Research Paper

Diversity of protease-producing marine bacteria from sub-antarctic environments

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From seawater and the intestines of benthonic organisms collected from the Beagle Channel, Argentina, 230 marine bacteria were isolated. Cultivable bacteria were characterized and classified as psychrotolerant, whereas few isolates were psychrophiles. These isolates were capable of producing proteases at 4 and 15 °C under neutral (pH 7.0), alkaline (pH 10.0) and acidic (pH 4.5) conditions on different media, revealing 62, 33 and 22% producers at cold and 84, 47 and 33% producers at low temperatures, respectively. More protease-producing strains (67%) were detected when isolated from benthic invertebrates as compared to seawater (33%), with protease production under neutral conditions resulting in milk protein hydrolysis halos between 27 and 30 ± 2 mm in diameter. Using sterile 0.22 μm membrane filters, 29 isolates exhibiting extracellular protease activity were detected. These were grouped into six operational taxonomic units by restriction analysis and identified based on 16S rDNA as γ-proteobacteria of the genera *Pseudoalteromonas*, *Pseudomonas*, *Shewanella*, *Alteromonas*, *Aeromonas*, and *Serratia*. Plasmids were found to be harbored by eight strains, mainly within the isolates from benthonic organisms.

Keywords: γ-Proteobacteria / Cold-active protease / Benthonic organisms / 16S rDNA / ARDRA

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Introduction

The industrial enzyme market has annual sales of US$ 2.3 billion, with applications in the detergents (34%), foods (27%), agriculture and feeds (16%), textiles (10%) and leather, chemicals, and pulp and paper industries (10%). The increased use of proteases in the industry has boosted the demand for new proteases that are better adapted to the specific conditions of production or processes [1]. Serine proteases used in washing powders account for US$ 200 million of this market [2]. An example of commercial application is seen with a cold-active protease from novozyme that is sold as an encapsulated detergent [3]. Proteolytic enzymes active at low temperatures provide an enormous biotechnological potential, offering economic and ecological advantages [4], and have a broad spectrum of application, e.g. in the food industry by improving the flavor of refrigerated treated meat, as well as in laundry products and leather processing [5, 6]. Besides, they can provide alternatives to mesophilic enzymes in the brewing and wine industry, in cheese manufacturing, animal feed production and in the fruit juice industry [7].

Marine environments represent an enormous pool of potential microbial biodiversity and exploitable biotechnology or "blue biotechnology". Novel enzymes with a wide range of enzymatic activities for biocatalytic processes have been obtained from cultured ma-

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rine bacteria [8]. Cold-adapted microorganisms (i.e. psychrophilic or psychrotolerant bacteria) have adapted to cold habitats, making them valuable sources for cold-active enzymes [9] with potential industrial applications [10]. The industrial use of cold-active proteases could offer economic benefits through energy saving because there is no need for expensive heating steps, and the enzymes also function in cold environments during the winter season. At the same time, cold-active enzymes provide good reaction yields, have an elevated level of stereospecificity, minimize undesired chemical reactions that can occur at higher temperatures and can be inactivated thermally if required [11]. In order to obtain new cold-active proteases, marine proteobacteria [4, 10] have been cultivated from seawater and sea ice [12]. Here, we examined marine psychrotolerant and psychrophilic bacteria isolated from the sub-antarctic Beagle Channel for their ability to produce extracellular proteases at different pH values. The producer strains were grouped by restriction analysis and identified based on 16S rDNA sequencing. Plasmid screening revealed similar banding patterns indicative of heterologous gene transfer, specifically within the invertebrate hosts.

Materials and methods

Sampling area

Samples were aseptically collected from Tierra del Fuego, South Argentina, in July 2001. Subsurface (20 m) seawater samples were taken from three coastal areas of the Beagle Channel (55°07′18″ S, 67°15′00″ W): Ushuaia Bay (54°50′01″ S, 68°15′48″ W), Ensenada Bay (54°51′11″ S, 68°29′59″ W), and Punta Segunda (54°51′27″ S, 68°27′41″ W). At the same locations, additional samples were taken from the intestines of benthonic organisms (*Munida subrugosa*, *Paralomis granulosa*, *Pseudoechinus magellanicus*, and *Nacella deaurarta*). The Beagle Channel is a sub-antarctic environment where habitats have an oceanic climate, with annual average air temperatures between 4 and 8 °C. The seawater temperature measured *in situ* ranged from 4 to 9 °C.

Enrichment and isolation of marine microorganisms

Microorganisms from seawater and intestines of benthonic organisms were enriched in liquid milk (containing in g/l: milk 10; and agar 15 for plates) and starch-casein agar (SCA; composition in g/l: casein 1, soluble starch 10, dibasic potassium phosphate 0.5, 1 M sodium hydroxide 5 ml, and agar 15) media. Casein was first dissolved in 5 ml 1 M sodium hydroxide. For milk medium, the pH (4.5, 7.0 and 10.0) was adjusted with 1 M chloridic acid or 1 M sodium hydroxide, and the milk medium was sterilized at 121 °C and 1 atm for 15 min. In addition, samples were enriched in liquid R2A (containing in g/l: yeast extract 0.5, proteose peptone 0.5, casamino acids 0.5, dextrose 0.5, soluble starch 0.5, sodium pyruvate 0.3, 75 vol% seawater; and agar 15 for plates) [13] and Luria-Bertani (LB) media according to Cristóbal *et al.* [9].

