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Extracellular hydrolytic enzyme production by proteolytic bacteria from the Antarctic

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Abstract: Cold-adapted marine bacteria producing extracellular hydrolytic enzymes are important for their industrial application and play a key role in degradation of particulate organic matter in their natural environment. In this work, members of a previously-obtained protease-producing bacterial collection isolated from different marine sources from Potter Cove (King George Island, South Shetlands) were taxonomically identified and screened for their ability to produce other economically relevant enzymes. Eighty-eight proteolytic bacterial isolates were grouped into 25 phylotypes based on their Amplified Ribosomal DNA Restriction Analysis profiles. The sequencing of the 16S rRNA genes from representative isolates of the phylotypes showed that the predominant culturable protease-producing bacteria belonged to the class Gammaproteobacteria and were affiliated to the genera *Pseudomonas*, *Shewanella*, *Colwellia*, and *Pseudoalteromonas*, the latter being the predominant group (64% of isolates). In addition, members of the classes Actinobacteria, Bacilli and Flavobacteria were found. Among the 88 isolates screened we detected producers of amylases (21), pectinases (67), cellulases (53), CM-cellulases (68), xylanases (55) and agarases (57). More than 85% of the isolates showed at least one of the extracellular enzymatic activities tested, with some of them producing up to six extracellular enzymes. Our results confirmed that using selective conditions to isolate producers of one extracellular

enzyme activity increases the probability of recovering bacteria that will also produce additional extracellular enzymes. This finding establishes a starting point for future programs oriented to the prospecting for biomolecules in Antarctica.

Key words: Antarctic, marine bacteria, cold enzymes, psychrophiles.

Introduction

The enzymes produced by microorganisms living in extreme environments are considered versatile tools for the development and improvement of a variety of industrial and biotechnological processes (Gomes and Steiner 2004) owing to their biodegradability, specific stability under extreme or non-conventional conditions, improved use of raw materials and lower amounts of waste products (Margesin *et al.* 2007; Kumar *et al.* 2011; Loperena *et al.* 2012). Cold-adapted enzymes in particular, are one of the main focuses of the bioprospecting for extremozymes, with a number of them currently used in industrial processes (Cavicchioli *et al*. 2011). Cold microbial proteases are of great commercial value, representing a significant fraction of the world market of cold-active enzymes (Kuddus and Ramteke 2012).

As a source of extremozymes, the Antarctic represents a vast and poorly explored cold environment suitable for bioprospecting for new cold-adapted biomolecules. In aquatic environments in general, it is known that the initial and rate-limiting step in carbon oxidation and nitrogen recycling from particulate organic matter (POM) is achieved through degradation processes mainly performed by bacterial enzymes (Talbot and Bianchi 1997; Huston and Deming 2002; Brunnegard *et al.* 2004; Hunter *et al.* 2006). In Antarctic coastal marine environments in particular, the main part of the POM consists of high molecular weight substances (Tatian *et al.* 2002) available for uptake by microorganisms only after their prior cleavage by these bacterial extracellular enzymes. Since proteins are one of the main components of sedimentary marine POM, protease-producing bacteria play a key role in the organic carbon and nitrogen metabolism in Antarctic coastal marine ecosystems (Vazquez *et al.* 2004; Dang *et al.* 2009; Srinivas *et al.* 2009; Zhou *et al.* 2009). In addition, other hydrolytic enzymes, such as those degrading the complex polysaccharides: xylan, cellulose, pectin and starch, produced by bacteria able to metabolize components of POM, as was observed for cold-adapted bacteria, producing polysaccharolytic enzymes (Dang *et al.* 2009; Dias *et al.* 2009; Srinivas *et al.* 2009).

In Antarctic surface soils and shallow marine waters strongly influenced by the terrestrial runoff, a significant fraction of the culturable bacteria are psychrotolerant rather than strict psychrophiles, when classified on the basis of their growth temperatures (Bakermans 2012). However, these psychrotolerant bacteria have enzymes exhibiting a fraction of their maximum catalytic activity at temperatures at which the true mesophilic enzymes have no activity whatsoever

