Extremophiles
DOI 10.1007/s00792-008-0203-5

ORIGINAL PAPER

Cold-adapted microorganisms as a source of new antimicrobials

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Received: 29 April 2008 / Accepted: 30 September 2008

Abstract Thirty out of 8,000 different colony morphotypes isolated from soil samples of Isla de los Estados were selected based on their ability to produce antimicrobials. The significant influence of culture media and incubation temperature on antimicrobial production was proved, being LB medium and 8°C the conditions of choice. Most of the psychrotolerant isolates were phylogenetically related to *Serratia proteamaculans* (96.4–97.9%) while the psychrophilic isolated 8H1 was closely related to *Pseudomonas* sp. (90–94% similarity). Produced antimicrobials showed a promising wide spectrum of activity both against gram-positive and gram-negative pathogenic bacteria. They were suspected to be microcin-like compounds (Mw <2,000 Da) and showed a marked tolerance to heat (1 h in boiling water bath) and pH-treatments (1–12). Antimicrobial compounds also showed to partially keep their activity even after overnight freezing at −20 and −80°C and displayed a negative net charge at pH 8.0, a common feature of class II microcins.

Keywords Psychrophiles · Psychrotolerant · Bacteriocins · Microcins · Isla de los estados

Introduction

The production of antimicrobial compounds by microorganisms has been widely studied for industrial applications and employed to treat infections caused by pathogenic bacteria. In particular, the production of antimicrobial compounds such as bacteriocins has become widely recognized and much interest has been put on developing novel applications for these natural agents in food, cosmetic and pharmaceutical industries.

Biodiversity screening programs seeking for therapeutic and anti-tumor drugs from natural products focus on biomolecules with unusual properties. Consequently, extremophiles, which produce biomolecules adapted to their unusual living conditions, have been recognized as valuable sources of novel bioproducts including antimicrobials (Da Costa et al. 1988; Horikoshi 1995; Ritzau et al. 1993). The antagonistic properties of cold-loving organisms have not been investigated as extensively as those of the mesophiles (O'Brien et al. 2004). Unlike the inhibitors produced by mesophiles; the antimicrobials produced in cold environments working at low temperatures give a competitive advantage to the producing microorganisms during their growth cycle (O'Brien et al. 2004). Such cold-active antimicrobial compounds may be exploitable in industrial applications including chilled-food preservation. There are a few reported data concerning the ability of extremophiles to produce active compounds in different environments (Horikoshi 1999; Rodriguez-Valera 1992). Evidence that extremophiles are capable of producing antibiotics also exists; a heat-labile β-lactamase has been purified from the psychrophile *Psychrobacter immobilis* (Feller et al. 1997). On the other hand, the ability of extremophiles to produce secondary metabolites with potential industrial interest has been also well documented; several archaea were found to produce archaeocins (halocins and sulfolobicins) that inhibit closely related species (Aravalli et al. 1998; Prangishvili et al. 2000).

Nowadays, there is a need for new antimicrobial agents due to the increase in drug resistance in many common

Communicated by H. Santos.

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bacterial pathogens, together with the emergence of new infections (Zahner and Fielder 1995; Davies and Webb 1998).

In this paper, we describe the isolation and identification of cold-loving microorganisms with the ability to produce cold-active antimicrobial compounds with potential application in chilled-food preservation.

Materials and methods

Bacterial strains and culture conditions

Isolates were grown in LB medium containing (in g l^{-1}): yeast extract 5; tryptone 10; and NaCl 5, and maintained at 4°C. Indicator bacteria used for antimicrobial activity detection listed in Table 1, were grown in LB medium at 37°C overnight.

Bacterial isolation and detection of inhibitor–producers

Soil samples were aseptically collected from Isla de los Estados Reservation (South Atlantic Argentina) at four locations: Observatory Island (54°39′S, 64°08′W) Crossley Bay (54°48′S, 64°41′W), Franklin Bay (54°53′S, 64°40′W) and San Juan del Salvamento Port (54°43′ S, 63°51′ W), and stored at −80°C until analysis.

For isolation of antimicrobial producers, soil samples (1 g) were defrosted and inoculated into 50 ml of either LB or M9 (in g l^{-1}: $CaCl_2$ 0.12, NaCl 0.5, NH_4Cl 1, Na_2HPO_4 12.8, KH_2PO_4 3, $MgSO_4{\cdot}7H_2O$ 0.5 and glucose 2). Flasks were incubated at 8 and 20°C in an orbital shaker (200 rpm) for 24 h. Turbid samples, indicating growth, were conveniently diluted and plated onto the same solid medium as liquid culture and incubated at the same temperatures for 2 days or longer when necessary, as described by O'Brien et al. (2004). Colonies were counted and their morphological characteristics recorded. Duplicate plates were made by randomly picking over 8,000 colony morphotypes.

