18SH was closely related to *Candida sake* (99.2%) based on the ITS1–5.8S rDNA–ITS2 and D1/D2 domain of 26S rDNA sequences (Table 6).

Influences of medium composition, temperature, and pH on antimicrobial production

The influence of culture medium on antimicrobial production was assayed for the selected isolates. Inhibition halos were clearer when LB-SW or M9 medium was used for isolates *H. titanicae* 2D, 5D, and 6D, while, despite growth, no activity was obtained in LB medium. In contrast, *Candida sp.* 18SH displayed the best antimicrobial activity growing on both LB and M9 media (Table 7). The highest antimicrobial activity was observed when isolates 2D, 5D, and 6D were cultivated at 15 °C, reaching 400 arbitrary units per milliliter (AU mL^{-1}), while *Candida sp.* 18SH showed the highest production at 20 °C (600 AU mL^{-1}) (Table 7). When the influence of the initial pH on antimicrobial production was evaluated, *Halomonas* isolates showed the highest activity around neutral pH (400 AU mL^{-1}), which became lower at basic pH (200 AU mL^{-1}). In contrast, *Candida sp.* 18SH showed the highest antimicrobial production at neutral pH (600 AU mL^{-1}) and a lower level under acidic conditions (100 AU mL^{-1}) (Table 7).

Antimicrobial spectrum of inhibitor-producing bacteria

The antimicrobial activity of selected bacteria was evaluated against several human pathogenic and phytopathogenic strains by a method based on antimicrobial diffusion through solid culture medium, previously inoculated with an indicator strain. To evaluate whether the antimicrobial production was influenced by nutrient depletion, minimal medium M9 was tested in addition to LB-SW.

The isolates *H. titanicae* 2D, 5D, and 6D showed a wide inhibition spectrum against both human pathogenic and phytopathogenic bacteria (Table 8). Among the tested phytopathogens, only *Xanthomonas* and *Erwinia* species were inhibited. In contrast, *Candida* sp. 18SH showed a narrow inhibition spectrum against most of the pathogens tested; nevertheless, it showed an antimicrobial effect against *Xanthomonas* species (Table 8).

Estimation of antimicrobial molecular weight and charge

The molecular weights of antimicrobials were estimated by using dialysis membranes with different molecular weight cutoffs (see Materials and Methods). Antimicrobials from isolates 2D, 5D, and 6D were able to diffuse through a 6000 MWCO dialysis membrane. However, they could not diffuse when the producer strain was grown on membranes with an MWCO of over 2000, indicating that their molecular mass was between 2 and 6 kDa. In contrast to bacterial isolates, the antagonistic compound produced by *Candida* sp. 18SH did not diffuse through the membrane with an MWCO of 12,000. Polonelli et al. (1983) reported similar results when studying yeast killer toxins with weights from 15,000 to 25,000 Da. These results were also confirmed using Centricon concentrator devices.

Table 4 Physiological features of isolates

Physiological features	Isolates			
	2D	5D	6D	18SH
Growth at				
Temperature (°C)				
5	±	±	±	±
15	+	+	+	+
25	+	+	+	+
30	+	+	+	+
37	±	±	±	±
45	–	–	–	–
pH				
4	–	–	–	+
5	+	+	+	+
6	+	+	+	+
9	+	+	+	–
10	–	–	–	–
Growth in				
NaCl % (w/v)				
0%	–	–	–	–
5%	+	+	+	+
10%	+	+	+	+
25%	–	–	–	–
Sodium Pyruvate—VP	+	+	+	+
Hippuric acid—HIP	–	–	–	–
Esculin Ferric Citrate—ESC	+	+	+	+
Pyroglutamic acid-β-naphthylamide—PYRA	–	–	–	–
6-bromo-2-naphthyl-α-D-galactopyranoside-α—GAL	+	+	+	–
Naphthol ASBI-glucuronic acid—βGUR	–	–	–	–
2-naphthyl-β-D-galactopyranoside—βGAL	+	+	+	–
2-naphthyl phosphate—PAL	+	+	+	–
L-leucine-β-naphthylamide—LAP	–	–	–	+
L-arginine—ADH	–	–	–	–
Potassium nitrate—NIT	+	+	+	–
Pirazinecarboxamide—PYZ	–	–	–	+
2-naphthyl-α-D-glucopyranoside—αGLU	+	+	+	+
1-Naphthyl-N-acetyl-βD-glucosaminide—βNAG	–	–	–	–
Urea—URE	–	–	–	–
Gelatin (hydrolysis)—GEL	–	–	–	–
Catalase—CAT	+	+	+	+
Production of acid from				
D-Lactose—LAC	–	–	–	–
D-trehalose—TRE	–	–	–	–
D-Glucose—GLU	–	–	–	+
D-Maltose—MAL	–	–	–	+
D-Xylose—XYL	–	–	–	–
D-Saccharose—SAC	–	–	–	+
Inulin—INU	–	–	–	–
D-raffinose—RAF	–	–	–	–
Starch—AMD	–	–	–	–
Glycogen—GLYG	–	–	–	–
D-ribose—RIB	+	–	–	+

