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# Phylogenentic and enzymatic characterization of psychrophilic and psychrotolerant marine bacteria belong to γ-Proteobacteria group isolated from the sub-Antarctic Beagle Channel, Argentina

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**Abstract** The phylogenetic and physiological characteristics of cultivable-dependent approaches were determined to establish the diversity of marine bacteria associated with the intestines of benthonic organisms and seawater samples from the Argentina's Beagle Channel. A total of 737 isolates were classified as psychrophlic and psychrotolerant culturable marine bacteria. These cold-adapted microorganisms are capable of producing cold-active glycosyl hydrolases, such as β-glucosidases, celulases, β-galactosidases, xylanases, chitinases, and proteases. These enzymes could have potential biotechnological applications for use in low-temperature manufacturing processes. According to polymerase chain reaction-restriction fragment length polymorphism analysis of part of genes encoding 16S ribosomal DNA (ARDRA) and DNA gyrase subunit B (*gyrB*-RFLP), 11 operational taxonomic units (OTU) were identified and clustered in known genera using InfoStat software. The 50 isolates selected were sequenced based on near full sequence analysis of 16S rDNA and *gyrB* sequences and identified by their nearest neighbors ranging between 96 and 99 % of identities. Phylogenetic analyses using both genes allowed relationships between members of the cultured marine bacteria belonging to the γ-Proteobacteria group (*Aeromonas*, *Halteromonas*, *Pseudomonas*, *Pseudoalteromonas*, *Shewanella*, *Serratia*, *Colwellia*, *Glacielocola*, and *Psychrobacter*) to be evaluated. Our research reveals a high diversity of hydrolytic bacteria, and their products actuality has an industrial use in several bioprocesses at low-temperature manufacturing.

**Electronic supplementary material** The online version of this article (doi:10.1007/s12223-014-0351-1) contains supplementary material, which is available to authorized users.

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## Introduction

Marine environments can possess enormous microbial biodiversity and therefore potential for the discovery of exploitable biotechnological resources. In fact, a wide range of enzymatic activities have already been obtained from cultured marine bacteria (Kennedy et al. 2008). In addition, marine microorganisms can be found as intracellular or extracellular symbionts in marine animals, such as vertebrates or invertebrates, where must possess a wide gamma of enzymes for to the requirements of the host organisms (Trincone 2011). The interest in the diversity of marine microorganisms and the demand of biocatalysts adapted to extreme conditions (low or high temperatures, acidic or basic solutions, and high salt concentration) have increased in the industry (Alvarenga et al. 2011). Therefore, isolation of biotechnologically relevant enzymes from extremophilic microbes has become a challenging task in recent years. The search for novel biological products, such as enzymes, dyes, antibiotics, and metabolites, has been spurred by with high potential to

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be used in biotechnological development (García-Echauri et al. 2011). Nowadays, many research institutes and companies have established collections of organisms from a variety of common and extreme environments (e.g., soils, seawater, hot springs, Antarctic ice, and alkaline lakes), yielding a variety of enzymes that catalyze reactions under normal or extreme conditions (Lee et al. 2010). Current applications of cold-active enzymes produced by psychrophilic microorganisms are economically important in numerous industrial applications (Cavicchioli et al. 2002; Cristóbal et al. 2011a), including the manufacture of cheese, animal feed, leather, indigo and linen, vinegar, beer, wine and fruit juice, chemicals, and biopolymers (Egorova and Antranikian 2005).

The enzyme industry worldwide is valued at US$5.1 billion and is predicted to show an annual increase in demand of 63 %; specialty enzymes used for animal feed processing and ethanol production are envisaged to have increased demand (Sarethy et al. 2011). Singh (2010) estimated value for enzymes in the global market is about US$2.3 billion/year, a value in which food enzymes constitute the major market share. On these bases of applications, enzymes are distributed to food (45 %), detergent (34 %), agriculture and feeds (16 %), textiles (11 %), leather (3 %), and pulp and paper (1.2 %) industries (Singh 2010; Demain and Dana 2007). Actually, many industrial microbial enzymes play important roles in modern biotechnology, improving or even replacing previously existing processes (Cristóbal et al. 2009, 2011b). Various companies, such as Novozymes and Genencor, utilize cold-active celluloses, xylanases, lipases, proteases, and other enzymes derived from bacteria in their products, including foods and detergents (Wang et al. 2008).

Phylogenetic systematic analyses based on 16S rDNA sequences have been widely used to characterize communities of microorganisms found in a variety of marine environments (Hagström et al. 2002) and have elucidated novel relationships between unknown microorganisms and existing bacterial taxa, e.g., *Proteobacteria* and *Cytophaga-Flexibacter-Bacteroides* (Junge et al. 2002). Studies of 16S rDNA sequences from bacteria-like γ-Proteobacteria have revealed the presence of specific mutations visible in the secondary structure of the molecule and have required polyphasic classification strategies (Hao et al. 2010). Therefore, for accurate classification of closely related organisms, use of other housekeeping genes such as *gyrB* and *rpo* becomes necessary and can also increase resolution to that provided by traditional taxonomy (Yamamoto et al. 2000). The 16S rDNA and *gyrB* genes can be used to established the degree of intra- or inter-relationship between species of marine microorganisms (Sanchez et al. 2008; Cristóbal et al. 2008). In the studies reported here, we characterize the cold-active glycosyl hydrolases produced by cultivable marine bacteria isolated from Argentina's Beagle Channel.

## Materials and methods

### Sampling area

Samples were collected in July 2001 and February 2002 in Tierra del Fuego, Argentina. Subsurface (20 m) seawater samples were taken from various coastal areas in the Beagle Channel (55° S, 67° 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 the following benthonic organisms: *Munida subrugosa*, *Paralomis granulosa*, *Pseudoechinus magellanicus*, and *Nacella deaurata* (Fig. 1) (Cristóbal et al. 2011a).

### Enrichment and isolation of marine microorganisms

To determine the size of the cultivable bacterial populations, 100 μL from a pool of each sample (seawater and intestines) were enriched in medium R2A liquid as was described by Reasoner and Geldreich (1985). Serial sixfold dilutions of these cultures were placed in seawater and physiological solution for seawater and intestine samples, respectively. The number of colony-forming units per milliliter (CFU/mL) for each sample was determined by spreading 100 μL subsamples onto agar R2A plates (agar, 15 g/L) and incubated at 28 °C, and CFU were counted for each sample after 72 h in triplicate.

Microorganism's isolation assays were carried out by enrichment of seawater and intestine samples in liquid R2A and modified Luria-Bertani (LB), descried previously by Cristóbal et al. (2008), and incubated at 4 and 20 °C in an orbital shaker (200 rpm) for 5 and 3 days, respectively. After turbidity indicated growth, 100 μL of each thus enriched culture were diluted in sterile distilled water and spread onto LB plates and R2A plates and both media incubated at 4 and 20 °C. The LB medium was supplemented separately with the following substrates in the final concentration of 10 g/L: cellobiose (LBC), lactose (LBL), xylan (LBX) or hemicellulose (bagasse from sugarcane, LBHm), chitin (LBCh), rhamnose (LBR), cellulose (LBCe, paper Whatman no. 1), and carboxymethyl cellulose (CMC). Proteolytic microorganisms were determined in two media: liquid milk and starch casein agar (SCA) as were described previously by Cristóbal et al. (2011b).

### Basic characterizations and qualitative methods for microbial enzyme determinations

Isolates were selected based on their hydrolytic activity, different colony morphology, Gram staining, and biochemical characterization, which were evaluated after grown in R2A at 20 °C by APIs (BioMérieux) strips. APIs identification strips

**Fig. 1** Benthonic organisms: **a** *Nacella deaurata*, **b** *Pseudoechinus magallanicus*, **c** *Munida subrrugosa*, and **d** *Paralomis granulosa*

(ZYM, 20NE and Coryne) tests were set up as manufacturer instructions and incubated at 20 °C for 72 h.

The β-glucosidase, celulase, and xylanase activities were evaluated on agar LBC, LBCe, and LBX or LBHm media plates, respectively. Enzymatic activities were made visible around of colonies with Congo red staining (0.1 % *w*/*v*), incubated for 40 min and washed with a 1 mol/L sodium chloride for 10 min. β-Galactosidase activity was evaluated on agar LBL media plates sprayed with 40 μL of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, 20 mg/mL) and 4 μL of isopropyl-β-D-thiogalactopyranoside (IPTG, 100 mg/mL). The chitinase and protease activities were evaluated on agar LBCh and on agar milk media plates, respectively. All assays were carried out in triplicate at 4 and 20 °C.

## Molecular characterizations

Total genomic DNA (gDNA) extraction was based on a protocol described by Weisburg et al. (1991). PCR amplifications of the 16S rDNA and *gyrB* genes were performed using an automated thermal cycler (Perkin-Elmer, model 9700, Applied Biosystems). A final volume of 25 μL was adjusted with distilled water, and the reaction mixtures contained 1 μL gDNA as template, 5 μL PCR buffer GoTaq (Promega), 0.2 μL of bovine serum albumin (BSA, 10 mg/mL), 0.5 μL dNTPs (10 μmol/L), 0.5 μL of forward and reverse primers (33 μmol/L), and 0.1 μL of GoTaq (2.5 U). PCR products were resolved by electrophoresis in 1 % (*w*/*v*) agarose gel in 1× TAE buffer and stained with ethidium bromide. All primers used in this study for different PCR assay are shown in Table 1.