The inoculated media were incubated at 4 and 15 °C in an orbital shaker (200 rpm) for 2 or 4 d. After turbidity-indicated growth, 100 μl culture was diluted in sterile distilled water and spread onto the same solid medium for incubation at the same temperatures.

Assimilation assays were evaluated on plates with saline medium MMB (DSMZ GmbH 2004; last accessed November 2010; http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium457.pdf) specifically supplemented (in g/l: sodium chloride 20, polymer 10 (cellobiose, lactose, xylan and chitin) as unique carbon source, and agar 15).

Qualitative determination of cold-active proteases

All qualitative proteolytic assays were carried out on milk agar plates and SCA plates by examination of hydrolysis halos around the colonies at 4 and 15 °C. Activities at neutral, alkaline or acidic pH were determined on agar milk plates. Extracellular neutral protease activities were determined on both kinds of medium plates covered with a sterile 0.22 μm membrane filter. All assays were performed in triplicate. Proteolytic activities were estimated by the dimensions of the hydrolysis halos between the colony center and the clear zone finish.

DNA extraction and 16S rDNA amplifications

Isolates were grown in liquid milk medium, and genomic DNA (gDNA) was extracted according to Cristóbal *et al.* [9]. gDNA was used as template for PCR amplifications, performed in a final volume of 25 μl. The PCR mixture consisted of 5 μl 5 × buffer (Go Taq, Promega), 0.5 μl dNTP mixture (10 μM), 1 μl of each universal primer (63f and 1389r; Weisburg *et al.* [14]), 1 μl gDNA, and 0.2 μl DNA polymerase (5 U/μl; Go Taq, Promega), in a thermal cycler (Perkin-Elmer, model 9700, Applied Biosystems), using an initial denaturation step of 4 min at 94 °C, followed by 30 amplification cycles of 94 °C for 1 min, 57 °C for 30 s and 70 °C for 1.5 min each. Reactions were completed with elongation at 72 °C for 7 min, followed by cooling to 4 °C. PCR products were analyzed by electrophoresis in a 1% (w/v)

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agarose gel with 1 × TAE buffer and stained with ethidium bromide using a 1 kb DNA ladder (Promega) as molecular weight marker. Images were visualized using a Bio-Rad Image Analyzer Gel Doc.

Amplified ribosomal DNA restriction analysis

To investigate the genetic heterogeneity, amplified ribosomal DNA restriction analysis (ARDRA) was employed: 16S rDNA PCR products were independently digested with *Cfo*I and *Hpa*II (Promega). Restriction analyses were carried out at 37 °C for 2 h in 15 μl incubation buffer containing: 0.5 μl restriction enzyme, 5 μl 1 × buffer, 0.2 μl bovine serum albumin (BSA 10 mg/ml) and 8 μl PCR product. All digestion fragments were analyzed by electrophoresis in 2% (w/v) agarose gels with 1 × TAE buffer at 50 V for 2.5 h and stained with ethidium bromide.

Phylogenetic analysis

The 16S rDNA regions were sequenced at Macrogen, Korea, by using previously described primers and procedures [14]. Phylogenetic analysis was based on 16S rDNA gene sequences (CSQ2, AY158039; LaSQ3, EU075116; Lac1, EU075115; 48X, EU100390; B2, EU100392; D2, DQ103509; F2, DQ103512; Lac4, EU075113; Lac5, EU75114; CSQ5, EU075119; En18, EU075121; 48XA, DQ103513; E3, AY745742); the sequences were edited and aligned using multiple alignments of DNA-MAN software version 4.03 (Lynnon BioSoft, Vandreuil, QB, Canada). Sequences belonging to the same genus or to validly published closely related species available in public databases (GenBank and RDP II) were aligned and a similarity matrix was calculated. Sequence comparisons were performed using the basic local alignment search tool (BLAST) program within the GenBank database [15]. A phylogenetic tree was constructed with 16S rDNA sequences according to the neighbor-joining (NJ) method using Mega4.1 [16] with Kimura's two-parameter correction. The ClustalW computer program [15] was used to align multiple sequences. Phylogenetic and molecular evolutionary analyses were evaluated with 1000-replication bootstrap analysis [17], and the consensus tree was analyzed and constructed with Mega4.1 software [18].

Plasmid screening

Plasmid DNA was isolated using the method described previously [9], separated by electrophoresis in 0.7% (w/v) agarose gels using 0.5 × TAE buffer, run at 90 V for 1–2 h and stained with ethidium bromide, using a 1 kb DNA ladder (Promega) as molecular weight marker. Plasmid DNA was visualized with UV light using a Bio-Rad Image Analyzer Gel Doc.

