RESEARCH/REVIEW ARTICLE

Culturable heterotrophic bacteria from Potter Cove, Antarctica, and their hydrolytic enzymes production

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Abstract

Affiliations of the dominant culturable bacteria isolated from Potter Cove, South Shetland Islands, Antarctica, were investigated together with their production of cold-active hydrolytic enzymes. A total of 189 aerobic heterotrophic bacterial isolates were obtained at 4°C and sorted into 63 phylotypes based on their amplified ribosomal DNA restriction analysis profiles. The sequencing of the 16S rRNA genes of representatives from each phylotype showed that the isolates belong to the phyla Proteobacteria (classes Alpha- and Gamma-proteobacteria), Bacteroidetes (class Flavobacteria), Actinobacteria (class Actinobacteria) and Firmicutes (class Bacilli). The predominant culturable group in the site studied belongs to the class Gammaproteobacteria, with 65 isolates affiliated to the genus *Pseudoalteromonas* and 58 to *Psychrobacter*. Among the 189 isolates screened, producers of amylases (9.5%), pectinases (22.8%), cellulases (14.8%), CM-cellulases (25.4%), xylanases (20.1%) and proteases (44.4%) were detected. More than 25% of the isolates produced at least one extracellular enzyme, with some of them producing up to six of the tested extracellular enzymatic activities. These results suggest that a high culturable bacterial diversity is present in Potter Cove and that this place represents a promising source of biomolecules.

Potter Cove is a shallow Antarctic marine environment, located in King George Island (Isla 25 de Mayo), South Shetland Islands. The weather in Potter Cove is not as cold as in continental Antarctica, with summer temperatures ranging from −3°C to 5°C and winter temperatures not lower than −15°C to −20°C. Carlini (Jubany) Argentinean Scientific Station (62° 14′ S 58° 40′ W) is located in an open area on the southern margin of the cove, which stays free of ice during summer (facilitating research and diving). With a unique climate and Antarctic location, Potter Cove and Potter Peninsula have frequently been the focus of interest for scientists looking for a site for long-term ecological research and monitoring programmes. In recent years, several studies have been conducted to evaluate the effect of UV radiation on bacteria isolated from the water column of Potter Cove (Hernández & Mac Cormack 2007; Hernández et al. 2009), to assess the biotechnological potential of

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autochthonous bacterial consortia for on-site bioremediation of hydrocarbon-contaminated soils (Ruberto et al. 2009; Vázquez et al. 2009; Ruberto et al. 2010), to detect the presence and diversity of naphthalene dioxygenase genes in soils (Flocco et al. 2009) and to screen for bacterial extracellular proteases for their use in the cleaning and food industries (Vázquez et al. 2004; Vázquez et al. 2005; Vázquez et al. 2008). A bacterial strain isolated from surface marine water of Potter Cove was described as a new species, *Bizionia argentinensis* (Bercovich et al. 2008), and its complete genome has been sequenced and is being thoroughly studied (Lanzarotti et al. 2011). This research has proven the ecological and biotechnological relevance of the culturable fraction of Potter Cove's bacterial community.

Because extracellular hydrolytic enzymes have diverse possible applications in a variety of industries, their detection and characterization from extremophilic microorganisms is one of the most active fields of applied microbiology research (Kumar et al. 2011). In psychrophilic and psychrotolerant bacteria, in particular, exoenzymes and other products are being studied for their use in a wide range of processes because of their potential to speed these up and to be active in processes requiring low temperatures, as well as their potential to reduce energy expenses, minimize the loss of volatile compounds and reduce contamination risks (Collins et al. 2007).

In this work, we report on the taxonomic affiliation of 189 bacterial isolates obtained from samples taken from Potter Cove, with an emphasis on providing an overview of the biodiversity of culturable bacteria and their capacity for hydrolytic enzyme activities with likely biotechnological applications.

Materials and methods

Sampling area

Samples of seawater, marine sediment, algae and different marine animals were taken from Potter Cove and its shore, near Carlini (Jubany) scientific station (62° 14′ S, 58° 40′ W) in King George Island (Isla 25 de Mayo), South Shetland Islands, Antarctica, during the Argentine summer Antarctic Research Expeditions in 1987, 2001, 2003 and 2005.

Isolation of bacteria

Small portions of the samples (except seawater, that was diluted directly) were placed in a screw-capped bottle containing 5 g of sterile sand and 15 ml of sterile diluent (1 g l^{-1} bacteriological peptone (Difco Laboratories, now BD Diagnostic Systems, Detroit, MI, USA) diluted in 75% v v^{-1} seawater) and vortexed for 5 min. After shaking, serial tenfold dilutions were prepared in the same diluent, and 0.1 ml of each dilution was spread onto the surface of half-strength marine agar 2216 (Difco) plates incubated for 96 h at 4°C. After incubation, the different morphotypes were picked out and isolates were obtained in pure culture by two successive transfers to the same media and finally preserved at −70°C in marine broth with 40% v v^{-1} glycerol.