Amplified 16S rDNA (ARDRA) and *gyrB* (RFLP-*gyrB*) were independently digested for 12 h at 37 °C in a total volume of 15 μL containing 12 μL each PCR product, 2.3 μL of buffer 1×, 0.2 μL BSA, and 0.5 μL of *Hae*III or *Hpa*II (Promega).

The restriction treatments were resolved by electrophoresis in 2.0 % (*w*/*v*) agarose gel in 1× TAE buffer at 50 V for 2.5 h and stained with ethidium bromide using 1 kb DNA Ladder (Promega) molecular weight markers for size comparisons. The images were visualized and digitalized as TIFF images using an Image Analyzer Gel Doc BIO-RAD. The banding patterns were analyzed and standardized with Quantity One (Bio Rad) software and converted to a two-dimensional binary matrix (1=presence of a band; 0=absence of a band) in each treatment. Cluster analyses of similarity matrices were performed using arithmetic averages with minimum variance criteria (Ward) and Euclidean distance according to Albuquerque et al. (2006). Dendrograms were obtained after cluster analyses for individual band patterns were statistically analyzed using a professional version of Infostat software. The procedure (DNA isolation, PCR amplification, and restriction analysis) was performed twice to assess reproducibility of the method.

**Table 1** Universal primers sequences used for different assay PCR reactions

| Primer's name | Nucleotide sequence 5′–3′ | Target gene (positions)[a] | Reference |
|---|---|---|---|
| 27f | AGAGTTTGATCMTGGCTCAG | 16S rDNA (8–27) | Weisburg et al. (1991) |
| 1492r | GGTTACCTTGTTACGACTT | 16S rDNA (1495–1510) | |
| 518r | CGTATTACCGCGGCTGCTGG | 16S rDNA (518–536) | Schmidt et al. (1991) |
| 63f | CAGGCCTAACACATGCAAGTC | 16S rDNA (63–83) | Cristóbal et al. (2008) |
| 1387r | GGGCGGWGTGTACAAGGC | 16S rDNA (1387–1404) | |
| UP-1 | GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA | *gyrB* | Yamamoto et al. (2000) |
| UP-2r | AGCAGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT | *gyrB* | |

*f* forward primer, *r* reverse primer, *16S rDNA* 16S subunit ribosomal DNA, *gyrB* gyrase subunit B

[a] *Escherischia coli* numbering positions on 16S rDNA

Phylogenetic analyses, based upon 16S rDNA and *gyrB* sequences, were compared with sequences belonging to the same genus or validly published closely related species available in public databases Ribosomal Database Project II and GenBank using the basic local alignment search tool (BLAST) program within the NCBI Web site (Thompson et al. 1994) were aligned, and a similarity matrix were calculated. The sequences closely related to studied genes were extracted and then aligned multiple sequences by ClustalW program from MEGA software version 5.0 (Tamura et al. 2011). Phylogenetic trees were inferred by the maximum likelihood method based on the Tamura-Nei model and constructed with MEGA software version 5.0. The bootstrap consensus trees were inferred from 1,000 replicates (Felsenstein 1985) to represent the evolutionary history of the taxa analyzed.

Nucleotide sequence accession numbers

In the database of the NCBI (http://www.ncbi.nlm.nih.gov/nucleotide), 16S rDNA and *gyrB* sequences from isolates identified in this study were reported and accession numbers are shown in the Table 2.

## Results

Isolation of marine bacteria based upon production of cold enzymes

The total counting of bacterial isolates in R2A medium from seawater and intestine samples is shown in Table 2, where CFU values from winter-collected samples were superior for intestine and seawater with an average of $2.53\times10^8$ and $1.32\times10^3$ CFU, respectively. The use of LB medium supplemented with different carbon sources allowed a higher diversity of isolation, suggesting that microorganisms were nutritionally favored. From a total of 737 isolates, 488 were obtained from seawater and 249 isolates from the intestines of benthonic organisms (Table 2). In addition, according to grown incubation at 4 and 20 °C, the isolated microorganisms were classified as moderate psychrophilic and psychrotolerant, respectively. The highest CFU values were observed in winter-collected samples taken from the intestines of *P. granulosa*, *P. magellanicus*, and *M. subrugosa*. On the other hand, summer-collected samples showed an inferior number of colonies in all cases, being Ushuaia Bay and *N. deaurata* the richest ones (Table 2).

Phenotypic analyses revealed many morphological differences. The majority of the bacteria were cocci (72 %) and bacilli (26 %), with a wide variety of colony morphologies. Most of the isolates were Gram negatives, both motile and non-motile. Colonies were cream, beige, white, and yellow, with a few red morphotypes. The results of biochemistry characteristics (API tests) of representative isolates of each genus are shown in the Supplementary Table 3.

Identification of cold-active enzymes from marine bacteria and their applications for commercial products

Enzyme activities were detected by substrate degradations clearing zone/color-diffusion or hydrolysis halo around of the colonies after grown at 4 or 20 °C; therefore, percentages of positive colonies were determined for β-galactosidases, β-glucosidases, xylanases, celulases, proteases, and chitinases (Fig. 2). Of the 249 isolates from intestines, 33 % of chitinases and 52 % of cellulases were from *Placopecten magallenicus*, while from *N. deaurata*, β-glucosidases (77 %) and β-galactosidases (95 %) were the most frequent enzymatic activities. *P. granulosa* and *M. subrrugosa* produced 95 % of proteolytic and 68 % of xylanolytic activities. For the total of 448 isolates from seawater, 55 and 58 % of the colonies showed cellulase activity from Ushuaía Bay and Punta Segunda, respectively. The latter location showed a 25, 42, 55, and 90 % of colonies exhibiting chitinases, β-glucosidases, proteases, and β-galactosidase activities, respectively. Only a few isolates displayed enzymatic activity against xylan from all seawater samples (Fig. 2).

*Cellulases and carboxymethyl cellulase (EC 3.2.1.4 or EC 3.2.1.91)* Of 60 positive isolates, 17 exhibited hydrolysis halos from 10 to 20±2 mm in diameter, and 11 (P8, P5, P2, B2, B3, B4, 48X, 48XR, 48P, PSC1, and EnC3) exhibited hydrolysis halos from 20 to 30±1 mm in diameter (Fig. 3a).

*Xylanases (EC 3.2.1.8)* A total of 40 isolates were selected on the basis of their hydrolytic ability to degrade xylan and hemicellulose. This property was detected with an assay utilizing Congo red, which caused the formation of hydrolysis halos between 10 and 27 mm in diameter around the colonies (Fig. 3b). The isolates labeled P8, 33, 34, 48, and 50 had hydrolysis halos between 10 and 24 mm.

*β-Glucosidases (βGs, EC 3.2.1.21)* Of 132 isolates, 87 displayed hydrolysis halos between 9 and 25 mm in diameter at 4 and 20 °C. Of these 87 colonies, 32 displayed hydrolysis halos between 15 and 25 mm in diameter (Fig. 3c).

*β-Galactosidases (3.2.1.23)* In our study, 44 positive colonies were selected based upon their blue color (Fig. 3d), due by degradation to X-gal substrate.

*Proteases (EC 3.4.21.11)* A total of 48 isolates were selected on the basis of their hydrolytic capacity (Fig. 3e), which were inoculated on sterile membrane filters of 0.22 μm on agar milk plates and 29 isolates excreting

**Table 2** A total of 737 marine bacteria were isolated from different samples (specific host or Bay) growing in several carbon sources to cold-active enzymes production