Table 4 (continued)

Physiological features	Isolates			
	2D	5D	6D	18SH
L-arabinose—ARA	+	–	–	–
D-mannitol—MAN	–	–	–	–
D-sorbitol—SOR	–	–	–	–

+ Positive reaction/growth, – No reaction/growth observed, ± weak reaction/growth

Table 5 Resistance/sensitivity to antibiotics for isolates 2D, 5D, 6D using antibiotic disks BGN1, BGN2, BGN3 and *Staphylococcus* A, B series

Antibiotics; µg	Isolates		
	2D	5D	6D
Penicillin 10 Units	R	R	R
Oxacillin; 1	R	R	R
Erythromycin; 15	S	S	R
Clindamycin; 2	R	R	R
Trimethoprim Sulfamethoxazole;(1.25/23.75)	S	S	S
Vancomycin; 30	R	R	R
Teicoplanin; 30	R	R	R
Levofloxacin; 5	S	S	S
Gentamicin; 10	S	S	S
Rifampicin; 5	S	S	S
Minocycline; 30	S	S	S
Chloramphenicol; 30	S	S	S
Imipenem; 10	R	R	S
Ceftazidime; 30	R	R	S
Ampicillin Sulfamethoxzole; 10/10	R	R	S
Cefotaxime; 30	R	R	S
Cefalotin; 30	R	R	R
Cefepim; 30	S	S	S
Ciprofloxacin; 5	S	S	S
Meropenem; 10	S	S	S
Amikacin; 30	S	S	S
Colistin; 10	R	R	R
Cefoperazone Sulbac.; (75/30)	S	S	S
Piperaciclin; 100	S	S	S
Aztreonam 30	S	S	S
Cefuroxim; 30	R	R	R

Analysis of the net charge of antimicrobials by horizontal electrophoresis on agarose gels using cell-free supernatants showed that all of the produced antimicrobial compounds from the selected isolates were negatively charged at pH 8.0 (Fig. 3).

Antimicrobial activity following enzymatic treatments

The sensitivity of the antimicrobial compounds to different enzymes was analyzed to gain an insight into their chemical

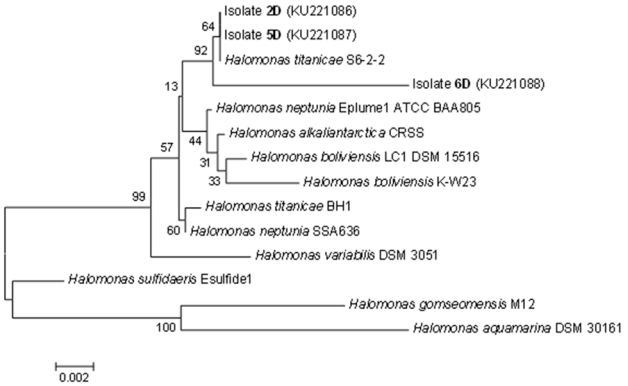

Fig. 2 Dendrogram based on 16S ribosomal gene sequences from isolates 2D, 5D, and 6D. The bootstrap support (100 replicates) is shown near each branch (Felsenstein 1985). Evolutionary distances were computed using the composite maximum likelihood method (Tamura et al. 2004) and are presented as base substitutions per site. All positions containing gaps (missing data) were deleted (*option* complete deletion)

Table 6 Identity values for isolates with the most closely related 16S rDNA sequences species obtained from GenBank