Identification of bacterial isolates

Isolates were identified on the basis of their 16S rRNA gene partial sequencing and phenotypic characteristics: colony and cell morphology, Gram stain affinity and metabolic profile, using standard tests as well as, in some cases, the analytical profile index API® 20 NE system (bioMérieux, Marcy l'Etoile, France).

For the molecular identification, isolates were grouped in phylotypes according to their amplified ribosomal DNA restriction analysis (ARDRA) profiles. Bacterial genomic DNA was extracted using the Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare, Waukesha, WI, USA) in accordance with the manufacturer's instructions. A fragment of the 16S rRNA gene of approximately 1500 bp was amplified using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). Polymerase chain reaction (PCR) reaction mixture (50 µl) contained 1 µg of genomic DNA, 100 µM of each deoxynucleoside triphosphate, 0.4 µM of each primer, 1.5 mM of $MgCl_2$ and 1 U of Taq DNA polymerase (Invitrogen, now Life Technologies, Carlsbad, CA, USA). Standard PCR consisted of an initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. PCR products were examined by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized under UV light. Amplified products were digested separately with the restriction enzymes *Alu*I and *Hpa*II, and the fragments obtained were further separated by electrophoresis on a 10% polyacrylamide gel stained with ethidium bromide and visualized under UV light. ARDRA profiles in different gels were normalized through the use of the molecular weight marker 100 bp ladder (Invitrogen). Isolates were grouped in phylotypes by visual comparison of their ARDRA profiles, considering that two isolates belonged to the same phylotype if they shared both (*Alu*I and *Hpa*II) restriction profiles. Distinct cleavage patterns were considered as different phylotypes. According to the size of each ARDRA group, one, two or more isolates from each unique phylotype were

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selected for sequencing. The selection was performed as to include at least one representative of each phenotypic pattern within each ARDRA group. Sequences from 16S rRNA genes were amplified from genomic DNA as described above and sent to Macrogen Inc. (Rockville, MD, USA) for further purification (Montage PCR Cleanup Kit; Millipore, Billerica, MA, USA) and sequencing (BigDye Terminator Cycle Sequencing Kit; Applied Biosystems, now Life Technologies, Carlsbad, CA, USA). Products were resolved on an Applied Biosystems 3730XL automated sequencer.

Sequence analysis

Partial 16S rRNA gene sequences (ranging from 800 to 1400 nt) were edited using Bioedit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and compared online with homologous sequences deposited in databases, using algorithms provided by FASTA (http://www.ebi.ac.uk/fasta33/nucleotide.html) and the Megablast option of BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences were further analysed by comparing them against the online database of 16S rRNA genes (type strains) using SeqMatch and Classifier tools from the Ribosomal Database Project Release 10 (http://rdp.cme.msu.edu/) and leBIBI web tool for bacteria identification (http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi). The integrative use of the above-mentioned tools allowed us to reliably identify the isolates to the genus level.

Screening for extracellular hydrolytic enzymes production

A detection assay based on growth on solid media with single substrates as a carbon source was conducted to evaluate the production of extracellular hydrolytic enzymes by the isolates. The strains were cultured by puncture in agar plates (75% v v^{-1} seawater and 1.7% w v^{-1} bacteriological agar) supplemented with 0.2% w v^{-1} $(NH_4)_2SO_4$, 0.01% w v^{-1} yeast extract and the following substrates as the sole carbon sources (0.5% w v^{-1}): crystalline cellulose and carboxymethyl-cellulose (CM-cellulose; Mallinckrodt Baker Inc., now Avantor Performance Materials, Center Valley, PA, USA) to detect cellulase production (Ulrich et al. 2007), xylan from birch wood (Sigma-Aldrich, St. Louis, MO, USA) to detect xylanase production (Li et al. 2008), citric pectin (Sigma-Aldrich) to detect pectinases (Sunnotel & Nigam 2002) and soluble starch (Mallinckrodt Baker Inc.) to detect amylases (Brizzio et al. 2007). In addition, extracellular protease detection was conducted by puncturing the isolates in agar plates (50% v v^{-1} seawater and 1.7% w v^{-1} bacteriological agar) supplemented with skim milk (2.5% w v^{-1}) (Dang et al. 2009). In all cases, pH was adjusted to 7.0–7.5 before sterilization and incubation was carried out at 16°C for five to seven days, except for crystalline cellulose plates, which were incubated for three weeks. For all the tested enzymes, a positive reaction was considered when a clear halo around the colony was observed after incubation at 16°C for the indicated number of days. The clear zones of hydrolysis in media containing cellulose, CM-cellulose and xylan were developed by flooding the agar surface with an aqueous solution of Congo Red dye (1 mg ml^{-1}) for 15 min at room temperature. The stain solution was then poured off and plates were further treated by flooding with 1 M NaCl for 15 min. The developed zones of hydrolysis were stabilized for at least two weeks by further flooding the agar with 1 M HCl, which changes the dye colour from red to blue and inhibits enzyme activity. In the case of starch and pectin, the hydrolysis zones were developed by flooding the agar media with an iodine solution (Sunnotel & Nigam 2002; Brizzio et al. 2007).