Table 1 CFU g^{-1} obtained from soil samples incubated on LB and M9 at 8°C and 20°C

Site	CFU g^{-1}			
	M9		LB	
	8°C	20°C	8°C	20°C
Observatory Island	523	1,435	1,198	4,228
Crossley Bay	468	1,169	981	3,582
Franklin Bay	289	982	785	3,025
San Juan de Salvamento Port	194	1,153	320	3,546
Total	1,474	4,739	3,284	14,381

The antimicrobial producers were identified by the deferred antagonism procedure of Gratia (1946) and Fredericq (1948) and also by a modified agar-well diffusion assay as described by Portrait et al. (1999). Putative producers were first grown at either 8 or 20°C for 3 days. Duplicate plates were overlaid with soft (0.8% w/v) LB medium seeded with 10^7 CFU ml^{-1} of *E. coli*, *S. typhimurium* or *Listeria monocytogenes* as indicator microorganisms. Clear zones (halos) around the producers, after overnight incubation at 25°C indicated the presence of an antagonistic compound.

Characterization of the isolates

Isolates were initially classified based on their gram staining. Cell morphology after 12–18 h of growth was examined under a Nikon Eclipse 80i (Nikon GmbH; Düsseldorf, Germany) phase contrast microscope at ×1,000 magnification. Catalase and oxidase activities were tested as previously described (Smibert and Krieg 1994). The tolerance range for growth at different temperatures, NaCl concentrations, and pHs were determined according to Labrenz et al. (1998). Antibiotic sensitivity was studied by the disk diffusion assay using the gram-negative 1, 2, 3 and *Staphylococcus* A and B series (Britania Laboratories; Buenos Aires, Argentina) antibiogram disks. Sugar fermentation pattern was evaluated using the API 50 CHB system (bioMérieux, France), and other biochemical characteristics were screened by API 20E system (bioMérieux, France), according to Logan and Berkeley (1984).

ISR amplifications and ARDRA

In order to group and differentiate antimicrobial producers, ISR-PCR (PCR amplification of Intergenic Spacer Regions between the 16S and 23S rDNA) and ARDRA analysis were conducted according to Benito et al. (2004). Genomic DNA was extracted and purified as described by Sambrook et al. (1989) and its purity was assessed from the A_{260}/A_{280} and A_{260}/A_{230} extinction ratios (Johnson 1994).

Universal primers corresponding to position 8–27 (AGA GTTTGATCCTGGCTCAG) and 1,492–1,509 (GGTTA CCTTGTTACGACTT) in the 16S rDNA sequence of *E. coli* were used to amplify the 16S rDNA by PCR, as previously described (Quillaguamán et al. 2004). Sequences belonging to the same genus or validly published closely related species, available through the public databases (GenBank and RDP II) were aligned and a similarity matrix was calculated (Maidak et al. 2000). The 16S rDNA sequence analysis was performed with the aid of the DNAMAN 4.03 software package by using the neighbor-joining method and the Jukes-Cantor distance correction method (Saitou and Nei 1987). Only

unambiguously aligned positions from all sequences were used, and gaps were not included in the match/mismatch count.

Due to 16S rDNA limitations, the isolates were also subjected to partial sequencing of their *gyrB* gene (Fukushima et al. 2002). The *gyrB* gene was amplified by PCR according to Kazunori et al. (2003). RFLP of amplified *gyrB* fragments using *Rsa*I and *Sau3*AI restriction enzymes was carried out according to Coenye et al. (2003).

Almost complete sequences of 16S rDNA and *gyrB* gene were obtained and deposited in the NCBI GenBank database under EU557334 to EU557353 accession numbers.

Influence of growth media composition on antimicrobial production

Effects of different culture media on the inhibitors production by the selected isolates were assessed by using liquid LB; M9; M63 (in g l^{-1}: KH_2PO_4 13.6, NH_4SO_4 8, $MgSO_4{\cdot}7H_2O$ 0.25, glucose 2, supplemented with vitamin B_1 1 mg l^{-1}); and R media (in g l^{-1}: $CaCl_2$ 0.24, NaCl 8, yeast extract 1, casein hydrolysate 10, glucose 1) after 72 h-incubation at 8 or 20°C. Antimicrobial activity in free cell supernatants was evaluated by the diffusion method through a semi-solid media, previously inoculated with *S. newport* or *L. monocytogenes* indicator strains.

Antimicrobials characterization

Enzymatic, pH, heat treatment sensitivity and size estimation

Antimicrobials nature was investigated according to O'Brien et al. (2004), using the following enzymes: trypsin (EC 3.4.21.4) and pronase E (EC 3.4.24.31) as proteases; catalase (EC 1.11.1.6) to eliminate inhibition by hydrogen peroxide; lipase (EC 3.1.1.3) and α-amylase (EC 3.2.1.1) to determine the presence of lipidic or glycogenic components in the inhibitory substances.