Results

Isolation of strains and characterization of proteolytic activity

A total of 230 isolates were obtained from seawater and intestine samples of benthonic organisms. All isolates were selected by their production of hydrolysis halos on plates as evidence of proteolytic activity. Of 230 isolates, 190 were isolated from the enriched samples on milk and SCA media; 40 isolates were from R2A and LB media. The majority of strains (62%) showed protease activity at 4 °C, and 84% at 15 °C (Fig. 1a). In a comparison of pH-dependent production of proteases, 33% of the strains showed protease activity under alkaline conditions at 4 °C and 47% at 15 °C (Fig. 1a), with hydrolysis halos of between 5 and 20 ± 2 mm. Under acidic conditions, 22% of the isolates at 4 °C and 33% of those at 15 °C exhibited hydrolysis halos of more than 10 ± 3 mm (Fig. 1a). Activities at neutral pH were seen with 67 and 33% of the isolated bacteria at the two temperatures, with halos between 27 and 30 ± 2 mm in diameter (Fig. 1a). Since the isolates were derived either directly from seawater or from the intestines of benthic organisms, the differences in the proteolytic capacities were checked. This revealed 96% of proteolytic colonies from intestine bacteria of *P. granulosa*, 68% for *P. magellanicus*, 64% for *M subrrugosa* and 45% for *N. deaurata*. Regarding the seawater samples, differences between samples from the three different sites were screened. This revealed 55% of proteolytic ability in colonies obtained from water at Punta Segunda, 25% for Ushuaia Bay and 22% for Ensenada Bay (Fig. 1b).

Selection of isolates with extracellular neutral protease activity

From the 230 colonies obtained as pure cultures, 135 showed protease activity. Of these colonies, 64% showed proteolytic activity at 4 °C after 5 days, and 84% at 15 °C after 2 days. Of these isolates, 51 were selected for further tests on the grounds that they exhibited hydrolysis halos between 10 and 30 ± 2 mm in diameter at 4 and 15 °C (Fig. 2a, b). Of these, 29 excreted proteases under neutral conditions, and these were divided into three groups by the diameters of their hydrolysis halos (Fig. 2c, d). Using 0.22 μm membrane filters on plates allows identification of the production of an extracellular enzyme. The pore size of these membranes does not allow the passage of cells,

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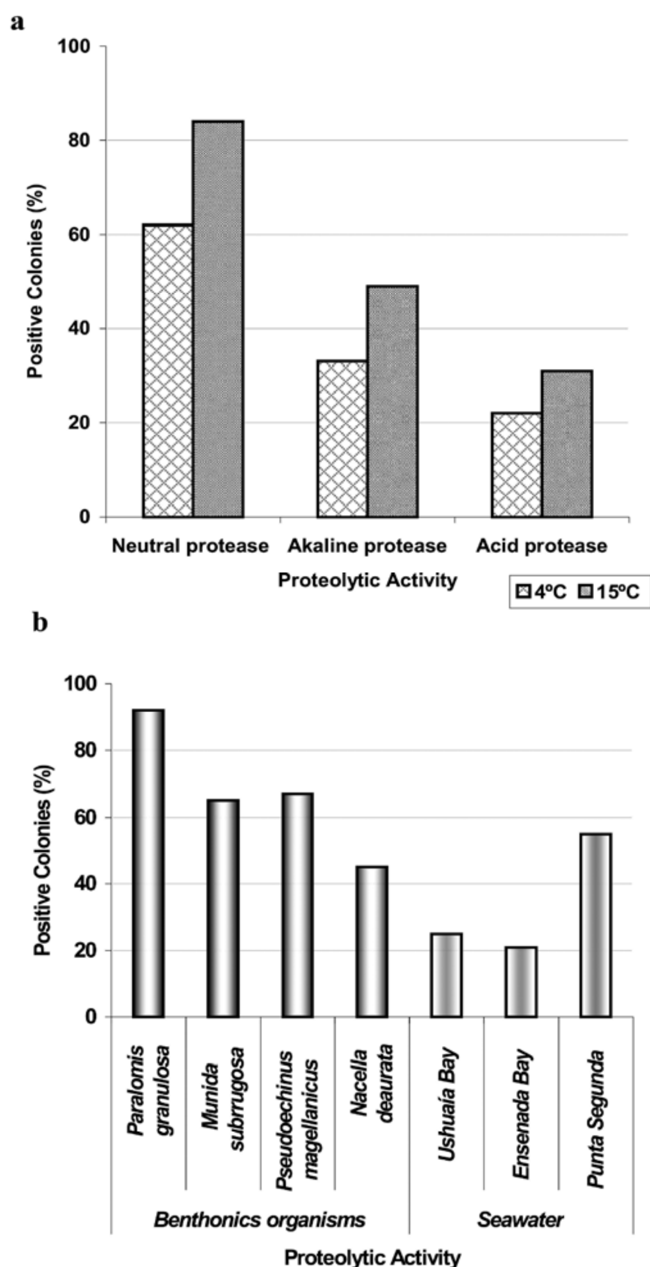

Figure 1. Percentage of positive colonies from 230 colonies. (a) Evidence of diversity of neutral, alkaline and acidic cold-active proteases, (b) distribution of colonies showing proteolytic activity obtained from each sample.