(Collins *et al.* 2007). Potter Cove, located at King George Island (Isla 25 de Mayo), South Shetland Islands, represents a typical Antarctic coastal marine environment. The weather is warmer than in the continental Antarctica, with air temperatures ranging from -3°C to 5°C in summer and seldom falling below -15°C in winter. Surface seawater temperatures in the cove are between 1.4°C and -1.7°C (Schloss *et al.* 2012). In a previous study performed on this cove focused on marine bacterial strains isolated without applying selective pressure for any exoenzyme production, we observed that, when the presence of one extracellular hydrolytic activity was detected, the production of other hydrolytic enzymes was frequently associated (Tropeano *et al.* 2012). As proteases were the most abundant extracellular enzymes detected, we postulated that a good strategy to retrieve producers of multiple activities could be to apply selective pressure for the production of proteases. Therefore, in this study we tested that hypothesis working with a previously-obtained collection of bacteria isolated from different marine biotopes in Potter Cove, using a culture media selective for protease producers. This strategy of isolation by selecting for one enzymatic activity as the first step in a screening program aimed to obtain multiple-enzyme producers results in an effort-reducing advantage.

In this study, we characterized a collection of 88 proteolytic isolates from Potter Cove in order to relate the species distribution of these culturable bacteria with their ability to produce additional hydrolytic enzymes other than proteases.

Sampling area

The samples were collected during five Argentine summer Antarctic Research Expeditions (ARE) between 1989 and 2006, near *Carlini* Station (ex-*Jubany*, 62°14' S, 58°40' W). This station is located in the southern margin of the cove and has held permanent human activity since 1952. All samples were taken from the Potter Cove marine environment, and corresponded to water, sediments, algae and tissues from different animals (Table 1).

Methods

Isolation and identification of bacterial isolates. — Suspensions from sediments, algae and animal tissues samples were treated as described previously (Tropeano *et al.* 2012). Ten-fold dilutions of the suspensions were spread onto the surface of skimmed-milk marine agar plates (Vazquez *et al.* 2005) and incubated for 96 h. Isolation temperature varied depending on the ARE in which the corresponding samples were taken, as shown in Table 1. After incubation, colonies with proteolytic activity, demonstrated as a clear zone due to casein hydrolysis, were

Table 1

Number of isolates obtained in each Antarctic Research Expedition (ARE) using marine skimmed-milk agar as selective medium for proteolytic bacteria.

ARE	Isolation temperature	Sample	Number of isolates	Laboratory reference-number of isolates
1989/90	10–13°C	fish tegument (*Notothenia rossi*)	3	273, 435, Piel-1
		elephant seal remains	2	Ele-2, Ele-3
1991/92	10–13°C	fish tegument (*Notothenia rossi*)	3	Prot-8, Prot-12, Prot-14
		surface seawater	2	Prot-4, Prot-5
		elephant seal remains	1	Prot-11
1994/95	20°C	surface marine sediment	2	P95-26, P95-28
1995/96	4°C	surface marine sediment	2	P96-25, P96-26
		surface seawater	8	P96-1, P96-3, P96-45, P96-46, P96-47, P96-48, P96-49, P96-50
		marine macroalgae (surface, unidentified)	3	P96-4, P96-5, P96-6
		fish stomach (*Notothenia rossi*)	1	P96-51
		fish tegument (*Notothenia rossi*)	2	P96-52, P96-56
		fish intestine (*Notothenia rossi*)	3	P96-53, P96-54, P96-55
2005/06	4°C	surface seawater	11	P06-27, P06-28, P06-29, P06-30, P06-31, P06-32, P06-33, P06-40, P06-41, P06-84, P06-114
		sessile medusa (whole tissue, unidentified)	2	P06-42, P06-43
		crustacean (surface, unidentified)	2	P06-63, P06-64
		limpet (*Nacella concinna*, whole tissue)	3	P06-44, P06-45, P06-46
		brittle star (*Ophiacantha vivipara*, whole tissue)	3	P06-60, P06-61, P06-62
		macroalgae (*Phaerus antarcticus*, surface)	3	P06-71, P06-72, P06-73
		macroalgae (*Ascoceira mirabilis*, surface)	2	P06-95, P06-96
		macroalgae (*Desmarestia anceps*, surface)	5	P06-66, P06-67, P06-68, P06-69, P06-70
		red algae (unidentified, surface)	2	P06-88, P06-89
		brown algae (unidentified, surface)	1	P06-74
		colonial ascidian (unidentified, whole tissue)	3	P06-85, P06-86, P06-87
		ascidian (*Aplidium radiatum*, whole tissue)	2	P06-92, P06-93
		isopod (*Serolis* sp., whole tissue)	2	P06-90, P06-91
		ice fish tegument (*Chaenocephalus aceratus*)	7	P06-81, P06-82, P06-99, P06-101, P06-102, P06-103, P06-104
		starfish (*Odontaster validus*, whole tissue)	8	P06-79, P06-80, P06-105, P06-106, P06-107, P06-108, P06-109, P06-110

re-streaked twice in the same media and finally preserved as pure cultures in nutrient broth (Merck KGaA) reconstituted with sea water and containing 40% v v^{-1} glycerol at -70°C. A total of 88 proteolytic isolates were selected for further characterization.