In order to evaluate pH stability, the pH of cell-free supernatants were adjusted within the range of 2.0–12.0 by adding HCl or NaOH, held for 1 h at room temperature, and then re-adjusted to 7.0 to examine the inhibitory residual activity as above described.

Thermo-stability was tested after freezing/heating cell-free supernatants up to −80, −20, 37, 45, 55, 85 during 8 h and 105°C for 15, 30, 45 and 60 min, respectively. Residual activity after autoclaving was also tested.

Antimicrobials size was estimated by growing the producers on top of different sterile dialysis membrane with 12,000, 6,000 and 2,000 MWCO. Diffusion through the membrane was analyzed by the above mentioned method using *S. newport* as indicator strain.

Induction assay

In order to establish microcin or bacteriocin behavior, a SOS inductor agent was used. The induction by mitomycin C is well documented as a test for differentiating colicins-like compounds from microcins (Gillor et al. 2004). Mitomicyn C at 0.25; 0.5 and 1 μg ml^{-1} was added to LB plates and let to diffuse overnight. Plates were thereafter inoculated with producer strains and incubated for 72 h at 8°C. Inhibition halos against *S. newport* were evaluated.

Net charge determination

Antimicrobials net charge was determined by observing the inhibition zone movement after an agarose gel electrophoresis. Drops of LB cell-free supernatants were deposited in the middle of an agarose gel and, after electrophoresis, the gel was covered with *S. newport* as indicator strain.

Results

Isolation from soil samples

Two types of media were used for the isolation of antimicrobial-producer bacteria: M9, to isolate bacteria from nutrient-deficient soils; and LB to isolate fastidious bacteria. In addition, two incubation temperatures were used, 8 and 20°C, in order to isolate both, psychrophilic and psychrotolerant microorganisms (Table 1).

As shown in Table 1, psychrotolerant were more frequently isolated than psychrophilic microorganisms for each isolation site. In addition, significant differences were observed depending on the culture media used. LB medium led to a higher rate of isolation, suggesting that microflora

Table 2 Inhibitor–producers detection

Site	CFU tested	Inhibitor–producers	
		LB	M9
Observatory Island	2,536	5	6
Crossley Bay	1,858	3	2
Franklin Bay	1,638	5	4
San Juan de Salvamento Port	1,825	3	2
Total	7,857	16	14

The inhibitor–producers were identified by clear zone formation around producer colonies against *E. coli*, *S. typhimurium* and *L. monocytogenes* used as indicator strain

Table 3 Physiological and morphological features of selected inhibitor–producers

Isolate	Gram	Oxidase	Catalase	Micro/macro-morphology	Growth optimum (range) °C
J49	−	+	−	Short rods; white, circular, convex, colonies; entire margin.	20 (4–25)
J437	−	+	+	Rods; white, circular, convex, mucoid colonies; entire margin.	20 (4–25)
J440	+	+	+	Single cocci; light pink, irregular colonies; lobate margin.	18 (4–25)
8HI	−	+	+	Long, in pairs rods; greenish, irregular mucoid colonies.	4 (4–15)
8HID	−	+	+	Short rods; white, circular, iridescent colonies.	20 (4–25)
25 N	−	+	−	Short rods; white, circular, convex, small colonies, entire margin.	20 (4–25)
136_1	−	+	+	Short, in chains rods; light cream, circular, small colonies, entire margin.	20 (4–25)
136_2	−	+	+	Rods; dry colonies.	20 (4–25)
226	+	+	+	Single cocci, colorless colony, polymer.	20 (4–25)
242_1B	−	+	+	Long rods; white, circular, convex colonies, lobate margin.	20 (4–25)
242_1I	−	+	−	Long rods; white, circular, lobate margin.	20 (4–25)

was nutritionally fastidious. It was not surprising, as marine soils often provide additional nutrients available from the macrofauna life processes (O'Brien et al. 2004).

Observatory Island was the site with the highest counts for psychrophilic and psychrotolerant microorganisms, probably due to anthropogenic influence, while soil samples from San Juan de Salvamento Port exhibited the minor isolation rate.

Antimicrobial screening

As shown in Table 2, around 8,000 colony morphotypes were picked out and screened for antimicrobial production against *E. coli*, *S. typhimurium* and *L. monocytogenes*. Sensitive strains were initially chosen as models of foodborne pathogens. Antimicrobial producers were found from all the isolation sites.

Thirty out of 8,000 isolates were able to produce antimicrobial compounds. Franklin Bay appeared to be the best source of inhibitor–producers, yielding 0.5% antimicrobial producers.

When the influence of culture media over the antimicrobial production was assayed, inhibition halos were clearer on LB than on R and M9 media, while no producers were obtained in M63 medium.