| Samples | Date | Specific host or bay | CFU/mL | Isolates | Carbon sources | Strains identified[a] |
|---|---|---|---|---|---|---|
| Intestine of benthonic organisms | 21 July 2001 | *Paralomis granulosa* | $2.53 \times 10^8$ | 5, 6, 7, 8, 9, 31<br>10,11,12,13 | Paper or carboxymethyl cellulose<br>Xylan or hemicellulose | CSQ5 (EU075119), CSQ1 (AY158034), CSQ2 (AY158039) |
| | | | | CSQ1 to CSQ8 | Chitin | |
| | | | | CQ1 to CQ8 | Cellobiose | |
| | | | | PG1 to PG8 | R2A medium | |
| | | *Nacella deaurata* | $1.65 \times 10^8$ | 25, 26, 27<br>28, 29, 30 | Xylan or hemicellulose<br>Paper or carboxymethyl cellulose | LaSQ8 (AY158031), LaQ2 (AY158032), LaSQ6 (AY158037), F2 (DQ103512) Lac4 (EU075113), Lac5 (EU075114), LaSQ1 (EU075115), LaSQ3 (EU075116), LaSQ7 (EU75117), CSQ2 (EU075118) |
| | | | | LaQ1 to LaQ8 and LaSQ1 to LaSQ8 | Chitin | |
| | | | | LaC1 to LaC8; LaSC1 to LaSC8 and F1 to F8 | Cellobiose | |
| | | | | La1 to La8 | MR2A medium | |
| | | *Munida subrrugosa* | $2.44 \times 10^7$ | 17, 18, 19, 20, 21<br>22, 23, 24 | Paper or carboxymethyl cellulose<br>Xylan or hemicellulose | G5 (AY398666), G8 (AY745741), LnSQ2 (AY158027), LnSQ6 (AY158028), LnSQ5 (AY158038) |
| | | | | LnQ1 to LnQ8 and LnSQ1 to LnSQ8 | Chitin | |
| | | | | LnC1 to LnC8; LnSC1 to LnSC8; and G1 to G8 | Cellobiose | |
| | | | | N1 to N8 | R2A medium | |
| | | *Pseudoechinus magellanicus* | $2.23 \times 10^8$ | 14, 15, 16<br>EQ1 to EQ8 and ESQ1 to ESQ8 | Carboxymethyl cellulose<br>Chitin | EQ2 (AY158029) |
| | | | | EC1 to EC8 and ESC1 to ESC8 | Cellobiose | |
| | | | | Ep1 to Ep16 | R2A medium | |
| Seawater | 21 July 2001 | Ushuaia Bay | $1.32 \times 10^3$ | U1 to U154 | R2A medium | B2 (EU100392), B4 (EU100393), U1 (AY100672), U7 |
| | | | | UQ1 to UQ16<br>UC1 to UC16 and B1 to B8 | Chitin<br>Cellobiose | (AY100673), U27 (AY100674), U32 (AY100675), U128 (AY100676), UQ1 (AY158036) |
| | | Lapataia Bay | $1.65 \times 10^3$ | L1-L84<br>1, 2, 3, 4 | R2A medium<br>Paper or carboxymethyl cellulose | L17 (AY158036), L1 (AY100677), L17 (AY100678), L20 (AY100679), L31 (AY100680), L38 (AY100681), L52 (AY100683), L63 (AY100684), L84 (AY100682), GU4 (AY100686), G3 (AY100685) |
| | | | | G3 and G4 | Glycerol | |
| | | | | A3 and A4 | Almidon | |
| | | | | Gu3 and Gu4 | L-Glutamico | |
| | | | | LtQ1 to LtQ16 | Chitin | |
| | | | | LtC1 to LtC16 | Cellobiose | |
| Intestine of benthonic organisms | 22 February 2002 | *P. granulosa* | $2.35 \times 10^7$ | CC1 to CC4<br>D1 to D16 | Paper or carboxymethyl cellulose<br>Cellobiose | D1 (DQ103511), D2 (DQ103509), D3 (AY45744), D5 (EU100389), D6 (DQ103510) |

**Table 2** (continued)

| Samples | Date | Specific host or bay | CFU/mL | Isolates | Carbon sources | Strains identified[a] |
|---|---|---|---|---|---|---|
| | | *N. deaurata* | $3.20\times10^9$ | LapC1-LapC4 | Cellulose | |
| | | | | F9 to F16 | Cellobiose | |
| | | *M. subrrugosa* | $1.82\times10^8$ | LnUC1-LnUC3 | Paper or carboxymethyl cellulose | |
| | | | | G9 to G16 | Cellobiose | |
| | | *P. magellanicus* | ND | LnEC1 to LnEC3 | Paper or carboxymethyl cellulose | E3 (AY745742) |
| | | | | E1 to E8 | Cellobiose | |
| Seawater | 22 February 2002 | Ushuaia Bay | $1.18\times10^3$ | U155 to U194 | R2A medium | |
| | | | | 32 to 42 | Xylan or hemicellulose | |
| | | | | UC1 to UC2 | Paper or carboxymethyl cellulose | |
| | | | | B9 to B16[a] | Cellobiose | |
| | | Ensenada Bay | 64 | En1 to En40 | R2A medium | A1 (DQ103508), A5 (AY745740), A7 (DQ103507), En18 (EU075121), En38 (EU075122), En40 (EU075123) |
| | | | | EnC1 to EnC4 | Paper or carboxymethyl cellulose | |
| | | | | A1 to A4 | Cellobiose | |
| | | Punta Segunda | $5.6\times10^2$ | P1 to P47 | R2A medium | P2 (EU100394), P8 (EU100395), P5, 48X (EU100390), 48P (EU100391), 48XA (DQ103513), C2 (EU075120) |
| | | | | 43-50-48X-48P-48XR-48XA | Xylan or hemicellulose | |
| | | | | PSC1 and PSC2 | Paper or carboxymethyl cellulose | |
| | | | | C1 to C8 | Cellobiose | |

Colony-forming units per milliliter (data were expressed as number of CFU/mL Proteobacteria of seawater and physiological solution) from all samples collected in July 2001 and February 2002 from seawater and intestines of benthonic organisms, on agar R2A and LB plates

*ND* data not available

[a] Strains identified from 16S rDNA gene sequencing, accession number of NCBI in parenthesis

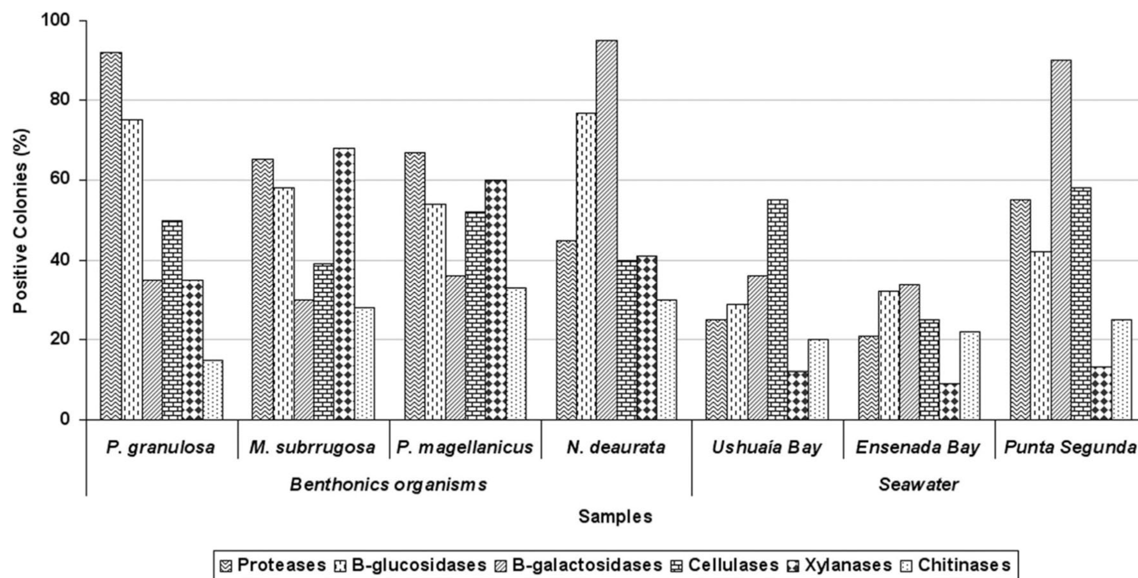



**Fig. 2** Enzymatic Activities: percentage of positive colonies from a total of 409 colonies from seawater and benthonic organisms: a) (▨) celluloses activity, b) (▨) xylanases activity, c) (▨) β-glucosidase activity, d) (▨) β-galactosidases activity, e) (▨) proteases activity, f) (▨) chitinase activity

neutral cold-active protease producer's halos between 27 and 30±2 mm of diameter. The pore size of these membranes does not allow passage of cells, thus allowing only enzymes to take nutrients from the medium to microorganism's growth (Cristóbal et al. 2011b).

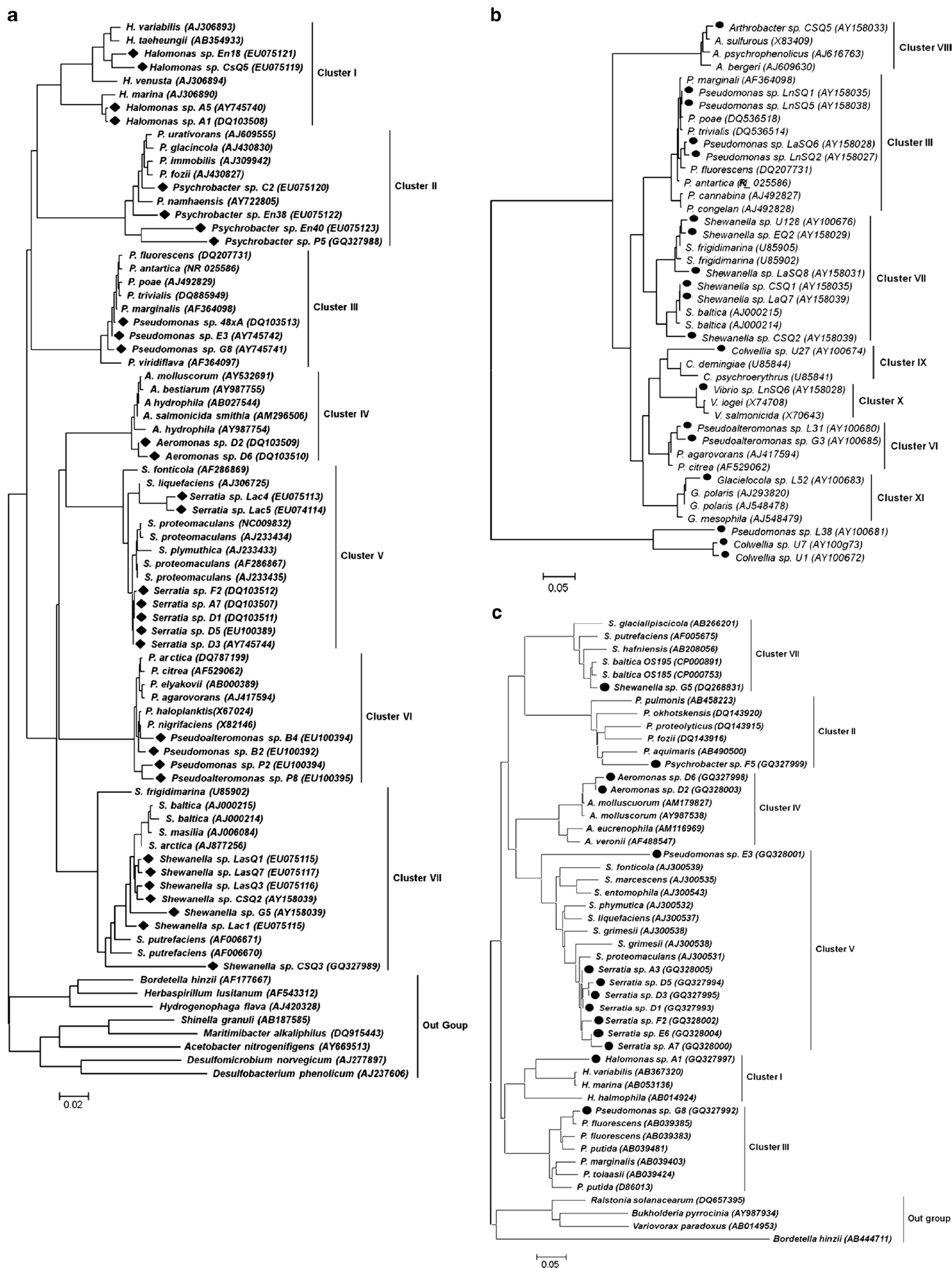
*Chitinase (EC 3.2.1.14)* A total of 35 colonies were selected on the basis of exhibiting hydrolysis halos between 10 and 24 mm in diameter (Fig. 3f).