Results

Diversity of marine isolates

Data about the 189 bacterial isolates obtained from the different biotopes explored are presented in Table 1. They were characterized on the basis of their ARDRA profiles obtained separately with two restriction enzymes, generating 63 distinct cleavage patterns, called phylotypes. A total of 79 representative isolates of all phylotypes were selected for their 16S rRNA gene sequencing (Table 1). Most of the sequences obtained shared quite a high similarity (99–100%) to their nearest-neighbour sequences deposited in databases. Most of them were from isolates or clones from cold marine environments under environmental conditions comparable to those present in the studied area. In addition, the identity with homologous sequences from the closest type strains was generally greater than 97% (Table 2).

The taxonomic identification of the representative isolates from each of the 63 phylotypes indicated that 49 of them were represented by gram-negative bacteria and 14 by gram-positive bacteria. Out of the 14 gram-positive phylotypes, 12 belonged to the phylum Actinobacteria, represented by the genera *Arthrobacter, Salinibacterium, Kocuria* and *Williamsia*, and two belonged to the phylum Firmicutes (class Bacilli), which was represented by the genus *Planococcus*. Conversely, out of the 49 gram-negative phylotypes, 45 belonged to the phylum

Table 1 Source, number of isolates obtained per sample in each summer Antarctic Research Expedition (ARE) using half-strength marine agar incubated at 4°C and their laboratory reference numbers.

ARE	Sample	Isolates	Laboratory reference number of isolates
1987	Fish (*Notothenia rossi*)		
	Stomach	8	3NRE3, 5NRE1, 5NRE2, 5NRE6, 5NRE7, 8NRE2, 9NRE2, 9NRE8
	Intestine	3	3NRI2, 3NRI4, 5NRI5
	Cloacae	3	1NRC2, 2NRC2, 10NRC2
	Tegument	6	2NRP2, 2NRP5, 3NRP10, 4NRP2, 4NRP4, 9NRP9
2001	Seaweed (*Adenocystis utricularis*)	3	S01-61, S01-62, S01-63
	Microalgae green mat	6	S01-64, S01-65, S01-66, S01-67, S01-68, S01-69
	Seawater	37	S01-70, S01-71, S01-72, S01-73, S01-74, S01-75, S01-76, S01-78, S01-99, S01-100, S01-101, S01-102, S01-103, S01-105, S01-106, S01-107, S01-108, S01-109, S01-110, S01-111, S01-112, S01-113, S01-114, S01-115, S01-116, S01-117, S01-118, S01-119, S01-120, S01-121, S01-122, S01-123, S01-124, S01-125, S01-126, S01-127, S01-128
	Surface marine sediment	8	S01-93, S01-94, S01-129, S01-130, S01-131, S01-132, S01-133, S01-134
	Bivalve (*Laternula elliptica*)	3	S01-96, S01-97, S01-98
	Fish (*Notothenia nudifrons*)		
	Stomach	1	S01-81
	Intestine	3	S01-82, S01-83, S01-84
	Fish (*Pagothenia bernacchii*)		
	Tegument	3	S01-85, S01-86, S01-87
	Stomach	1	S01-88
	Intestine	3	S01-89, S01-90, S01-91
	Limpet (*Nacella concinna*)	2	S01-79, S01-80
2003	Bivalve (*Laternula elliptica*)	9	S03-1, S03-2, S03-3, S03-4, S03-5, S03-6, S03-7, S03-8, S03-11
	Surface marine sediment	6	S03-9, S03-10, S03-12, S03-13, S03-14, S03-15
	Seawater	4	S03-21, S03-22, S03-23, S03-24
2005	Pelagic tunicate (*Salpa thompsoni*)	3	S05-48, S05-49, S05-53
	Surface marine sediment	9	S05-56, S05-60, S05-61, S05-62, S05-70, S05-72, S05-73, S05-74, S05-152
	Seawater	9	S05-88, S05-89, S05-90, S05-97, S05-99, S05-147, S05-148, S05-220, S05-221
	Fish (*Notothenia coriceps*)		
	Gills	4	S05-105, S05-106, S05-107, S05-109
	Fins	6	S05-112, S05-113, S05-115, S05-116, S05-118, S05-119
	Tegument	10	S05-120, S05-121, S05-122, S05-123, S05-124, S05-125, S05-126, S05-127, S05-128,
	Stomach	5	S05-129
	Intestine	2	S05-132, S05-133, S05-135, S05-136, S05-137, S05-140, S05-145
	Polichaete	4	S05-153, S05-154, S05-155, S05-156
	Starfish (*Odontaster validus*)	4	S05-163, S05-164, S05-165, S05-166
	Bivalve (*Laternula elliptica*)	5	S05-178, S05-179, S05-180, S05-185, S05-187
	Microalgae red mat	4	S05-207, S05-208, S05-209, S05-210
	Amphipod	2	S05-215, S05-216
	Gastropod	3	S05-217, S05-218, S05-219
	Isopod	7	S05-173, S05-174, S05-175, S05-222, S05-223, S05-224, S05-225
	Giant isopod (*Glyptonotus antarcticus*)	2	S05-158, S05-159
	Ascidian (*Molgula pedunculata*)	1	S05-94