On the other hand, antimicrobial production was only observed when the microorganisms were grown at 8°C. This fact suggested that the inhibitory compounds production might be associated to low incubation temperatures.

Isolates preliminary differentiation

Selected bacteria were characterized by both conventional and molecular methods.

ISR pattern and ARDRA analysis allowed us to group the isolated producers within 11 OTUs (operational taxonomic units). Only one microorganism from each representative group was chosen for further studies. Isolates were arbitrary named as: J49, J437, J440, 8HI, 8HID, 25N, 136_1, 136_2, 226, 242_1B, 242_1I.

Bacteria were first characterized based on their gram staining, morphology, catalase and oxidase reactions. Table 3 shows some physiological and morphological features of the isolates.

All of the isolates shared the gram-negative characteristic and only one (8HI) was strictly psychrophilic with an optimal growth temperature of 4°C.

While most of the colonies of inhibitor-producing bacteria were white and cream, 8HI isolate displayed a greenish color and mucoid phenotype on agar plates, probably due to exopolysaccharide formation; and isolate 136_2 showed dry and hard colonies. Isolates sugar fermentation profiles and further features are shown in Table 4. Physiology showed a certain degree of similarity between strains phylogenetically clustered within the same group (API 20E and API 50 test). Strain 8HI was the one that showed the most divergent profile as compared to the other isolates.

Isolates Identification

Sequencing of 16S rDNA is considered as standard tool not only for elucidating phylogenetic relatedness but also as a mean of bacterial identification (Weisburg et al. 1991). 16S rDNA partial sequences (1,460 base pairs) showed about 99% similarities with different unidentified ''gamma proteobacteria" for isolates J49, J437, 25N, 136_1, 136_2, 226, 242_1I and 242_1B being *Serratia* members their closest relatives. However, all these isolates showed several physiological and morphological differences. The 16S rDNA sequence analysis of isolate 8HI showed 99% identity with an uncultured swine manure bacterium and other non-cultured bacteria, being *Pseudomonas* the closest genus with 90% identities, being probably a not so far described microorganism.

Table 4 API 20E and API 50CH tests. Only the cases with a differentiated reaction are shown

Isolate	ONPG	ODC	CIT	GEL	GLU	MAN	INO	SOR	SAC	XYL	ADO	XLT	MAL	TRE	MLZ	RAF	ARA	NO_3
J49	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−
J437	−	+	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	+
J440	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+	+	+
8HI	−	+	W	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−
8HID	−	+	W	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−
25 N	−	−	+	−	+	+	−	−	−	+	−	−	+	+	+	+	+	+
136_1	+	+	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	+
136_2	+	+	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	+
226	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+	+	+
242_1B	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+
242_1I	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+

+ positive reaction, − negative reaction, *w* weak reaction

Table 5 Inhibition spectrum of antimicrobial producers against pathogenic strains

Sensitive strain	Isolate										
	J49	J437	J440	8HI	8HID	25 N	136_1	136_2	226	242_1B	242_1I
S. flexneri	+	+	+	+	+	+	+	+	+	+	+
S. aureus ATCC 29213	+	+	+	+	+	+	+	+	+	+	+
S. aureus ATCC 25923	+	+	+	+	+	+	+	+	+	+	+
M. luteus	+	+	+	−	+	+	+	+	+	+	+
B. subtilis 168	+	+	+	−	+	+	+	+	+	+	+
S. epidermidis	+	+	+	+	+	+	+	+	+	+	+
S. newport	+	+	+	−	+	+	+	+	+	+	+
L. monocytogenes	+	+	+	+	+	+	+	+	+	+	+
S. sonnei	+	+	+	+	+	+	+	+	+	+	+
E. coli AB1133	+	+	+	−	+	+	+	+	+	+	+
E. coli ATCC 35218	+	+	+	+	+	+	+	+	+	+	+
E. coli O157:H7 (stx1,stx2)	+	+	+	−	+	+	+	+	+	+	+
E. coli O26:H11	−	−	−	+	−	−	−	−	−	−	−
E. coli O15:NM	+	+	+	−	+	+	+	+	+	+	+
E. coli ATCC 25922	+	+	+	+	+	+	+	+	+	+	+
C. freundii	+	+	+	+	+	+	+	+	+	+	+
E. aerogenes	−	−	−	+	−	−	−	−	−	−	−
E. cloacae	+	+	+	+	+	+	+	+	+	+	+
P. aeruginosa ATCC 27853	+	+	+	+	+	+	+	+	+	+	+
S. typhimurium	+	+	+	+	+	+	+	+	+	+	+
S. enteritidis	+	+	+	−	+	+	+	+	+	+	+
P. mirabilis	+	+	+	+	+	+	+	+	+	+	+
P. vulgaricus	+	+	+	−	+	+	+	+	+	+	+

In order to obtain a more accurate grouping, a RFLP analysis of the amplified *gyrB* gene was carried out (Fig. 1). This allowed us to group the isolates within three different groups: (I) Isolates: J49, J437, J440, 136_1, 136_2, 226, 8HID, with a similar enzyme restriction pattern; (II) 25 N, 242_1B and 242_1I; (III) 8HI isolate with a unique restriction pattern, as later confirmed by *gyrB* gene sequencing.