Isolates	Species	% Similarity		
		16S rDNA	RPS*	*Gyr*B gene
2D	*Halomonas titanicae* BH1$^{(T)}$	99.8	–	99.7
	H. neptunia Eplume1$^{(T)}$	99.0	–	98.9
	H. alkaliantartica CRSS$^{(T)}$	99.0	–	99.1
5D	*H. titanicae* BH1$^{(T)}$	98.9	–	99.3
	H. alkaliantartica CRSS$^{(T)}$	98.1	–	99.1
	H. neptunia Eplume1$^{(T)}$	98.0	–	99.0
6D	*H. titanicae* BH1$^{(T)}$	96.7	–	98.3
	H. neptunia Eplume1$^{(T)}$	96.2	–	96.5
	H. alkaliantartica CRSS(T)	96.1	–	96.0
18SH*	*Candida sake* CBS159	–	99.2	–
	Candida sp. K2	–	99.0	–

Alignments were done in pairs by using Mega 6 software

*Data represent the identity value of ITS1-5.8S rDNA-ITS2 and D1/D2 domain of the 26S rDNA sequences

Table 7 Influence of media, temperature and pH of incubation on antibiotic units per milliliter (AU mL^{-1}) against *S. enterica* ser. Enteritidis after 24 h of incubation at 37 °C. *Incubated at 15 °C for 2D, 5D and 6D and 20 °C for 18SH. **Grown on LB-SW medium for 2D, 5D and 6D and LB for 18SH. *** Incubated at 15 °C for 2D, 5D and 6D and 20 °C for 18SH. (−): No inhibition activity was observed

	Arbitrary units per milliliter (AU mL^{-1})			
	2D	5D	6D	18SH
Medium*				
M9	200	200	200	600
LB-SW	400	400	200	100
M63	100	100	100	–
LB	–	–	–	600
Temperature (°C)**				
5	–	–	–	–
15	400	400 ± 1	400	–
20	200	200	200	600
25	100	100	100	400
30	100	100	100	–
40	–	–	–	–
pH***				
4	–	–	–	100
6	400	400	400	600
9	200	200	200	–
10	–	–	–	–

nature. None of the activities produced by isolates *H. titanicae* 2D, 5D, and 6D and *Candida sp.* 18SH were sensitive to catalase, lipase, or amylase treatment under the assayed conditions. Therefore, it could be inferred that these activities would not be due to hydrogen peroxide production, or lipid or glycan moieties within the antimicrobial molecule. The inhibition of sensitive strain growth by acid production could also be ruled out after the neutralization of cell-free supernatants (pH 7.0). In contrast, *Candida* 18SH antimicrobial activity was lost after chloroform treatment, which suggested the proteinaceous nature of this compound. Meanwhile, the antimicrobial activities from *Halomonas* isolates 2D, 5D, and 6D were not affected by the proteases tested, probably owing to their small molecular size (2,000–6000 Da). This may have been related to the presence of microcin-like compounds or a low-molecular-weight secondary metabolite.

Effects of temperature and pH on antimicrobial activity

The antimicrobials produced by *H. titanicae* 2D, 5D, and 6D were stable in the pH range tested (pH 2–12), retaining 100% of their activity in all cell-free supernatants (~400 AU mL^{-1}). Nevertheless, loss of activity was observed for the antimicrobial produced by *Candida sp.* 18SH after 1 h of incubation at pH 4.0, 8.0, 9.0, or 10.0 at 20 °C. The antimicrobial activity of 18SH was depressed by ~92% after incubation at pH 4.0, 9.0, and 10.0 (from 600 UA mL^{-1} to 50 AU mL^{-1}), while 17% of activity (100 AU mL^{-1}) remained after incubation at pH 8.0.

When different temperatures and incubation times where evaluated on cell-free supernatants, a loss of 50% of antimicrobial activity was observed after 30 min of incubation at temperatures above 55 °C for antimicrobials produced by *H. titanicae* 2D, 5D, and 6D (200 AU mL^{-1}), and *Candida sp.* 18SH (300 AU mL^{-1}). However, incubation for 60 min in the temperature range of 8–45 °C did not affect the activity of any of the antimicrobials evaluated. When supernatants of both *Halomonas* and *Candida* were frozen at − 20 °C, the activity remained at 100%. Interestingly, inhibition of the psychrotolerant *Pseudomonas yamanorum* $8H1^T$ strain during growth at 8 °C was observed for all of the antimicrobials tested, giving evidence that the antimicrobial activity remains in all cases, even at a low temperature. Unsurprisingly, antimicrobial activity was completely lost after incubation in boiling water and also after autoclaving at 121 °C for 15 min, for all cell-free supernatants.