Strains were identified on the basis of their 16S rRNA gene-sequencing and phenotypic characterization: colony and cell morphology, Gram-stain affinity and metabolic profile (using standard tests and, in some cases, also the Analytical Profile Index API® 20 NE system, bioMérieux).

For the molecular identification isolates were grouped into 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) and the 16S rRNA gene was amplified using the primers 27F and 1492R as described previously (Vazquez *et al.* 2005). ARDRA profiles were obtained and analysed as described in Tropeano *et al.* (2012) to group the isolates into phylotypes. According to the size of each ARDRA group, one or more isolates from each unique phylotype were selected for sequencing. In this selection, at least one representative of each phenotypic pattern present in each ARDRA group was included. Sequences from 16S rRNA genes were amplified from genomic DNA as described above and sent to Macrogen Inc., USA, for sequencing.

Sequence analysis. — Partial 16S rRNA genes sequences (ranging from 800 to 1400 nucleotides) were edited using the BIOEDIT software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and compared online with related sequences present in databases, using BLAST (Megablast) (http://www.ncbi.nlm.nih.gov/BLAST/) algorithm. The isolates were identified using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim *et al.* 2012) on the basis of the similarity of their 16S rRNA sequences compared against the database containing type strains with validly published prokaryotic names according to the List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.net) (Euzéby 1997).

Screening for production of extracellular hydrolytic enzymes. — A culture-dependent detection assay was conducted on agar-plates to evaluate the production of other extracellular hydrolytic enzymes by the already protease-producing isolates. The strains were cultured by puncturing 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 sole carbon sources (0.5% w v^{-1}): crystalline cellulose and carboxymethyl-cellulose (CM-cellulose) (Mallinkrodt Baker Inc., now Avantor Performance Materials) to detect cellulase production (Ulrich *et al.* 2007), xylan from birchwood (Sigma-Aldrich) to detect xylanase production (Li *et al.* 2008), citric pectin (Sigma-Aldrich) to detect pectinases (Sunnotel and Nigam 2002) and soluble starch (Mallinkrodt Baker Inc.) to detect amylases (Brizzio *et al.* 2007). In all cases, pH was adjusted to 7.0–7.5 before sterilization. For all tested enzymes, reaction was considered positive when a clear halo around the colony was observed after incubation at 16°C for 5–7 days, except for crystalline cellulose plates which were incubated for 3 weeks. In addition, a softening of the agar observed as a pit around the colony after incubation in marine agar at 16°C for 5–7 days was considered as evidence of agarase production. The clear

zones of hydrolysis in media formulated with 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 zones of hydrolysis were stabilized for at least 2 weeks by further flooding the agar with 1 M HCl, which changes the dye colour from red to blue and inhibits further enzyme activity. In the case of starch and pectin, the hydrolysis zones were developed by flooding the agar media with an iodine solution (Brizzio *et al.* 2007; Sunnotel and Nigam 2002).

Results

Identification of protease-producing bacterial isolates. — Based on both individual *Alu*I and *Hpa*II ARDRA profiles, a total of 88 proteolytic isolates was grouped into 25 distinct phylotypes. Forty-eight isolates, representatives of all phylotypes, were selected for 16S rRNA gene sequencing (Table 2). Most of the sequences obtained were highly similar (usually 99–100% of identity) to their nearest-neighbour sequences, almost all of them derived from isolates or clones obtained from cold marine environments, which was consistent with the origin of our isolates. Likewise, the percentages of identity shared with the closest 16S rRNA gene sequences from type strains were generally greater than 98%, supporting the affiliation to genus level (Stackebrandt and Goebel 1994).