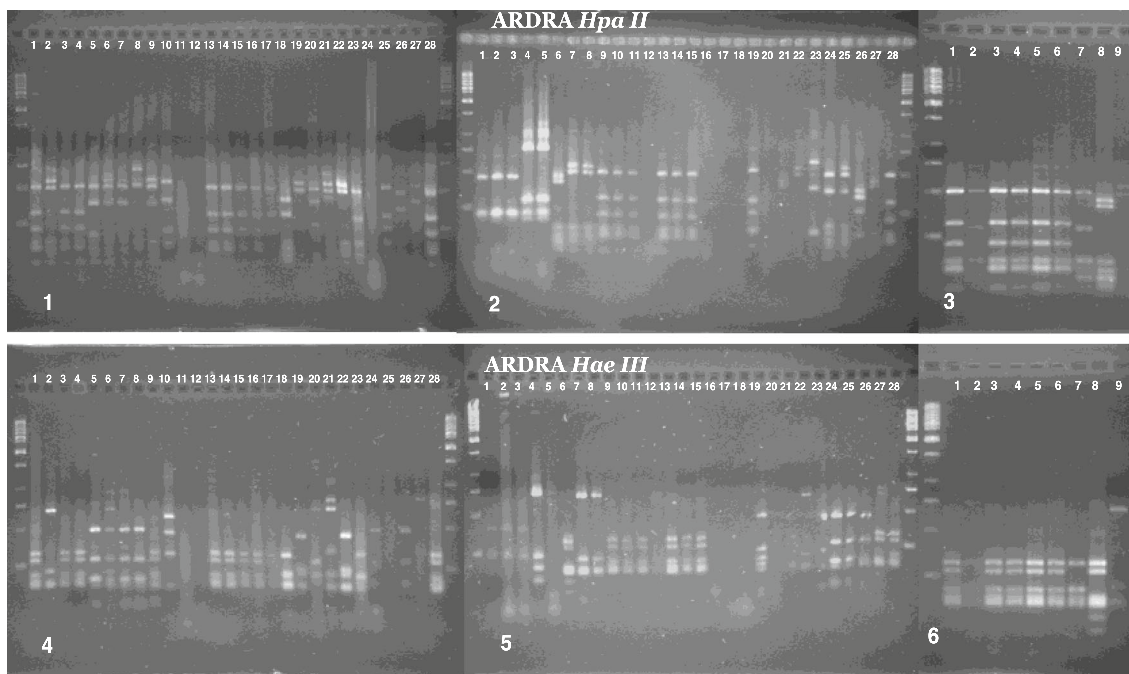
## Molecular and phylogenetic analyses

ARDRA-16S rDNA (Fig. 4a) and RFLP-*gyrB* (Fig. 4c) analyses allowed grouping cultured marine bacteria into 11 OTUs, which was defined as a cluster establishing a clear taxonomic group. One or more microorganisms from each representative group were selected for 16S rDNA and *gyrB* sequencing in order to elucidate the phylogenetic relationships between them. Statistical analyses based on all band patterns allowed establishment of the first branching between the isolates in the dendrograms using InfoStat software. In our study, the first tree was produced based on ARDRA analysis, allowing differentiation of microorganisms belonging to the genera *Pseudoalteromonas*, *Pseudomonas*, *Halomonas*, and *Psycrobacter*. However, isolates related to the genera *Serratia*, *Shewanella*, *Aeromonas*, and *Halomonas*, which had been previously incorporated into different OTUs (Fig. 4b). The second dendrogram was constructed based on RFLP-*gyrB* data analysis, which revealed a higher resolution and differentiation between relationships of isolates in the majority of the OTU (Fig. 4d).

Results established a predominance of cultured marine bacteria belonged to the gamma (γ) subgroup of the phylum Proteobacteria. Our broad phylogenetic analysis, based on 16S rDNA and *gyrB* sequences, confirmed that 50 of the strains studied belong to this robust, well-defined monophyletic taxon, with results separated into 11 clusters, defined in clusters I to XI (Fig. 5). Phylogenetic positioning of the first seven clusters allowed establishment of relationships with the following genera: *Halomonas* (cluster I), *Psychrobacter* (cluster II), *Pseudomonas* (cluster III), *Aeromona* (cluster IV), *Serratia* (cluster V), *Pseudoalteromonas* (cluster VI), and *Shewanella* (cluster VII). These seven clusters were established from 31 strains based upon sequencing of nearly full-length segments of 16S rDNA (Fig. 5a). The other clusters (VIII to XI) were established using only the first 500 bp of 16S rDNA sequences from 19 strains (Fig. 5b), an approach based upon that of Schmidt et al. (1991). The identified microorganisms in these remaining clusters showed relationships with the following genera: *Arthrobacter* (cluster VIII), *Colwellia* (cluster IX), *Vibrio* (cluster X), and *Glaciecola* (cluster XI). Phylogenetic analysis based on approximately 1,200 nucleotides of *gyrB* sequences allowed corroboration of the relationships of 15 strains belong to the clusters I, II, and VII (Fig. 5c).

**Fig. 3** Qualitative methods for identification of microbial enzymes at 4 and 20 °C on agar LB plates: a activity of celluloses from the P8 and P5 strains on agar LBCe plate, exhibiting hydrolysis halos of 30 mm in diameter; b bacteria shown on agar LBX plate with activity of xylanases indicated by hydrolysis halos on hemicellulose; c bacteria with β-glucosidase activity capable of hydrolyzing cellobiose on agar LBC plate; d blue-colored colonies from Punta Segunda showing the activity of β-galactosidases on agar LBL plate sprayed with 4 mL of X-gal and 20 mL of IPTG; e extracellular proteases obtained from 29 selected strains on agar milk plate; and f chitinase activity from 48× strains on agar LBCh plate, exhibiting a hydrolysis halo of 24 mm in diameter ▶