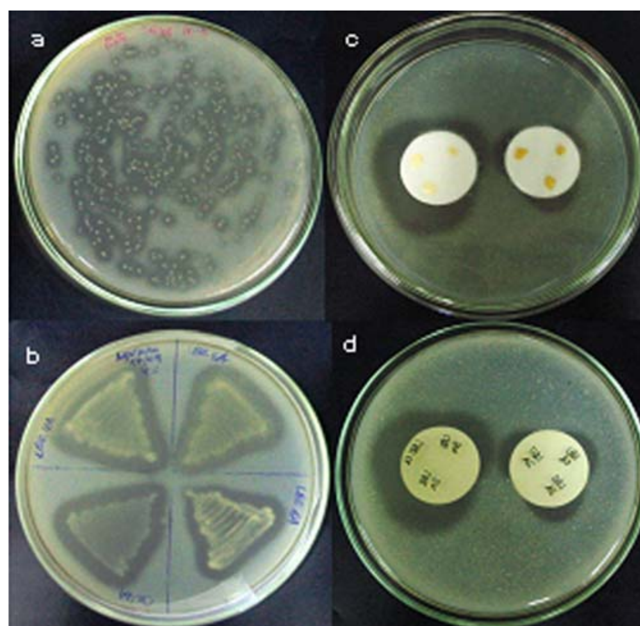

Figure 2. Qualitative assays to detect proteolytic activities from intestine and seawater samples: (a) isolates from *M. subrrugosa* on agar milk plates, (b) isolated bacteria showing hydrolysis halos, (c) extracellular neutral protease produced by inoculated colonies on sterile membrane filters at 4 °C, and (d) hydrolysis halo of the extracellular activities observed on the plates bearing the colonies (under the membrane).

thus allowing only the extracellular enzymes to access nutrients from the medium in order to support growth of the microorganisms.

Samples taken from subsurface seawaters of Tierra del Fuego, Argentina, were used to isolate marine bacteria to be tested for cold-adaptation of isolates and their respective enzymes (Table 1). Mostly, the isolated bacteria were coccoid or rod-formed gram-negative bacteria, both motile or without flagellum, and colony colors were cream, beige, or white to yellow, with few red morphotypes. Halotolerance and growth at higher temperature showed that, from the 51 isolates selected, 28 were capable of growth at both 4 and 37 °C, revealing a high temperature range of growth, and 39 isolates grew at 6% NaCl, demonstrating moderate halophilicity (Table 1). The assays for growth on different carbon sources showed significant differences in the numbers of positive colonies depending on the carbon source employed.

ARDRA assaying and phylogenetic analysis based on the 16S rDNA gene

The 16S rDNA PCR product from 29 isolates with hydrolysis halos of 27 to 30 ± 2 mm were subjected to ARDRA classification into six operational taxonomic units (OTU; Table 2). In this study, we defined an OTU as a cluster establishing a clear group. Nine isolates with the same band profile were classified into group I, two into group II with a profile most similar to group I but with one additional band, while group III consisted of only one strain that exhibited a unique profile. Groups IV–VI represented different profiles with eight, five and four isolates, respectively (Fig. 3a).

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Table 1. Morphological, physiological and biochemical properties of protease-producing bacteria from sub-antarctic environments. Extracellular proteases from marine bacteria selected at 4 and 15 °C. 51 isolates: 32 from milk and SCA media and 19 isolates from LB and R2A media. From these 51 isolates, 29 (in bold) were selected by proteolytic activity testing and plasmid screening. Three groups of strains (in bold) were determined according to their hydrolysis halo diameter.

Media	Milk and SCA[a]																								
Source	*P. granulosa*						*M. subrrugosa*							*N. deaurata*					*P. magallanicus*					Ushuaia Bay	
Isolates	**Ce1A** *	**Ce3A** **	**Ce5A** **	**Ce3B** ***	**Ce6B** *	**Ce8B** *	**L1A** **	**L5A** *	**L7A** ***	**L8A** *	**L2B** **	**L3B** ***	**L4B** *	**La2A** **	**La6A** *	**La7A** *	**La3B** **	**La6B** ***	**E4A** ***	**E5A** **	**E8A** *	**E2B** *	**E7B** *	**UB1** **	**UB5** *
Colony color	cream	cream	beige	cream	white	cream	cream	cream	beige	beige	cream	red	cream	cream	white	white	cream	cream	cream	white	cream	cream	cream	white	cream
Growth at																									
4 °C	+	+	+	+	−	+	+	−	−	−	+	+	+	ND	+	+	−	+	+	+	+	+	+	+	+
37 °C	+	−	ND	−	+	+	ND	+	+	+	−	−	+	+	+	+	+	−	−	−	+	+	+	−	+
3% (w/v) NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% (w/v) NaCl	+	−	+	+	+	−	−	+	+	+	+	ND	+	−	+	+	+	+	−	−	ND	ND	−	+	+
Assimilation																									
Cellobiose	ND	−	−	+	+	+	−	+	+	+	−	−	+	+	+	+	+	+	−	−	+	+	+	+	−
Lactose	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylan	−	−	−	−	ND	ND	−	−	+	+	−	−	−	−	−	−	−	ND	+	+	+	ND	+	−	−
Chitin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Media	Milk and SCA[a]							LB and R2A																		
Source	Ensenada Bay		Punta Segunda					*P. granulosa*			*M. subrru-gosa*		*N. deaurata*					*P. magalla-nicus*		Ushuaia Bay			Ensenada Bay		Punta Segunda	
Isolates	**EB2** *	**EB7** **	**PS1** ***	**PS3** *	**PS6** *	**PS7** *	**PS11** **	**CSQ2** *	**CSQ5** *	**D2** *	**G1** ***	**LnSQ8** ***	**Lac1** *	**Lac4** *	**Lac5** *	**F2** *	**LaSQ3** *	**E3** *	**EQ3** ***	**B2** *	**UQ1** ***	**UC2** ***	**En18** *	**En3** ***	**48X** *	**48XA** *
Colony color	white	cream	cream	cream	white	white	cream	beige	cream	white	cream	white	beige	cream	white	cream	beige	yellow	red	beige	white	beige	yellow	white	red	yelow
Growth at																										
4 °C	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37 °C	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+
3% (w/v) NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% (w/v) NaCl	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Assimilation																										
Cellobiose	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Lactose	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Xylan	−	−	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+
Chitin	−	−	+	+	−	−	−	−	−	+	+	+	+	+	+	+	−	+	+	−	−	−	+	+	−	+