The taxonomic affiliation of the representative isolates from each of the 25 phylotypes showed that 22 of them were Gram-negative bacteria and only three were Gram-positive. Out of the 22 Gram-negative phylotypes, 14 belonged to the class Gammaproteobacteria and 8 to the class Flavobacteria. Among the Gammaproteobacteria, a total of four different genera were identified (*Pseudoalteromonas*, *Pseudomonas*, *Shewanella* and *Colwellia*), with *Pseudoalteromonas* representing the largest proportion of isolates (56 of 88 isolates, Table 2). In the Flavobacteria group, five different genera were found, including *Nonlabens*, *Flavobacterium*, *Cellulophaga*, *Lacinutrix* and *Olleya*. Out of the three Gram-positive phylotypes, one was affiliated to the class Actinobacteria and two to the class Bacilli. The first one was represented by the genus *Arthrobacter*, while the second one was represented by the *Sporosarcina* and *Planomicrobium* genera.

Detection of extracellular hydrolytic enzymes production. — The production of extracellular hydrolytic enzymes by the 88 isolates is summarized in Table 2 and Fig. 1. Pectinase and CM-cellulase were the activities most frequently detected. They were found in 67 (76%) and 68 (77%) isolates respectively, belonging to the genera *Pseudoalteromonas*, *Pseudomonas*, *Olleya*, *Flavobacterium*, *Cellulophaga* and *Lacinutrix*. In addition, 53 (60%) out of those 88 isolates produced cellulases and 55 (62%) were positive for xylanases, being mainly members of the

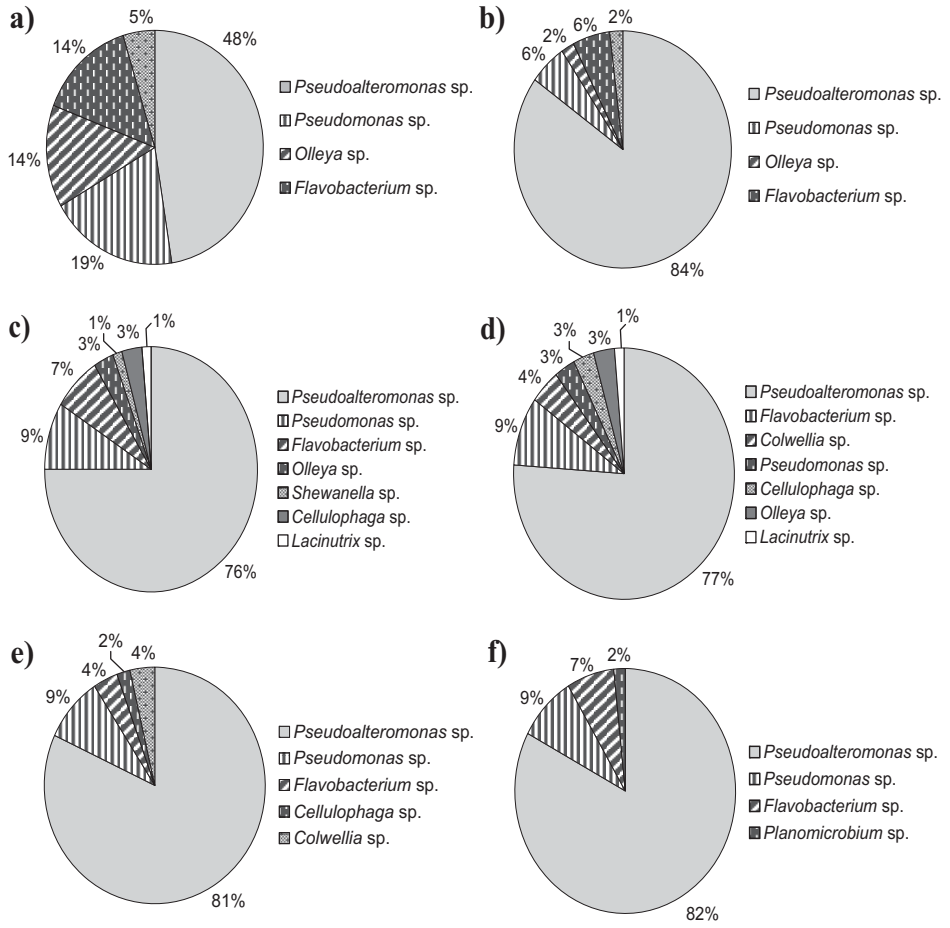

Fig. 1. Distribution of the abundance (%) of the different genera recovered producing each of the detected extracellular enzymatic activities. **a**, amylase; **b**, cellulase; **c**, carboxymethyl-cellulase; **d**, pectinase; **e**, xylanase; **f**, agarase.