Hemolytic, bio-emulsifying, and bio-surfactant activities

Lysis of erythrocytes due to hemolytic activity was not observed for all supernatants with antimicrobial activity (~400 AU mL^{-1}), even for directly streaked LB plates supplemented with fresh blood. In any case, hemolysis halos were observed around neither the colonies of *H. titanicae* 2D, 5D, and 6D nor those of *Candida sp.* 18SH.

The treatment with kerosene resulted in the formation of perdurable emulsions when cell-free supernatants of *H. titanicae* 2D, 5D, and 6D were evaluated. The emulsifying indexes after 24 h (E_{24}) were 21.0, 40.9, and 27.9, respectively. However, drops of cell-free supernatants did not collapse and appeared like firm drops on tested oils, in contrast to the case upon the addition of a surfactant (like SDS), which normally causes drops to spread out. These results showed emulsifying activity but no bio-surfactant ability.

Discussion

To survive stressful conditions, microorganisms have developed genetic and physiological mechanisms to thrive and colonize under unfavorable and extreme conditions (Beales 2004). Therefore, some microorganisms have developed the ability to produce antimicrobial compounds as a defensive mechanism. The biosynthesis of different compounds with antibacterial effects is regarded as an efficient strategy to inhibit the growth of neighboring microorganisms, providing

Table 8 Inhibition spectrum of isolates 2D, 5D and 6D in LB-SW and M9 media against both pathogenic and phytopathogenic bacteria

Indicator Strain	Isolate							
	LB-SW*				M9			
	2D	5D	6D	18SH	2D	5D	6D	18SH
Salmonella enterica ser. Tiphymurium PA01	+	+	+	–	+	+	+	–
Salmonella enterica ser. Newport PA02	+	+	+	–	+	+	+	–
Salmonella enterica ser. Enteritidis	+	+	+	+	+	+	+	–
Escherichia coli 35218	+	+	+	–	+	+	+	–
Escherichia coli 25922	+	+	+	+	+	+	+	–
Escherichia coli ser. O15:NM PA04	+	+	+	–	+	+	+	–
Enterobacter aerogenes PA14	+	+	+	–	+	+	+	–
Citrobacter freundii PA15	+	+	+	+	+	+	+	–
Serratia marscecens ATCC 13880	+	+	+	–	–	–	–	–
Shigella flexnerii PA09	+	+	+	–	+	+	+	–
Shigella sonnei PA10	+	+	+	–	+	+	+	–
Enterococcus faecalis ATCC 29212	+	+	+	–	–	–	–	–
Lysteria monocytogenes PA07	+	+	+	–	+	+	+	–
Staphylococcus aureus ATCC 29213	+	+	+	–	+	+	+	–
Staphyloccus aureus ATCC 25923	+	+	+	+	+	+	+	–
Staphylocccus epidermidis PA17	+	+	+	+	+	+	+	–
Bacillus subtilis 168	+	+	+	–	+	+	+	–
Xanthomonas axonopodis pv. *citri*	+	+	–	+	+	+	–	+
Xanthomonas axonopodis pv. *phaseoli*	+	+	+	+	+	+	+	+
Xanthomonas albilineans	+	+	+	+	+	+	+	+
Acidovorax avenae	–	–	–	–	–	–	–	–
Agrobacteirum tumefaciens	–	–	–	–	–	–	–	–
Erwinia stewartii	+	+	+	–	+	+	+	–
Erwinia amylovorans	+	+	+	–	+	+	+	–
Clavibacter michiganensis	–	–	–	–	–	–	–	–
Pseudomonas syringae pv. *syringae*	–	–	–	–	–	–	–	–

*For isolate 18SH inhibition spectrum analysis was carried out in LB medium, instead of LB-SW

advantages for effective colonization and competition for nutrients in environments where they are scarce (Grossart et al. 2003; Lo Giudice et al. 2007).