a
H. variabilis (AJ306893)
H. taeheungii (AB354933)
Halomonas sp. En18 (EU075121)
Halomonas sp. CsQ5 (EU075119)
H. venusta (AJ306894)
H. marina (AJ306890)
Halomonas sp. A5 (AY745740)
Halomonas sp. A1 (DQ103508)
Cluster I
P. urativorans (AJ609555)
P. glacincola (AJ430830)
P. immobilis (AJ309942)
P. fozii (AJ430827)
Psychrobacter sp. C2 (EU075120)
P. namhaensis (AY722805)
Psychrobacter sp. En38 (EU075122)
Psychrobacter sp. En40 (EU075123)
Psychrobacter sp. P5 (GQ327988)
Cluster II
P. fluorescens (DQ207731)
P. antartica (NR 025586)
P. poae (AJ492829)
P. trivialis (DQ885949)
P. marginalis (AF364098)
Pseudomonas sp. 48xA (DQ103513)
Pseudomonas sp. E3 (AY745742)
Pseudomonas sp. G8 (AY745741)
P. viridiflava (AF364097)
Cluster III
A. molluscorum (AY532691)
A. bestiarum (AY987755)
A hydrophila (AB027544)
A. salmonicida smithia (AM296506)
A. hydrophila (AY987754)
Aeromonas sp. D2 (DQ103509)
Aeromonas sp. D6 (DQ103510)
Cluster IV
S. fonticola (AF286869)
S. liquefaciens (AJ306725)
Serratia sp. Lac4 (EU075113)
Serratia sp. Lac5 (EU074114)
S. proteomaculans (NC009832)
S. proteomaculans (AJ233434)
S. plymuthica (AJ233433)
S. proteomaculans (AF286867)
S. proteomaculans (AJ233435)
Serratia sp. F2 (DQ103512)
Serratia sp. A7 (DQ103507)
Serratia sp. D1 (DQ103511)
Serratia sp. D5 (EU100389)
Serratia sp. D3 (AY745744)
Cluster V
P. arctica (DQ787199)
P. citrea (AF529062)
P. elyakovii (AB000389)
P. agarovorans (AJ417594)
P. haloplanktis(X67024)
P. nigrifaciens (X82146)
Pseudoalteromonas sp. B4 (EU100394)
Pseudomonas sp. B2 (EU100392)
Pseudomonas sp. P2 (EU100394)
Pseudoalteromonas sp. P8 (EU100395)
Cluster VI
S. frigidimarina (U85902)
S. baltica (AJ000215)
S. baltica (AJ000214)
S. masilia (AJ006084)
S. arctica (AJ877256)
Shewanella sp. LasQ1 (EU075115)
Shewanella sp. LasQ7 (EU075117)
Shewanella sp. LasQ3 (EU075116)
Shewanella sp. CSQ2 (AY158039)
Shewanella sp. G5 (AY158039)
Shewanella sp. Lac1 (EU075115)
S. putrefaciens (AF006671)
S. putrefaciens (AF006670)
Shewanella sp. CSQ3 (GQ327989)
Cluster VII
Bordetella hinzii (AF177667)
Herbaspirillum lusitanum (AF543312)
Hydrogenophaga flava (AJ420328)
Shinella granuli (AB187585)
Maritimibacter alkaliphilus (DQ915443)
Acetobacter nitrogenifigens (AY669513)
Desulfomicrobium norvegicum (AJ277897)
Desulfobacterium phenolicum (AJ237606)
Out Goup
0.02
b
Arthrobacter sp. CSQ5 (AY158033)
A. sulfurous (X83409)
A. psychrophenolicus (AJ616763)
A. bergeri (AJ609630)
Cluster VIII
P. marginali (AF364098)
Pseudomonas sp. LnSQ1 (AY158035)
Pseudomonas sp. LnSQ5 (AY158038)
P. poae (DQ536518)
P. trivialis (DQ536514)
Pseudomonas sp. LaSQ6 (AY158028)
Pseudomonas sp. LnSQ2 (AY158027)
P. fluorescens (DQ207731)
P. antartica (RL 025586)
P. cannabina (AJ492827)
P. congelan (AJ492828)
Cluster III
Shewanella sp. U128 (AY100676)
Shewanella sp. EQ2 (AY158029)
S. frigidimarina (U85905)
S. frigidimarina (U85902)
Shewanella sp. LaSQ8 (AY158031)
Shewanella sp. CSQ1 (AY158035)
Shewanella sp. LaQ7 (AY158039)
S. baltica (AJ000215)
S. baltica (AJ000214)
Shewanella sp. CSQ2 (AY158039)
Cluster VII
Colwellia sp. U27 (AY100674)
C. demingiae (U85844)
C. psychroerythrus (U85841)
Cluster IX
Vibrio sp. LnSQ6 (AY158028)
V. logei (X74708)
V. salmonicida (X70643)
Cluster X
Pseudoalteromonas sp. L31 (AY100680)
Pseudoalteromonas sp. G3 (AY100685)
P. agarovorans (AJ417594)
P. citrea (AF529062)
Cluster VI
Glacielocola sp. L52 (AY100683)
G. polaris (AJ293820)
G. polaris (AJ548478)
G. mesophila (AJ548479)
Cluster XI
Pseudomonas sp. L38 (AY100681)
Colwellia sp. U7 (AY100g73)
Colwellia sp. U1 (AY100672)
0.05
c
S. glacialipiscicola (AB266201)
S. putrefaciens (AF005675)
S. hafniensis (AB208056)
S. baltica OS195 (CP000891)
S. baltica OS185 (CP000753)
Shewanella sp. G5 (DQ268831)
Cluster VII
P. pulmonis (AB458223)
P. okhotskensis (DQ143920)
P. proteolyticus (DQ143915)
P. fozii (DQ143916)
P. aquimaris (AB490500)
Psychrobacter sp. F5 (GQ327999)
Cluster II
Aeromonas sp. D6 (GQ327998)
Aeromonas sp. D2 (GQ328003)
A. molluscuorum (AM179827)
A. molluscorum (AY987538)
A. eucrenophila (AM116969)
A. veronii (AF488547)
Cluster IV
Pseudomonas sp. E3 (GQ328001)
S. fonticola (AJ300539)
S. marcescens (AJ300535)
S. entomophila (AJ300543)
S. phymutica (AJ300532)
S. liquefaciens (AJ300537)
S. grimesii (AJ300538)
S. grimesii (AJ300538)
S. proteomaculans (AJ300531)
Serratia sp. A3 (GQ328005)
Serratia sp. D5 (GQ327994)
Serratia sp. D3 (GQ327995)
Serratia sp. D1 (GQ327993)
Serratia sp. F2 (GQ328002)
Serratia sp. E6 (GQ328004)
Serratia sp. A7 (GQ328000)
Cluster V
Halomonas sp. A1 (GQ327997)
H. variabilis (AB367320)
H. marina (AB053136)
H. halmophila (AB014924)
Cluster I
Pseudomonas sp. G8 (GQ327992)
P. fluorescens (AB039385)
P. fluorescens (AB039383)
P. putida (AB039481)
P. marginalis (AB039403)
P. tolaasii (AB039424)
P. putida (D86013)
Cluster III
Ralstonia solanacearum (DQ657395)
Bukholderia pyrrocinia (AY987934)
Variovorax paradoxus (AB014953)
Bordetella hinzii (AB444711)
Out group
0.05




**Gel 1**: 1- A3, 2- A4, 3- A5, 4- A7, 5- B2, 6- B3, 7- B4, 8- B8, 9- B6, 10- C6, 11- CsQ2, 12- CsQ5, 13- D1, 14- D2, 15- D3, 16- D4, 17- D5, 18- D6, 19- D8, 20- D10, 21- D13, 22- 48xA, 23- 48xR, 24- P2, 25- P5, 26- P8, 27- E2 Y 28- E3. **Gel 2**: 1- Lac1, 2- Lac4, 3- Lac5, 4- LasQ1, 5- LasQ3, 6- LasQ7, 7- L1, 8- L38, 9- LnsQ2, 10- D11, 11- D12, 12- D15, 13- E4, 14- E6, 15- E8, 16- En11, 17- En13, 18- En18, 19- En38, 20- En40, 21- G1, 22- G2, 23- G3, 24-B5, 25- B7, 26- C3, 27- CsQ3 Y 28- D7. **Gel 3:** 1- F2, 2- F3, 3- F4, 4- F5, 5- F6, 6- F7, 7- F8, 8- G5 Y 9- G8. **Gel 4**: 1- A3, 2- A4, 3- A5, 4- A7, 5- B2, 6- B3, 7- B4, 8- B8, 9- B6, 10- C6, 11- CsQ2, 12- CsQ5, 13- D1, 14- D2, 15- D3, 16- D4, 17- D5, 18- D6, 19- D8, 20- D10, 21- D13, 22- 48xA, 23- 48xR, 24- P2, 25- P5, 26- P8, 27- E2 Y 28- E3. **Gel 5**: 1- Lac1, 2- Lac4, 3- Lac5, 4- LasQ1, 5- LasQ3, 6- LasQ7, 7- L1, 8- L38, 9- LnsQ2, 10- D11, 11- D12, 12- D15, 13- E4, 14- E6, 15- E8, 16- En11, 17- En13, 18- En18, 19- En38, 20- En40, 21- G1, 22- G2, 23- G3, 24- B5, 25- B7, 26- C3, 27- CsQ3 Y 28- D7. **Gel 6**: 1- F2, 2- F3, 3- F4, 4- F5, 5- F6, 6- F7, 7- F8, 8- G5 Y 9- G8. B5, 25- B7, 26- C3, 27- CsQ3 Y 28- D7.

**Fig. 4** Restriction analysis patterns obtained by digestion with the enzymes *Hae*III and *Hpa*I separated in 2 % agarose gels: **a** ARDRA analysis and **c** *gyrB*-RFLP analysis. Dendrograms based on data from ARDRA and *gyrB*-RFLP fingerprints, followed by cluster analysis with Ward distance through InfoStat software: **b** based on ARDRA analysis and **e** based on *gyrB*-RFLP analysis