A 1,200 bp nucleotide sequence of the *gyrB* gene was obtained for the 11 isolates. The *gyrB* sequence analysis allowed us to differentiate isolates that 16S rDNA sequence analysis could not, showing 97–99% identities

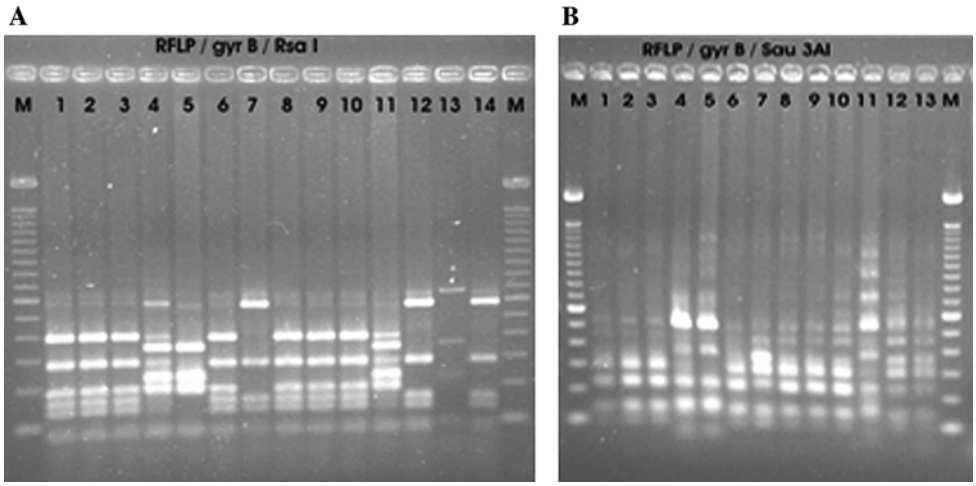

Fig. 1 RFLP of partial amplified *gyrB* gene by using *Rsa*I (**a**) and *Sau*3AI (**b**) restriction enzymes. **a** *M* molecular weight marker 1 kb (Promega), *1* J49, *2* J437, *3* J440, *4* J441, *5* 8HI, *6* 8HID, *7* 25N, *8* 136_1, *9* 136_2, *10* 226, *11* 226_2, *12* 242_1B, *13* F, *14* 242_1I. **b** *M* molecular weight marker 1 kb, *1* J49, *2* J437, *3* J440, *4* J441, *5* 8HI, *6* 8HID, *7* 25N, *8* 136_1, *9* 136_2, *10* 226, *11* 226_2, *12* 242_1B, *13* 242_1I