Sub-Antarctic and Antarctic regions, which are remote and largely pristine, contain microbial communities adapted to extreme conditions that are remarkably suitable for bioprospection and novel species discovery (Carrión et al. 2011; Dionisi et al. 2012b). Since LB-SW medium is considered to be richer in nutrients than R_2A (Mandal et al. 2013), we expected to isolate greater number of fastidious bacteria in this rich medium, as previously observed by O'Brien et al. (2004). However, our records did not show significant differences between the number of isolates in the two-culture media (R_2A vs. LB-SW) at the temperatures tested (5 °C and 15 °C).

Psychrotolerant bacteria were more frequently isolated than psychrophilic ones. This may have been due to the fact that, during summer, soil heats up to 20–25 °C, which allows psychrotolerant microorganisms to grow faster in a short period of time than psychrophilic ones, thus promoting their dominance in these environments (White et al. 2000). The samples processed in this work were collected during Antarctic summer (January and February) when psychrotolerant microorganisms are predominant, increasing the likelihood that this type of microorganism would be isolated (O'Brien et al. 2004; Sánchez et al. 2009; Arnau et al. 2016).

Initially, 63 isolates were shown to be capable of inhibiting the growth of sensitive strains. However, when antimicrobial production was re-evaluated, this activity could be confirmed for only 27 of the isolates. The loss of inhibitory activity after secondary isolation was reported by O'Brien et al. (2004), who mentioned that overpopulation of colonies on the plates in the first isolation may mask the zones of inhibition and thus produce false positive results. In addition, antimicrobial production can be encoded by a plasmid, which might eventually be lost after repeated subculturing.

The rate of detection of antimicrobial production was 0.44% in this study, which is in accordance with previously

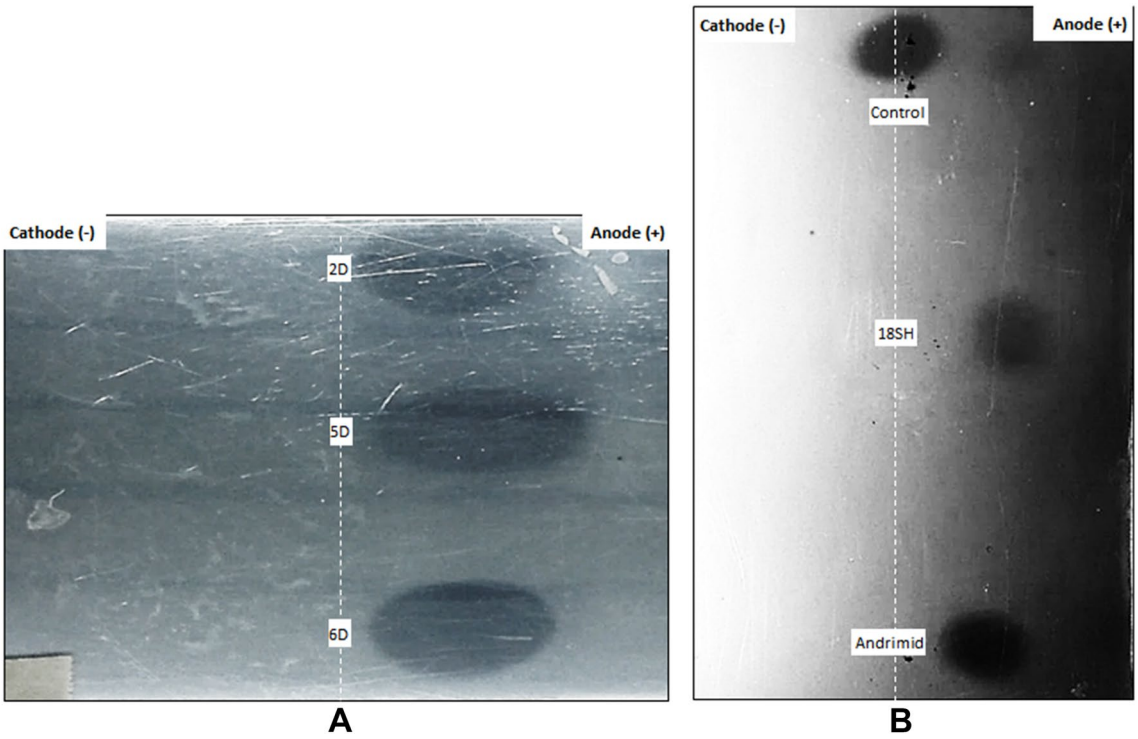

Fig. 3 Agarose gel electrophoresis showing antimicrobial net charge. The white line indicates the loading point. **a** Inhibition zone of *Salmonella enterica* ser. Enteritidis produced by antimicrobial compounds of *Halomonas sp.* 2D, 5D, and 6D and *Candida* sp. 18SH moved toward the anode after electrophoresis. **b** Andrimid produced by *Serratia proteamaculans* and an uncharged molecule were used as controls