genera *Pseudoalteromonas* or *Cellulophaga*. Although amylase was detected less frequently than the other screened activities, the taxonomic affiliation of producing strains was rather diverse and comprised 21 (24%) isolates belonging to the genera *Pseudoalteromonas*, *Pseudomonas*, *Olleya*, *Flavobacterium*, and *Cellulophaga*. Furthermore, agarolytic isolates predominantly belonged to the genera *Pseudoalteromonas*, *Pseudomonas* and *Flavobacterium*. In contrast to the variety of hydrolytic activities found among the Gram-negative isolates, in the Gram-positive group (*Sporosarcina, Arthrobacter* and *Planomicrobium*) no member produced any of the extracellular hydrolytic enzymes screened. The only exception was a weak agarolytic activity produced by *Planomicrobium* sp. P95-26 that was demonstrated as a softening of the agar more than as a clear pit around the colony. Several isolates produced more than one extracellular enzyme; nine were positive

Table 2

Distribution of the 88 proteolytic bacterial isolates into 25 ARDRA phylotypes and 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	26	P96-49, **P96-52, P06-32, P06-62, Prot-8**, Prot-14, P06-27, P06-28 **P06-29,** P06-40, **P06-42**, P06-44	S, X, P, C, CMC, A	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (99.6–100%)
		P06-86, **P06-90, P06-91,** P06-96, P06-99, P06-101, P06-105, P06-109	X, P, C, CMC, A		
		P06-60, **P06-61,** P06-84, P06-85	X, P, CMC, A		
		P06-87	X, P, C, CMC		
		P06-106	P		
F2	6	**P96-47**, P96-54, 435	ND	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (100%)
		Piel-1	X, P, C, CMC, A		
		P96-55	S, P, C, CMC, A		
		Prot-5	X, CMC		
F3	3	P96-4, **P96-5**, **P06-114**	X, P, C, CMC, A	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (99.9%)
F4	1	**P96-3**	P, C, CMC, A	*Pseudoalteromonas* sp.	*P. arctica* (DQ787199) (99.4%)
F5	9	**P96-46, P06-45**, P06-46, P06-63, P06-66, P06-80, P06-81, P06-82, P06-89	X, P, C, CMC, A	*Pseudoalteromonas* sp.	*P. nigrifaciens* (X82146) (99.8%)
F6	1	**P96-6**	ND	*Pseudoalteromonas* sp.	*P. nigrifaciens* (X82146) (99.8%)
F7	2	P06-71	S, P, C, CMC, A	*Pseudoalteromonas* sp.	*P. translucida* (AY040230) (99.3%)
		P06-74	X, P, C, CMC, A		
F8	8	P06-33, **P06-79**	S, X, P, C, CMC, A	*Pseudoalteromonas* sp.	*P. translucida* (AY040230) (99.3%)
		P06-92, P06-93	X, P, C, CMC, A		
		P06-108	X, P, C, CMC		
		P06-64	X, P, CMC, A		
		P06-68	S, P, C, CMC, A		
		P06-110	P, C, CMC, A		
F9	5	P96-48, P96-51	S, X, P, C, CMC, A	*Pseudomonas* sp.	*P. gessardii* (AF074384) (99.9%)
		P96-50, P96-53	X, CMC, A		
		Prot-4	ND		