ND, Data not available.
[a] Media used for the proteolytic activity assay.
Diameter of hydrolysis halo: * 27 to 30 ± 2 mm; ** 24 to 26 ± 2 mm and *** <23 ± 1 mm.

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Table 2. Classification of 29 microorganisms according to ARDRA.

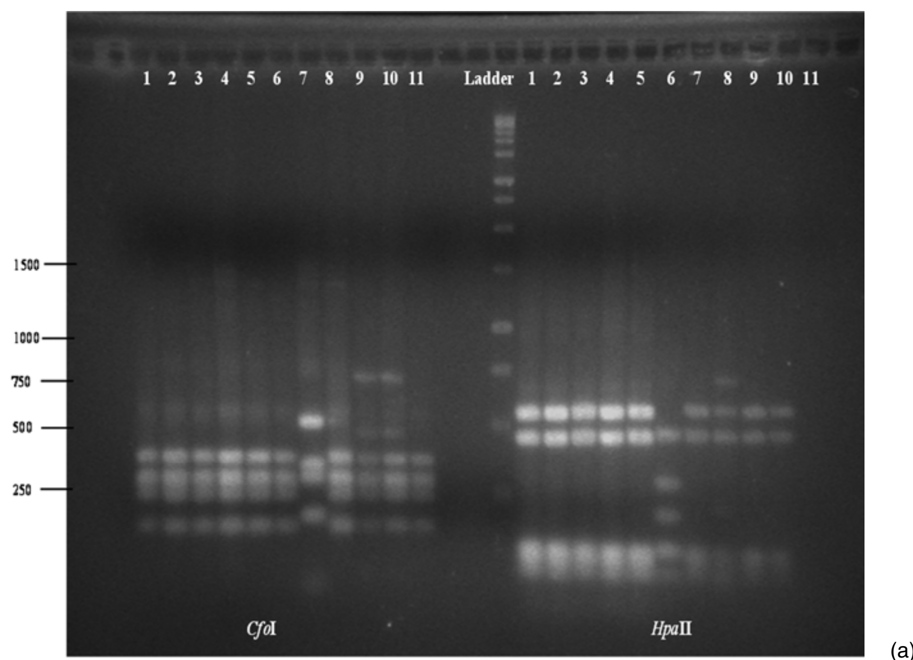
OTU	Strains[a]	Related genera
I	**Lac4**, **Lac5**, **F2**, Ce1A, Ce6B, L4B, La6A, La7A, E8A	*Serratia*
II	**48X**, **B2**	*Pseuadolteromonas*
III	**D2**	*Aeromonas*
IV	**48XA**, **E3**, L5A, Ce8B, L8A, UB5, EB2, PS3	*Pseudomonas*
V	**Lac1**, **CSQ2**, **LnSQ3**, PS6, PS7	*Shewanella*
VI	CSQ5, **En18**, E2B, E7B	*Halomonas*

[a] Strains in bold were selected for 16S rDNA sequencing.

One or two members of each OUT were selected for 16S rDNA-based identification. All 29 isolates belonged to the class γ-proteobacteria (Fig. 3b), identifying the six OTU as genera within defined taxa as: *Shewanella* (cluster I), *Pseudoalteromonas* (cluster II), *Aeromonas* (cluster III), *Serratia* (cluster IV), *Halomonas* (cluster V), and *Pseudomonas* (cluster VI). In cluster I, *Shewanella* sp. CSQ2, LasQ3, and Lac1 were closely related to *S. putrefaciens* ACAM-576 (99, 98, and 97%, respectively) and *S. arctica* 40-3 (98, 97, and 96%, respectively). In cluster II, *Pseudoalteromonas* sp. 48X was associated with *P. arctica* A-37 (98%) and *P. citrea* CIP 105339 (97%), and *Pseudoalteromonas* sp. B2 was closely related to *P. elyakovii* IAM 14594 (99.5%) and supported by a bootstrap of 100%. In cluster III, *Aeromonas* sp. D2 was closely associated with *A. molluscorum* LMG 22214 (99%). In cluster IV, *Serratia* sp. F2 was closely related to *S. proteomaculans* LMG 7887 (99%). *Serratia* sp. Lac4 and Lac5 were closely related to *S. grimesii DSM 30063* (98 and 99%, respectively). In cluster V, *Halomonas* sp. CSQ5 and En18 were very similar to *H. variabilis* DSM 3051 (98.1 and 99.2%, respectively). In cluster VI, *Pseudomonas* sp. E3 was related to *P. meridiana* CMS 38 (99%) and *P. antarctica* CMS 35 (98%), and *Pseudomonas* sp. 48XA was related to *P. veronii* CIP 104664 (98%).