Proteobacteria and four to the phylum Bacteroidetes. The phylum Bacteroidetes was represented for the class Flavobacteria, with isolates belonging to the genera *Polaribacter, Cellulophaga* and *Flavobacterium*. The phylum Proteobacteria was represented by 43 phylotypes belonging to the class Gammaproteobacteria, one to the class Alphaproteobacteria and one to the class Betaproteobacteria. Among the Gammaproteobacteria, a total of 12 different genera were identified (Table 2). Of these, *Pseudoalteromonas* and *Psychrobacter* were the most frequently recovered, representing the largest groups in terms of the number of isolates (65 isolates belonged to the genus *Psychrobacter* and 58 belonged to the genus *Pseudoalteromonas*). These two genera proved to be ubiquitous in Potter Cove, being recovered from almost all the samples screened: sediments, seawater, crustaceans, bivalves, ascidians, isopods, salps, amphipods, polychaetes, starfish, different fish species and algae.

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Table 2 Distribution of the 189 proteolytic bacterial isolates into 63 amplified ribosomal DNA restriction analysis (ARDRA) phylotypes, indicating their taxonomic affiliation and hydrolysed substrates. The isolates selected for 16S rRNA gene sequencing are indicated in boldface.

ARDRA phylotype	Number of isolates	Laboratory reference number of isolates	Hydrolysed substrates[a]	Taxonomic affiliation	Closest relative type strain 16S rRNA gene (% sequence identity)
F1	8	3NRE3, 5NRE1, **5NRE6**, 8NRE2, 5NRI5, 2NRP2, **9NRE8**, 9NRE2	ND	*Psychrobacter* sp.	*P. cibarius* (AY639871) (100%)
					P. urativorans (AJ609555) (99.9%)
F2	5	**3NRI2**, 3NRI4, 10NRC2, 2NRP5, **9NRP9**	ND	*Psychrobacter* sp.	*P. aquimaris* (AY722804) (99.1%)
F3	1	**1NRC2**	ND	*Psychrobacter* sp.	*P. fozii* (AJ430827) (99%)
F4	1	**4NRP2**	ND	*Psychrobacter* sp.	*P. luti* (AJ430828) (99.7%)
F5	7	**S01-115**, S01-120, S01-122, **S01-127**,	S, SM	*Psychrobacter* sp.	*P. nivimaris* (AJ313425) (99.6-99.9%)
		S01-128, S01-134	SM		
		S01-123			
F6	34	**S05-48**, S05-105, S05-148, S05-121,	SM	*Psychrobacter* sp.	*P. fozii* (AJ430827) (99-99.2%)
		S01-124	ND		
		S05-70, S05-94, S05-106, S05-107, S05-109, S05-113, S05-115, S05-116, S05-120, S05-122, S05-123, S05-125, S05-126, S05-127, S05-128, S05-129, S05-132, S05-133, S05-135, S05-136, **S05-137**, S05-140, S05-145, **S05-152**,			
		S05-185			*P. cryohalolentis* (CP000323) (99.7-100%)
		S05-187, S05-147, S05-220, S05-221			*P. aquimaris* (AY722804) (99.7%)
F7	5	**S05-88**	ND	*Psychrobacter* sp.	*P. submarinus* (AJ309940) (99.7%)
		S05-89			*P. faecalis* (AJ421528) (99.8-99.6%)
		S05-156, S05-60, S05-61			*P. pulmonis* (AJ437696) (99.8%)
F8	3	**S05-56**, S05-119	ND	*Psychrobacter* sp.	*P. cryohalolentis* (CP000323) (99%)
		S05-118	SM		
F9	1	S05-112	ND	*Psychrobacter* sp.	*P. arcticus* (AY444822) (98.5%)
F10	11	**S01-65**	C, CMC, SM	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (100%)
		S01-61, S01-125	S, C, CMC, P, X, SM		*P. paragorgicola* (AY040229) (99.8%)
		S01-64, S01-67, S01-68, S01-71,			*P. elyakovii* (AF082562) (99.8%)
		S01-99	C, CMC, P, X, SM		
		S01-70	S, C, CMC, P, SM		
		S01-113	CMC, P, X, SM		
		S01-86	SM		
F11	7	S01-84, S01-110, **S01-117**	SM	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (99.6%)
		S01-111	CMC, X, SM		*P. translucida* (AY040230) (99.2%)
		S01-132	SM		
		S01-129, S01-130	C, CMC, SM		
F12	4	**S01-121**	C, CMC, X, SM	*Pseudoalteromonas* sp.	*P. elyakovii* (AF082562) (99.7%)
		S01-66, S01-103	C, CMC, P, X, SM		
		S01-98	SM		
F13	9	**S01-81**, S01-82, S01-83, S01-88, S01-89, S01-90, S01-91, S01-133	SM	*Pseudoalteromonas* sp.	*P. translucida* (AY040230) (99.4%)
		S01-114	CMC, SM		
F14	5	**S01-105**	CMC, P, SM	*Pseudoalteromonas* sp.	*P. elyakovii* (AF082562) (99.9%)
		S01-108	CMC, SM		
		S01-109	C, CMC, SM		
		S01-118	C, CMC, X, SM		
		S01-119	C, CMC, P, X, SM		
F15	3	**S01-124**	S, P, SM	*Pseudoalteromonas* sp.	*P. translucida* (AY040230) (99.1%)
		S01-100	S, C, CMC, P, SM		
		S01-126	P, SM		
F16	5	**S03-13**	CMC, P, X, SM	*Pseudoalteromonas* sp.	*P. translucida* (AY040230) (99.1%)
		S03-1, S03-23	S, CMC, P, X, SM		
		S03-11	S, CMC		
		S03-22	S, CMC, P, SM		