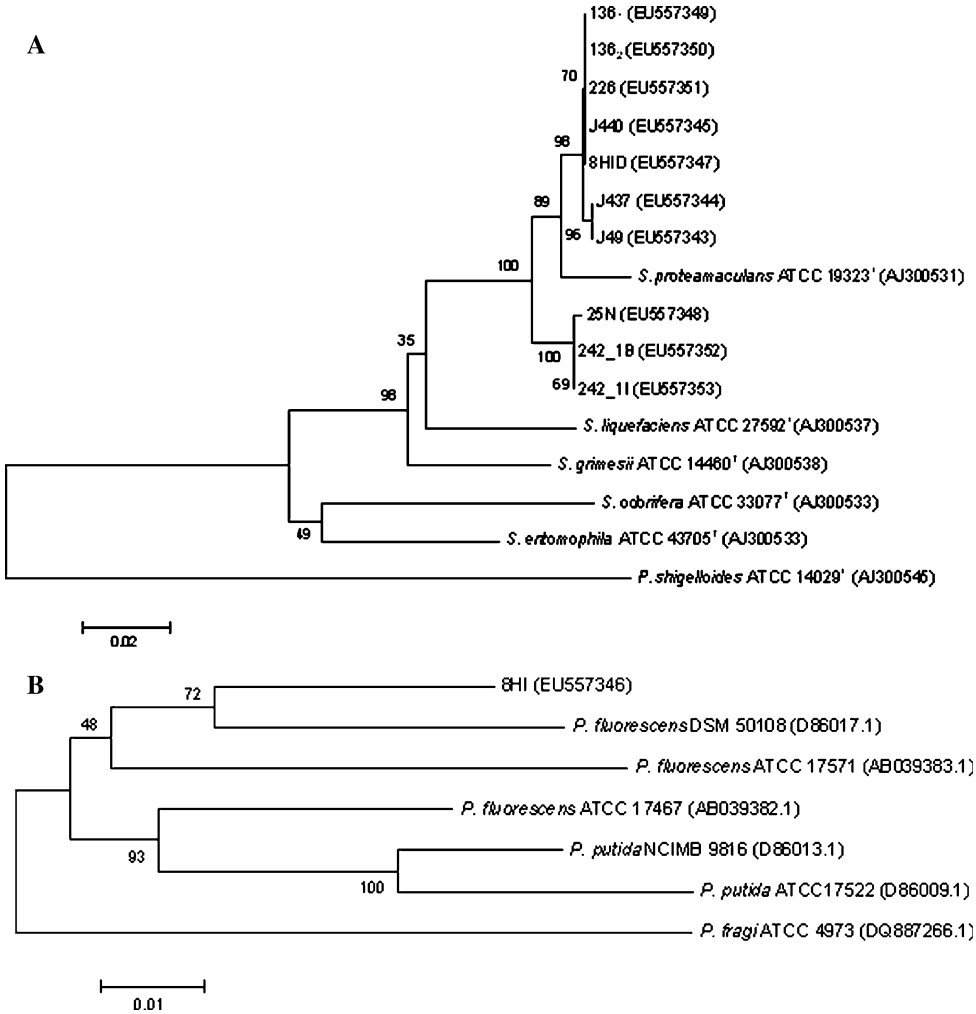

Fig. 2 **a**, **b** The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The *optimal tree* with the sum of branch length = 0.48362477 is shown for *tree* **a** and 0.29712427 for **b**. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). *Phylogenetic tree* derived from *gyrB* gene sequence consider 948 bp for *tree* **a** and 1,125 for *tree* **b**. *Plesiomonas shigelloides* ATCC 14029^T *gyrB* gene sequence was used in tree A as out-group (Brenner 1981)

for *Serratia* and *Pseudomonas* members, with *S. proteomaculans* (Fig. 2a) and *Pseudomonas fluorescens* (Fig. 2b) as the closest related species.

According to the phylogenetic method employed, the closest species was *S. proteamaculans,* with 96.4–97.9% identities for all the isolates. *S. grimesii* and *S. liquefaciens* displayed to be closely related with identity values around 92.4–94%. Figure 2a demonstrates a monophyletic cluster formed by isolates (8HID, 226, J440, 136_1, 136_2) (J437, J49); and (242_1I, 242_1B, 25 N) validated with a significant bootstrap value branching out the other *Serratia* sp.

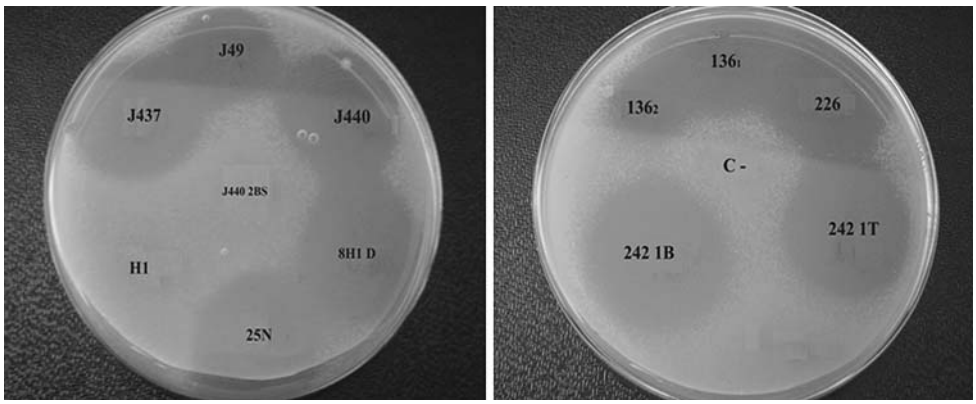

Fig. 3 Inhibition halos of the inhibitor–producers against *S. newport*

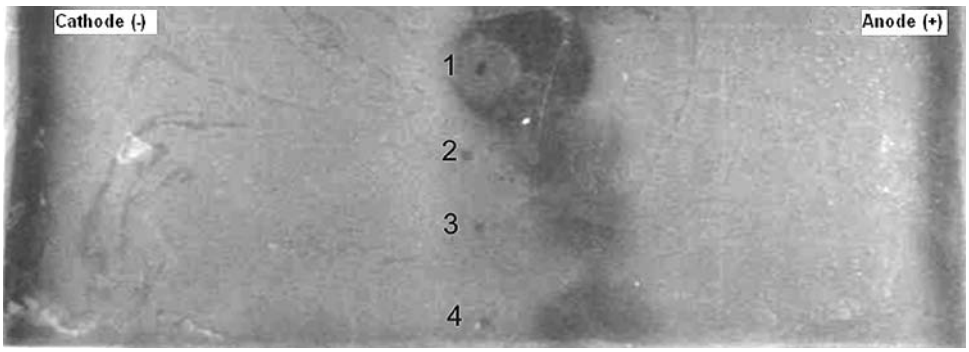

Fig. 4 Agarose gel electrophoresis showing the antimicrobials net charge. Numbers indicates the loading point. *1* MccJ25 (microcin J25) used as control, *2* J49, *3* 242_1I, *4* 136_1 isolates cell-free supernatants