In cluster I, *Shewanella* sp. LasQ1 was strongly related to *Shewanella arctica* 40-3$^T$ (99 % of identities according to the BLAST analysis), but also associated to *Shewanella putrefaciens* ACAM-576$^T$ (98 %) and *Shewanella baltica* NCTC 10735$^T$ (97 %). All *Shewanella* related isolates such as G5, CSQ3, CSQ2, LasQ3, LasQ7, and Lac1 were closely related to *S. baltica* NCTC 10735$^T$ with 98–99 % of identities; but those strains were also associated to *S. arctica* 40-3$^T$ and *S. putrefaciens* ACAM-576$^T$ between 96 and 98 %. *Pseudoalteromonas* sp. 48X from cluster II was associated to *P. arctica* A-37$^T$ (98 %), while *Pseudoalteromonas* sp. P8 was associated to *P. citrea* CIP105339$^T$ (99 %) and *Pseudoalteromonas* sp. P2, B4, and B2 to *Pseudoalteromonas elyakovii* IAM 14594$^T$ (99 %) and *Pseudoalteromonas paragorgicola* KMM 3548$^T$ (99, 98, and 99 %). In cluster III, *Aeromonas* sp. D6 was closely related to *Aeromonas encheleia* A1881$^T$ (99 %), and *Aeromonas* sp. D2 was closely associated to *Aeromonas molluscorum* LMG 22214$^T$ (99 %). *Serratia* sp. 48P, contained by cluster IV, was related to *Serratia nematodiphila* DZ0503SBS1$^T$ (98 %); *Serratia* sp. Lac4 and Lac5 were related to *S erratia grimesii* DSM 30063$^T$ (98 and 99 %, respectively); and *Serratia* sp. F2, F6, A7, D3, D1, D5 were closely related to *Serratia proteomaculans* LMG 7887$^T$ (98–99 %). In cluster V, *Cobetia* sp. A5 and A1 were strictly related to *Cobetia crustatorum* JO1$^T$ (both with 99 %). *Halomonas* sp. CSQ5 and En18 were very close to *Halomonas variabilis* DSM 3051$^T$ (98 and 99 %) and *Halomonas bolivoensis* LC1$^T$ (97 and 98 %), respectively. *Psychrobacter* sp. En38 was closely related to *Psychrobacter pulmonis* CECT 5989$^T$ (99 %) and *Psychrobacter* sp. P5 was associated with an identity of 97 % to *Psychrobacter namhaensis* SW-242$^T$. Within this cluster, *Psychrobacter* sp. En40 and C2 were closely associated to *Psychrobacter proteolyticus* 116$^T$ (99 %) and *Psychrobacter cibarius* JG-219$^T$ (99 %), respectively. Members of cluster VIII, *Pseudomonas* sp. E3 was found to be close to *Pseudomonas meridiana* CMS 38$^T$ (99 %) and *Pseudomonas antarctica* CMS 35$^T$ (98 %), *Pseudomonas* sp. 48XA to *Pseudomonas veronii* CIP 104664$^T$ (98 %) and *Pseudomonas* sp. G33, G8, and B3 were found to be close to *Pseudomonas proteolytica* CMS64$^T$ and *Pseudomonas fulvagene* AJ2129$^T$ (98 and 97 %), respectively. In the cluster IX, *Arthrobacter* encompassing *Arthrobacter* sp. CSQ5 related to *Arthrobacter sulfureus* DSM 20167$^T$ (98 %) and *Arthrobacter kerguelensis* KGN15$^T$ (97 %). Cluster X included *Colwellia* sp. U27 displaying 98 % of identities with *Colwellia polaris* BSi20537$^T$. Strain U7 and U1 were found to be related to *Colwellia* genus when the RDP database was used for sequence

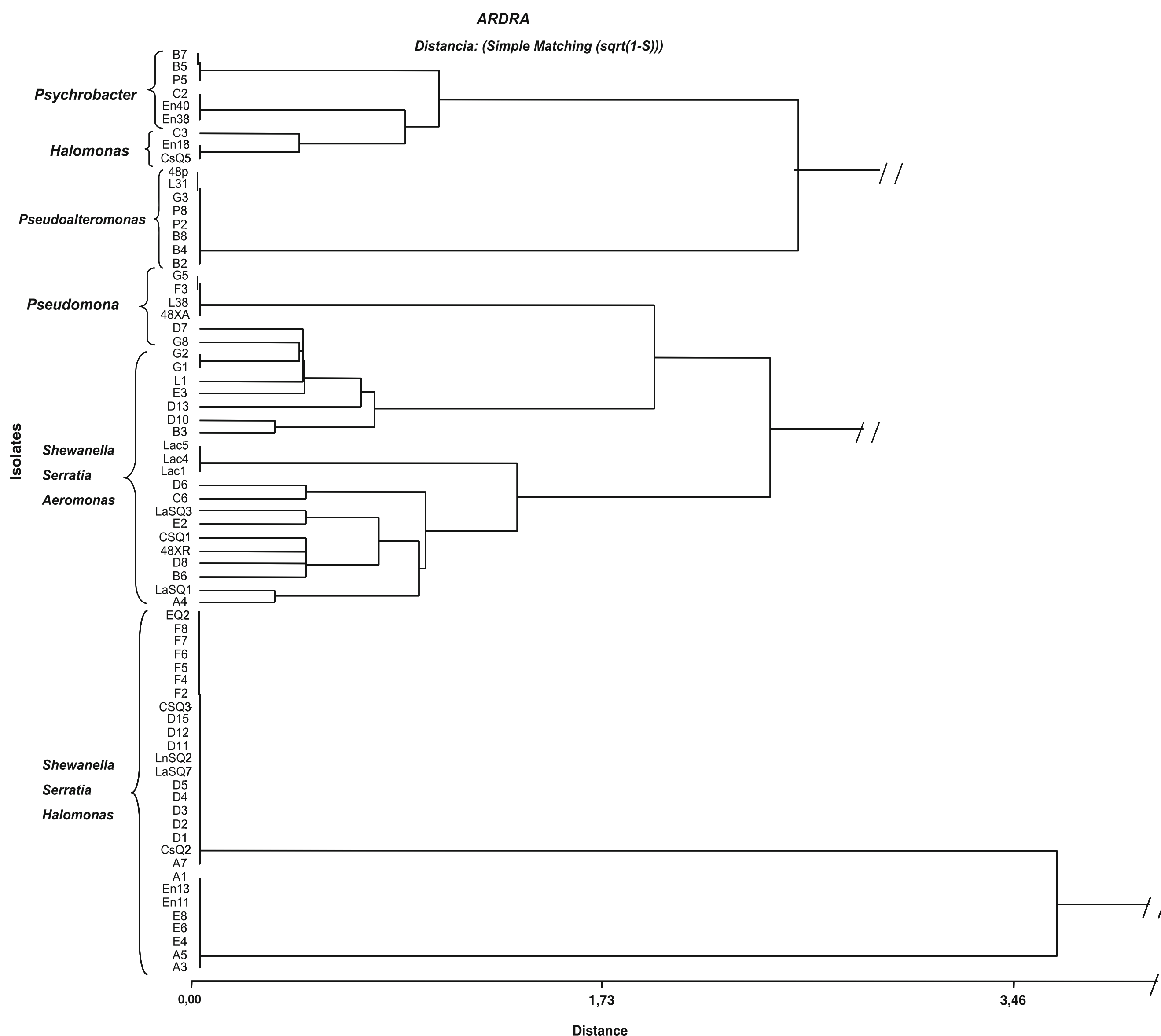


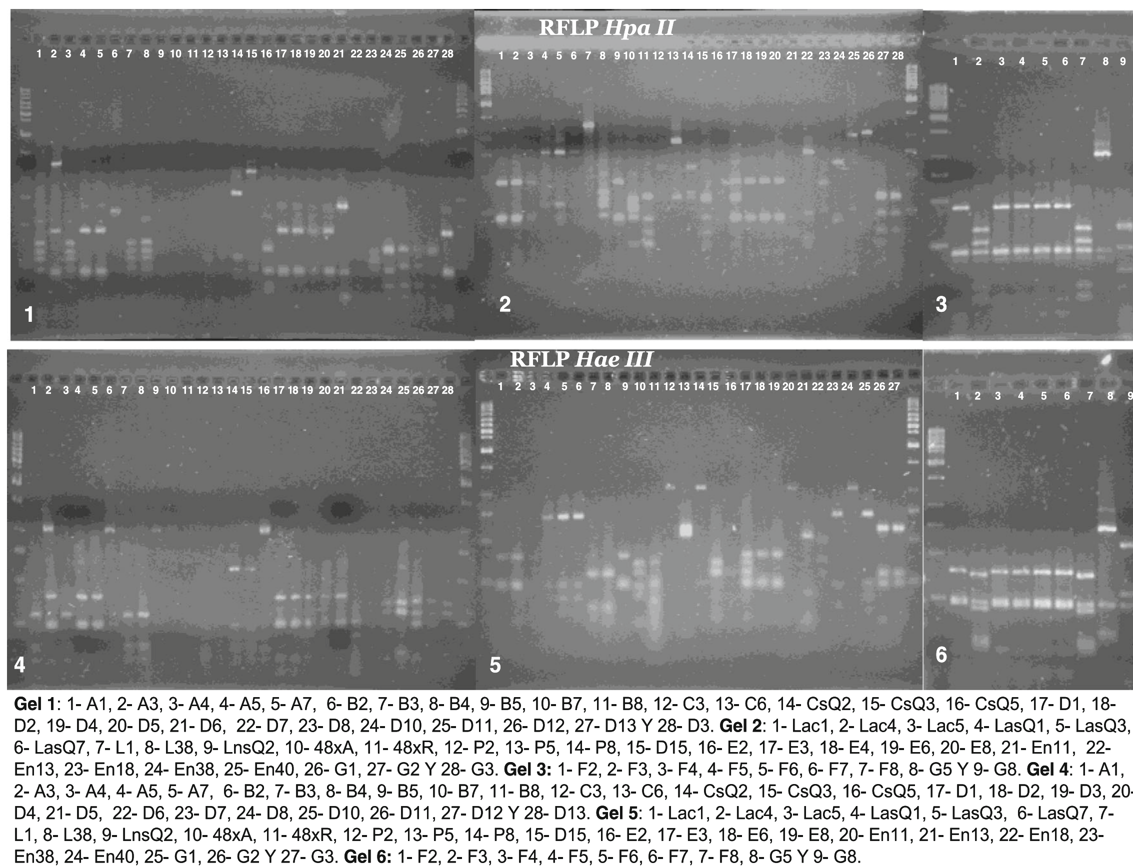

**Fig. 4** (continued)

alignments, particularly associated to *C. polaris* BSi20537$^T$ (97 and 98 % respectively), but through the methods employed for phylogenetic analysis were grouped outside of this group. Regrettably, *gyrB* amplification was unsuccessful for these strains and can be seen in Fig. 4c that *Colwiella* sp. A1 grouped with *Halomonas* spp., pointing out the need of further analyses to identify them. *Aliivibrio* sp. LnSQ6, cluster XI, was closely related to *Aliivibrio logei* NCIMB 2252$^T$ (99 %) and *Aliivibrio salmonicida* NCIMB 2262$^T$ (98 %) and *Glacielocola* sp. L52, cluster XII, was closely associated to *G. polaris* LMG 21857$^T$ (98 %).