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)
F10	1	**P96-1**	X, CMC	*Pseudomonas* sp.	*P. gessardii* (AF074384) (99.8%)
F11	1	**P96-45**	CMC, A	*Pseudomonas* sp.	*P. libanensis* (AF057645) (100%)
F12	4	**Prot-12, Prot-11**, Ele-2, Ele-3	ND	*Pseudomonas* sp.	*P. gessardii* (AF074384) (100%)
F13	2	P06-30	S, P, CMC	*Olleya* sp.	*O. namahensis* (JQ327134) (98%)
		P06-69	S		
F14	1	**P06-67**	S, P, C, CMC	*Olleya* sp.	*O. namahensis* (JQ327134) (97.5%)
F15	1	**P96-25**	S, CMC	*Flavobacterium* sp.	*F. frigidarium* (AF162266) (97.3%)
F16	6	**P06-41**	S, X, P, C, CMC, A	*Flavobacterium* sp.	*F. frigidarium* (AF162266) (98-5%)
		P06-43**,** P06-73	P		
		P06-70	S, P, C, CMC, A		
		P06-88	X, P, CMC, A		
		P06-107	P, C, CMC, A		
F17	1	**P96-26**	S, X, P, C, CMC	*Cellulophaga* sp.	*C. algicola* (CP002453) (99.9%)
F18	1	**P06-31**	P, CMC	*Cellulophaga* sp.	*C. fucicola* (AJ005973) (99.6%)
F19	1	**P95-28**	ND	*Sporosarcina* sp.	*S. koreensis* (DQ073393) (99%)
F20	1	**273**	ND	*Arthrobacter* sp.	*A. bergerei* (AJ609630) (99.8%)
F21	1	**P95-26**	A	*Planomicrobium* sp.	*P. okeanokoites* (D55729) (99.7%)
F22	1	**P96-56**	CMC	*Shewanella* sp.	*S. arctica* (GU564402) (100%)
F23	1	**P06-102**	P, CMC	*Lacinutrix* sp.	*L. algicola* (DQ167238) (99.6%)
F24	1	**P06-103**	ND	*Nonlabens* sp.	*N. xylanidelens* (FR733714) (99.1%)
F25	3	P06-72, **P06-95**	X, P	*Colwellia* sp.	*C. aestuarii* (DQ055844) (98%)
		P06-104	P		

[a] hydrolysed substrates; S: starch; X: xylan; P: pectin; C: cellulose; CMC: carboxymethyl-cellulose; A: agar; ND: none enzymatic activity detected.

for the six enzymatic activities screened, 37 were positive for five of them, 12 produced only four of the hydrolytic enzymes tested and 18 isolates only produced one to three. In addition, the two predominant genera, *Pseudoalteromonas* and *Pseudomonas*, were also the ones with the highest number of enzyme-producers. Among the 56 *Pseudoalteromonas* isolates, 52 produced at least one hydrolytic enzyme activity. The group of isolates producing five hydrolytic activities represented the most abundant one, with 37 isolates positive for extracellular pectinases, cellulases, CM-cellulases, and agarases, with some positive also for xylanases, while the others produced amylases. On the other hand, most of the *Pseudomonas* isolates produced both, CM-cellulases and xylanases, while 6 produced at least one enzyme. All the isolates belonging to the genus *Flavobacterium* produced at least one enzymatic activity, with pectinases and CM-cellulases being the most frequently detected.

Discussion

The results obtained in this work highlight the biotechnological potential of Potter Cove as they showed that a high proportion of the isolates recovered for their ability of producing proteases also produced all the other screened enzymatic activities. This also gives evidence of the versatility of the culturable microbiota from Potter Cove to take advantage of the nutrient sources, which are sometimes scarce in the Antarctic. In fact, *Pseudoalteromonas* isolates showed a great potential for the bioprospection of a great number of enzymes, in accordance with other reports (Holmström and Kjelleberg 1999; Truong *et al.* 2001; Tutino *et al.* 2002; Van Petegem *et al.* 2003; Zeng *et al.* 2006). The versatile hydrolytic capacity exhibited by this genus and its remarkable abundance among the producers of cold enzymes suggest its relevance to the recycling of organic matter. Since the coastal marine environments in the Antarctic can be considered rather changeable from the point of view of the substrate availability for bacterial metabolism, it can be argued that those bacteria which produce a wide spectrum of bioactive molecules are also the ones with the greatest possibilities for successful adaptation to live there. To thrive in these ecosystems, the microorganisms should be able to uptake many different substrates as nutrients. In this sense, our present results confirm our previous hypothesis (Tropeano *et al.* 2012) that it is possible to obtain a high proportion of multiple-hydrolytic-enzymes producers among a group of microorganisms selected by the presence of a single activity, in the case of this work, proteolytic activity. Following this concept, whenever the purpose of a screening program were the isolation of bacteria producing multiple enzymatic activities, a faster and less laborious strategy would be to perform an initial isolation using a culture medium selective for a single activity. This strategy would avoid an initial isolation followed by a second screen-

ing step, where a different selective medium for each enzymatic activity would have to be used simultaneously.