Table 2 *Continued*

ARDRA phylotype	Number of isolates	Laboratory reference number of isolates	Hydrolysed substrates[a]	Taxonomic affiliation	Closest relative type strain 16S rRNA gene (% sequence identity)
F17	5	**S03-12**	SM	*Pseudoalteromonas* sp.	*P. translucida* (AY040230) (99%)
		S03-2, S03-10	CMC, P, X, SM		
		S03-4	CMC, P, SM	*Pseudoalteromonas* sp.	*P. translucida* (AY040230) (99%)
		S03-15	S, CMC, P, X, SM		
F18	1	**S03-24**	S, CMC, P, SM	*Pseudoalteromonas* sp.	*P. arctica* (AF529062) (99.7%)
F19	6	**S05-154**, S05-155, S05-173, S05-174	SM	*Pseudoalteromonas* sp.	*P. arctica* (AF529062) (99.7%)
		S05-153	P, SM		
		S05-180	S, P, SM		
F20	1	**S05-175**	SM	*Pseudoalteromonas* sp.	*P. arctica* (AF529062) (99.7%)
F21	1	**S05-223**	C, CMC, P, X, SM	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (100%)
F22	9	**S01-63**, S01-79	ND	*Psychromonas* sp.	*P. arctica* (AF374385) (99.7%)
		S01-72	C, CMC, X		
		S01-116	P		
		S01-96, S01-106, S01-107, S01-112, S01-131	X		
F23	2	**S01-62**	C, CMC	*Psychromonas* sp.	*P. arctica* (AF374385) (98.4-98.7%)
		S01-73	CMC		
F24	1	**S01-101**	C, CMC, P, X	*Psychromonas* sp.	*P. arctica* (AF374385) (99.2%)
F25	1	**S03-3**	ND	*Psychromonas* sp.	*P. arctica* (AF374385) (97.4%)
F26	2	**S05-166**	X	*Psychromonas* sp.	*P. arctica* (AF374385) (99.5%)
		S05-164	P, X		
F27	1	**S05-218**	ND	*Psychromonas* sp.	*P. arctica* (AF374385) (97.7%)
F28	1	**S05-224**	P, X	*Psychromonas* sp.	*P. arctica* (AF374385) (98.9%)
F29	1	**S01-85**	ND	*Pseudomonas* sp.	*P. migulae* (AF074383) (99.6%)
F30	4	**S05-49**	P	*Pseudomonas* sp.	*P. cedrella* (AF064461) (99%)
		S05-97, S05-99	SM		
		S05-178	ND		
F31	2	**S05-207**, S05-208	SM	*Pseudomonas* sp.	*P. brenneri* (AF268968) (99.6%)
F32	1	**5NRE2**	ND	*Arthrobacter* sp.	*A. gangotriensis* (AJ606061) (99.1%)
F33	1	**S01-102**	S, P	*Arthrobacter* sp.	*A. bergerei* (AJ609630) (98.6%)
F34	1	**S05-72**	ND	*Arthrobacter* sp.	*A. stackebrandtii* (AJ640198) (97.4%)
F35	1	**S05-210**	ND	*Arthrobacter* sp.	*A. gangotriensis* (AJ606061) (98.4%)
F36	1	**S05-215**	ND	*Arthrobacter* sp.	*A. gangotriensis* (AJ606061) (98.2%)
F37	1	**S05-163**	C, CMC, P, X, SM	*Colwellia* sp.	*C. aestuarii* (DQ055844) (97.7%)
F38	1	**S05-222**	ND	*Colwellia* sp.	*C. psychrerythraea* (AF001375) (99.4%)
F39	1	**S05-225**	C, CMC, P, X, SM	*Colwellia* sp.	*C. aestuarii* (DQ055844) (97.8%)
F40	2	**S01-93**	C, CMC, SM	*Colwellia* sp.	*C. aestuarii* (DQ055844) (97.6%)
		S01-94	C, CMC, X, SM		
F41	2	**S03-14**	S, CMC, X, SM	*Salinibacterium* sp.	*S. amurskyense* (AF539697) (99.9%)
		S03-9	CMC, P, X, SM		
F42	2	**3NRP10**, 5NRE7	ND	*Salinibacterium* sp.	*S. amurskyense* (AF539697) (99.8%)
F43	1	**S05-216**	ND	*Salinibacterium* sp.	*S. xinjiangense* (DQ515964) (98.2%)
F44	1	**S05-53**	P	*Salinibacterium* sp.	*S. amurskyense* (AF539697) (98.9%)
F45	1	**S05-73**	ND	*Salinibacterium* sp.	*S. amurskyense* (AF539697) (98.8%)
F46	4	**S05-158**, S05-209	SM	*Shewanella sp.*	*S. canadensis* (AY579749) (98.9%)
		S05-90	ND		
		S05-165	S, P, SM		
F47	2	**S01-69**, S01-87	C, CMC	*Polaribacter* sp.	*P. irgensii* (M61002) (97.3%)
F48	1	**S01-97**	ND	*Polaribacter* sp.	*P. irgensii* (M61002) (97.6%)
F49	2	**S05-62**	S, P	*Planococcus sp.*	*P. antarcticus* (AJ314745) (98.3–99.4%)
		S05-74	S		
F50	1	**4NRP4**	ND	*Planococcus* sp.	*P. antarcticus* (AJ314745) (98.6%)
F51	1	**S05-159**	P	*Photobacterium* sp.	*P. indicum* (AB016982) (99.8%)
F52	1	**S05-179**	ND	*Photobacterium* sp.	*P. frigidiphilum* (AY538749) (99.1%)
F53	2	**S01-76**	P	*Kocuria* sp.	*K. palustris* (Y16263) (99.3%)
		S01-78	ND		