The gyrB gene partial nucleotide sequence of 8HI showed a low level (Fig. 2b) of identity with the closer related species *P. fluorescences* DSM 50108 (94%) and ATCC 17571^T (92%); *P. putida* ATCC 17484^T (90%) accession numbers D86017, AB039383.1 and D86007, respectively.

Antimicrobial spectrum of inhibitor-producing bacteria

Antimicrobial activity of the isolated bacteria was evaluated against several pathogenic strains by a method based on the bacteriocin diffusion through solid or semisolid culture media, previously inoculated with an indicator strain. To ensure the sterility of the agar surface, bacteriocin-producing strain was killed by chloroform vapors before inoculating sensitive strains.

Figure 3 shows the inhibition halos produced by the 11 antimicrobial producers against *Salmonella newport* as sensitive tested strain. All the isolates showed a wide spectrum of inhibitory activity against most of the indicator strains (Table 5) though 8HI exhibited a narrow range of inhibitory action.

Induction assays

The inhibition halos observed in LB agar plates containing 0.25; 0.5 and 1 μg ml^{-1} of Mitomycin C inductor agent (Hardy and Meynell 1972) were identical to the control plate. Therefore, the antimicrobial production was not-inducible by agents that activate the SOS-response system, oppositely to colicins whose production is amplifiable by mitomycin C.

Antimicrobials size and charge estimation

Since microcins share the low molecular weight characteristic, we evaluated if antimicrobials could pass through different MW-cut off dialysis membranes ("Materials and methods"). The antimicrobials produced from isolates were able to pass through a 2,000 MWCO-dialysis membrane, indicating that their molecular mass would be lower than 2 kDa.

Antimicrobials net charge was determined on horizontal agarose gel electrophoresis. All the antimicrobial compounds from cell-free supernatants showed to be negatively loaded at pH 8, a common feature of class II microcins (Pons et al. 2002). Inhibition zones moved toward the anode, as shown in Fig. 4.

Enzymatic treatments

The sensitivity of the antimicrobials to enzymes was tested in order to gain an insight into their chemical structure. None of the inhibitors produced by the 11 isolates were sensitive to the treatment with the tested enzymes. This fact indicated that the activities were not due to hydrogen peroxide, lipid or glycan moieties. Inhibition of growth by

Table 6 Residual antimicrobial activity after freezing and different incubation temperatures during 8 h and boiling water incubation

Isolate	Freezing (°C)		Temperature (°C)					Relative activity after incubation in boiling water (min)			
	−20	−80	37	45	55	85	105	15	30	45	60
J49	+	+	+	+	+	−	−	100	85	20	20
8HID	+	+	+	+	+	−	−	100	40	20	20
136_1	+	+	+	+	+	−	−	100	80	80	20
242_1B	+	+	+	+	+	−	−	100	80	25	20

acid production was also discarded after neutralization (pH 7.0) of the inhibitors. On the other hand, the activities were lost after chloroform treatment of the inhibitors, leading to suspect proteinaceous nature of the inhibitors. As they were not affected by the proteases tested, a fact probably due to the small molecular size (<2,000 Da), they would probably be microcin-like compounds.

Effect of temperature and pH on antimicrobials activity

The effect of temperature on the antimicrobials activity was evaluated. Isolates J49, 8HI, 136_1 and 242_1B were selected as the highest bacteriocin-producer strains, being representatives of every group formed according to gyrB sequence analysis. Table 6 shows temperature range over which the antimicrobials were active, including 1 h-incubation in boiling water and after freezing at −80 and −20 overnight. Due to the small molecular size they could be not affected by low or high temperatures, facilitating downstream processing by means of denaturalization of the accompanying proteins. Furthermore, the wide pH changes at which culture supernatants were subjected, had no effect in the bacteriocin activity, being active in the 1–12 pH range.

Discussion and conclusions

The use of classical antimicrobials for application in different industries has been well documented, but the need for novel antimicrobial compounds appears particularly significant as many pathogenic bacteria become resistant to the conventional antibiotics. Within the biotechnological industries, food industry would be the one more susceptible to bacteriocins utilization as natural preservatives. However, relative few of them have been developed for their use in meat and dairy products. Currently, the unique bacteriocins employed in food preservation are those produced by lactic acid bacteria (LAB), and a limitation is found due to their limited inhibition of only gram-positive bacteria (Rodgers 2001). As a consequence, gram-negative bacteria that are frequently found in the most common spoilage-food diseases, such as *Escherichia coli*, *Salmonella, Listeria, Pseudomonas* among others, are rarely sensitive to LAB bacteriocins unless they suffer additional stress (Helander et al. 1997). In this study, we have focused in *extremophiles* from cold environments as a potential source for novel antimicrobial compounds.