Behind the biotechnological relevance, our results reveal some observations on the occurrence and taxonomic affiliation of the proteolytic culturable bacteria from Potter Cove. One of them is the predominance of the class Gammaproteobacteria (more than 76% of the total isolates), mainly represented by *Pseudoalteromonas* and *Pseudomonas* as the two dominant genera, isolated from almost all the explored biotopes. This predominance could be attributed to the selective conditions used for isolation, as the extracellular proteolytic activity seems to be well represented among members of these two genera (Margesin and Schinner *et al.* 1992; Holmström and Kjelleberg 1999; Vázquez *et al.* 2004; Vázquez *et al.* 2008; Xiong *et al.* 2007). For instance, Konieczna *et al.* (2011) reported that 70% of the total aerobic bacteria that they cultured from marine sediment samples at Spitsbergen, in the Arctic Ocean, revealed proteolytic activity and that *Pseudoalteromonas* was the most frequently occurring genus within their isolates. Also in the opposite pole, in a Sub-Antarctic area in the Southern Ocean, Olivera *et al.* (2007) reported that Gammaproteobacteria was the main group among protease-producing bacteria isolated from marine sediments, represented by members of the genera *Pseudoalteromonas, Shewanella, Colwellia* and *Planococcus*. Although they reported the taxonomic affiliation of few (19) selected strains, the similarity to our results, in relation to the dominant genera among the protease-producing isolates, is remarkable. Even when the geographic areas explored in both studies are located in the Southern Ocean, they are separated by the Polar Front, which represents a strong 25-million-year-old barrier for the free latitudinal exchange of water and is considered a distinctive biogeographical discontinuity (Clarke *et al.* 2005). The fact that the same bacterial genera was found to dominate the culturable microbiota at both sides of the Polar Front represents an example of the above mentioned effect of the same culture conditions used for isolation. When equal selective conditions (cold marine waters and protease production) were present, the dominance of the same bacterial groups was observed despite their physical isolation. It is worth mentioning that, as these studies were restricted to the culturable heterotrophic fraction of the bacteria present in the explored biotopes, the taxa recovered probably differ from those found when the bacterial diversity was described using culture-independent methods, since it has been clearly stated that certain populations that are detected by culture-independent methods cannot be recovered in culture (Cowan and Tow 2004).

The class Flavobacteria was the second commonest taxonomic group (16%), with all the isolates belonging to the family Flavobacteriaceae. Members of this family are surprisingly widely present all around the world (Bernardet and Nakagawa 2006), and marine habitats seem to be most commonly colonized by this group (Kirchman 2002; Grossart *et al.* 2005). Flavobacteria utilize a broad spectrum of organic carbon sources, from complex polymers (such as exopolysaccharides and pro-

teins) to simple molecules (such as amino acids and monosaccharides), having an important role in the assimilation of the high-molecular-weight portion of dissolved organic carbon (Kirchman et al 2004). Their prevalence among the exoenzyme-producing isolates from Potter Cove not only supports the ecological role attributed to these microorganisms but also reinforces the biotechnological potential of this group as a source of cold-active bioproducts (Secades *et al.* 2003; Williams 2009; Chen *et al.* 2013).

It is important to remark that the bacterial taxa isolated here seem to be ubiquitous within the microbiota of Potter Cove, with no particular pattern of distribution among the explored biotopes. Moreover, the bacterial diversity found in Potter Cove undoubtedly contributes to the mineralization and nutrient recycling of organic matter in this ecosystem, conditioning the exceptional biodiversity of phytoplanktonic, zooplanktonic, benthic and pelagic organisms that characterizes the cove and its shoreline and that led the Antarctic Treaty Consultative Meeting (ATCM 2002) to designate the coastal area of Potter Peninsula as Specially Protected Area (ASPA 132).

In conclusion, the present work has demonstrated that the heterotrophic culturable microbiota of Potter Cove, a place in the Antarctic that is easy to access, is a promising source of biomolecules of industrial interest and also highlights the diversity and ecological relevance of the heterotrophic culturable fraction of its marine bacterial community. In our opinion, this work establishes a starting point for future programs oriented to the prospecting for biomolecules in Antarctica. Such programs may not necessarily look for the same activities detected here, since it was demonstrated that many isolates were producers of multiple extracellular enzymes. In this sense, when the selection of bacteria producing one certain extracellular enzyme activity is performed, many of the obtained isolates will be likely to produce additional extracellular enzymes. Further studies are underway to assess the potential of the isolates for their use in biotechnological applications.

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