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Table 2 *Continued*

ARDRA phylotype	Number of isolates	Laboratory reference number of isolates	Hydrolysed substrates[a]	Taxonomic affiliation	Closest relative type strain 16S rRNA gene (% sequence identity)
F54	2	**S03-6**	CMC	*Cellulophaga* sp.	*C. algicola* (AF001366) (98.5%)
		S03-8	P, SM		
F55	1	**2NRC2**	ND	*Enterobacter* sp.	*E. ludwigii* (AJ853891) (98.4%)
F56	1	**S01-74**	ND	*Acinetobacter* sp.	*A. lwoffii* (X81665) (99.6%)
F57	1	**S01-75**	ND	*Williamsia* sp.	*W. muralis* (Y17384) (99.9%)
F58	1	**S01-80**	ND	*Vibrio* sp.	*V. logei* (AJ437616) (99.7%)
F59	1	**S03-5**	CMC, X	*Flavobacterium* sp.	*F. algicola* (AB455265) (98.3%)
F60	1	**S03-7**	X, SM	*Hydrogenophaga* sp.	*H. taeniospiralis* (AF078768) (98.8%)
F61	1	**S03-21**	ND	*Cobetia* sp.	*C. marina* (AJ306890) (98.9%)
F62	1	**S05-217**	ND	*Sulfitobacter* sp.	*S. litoralis* (DQ097527) (99.8%)
F63	1	**S05-219**	ND	*Marinobacter* sp.	*M. maritimus* (AJ704395) (99.5%)

[a]SM: skim milk; S: starch; X: xylan; P: pectin; C: cellulose; CMC: carboxymethyl-cellulose; ND: no enzymatic activity detected.

Detection of hydrolytic enzyme activities

The production of extracellular hydrolytic enzymes by the 189 isolates is summarized in Table 2. The distribution of isolates producing the different extracellular hydrolytic enzymes screened among the recovered genera is shown in Fig. 1. Isolates that were able to hydrolyse starch, cellulose, CM-cellulose, pectin, xylan and casein represented 9.5, 14.8, 25.4, 22.8, 20.1 and 44.4% of the total, respectively. Proteolysis was the most frequently detected activity, expressed by 83 isolates belonging to the genera *Psychrobacter, Pseudoalteromonas, Pseudomonas, Colwellia, Salinibacterium, Shewanella, Cellulophaga* and *Hydrogenophaga* (Fig. 1). However, pectinase activity was detected in a greater number of genera than was protease activity, with 43 isolates distributed in 11 different genera. The production of amylase was less frequently detected than the other hydrolytic activities. Nevertheless, the amylolytic group was quite diverse, represented by 18 isolates belonging to the genera *Psychrobacter, Pseudoalteromonas, Arthrobacter, Salinibacterium, Shewanella* and *Planococcus*. The group able to hydrolyse crystalline cellulose was less abundant than the one with CM-cellulose hydrolytic activity, with 28 and 48 representative isolates, respectively. Moreover, while the cellulase-producing isolates were distributed between *Pseudoalteromonas, Psychromonas, Colwellia* and *Polaribacter* genera, the CM-cellulase producers belonged to these four genera plus *Salinibacterium, Cellulophaga* and *Flavobacterium*. Although the detection of agarase production was not intended, the agarolytic activity of some isolates was evidenced as a softening of the agar around the colony observed after incubation in half-strength marine agar. This activity was found mainly in *Pseudoalteromonas* isolates from diverse biotopes.