Plasmid screening

We investigated both free-living and intestinal bacteria with the rationale that specific adaptation in the symbiotic association could be linked to the ingestion or



(a)

Figure 3. (a) ARDRA. Agarose gel (2% w/v) electrophoresis showing the 16S rDNA PCR products of individual selected isolates treated with either *Cfo*I or *Hpa*II. A 1-kb DNA ladder (Promega) was used (M). Legends: (1) Ce1A, (2) Ce6B, (3) La6A, (4) La7A, (5) L4B, (6) Ea8, (7) D2, (8) F2, (9) 48X, (10) B2, and (11) Lac4. (b) Evolutionary relationship of the 51 taxa. The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The optimal tree with the sum of branch lengths = 0.94840370 is shown. 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 and are shown in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 1161 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.1.

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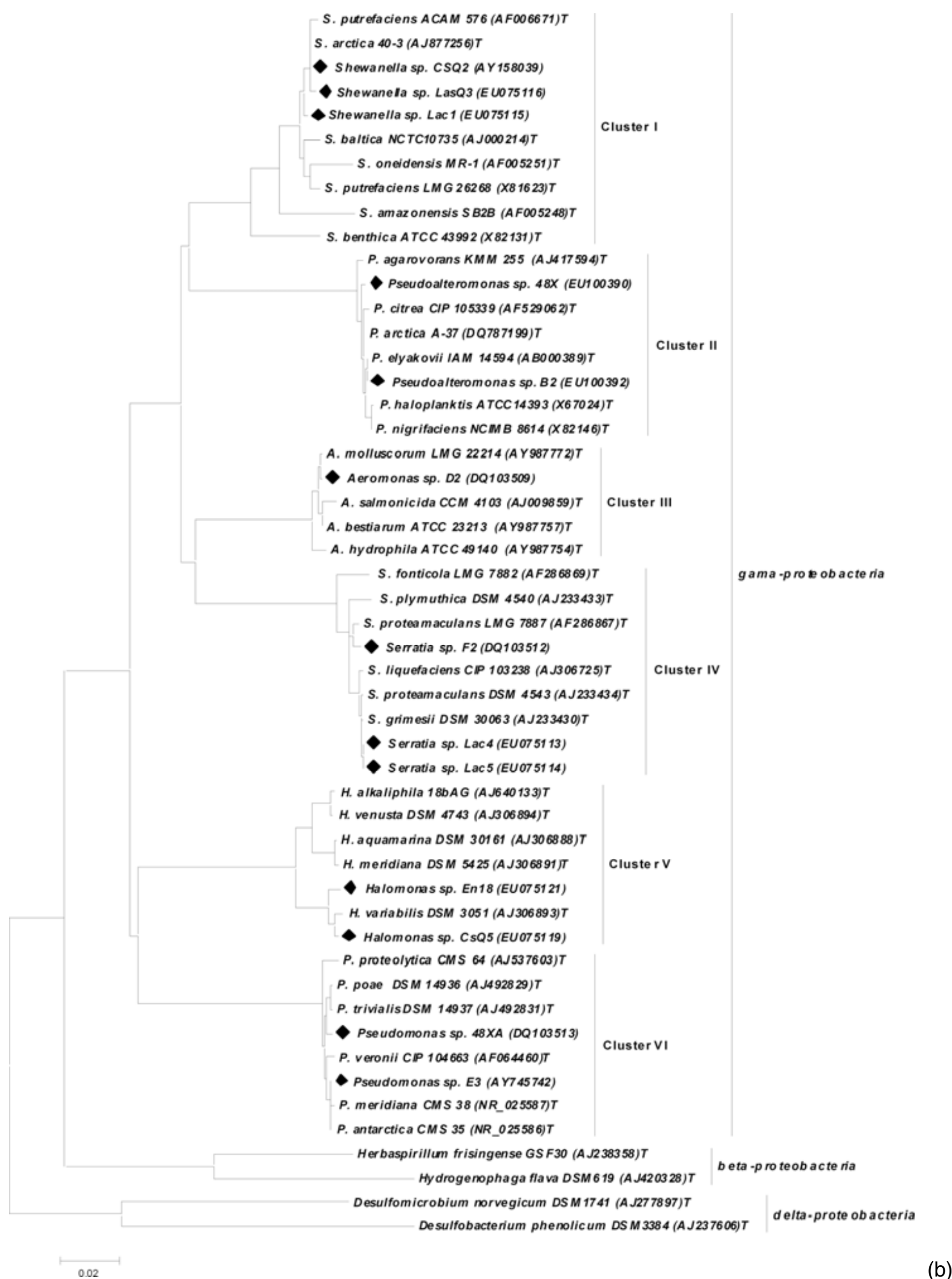

Figure 3. (Continued)

presence of particular nutrients (*e.g.* chitin, cellulose, hemicellulose, *etc.*), in the intestines of benthonic organisms. Horizontal gene transfer (HGT) might act to allow for easy adaptation. Thus, plasmid analysis was performed on the selected 29 isolates obtained from intestines and from seawater.