reported results (O'Brien et al. 2004; Sánchez et al. 2009); however, this rate strongly depends on the criteria used for selection. Some studies reported a 0.20% detection rate for bacteriocin producers using direct seeding methods. However, from 83,000 colonies isolated from fish and vegetables using enrichment methods, the detection rate for bacteriocin producers was 3.4% (Coventry et al. 1997). These studies focused on the isolation of antimicrobial peptides from mesophilic GRAS (generally recognized as safe) microorganisms. Studies based on general antimicrobial activities reported higher detection rates. For example, Hentschel et al. (2001) reported that, from 200 bacteria isolated from sponges from the Mediterranean Sea, 11.3% showed antimicrobial activity. In contrast, searches for antimicrobials produced by microorganisms from cold environments seem to have resulted in lower detection rates, for example, 0.29% for antimicrobials from Antarctic soil samples (O'Brien et al. 2004) and 0.18% for those from sub-Antarctic soil samples (Sánchez et al. 2009).

In accordance with our findings, Wong et al. (2011) described the isolation and characterization of bacterial isolates producing antimicrobial compounds from the South Shetland Islands, King George Island, and Antarctica. Out of 2465 bacterial isolates recovered from soil samples, only six demonstrated inhibitory effects on the growth of Gram-negative or Gram-positive indicator foodborne pathogens. Moreover, Tomova et al. (2015) studied the antimicrobial activity produced by 24 bacterial strains isolated from sediment and soil samples of Deception and Galindez islands. All of the bacteria isolated in the Antarctic were found to inhibit the growth of the indicator bacteria tested, showing a broad inhibition spectrum against foodborne pathogens and yeast cultures.

In the present work, three selected strains arbitrarily identified as 2D, 5D, and 6D isolated from soil samples around Deception Island were identified as *H. titanicae,* while the 18SH yeast strain isolated from South Shetland Islands was found to be closely related to *Candida sp.* Little information is available about antimicrobial activity from the genus *Halomonas*. However, Bitzer et al. (2006) described elucidation of the molecular structure of novel aminophenoxazinones produced by marine *Halomonas sp.* and their antibacterial activity. In addition, Radjasa et al. (2007) described significant antimicrobial activity against multi-drug-resistant bacteria produced by a *Halomonas* strain associated with the sponge *Aaptos* sp., collected from North Java Sea, Asia. Later, Chen et al. (2009) reported several halophilic bacterial strains isolated during a bioprospecting project in Weihai Solar Saltern, China, which showed potent activities

against Gram-positive bacteria, human pathogenic fungi, and also plant pathogenic fungi. Among these isolates, 28 were related to the genus *Halomonas* and classified into five species: *H. salina*, *H. denitrificans*, *H. ventosae*, *H. saccharevitans*, and *H. taeanensis*. Similarly, Ravikumar et al. (2016) described the antimicrobial potential of carotenoids extracted from halobacteria isolated from salt pan sediments. One of these isolates was closely related to *Halomonas sp*. and showed significant antimicrobial activity against antimicrobial-resistant pathogens such as *Klebsiella* sp., *S. aureus*, and *P. aeruginosa*.

The strains of *H. titanicae* with antimicrobial activity tested in the present study were isolated from Antarctic environments using a modified medium that was enriched in marine artificial salts, in order to mimic seawater. Recently, Zaccai et al. (2016) described an *H. titanicae* strain isolated from the marine Arctic environment surrounding the hull of the Titanic, for which the authors reported the production of ectoine (a compatible solute) in order to compensate for fluctuations in medium osmolarity. Through this adaptive regulatory process in its cytosol, *H. titanicae* is known to be halotolerant over a broad range of salt concentrations (e.g., 0.5–25% w/w NaCl).