It is important to remark that many isolates produced more than one extracellular enzyme: two were positive for the six enzymatic activities screened, 16 were positive for five of them, 12 produced three to four of the hydrolytic enzymes tested and 16 were positive for two of them. A total of 51 isolates produced only one enzyme activity. Consequently, we considered the hypothesis that the success in the isolation of bacteria producing multiple hydrolytic enzymes can be optimized by applying selective pressure for a single enzyme activity at the initial isolation step, and then to test the other activities of interest. To assess this, the results from our screening were used to calculate, for the total isolates producing each enzymatic activity tested, the percentage that also produced the other enzymes screened (Fig. 2). In this way, for example, all the isolates that in our screening were positive for amylase were considered as the 100% of a hypothetic screening using starch as sole carbon source; and then the percentages of isolates capable of utilizing each one of the other substrates (cellulose, pectin, casein, etc.) were calculated. The analysis showed that protease was the most frequently found activity within the groups that had been obtained if initial isolation had been made on media selective for amylase, CM-cellulase and pectinase hydrolytic activities. In the groups simulating direct selection for cellulase and xylanase producers, CM-cellulase was the most frequent activity and protease activity was the second one. The uptake and hydrolysis of crystalline cellulose is more difficult for bacteria than that of its soluble derivative CM-cellulase (Ulrich et al. 2007), so it is not surprising that isolates producing cellulases were all able to hydrolyse CM-cellulase. Therefore, the production of proteases represented the main secondary activity produced by the isolates recovered from four out of the five simulated selective isolation conditions.

Discussion

The microorganisms used in this study were isolated over a period of 10 years during different summer

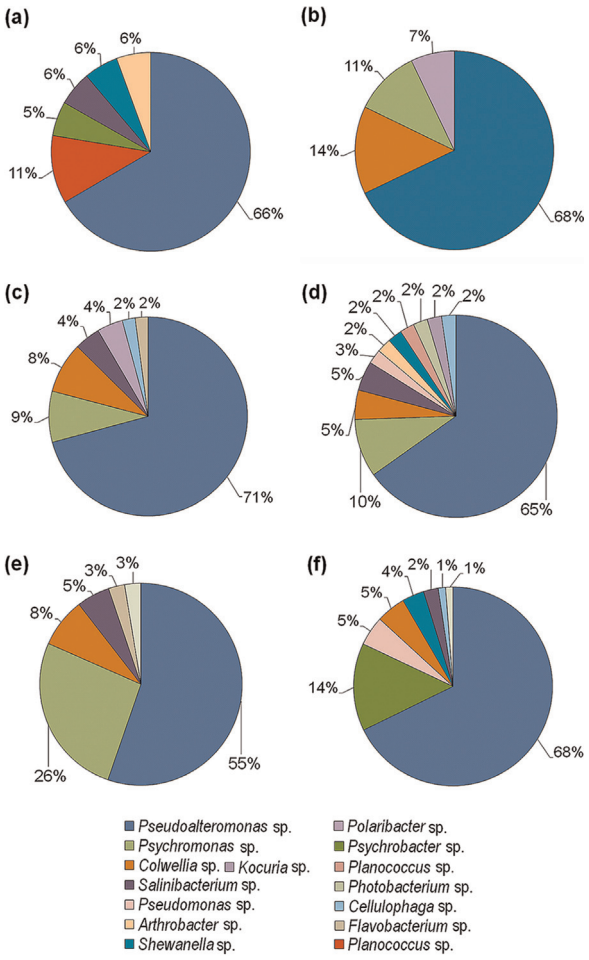

Fig. 1 Abundance (%) of isolates affiliated with the different genera recovered for each detected enzymatic activity: (a) amylase, (b) cellulase, (c) carboxymethyl (CM)-cellulase, (d) pectinase, (e) xylanase and (f) protease.

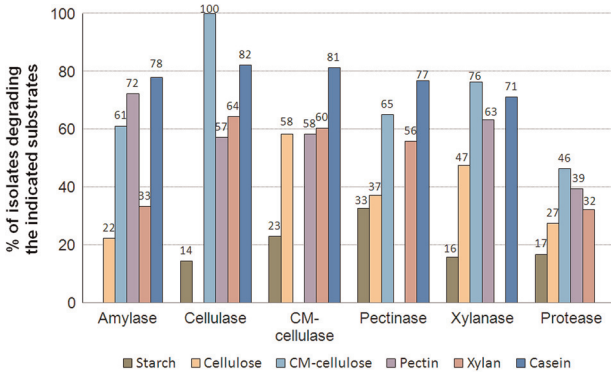

Fig. 2 Percentage of isolates degrading additional substrates within the groups of producers of a particular enzymatic activity.