Only eight strains revealed the presence of plasmids (Fig. 4). *Serratia* sp. Ce1A and Ce6B as well as *Pseudomonas* sp. Ce8B from benthic *P. granulosa* presented partially overlapping plasmid profiles. The *Serratia* sp. La6A strain isolated from *N. deaurata* (with a plasmid profile most similar to *Serratia* Ce1A) and *Halomonas* sp. E2B isolated from *P. magallanicus* (with higher similarities to *Serratia* sp. Ce1A and Ce6B) like the strains mentioned before share a 10 kb plasmid (Fig. 4). *Pseudomonas* sp. PS3 isolated from seawater (Punta Segunda) showed a unique plasmid (>10 kb). *Pseudomonas* sp. L5A and L4B isolated from *M. subrrugosa* revealed the presence of plasmids from a separate group according to the plasmid profiles (Fig. 4).

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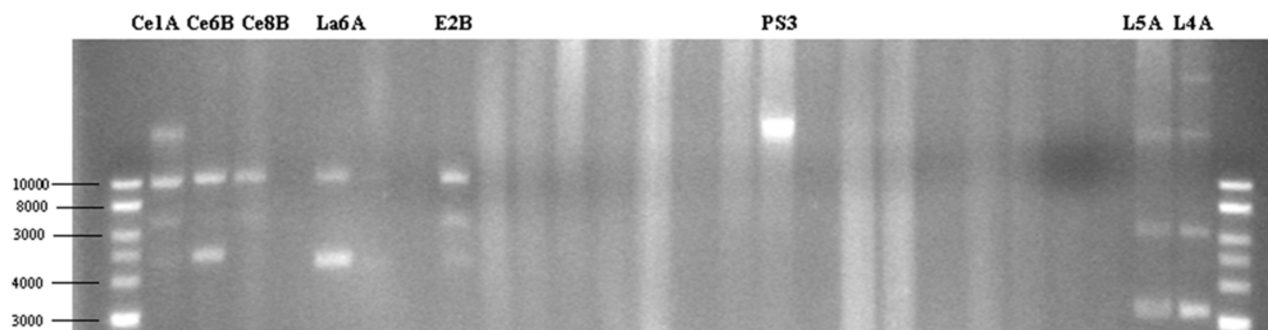

Figure 4. Plasmid screening. Agarose gel (0.8% w/v) electrophoresis showing the presence of plasmids in several bacteria isolated from intestine and seawater. A 1-kb DNA ladder (Promega) was used. In the figure, the isolates that exhibit plasmid DNA are indicated.

Discussion

Kennedy *et al.* [8] explained the interest in newly identified marine microorganisms by the key role they play in marine food webs, in biogeochemical cycling, and their ability to produce novel enzymes, metabolites or compounds with potential biotechnological applications. Our results on proteolytic activities detected in marine γ-proteobacteria are consistent with other reports [19–22]. The experimental work presented here provides a qualitative screening on plates for the detection of proteolytic bacteria. Other, similar studies employing enrichment procedures before detection report detection from cultivation media and product sources [10, 23–25]. Of the 230 proteolytic marine bacteria, 29 were selected by their ability to produce proteases on agar milk and SCA, with hydrolysis halos ranging from 10 to 30 ± 2 mm in diameter at 4 and 15 °C (Table 1).

In order to obtain enzymes active at low temperature, we chose samples from the sub-antarctic Beagle Channel. Several power stations have led to the enrichment of microbial populations that are adapted to a relative abundance of different nutrients, which makes them apt to be easily adapted to biotechnological processes and fermentation. Olivera *et al.* [23] reported bacteria isolated from various species of crustaceans and molluscs of Tierra del Fuego (Argentina), with a wide range of enzymatic activities. A great proportion of these invertebrates had incorporated bacteria with hydrolytic enzymes, mainly proteases. Thus, we included isolates from benthonic invertebrates in our study. They also reported that the coast of Tierra del Fuego (Argentina) as a cold environment (with temperatures from 9.7 °C in summer to 4.5 °C in winter) is a very suitable environment for the isolation of psychrophilic microorganisms. Microorganisms adapted to cold environments have been classified into two different physiological groups: psychrophiles and psychrotrophs [26]. This definition considers that psychrophilic microorganisms have an optimal temperature for growth at about 15 °C, with a maximal temperature for growth of 20 °C and a minimal growth temperature of 0 °C or below, while psychrotolerant microorganisms can grow at 0 °C but prefer higher temperatures. In this work, from 51 isolates, 36 were classified as psychrotolerant, among them 28 isolates growing at 4 and 37 °C and 8 only at 37 °C. In addition, 15 isolates grew only at 4 °C and were therefore classified as psychrophiles (Table 1). Vázquez *et al.* [10] isolated eight psychrotrophic protease-producing *Pseudomonas* sp. strains from the antarctic coast, with optimal activity at 20 °C. They explained the importance to evaluate an extracellular protease, which was classified as a neutral metalloprotease, for its possible industrial applications. In addition, Wang *et al.* [3] purified an extracellular cold-active protease from the psychrophilic bacterium *Pseudoalteromonas* sp. NJ276 selected from 260 bacteria from the antarctic region. These enzymes had broad substrate specificities over a wide pH range (5–12) and their protease activities exhibited nearly 30% of the respective maximum activity at 0 °C. Their properties would make these enzyme potentially useful for industrial applications [3]. Several studies focused on the role played by the bacterial community in heterotrophic niches, in the cycling of matter, and in the trophic dynamics of food webs [24, 25]. We detected differences in the percentages of proteolytic bacteria from intestine (67%) and seawater (33%) samples.