Bourbouli et al. (2015) reported antimicrobial activity from a *Halomonas* strain isolated from Kolumbo submarine volcano in the Aegean Sea. This isolate showed potent inhibition of *Enterococcus faecalis* and *Streptococcus pneumoniae*. Moreover, Azemin et al. (2015) described a bacteriocin produced by *Halomonas sp.* strain M3 as a bacteriostatic agent. Specifically, the marine halophile was isolated from the Straits of Johor, Malaysia, and was shown to produce bacteriocin CC. In agreement with our results for *H. titanicae* 2D, 5D, and 6D, characterization of *Halomonas sp.* M3 demonstrated optimal growth and bacteriocin production at 25 °C and the pH range of 8–8.5 in nutrient broth medium supplemented with 2.9% (w/v) NaCI to mimic saltwater conditions. However, bacteriocin CC was shown to be heat-labile (35–50 °C) and sensitive to proteolytic enzyme treatment, indicating the proteinaceous nature of this antimicrobial compound.

The antimicrobial produced by *Candida sp.* 18SH lost its activity when the pH of the solution diverged from neutrality. Variations in pH were shown to cause significant changes in the ionization state of killer toxins and might be the reason for altered structural conformations, affecting both activity and stability (Bajaj et al. 2012). Most killer toxins are irreversibly inactivated above pH 5.0 and seem to be stable only within a narrow pH range (Chen et al. 2000; Marquina et al. 2001). However, a killer toxin produced by *Hansenula saturnus* (Ohta et al. 1984) was reported to be stable over a wide pH range.

Regarding the effect of temperature on antimicrobial stability, all antimicrobials lost 50% of their activity after 30 min of incubation at temperatures above 55 °C. Nevertheless, antimicrobials retained 100% of their activity after 60 min of incubation in the temperature range of 8–45 °C, indicating that antimicrobials produced by *H. titanicae* 2D, 5D, and 6D, and *Candida sp*. 18SH are compounds with intermediate thermostability. Pavlova and Severinov (2006) described low-molecular-weight antimicrobial compounds (microcin-like) that were thermostable and tolerant to high temperatures and some proteases. This could facilitate downstream processing by denaturalization of the accompanying proteins. In contrast, the stability of all killer toxins largely depends on temperature owing to their proteinaceous nature. Bajaj et al. (2012) reported results similar to those obtained in this study with regard to a killer toxin produced by *Pichia kudriavzevii* RY55, which showed good thermostability in the temperature range of 4–40 °C for short periods, while at 50 °C, the activity considerably decreased, and no activity was observed at higher temperatures. In contrast, a killer toxin isolated from *Hansenula mrakii* showed high heat stability after incubation at 100 °C for 10 min, retaining 100% of its activity (Yamamoto et al. 1988).

The antimicrobial compounds produced by *H. titanicae* 2D, 5D, and 6D and *Candida sp.* 18SH were also shown to be negatively charged at pH 8.0, a common feature of class II microcins (Pons et al. 2002). The inhibition zones moved towards the anode, as shown in Fig. 3. Similar results were obtained in an evaluation by Sánchez et al. (2009) of the net charge of antimicrobials produced by bacterial species (isolated from Isla de los Estados, Ushuaia, Argentina).

Cold-adapted microorganisms isolated from soil samples from sub-Antarctic regions (Isla de los Estados, Ushuaia, Argentina) were described according to antimicrobial production (Sánchez et al. 2013). Similarly, antimicrobial activities from Antarctic microorganisms, especially bacteria, have been reported here. Most of the isolates showed a wide spectrum of activity against both Gram-positive and Gram-negative enteropathogenic bacteria. Antimicrobials were suspected to be microcin-like compounds and exhibited activity even after freezing at − 20 and − 80 °C. Those cold-living microorganisms with the ability to produce cold-active compounds have potential application in the preservation of chilled food (Sánchez et al. 2009). Microorganisms living in Antarctic environments and surrounding islands have acquired some adaptive strategies that enable them to thrive under the extreme conditions that prevail there. In line with this, some microorganisms have developed the ability to release antimicrobial compounds into the environment to inhibit the growth of their competitors (Lo Giudice et al. 2007).

Cold-adapted microorganisms are considered to be a promising source of novel antimicrobial metabolites with potential application in different industrial sectors, such as food processing, cosmetics, pharmaceuticals, and even for

microbial biopesticides (Ravot et al. 2006; Sánchez et al. 2009, 2010).

Our results support the hypothesis that cold-adapted bacteria are a promising source of novel bioactive compounds. Further studies are planned to identify the compounds produced by the isolates described herein that are responsible for their biological activities.

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