Antarctic Research Expeditions focused on different research objectives. The samples from which the bacterial isolates were obtained were taken from numerous and diverse marine sites and processed under different culture conditions, resulting in a different number of isolates from each studied biotope. Therefore, it has not been possible in the present work to perform a quantitative description of diversity based on statistical analyses, like those reported in publications where microbial communities from unique biotopes have been studied (Brinkmeyer et al. 2003; Schulze et al. 2006; Dias et al. 2009; Srinivas et al. 2009). In contrast, our results allowed a qualitative description at the genus level of the diversity of culturable heterotrophic bacteria present in numerous marine biotopes, something that has not been previously assessed in Potter Cove. Our approach was successful in disclosing the huge diversity of bacteria able to be cultured and producing bioactive molecules of industrial relevance. In this context, there are some points that can be highlighted from the present study. One of them is the predominance of the class Gammaproteobacteria over the other classes recovered. This predominance, together with the presence of the classes Flavobacteria and Bacilli, agrees with that found in detailed studies of the bacterial Antarctic communities from seawater and marine ice (Bowman et al. 1997; Acinas et al. 1999; Brown & Bowman 2001; Brinkmeyer et al. 2003) and also from the Arctic coastal waters (Groudieva et al. 2004). Gammaproteobacteria is an important and widely distributed group in marine environments, frequently also detected in the analysis of the bacterial diversity from marine sediments (Bowman et al. 2003, Olivera et al. 2007; Srinivas et al. 2009; Zhou et al. 2009; Yu et al. 2011). In addition, the isolates belonging to the most commonly recovered genera, *Pseudoalteromonas* and *Psychrobacter,* were retrieved from almost all the samples tested, showing their ubiquitous distribution in the coastal ecosystems of Potter Cove. These results agree with those reported by other authors who have isolated several species of these genera from many Antarctic environments (Bozal et al. 1997; Bowman 1998; Bozal et al. 2003; Vynne et al. 2011).

Among the isolates obtained in each Antarctic expedition, we found enzyme producers in all groups except NR, in which all isolates were obtained from the fish *Notothenia rossi*. A high proportion of the enzyme producers were affiliated with the more commonly represented genera *Pseudoalteromonas*, *Pseudomonas* and *Psychromonas*, while only a few belonged to *Psychrobacter*. This last genus was almost the only one recovered from fishes, mainly *Notothenia*, explaining the absence of enzyme producers within the NR group as well as in almost all the *Notothenia coriceps* isolates obtained in 2005 expedition. Members of genus *Psychrobacter* were also reported as dominant

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among isolates from deep-sea sediments from the western Pacific (Dang et al. 2009), but those isolates were found to produce extracellular enzymes, mainly lipases and proteases, probably because the biotope represents an important site for particulate organic matter deposit that may stimulate the metabolic activity of sedimentary heterotrophic microorganisms.

In this work, we detected a high proportion of isolates that were positive for all the enzymatic activities screened. This provides only a small glimpse into the exceptional adaptation of the microbiota to the Antarctic environment. The *Pseudoalteromonas* isolates in particular showed great potential for bioprospecting of all screened enzymatic activities, a result that agrees with those from previous studies (Holmström & Kjelleberg 1999; Hoyoux et al. 2001; Truong et al. 2001; Tutino et al. 2002; Zeng et al. 2006). Beyond the biotechnological potential, the high proportion of isolates belonging to the genus *Pseudoalteromonas* and the versatile hydrolytic activities detected in this group also suggest that these organisms may play an important role in polymer hydrolysis in cold environments. Considering only the marine environment where these microorganisms thrive, it can be argued that those who express a great variety of bioactive molecules are those having the higher chances of adaptation and survival in a rapidly changing environment in terms of the availability of substrates for bacterial metabolism. These multiple-enzyme producers will be able to take advantage of a broader spectrum of substrates to incorporate as nutrients. Assuming that the purpose of a screening programme is to screen for several enzymatic activities, the faster and less labourious strategy would be to perform the initial isolation pushing for a single activity rather than performing the initial isolation in parallel using a different selective medium for each enzymatic activity. In the studied environment, the selective pressure for protease production can be a good choice for carrying out the isolation of multiple producers since it proved to be the most frequently detected extracellular enzyme in bacteria obtained from the explored biotopes. This fact was also observed by Groudieva et al. (2004) for bacteria from cold Arctic fjords, where protease was the most abundant hydrolytic enzyme produced by bacteria isolated from sea ice.

Our results demonstrate the ecological relevance of the marine culturable heterotrophic bacterial community from Potter Cove and also highlight its diversity as representative isolates from several distinct bacterial genera were recovered from it. Furthermore, the majority of the retrieved genera were ubiquitous, not displaying any evident distribution pattern among the explored biotopes. This brings to the light the presence of a remarkable bacterial diversity in Potter Cove, which undoubtedly contributes to varied processes of organic matter mineralization and nutrient recycling in the ecosystem, partly conditioning the biodiversity of phytoplanktonic, zooplanktonic, benthic and pelagic organisms that characterize the cove and its shore (Schloss et al. 2002; Raes et al. 2009).

We hope that this work establishes a starting point for future efforts toward biomolecular discovery. Such studies could target molecules detected in this study, or they could look for other molecules: we have demonstrated that many isolates were producers of multiple bioactivities and the possibility that they may produce other interesting molecules cannot be ruled out. We have also shown that the heterotrophic culturable microbiota of Potter Cove, an Antarctic location relatively easy to access and study on account of the Argentinean Scientific Station Carlini there, is a promising source of biomolecules with industrial potential. Further studies are being conducted to assess the potential of these marine isolates in biotechnological applications.

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