Proteases active at neutral, alkaline and acidic pH were detected on agar milk plates, with 62% of the strains displaying proteolytic activity at 4 °C and 84% at 15 °C for neutral pH, specifically for the organisms originating from benthonic organisms. These results are in agreement with the fact that the intestines supply an appropriate and varied nutrient source in a neutrophilic environment. Leon *et al.* [22] established that

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62.7% of the isolates showed proteolytic activity on SCA, suggesting the possibility of using native isolates for biotechnological purposes.

Since extracellular proteases are of special interest for possible industrial applications [10], a fast and easy screening for the detection of protease activity on plates was used [19, 23]. On agar milk plates and SCA, 29 isolates displayed extracellular neutral protease activities, as seen by applying them onto a sterile 0.22 μm membrane filter. This membrane allows only extracellular proteases to diffuse through and to lead to the hydrolysis halos observed. For a (semi)quantitative measure of protease production and activity, the hydrolysis halo diameter was used. In a similar assay, Esteves *et al.* [27] could show extracellular protease activity of marine yeasts on potato dextrose agar enriched with 1% casein, with halos ranging from 11 to 20 mm in diameter.

Restriction analysis (ARDRA) was carried out to produce genetic fingerprints for the clustering of isolates. The patterns allowed us to assign six OTU, which corresponded to six γ-proteobacterium genera (Table 2).

Therefore, this method is useful for the detection of any structural changes in such communities and their diversity [28]. Molecular phylogenetic relationships based on 16S rDNA and *gyrB* genes have been widely used to characterize communities of microorganisms belong to the γ-proteobacteria from the Beagle Channel and the Isla de los Estados [25, 26]. In our phylogenetic study, 16S rDNA sequence analysis allowed us to differentiate and identify 13 isolates belonging to the γ-proteobacteria class. These sequences showed between 97 and 99% similarity, calculated from GenBank and RDP II, with type strains of the following genera: *Shewanella*, *Pseudoalteromonas*, *Aeromonas*, *Serratia*, *Halomonas*, and *Pseudomonas* (Fig. 3b). Thus, this molecular identification allows established the genera assigned at each OTU and, therefore, their taxonomic classification of the other strain described in the Table 2. All genera found in this study were reported as protease-producing bacteria and to be present in different habitats of the marine environment, such as sediments, seawater, sea ice, and the intestines of invertebrates [10, 20, 23–25].

Esteves *et al.* [27] and Prabagaran *et al.* [29] analyzed hydrocarbon-degrading communities from seawater reported to be polluted with crude oil in Tierra del Fuego. They identified γ- and α-proteobacteria, cytophaga-flavobacterium-bacteroides and non-cultivable bacteria to be the dominant taxa in seawater [30, 31].

The benthonic organisms were taking into account nutritional habits, the distribution, and the different roles in the ecosystem of the Beagle Channel [24]. It should be noted that variations in the expression of genes that regulate a diversity of enzyme-producing bacteria could be linked to the ingestion or presence of particular nutrients [23, 24]. Therefore, this effect observed in the assimilation of several carbon sources (Table 1) allowed the establishment of symbiosis between several clusters of bacteria and benthonic organisms. A possible hypothesis could be that a certain mechanism of adaptation may be acting [32]. One of them, heterologous gene transfer proposes that the plasmids play a main role in the gene transfer between some bacteria [33].

We used plasmid screening to identify similar or different plasmid profiles for microorganisms of different genera. These strains exhibited various enzymatic activities and were isolated from different benthonic organisms or from seawater. Similar banding profiles may indicate HGT among these bacteria. Numerous reports on HGT show high incidences in naturally occurring microbial communities and indicate that genetic exchange plays a significant role in the evolution and ecological impact of terrestrial and aquatic microorganisms [32, 33].

In conclusion, cold-adapted microorganisms were cultured from sub-antarctic seawater and benthic organisms. They were able to produce proteases active under neutral, acidic and alkaline conditions at 4 and 15 °C. High extracellular protease activities were shown in 29 isolates; these enzymes may have biotechnological potential for use in industrial processes. 16S rDNA gene and ARDRA analysis were confirmed as powerful markers for elucidating phylogenetic relationships between γ-proteobacteria. The plasmid profiles found in these bacteria may have been modified by HGT between the benthic hosts and may be involved in adaptation to the environment as a result of novel bacterial properties [33].

The economic importance of studies of cold-active proteases is exemplified by numerous applications of these enzymes in commercial products. For example Novozyme utilizes a protease sold as an encapsulated detergent; the Japan Advanced Institute of Science and Technology holds two patents for cold-active proteases: CP-58 and CP-70. Another Japanese company, Kao Corporation, has filed a number of patents for cold-active proteases for their use in detergents [3]. The results obtained in this study contribute to the knowledge of the marine bacterial diversity in the Beagle Channel and to the characterization of proteases active in cold environments, which could be potentially useful for developing biotechnology processes.

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