From over 20,000 isolated microorganisms, cold-active isolates from Isla de los Estados Reservation were predominantly psychrotolerant (80%), a common finding in permanently cold environments, and psychrophilic (20%) in a minor extent. From the 8,000 colonies picked out, the detection rate for antimicrobial producers was 0.16%, a comparable value to those reported in the literature (O'Brien et al. 2004). Other similar studies employing enrichment procedures before detection, report detection rates of 0.2% from meat and dairy products sources. A 3.4% detection rate was also obtained from fish and vegetables sources (Coventry et al. 1997). The yield of psychrophilic and psychrotrophic bacteria isolation was influenced by the growth media used, in the same way of the antimicrobial compound production, being LB the medium which rendered major number of antimicrobial producers (expressed as CFU g^{-1}), probably due to fastidious nutritional requirements. The dependence of the cultivation medium in the antimicrobials production has been well documented; i.e., Bizani and Brandelli (2004) determined that the bacteriocin production from a *Bacillus cereus* strain is influenced by cultivation medium and temperature.

Despite 16S rDNA sequencing is considered as a standard tool not only for elucidating phylogenetic relatedness but also as a mean for bacterial identification (Weisburg et al. 1991), its variation is not sufficient to differentiate between some bacterial genera (Fukushima et al. 2002) and may result from slight differences between multiple gene copies within a strain (Dauga et al. 1990).

The sequence of *gyrB* gene, which encodes the subunit B of DNA gyrase has been previously used for describing phylogenetic relationships within closely related species by Dauga (2002). In comparison with 16S rDNA, the *gyrB* gene has a greater evolutionary divergence and has been proposed as a suitable phylogenetic marker for bacterial identification and classification (Yamamoto and Harayama 1996). Due to the 16S rDNA sequencing limitations, we

have used *gyrB* gene sequencing to obtain a complementary taxonomy technique in addition to further phenotypic methods to identify our isolates. By means the RFLP analysis of the amplified *gyrB* gene, the isolates could be classified into three different groups, affiliation that was later confirmed by *gyrB* gene sequencing. In this way, *gyrB* sequence analysis allowed us to differentiate isolates not distinguishable by 16S rDNA sequence analysis, showing 97–99% identities with members of the genera *Serratia* and *Pseudomonas.*

Antimicrobial activity of the selected isolates showed a similar wide-range spectrum for almost all of them concerning the target pathogenic bacteria and pH. Antimicrobials produced would exhibit microcin-like features such as their peptidic nature and activity against *E. coli* and its close relatives (Asensio and Pérez-Diaz 1976). The antimicrobial agents produced by the herein described cold-adapted microorganisms would be different from colicins according to their size, since colicins range from 25 to 80 kDa while microcins are smaller than 10 kDa. In the present work all the antimicrobials were able to diffuse through a 2,000 MWCO-dialysis membrane, a method previously used for microcin isolation i.e., MccJ25 (Salomón and Farías 1999) and MccH47 (Laviña et al. 1990).

On other hand, the production of the antimicrobials was not lethal to the producer strains and was not-inducible by SOS system when cells were incubated with mitomycin C, characteristics used to differentiate microcins from colicins.

Most of the isolates herein selected, closely related to *Serratia* sp., showed a wide inhibition spectrum against common pathogenic bacteria. Slightly different was the case of the isolate 8HI which showed some resistant bacterial targets (Table 5), and which would probably be a so far not described microorganism. A particularly interesting result was the inhibition against *E. coli* O157:H7, a shiga-toxin producing strain causing hemorrhagic colitis and hemolytic uremic syndrome in humans (Phillips 1999). Additionally, swine, cattle, poultry meat are commercial livestock that might also become infected by the pathogenic enteric microbes evaluated like *E. coli* virulent strains and *S. enteritidis* serotype t*yphimurium*, microorganism able to cause intestinal and extra-intestinal infection in humans as well as in animals, thus requiring antimicrobial therapy in livestock (Barton and Hart 2001).

In conclusion, this study has shown that cold-adapted isolated bacteria from Isla de los Estados, Ushuaia, Argentina, can serve as a valuable source of novel antibiotics. Produced antimicrobials demonstrated to be active against diverse pathogenic mesophile strains, known etiological agents of common infections and food-borne illnesses. Therefore, these cold-active antimicrobials could be important for applications on products that involve "cold-steps" during manufacture, processing or preservation.

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