Phylogenetic analyses based on *gyrB* gene are shown in Fig. 5c. *Sewanella* sp. G5 (cluster I) was highly associated to *S. baltica* NCTC 1035$^T$ (98 % of identity); *Psychrobacter* sp. F5 (cluster II) related to *Psychrobacter okhotskensis* MD17$^T$ (98 %); *Cobetia* sp. A1 (Cluster III) associated to *Cobetia marina* DSM 4741 (99 %); *Pseudomonas* sp. G8 and E3 (Cluster IV) related to *P. proteolytica* CIP 108464$^T$, 98 and 97 % of identities, respectively. *Aeromonas* sp. D6 and D2 (cluster V) were associated to *A. molluscorum* MDC20$^T$ (97 and 98 %, respectively), and isolates grouped in cluster VI, *Serratia* sp. F2, A7, A6, E6, A3, D3, D1, and D5, were all linked to *S. proteomaculans* ATCC 19323$^T$ (98–99 %).

## Discussion

Our studies were focused on the role of the bacterial community as a whole in heterotrophic production and on the enzymatic activities that determine the importance of the bacterial flora in the food web as a resource of metabolites with




**Gel 1**: 1- A1, 2- A3, 3- A4, 4- A5, 5- A7, 6- B2, 7- B3, 8- B4, 9- B5, 10- B7, 11- B8, 12- C3, 13- C6, 14- CsQ2, 15- CsQ3, 16- CsQ5, 17- D1, 18- D2, 19- D4, 20- D5, 21- D6, 22- D7, 23- D8, 24- D10, 25- D11, 26- D12, 27- D13 Y 28- D3. **Gel 2**: 1- Lac1, 2- Lac4, 3- Lac5, 4- LasQ1, 5- LasQ3, 6- LasQ7, 7- L1, 8- L38, 9- LnsQ2, 10- 48xA, 11- 48xR, 12- P2, 13- P5, 14- P8, 15- D15, 16- E2, 17- E3, 18- E4, 19- E6, 20- E8, 21- En11, 22- En13, 23- En18, 24- En38, 25- En40, 26- G1, 27- G2 Y 28- G3. **Gel 3:** 1- F2, 2- F3, 3- F4, 4- F5, 5- F6, 6- F7, 7- F8, 8- G5 Y 9- G8. **Gel 4**: 1- A1, 2- A3, 3- A4, 4- A5, 5- A7, 6- B2, 7- B3, 8- B4, 9- B5, 10- B7, 11- B8, 12- C3, 13- C6, 14- CsQ2, 15- CsQ3, 16- CsQ5, 17- D1, 18- D2, 19- D3, 20- D4, 21- D5, 22- D6, 23- D7, 24- D8, 25- D10, 26- D11, 27- D12 Y 28- D13. **Gel 5**: 1- Lac1, 2- Lac4, 3- Lac5, 4- LasQ1, 5- LasQ3, 6- LasQ7, 7- L1, 8- L38, 9- LnsQ2, 10- 48xA, 11- 48xR, 12- P2, 13- P5, 14- P8, 15- D15, 16- E2, 17- E3, 18- E6, 19- E8, 20- En11, 21- En13, 22- En18, 23- En38, 24- En40, 25- G1, 26- G2 Y 27- G3. **Gel 6:** 1- F2, 2- F3, 3- F4, 4- F5, 5- F6, 6- F7, 7- F8, 8- G5 Y 9- G8.

**Fig. 4** (continued)

biotechnological potential. In our work, 737 cultural marine bacteria were isolated from the sub-Antarctic environment Beagle Channel as a potential to isolate novel enzymes. Therefore, seawater and intestines of invertebrate's samples are a great source of cold-adapted microorganisms able to produce enzymes with high activity at low temperatures. While our assays were carried out at 4 and 20 °C, the most microorganisms isolated were classified as psychrotolerant, which can grow on the widely range of temperatures (4 to 37 °C). Our result are consistent with those of Trincone (2011) and Olivera et al. (2007), who reported on bacteria that were isolated from crustaceans and mollusks and which produced several hydrolytic enzymes, mainly proteases. Marine microorganisms are of great interest to explain its function as key factors in the recycling of organic matter and a source of novel enzymes, metabolites, or compounds with potential biotechnological applications (Kennedy et al. 2008). Olivera et al. (2007) 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 isolation of psychrophilic microorganisms. In addition, García-Echauri et al. (2011) reported the isolated of 260 culturable psychrophilic bacteria obtained from Antarctic environmental; these bacteria were capable to growth at 4 °C in different culture media and visualized as a potential source for biotechnology products, such as cold-active enzymes and metabolites.

The wide 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 in the ecosystem of the Beagle Channel (León et al. 2000; Cristóbal et al. 2011a, b). Therefore, this effect was observed in previous studies by the assimilation of several carbon source establishments' symbiosis mechanisms between several clusters of bacteria and benthonic organisms (Fig. 1) (Trincone 2011; Cristóbal et al. 2011b). A possible hypothesis could be explained by a certain mechanism of adaptation that could be a horizontal gene transfer (HGT) that proposes that the plasmids play a main role on the gene transfer that could be acquired between some bacteria (Ochman et al. 2000; Thomas and Nielsen 2005). In other studies, different plasmid profiles were observed from microorganisms belonging to several Bacteria phylum isolated from benthonic organisms and seawater. These strains exhibited various enzymatic activities, however, were described as proteolitic bacteria. The presence of plasmid banding profiles may be indicating HGT among these bacteria (Cristóbal et al. 2011b). Numerous reports on HGT show high incidences in naturally occurring microbial

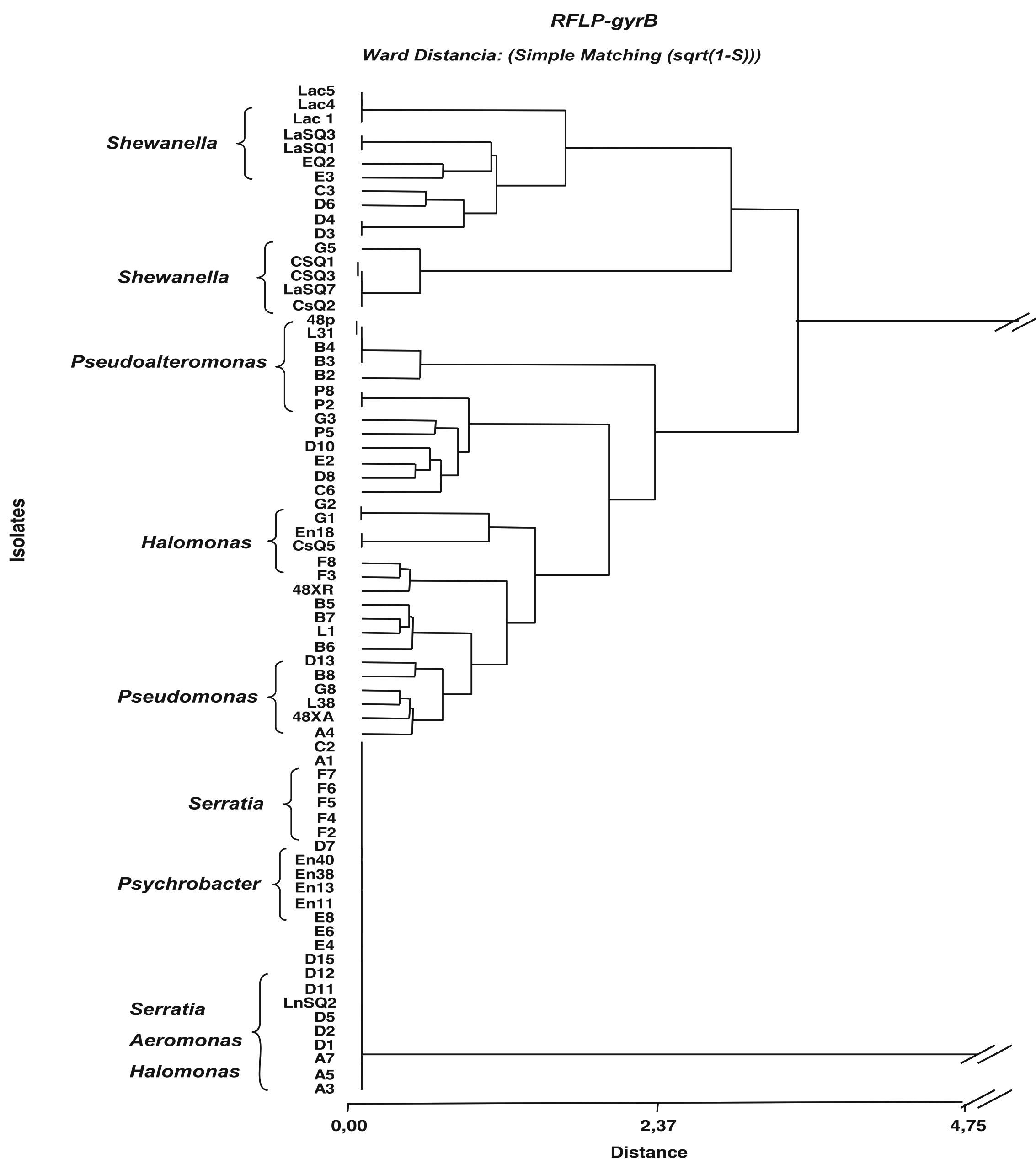



**Fig. 4** (continued)

communities and indicate that genetic exchange plays a significant role in the evolution and ecological impact of terrestrial and aquatic microorganisms (Ochman et al. 2000; Thomas and Nielsen 2005).

The number of heterotrophic bacteria in seawater was reported to be in the order of $1\times10^3$ to $1\times10^4$ cells per milliliter (Boniek et al. 2010). Expectedly, the number of total CFU in our work was $1\times10^3$ in seawater samples (Table 1). It was continually reported that in many environments, only a small fraction (<1 %) of cells observed by direct microscopy can be recovered as CFU on laboratory conditions (Hagström et al. 2002). Sanchez et al. (2008) reported that 8,000 CFU/g of bacteriocin producers isolated from soil of Isla de los Estados, Argentina. Our approach resulted in a major population retrieved from enrichments in R2A medium from intestines collected during winter and summer. Seawater samples did not produce marked differences in the CFU/mL between winter and summer (Fig. 2). Even when a correlation between the bacterial content of the samples is not possible, the results obtained are explained by the fact that intestine samples carry a higher microbial concentration.

According to the API tests, Supplementary Table 3, the characterization of marine bacteria was rapid and effective in supporting the biochemical and physiology makeup with

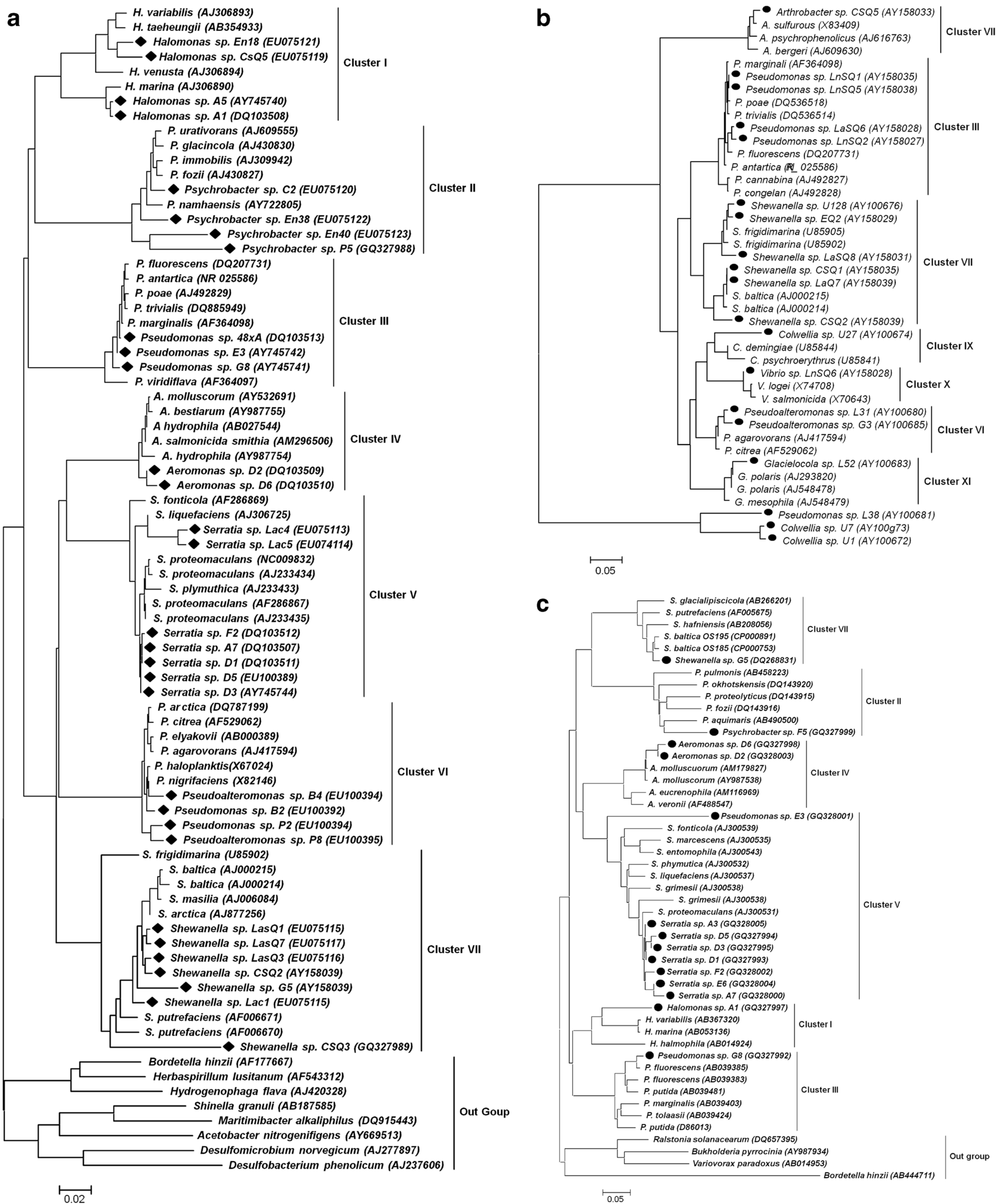
other studies. Vogel et al. (2005) and Cristóbal et al. (2009) described a simple phenotypic scheme derived from various biochemical tests, which were used in the classification of several strains, as belonging to the genus *Shewanella*. The report of Sanchez et al. (2008), though the use of APIs, tests the characteristic of members of *Serratia* group. Our results allowed us to identify members of *Pseudomonas*, *Pseudoalteromonas*, *Serratia*, *Shewanella*, *Alteromonas*, and *Colwellia* genera.

Several cultivable marine bacteria were able to survive the selection imposed by the presence of a carbon source and metabolically efficient to produce enzyme which hydrolyses the substrates (Table 2). In this approach, we select the best bacteria able to produce β-glucosidases, celulases, β-galactosidases, β-xylanases, proteases, and chitinases (Fig. 2). These enzymes are employed in a wide range of industrial processes (Satyanarayana et al. 2005; Cavicchioli et al. 2002). Furthermore, there is today an expanding demand for novel enzymes useful for degrading natural polymers, including starch, cellulose, and proteins, or useful for the chemical and pharmaceutical industries (Trincone 2011; Kennedy et al. 2008; Lee et al. 2010). The products of Antarctic microorganisms including enzymes, metabolic cofactors, light-harvesting complexes, and ether-linked lipids have potential biotechnological applications (Satyanarayana et al. 2005).

In our enzymatic assay, 11 isolates were able to produce cellulases and carboxymethyl cellulose by hydrolysis of paper and CMC, respectively, and exhibit the best hydrolysis halos (20 to 30±1 mm in diameter, Fig. 3a); these important enzymes are in demand for a variety of applications including alcohol production, improvement of juice yield, extraction of color from juices, improves color brightening in detergents, softening and soil particle removal, etc. (Egorova and Antranikian 2005). Commercial products have been developed by Novozymes include Celluzyme®, a cellulase capable of efficient hydrolysis at 15 °C (Wang et al. 2008). While with xylanase activity, 40 isolates were capable to hydrolyze the xylan and bagasse from sugarcane, leaving a clear zone by substrate degradations (Fig. 3b); this enzyme has a substantial market value of US$200 million (Sarethy et al. 2011). Commercial interest in developing applications for xylanases, which degrade hemicellulose from the plant cell wall fraction, is based on their ability to play a key role as quality- and yield-improving agents in the food, feed, pulp, baking, and paper industries (Wang et al. 2008). For β-glucosidase activity, 32 best isolates could hydrolyze the cellobiose, revealing with Congo red dye its degradation (Fig. 3c). The Congo red dye allowed disclose enzymatic activity, which is capable of binding to the polymeric compounds (e.g., cellulose, xylan, and cellobiose), leaving a clear zone due to the presence of a monomeric compound (e.g., glucose). Several β-glucosidases from microbial sources have been determined to have a commercial application in increasing the aroma of wine (Palmieri and Spagna 2007; Cristóbal et al. 2008). In the case of β-galactosidase activity, the isolates were able to gown on lactose and revealing with blue colonies by X-gal degradation (Fig. 3d). The importance of cold-adapted β-galactosidases operating at neutral pH levels can improve the digestibility of dairy products for lactose-intolerant consumers and also enhance sweetness at temperatures where contamination can be minimized (Cristóbal et al. 2009). It is an important remark that the protease activity in agar milk was observed by the casein degradation as source of carbon (Fig. 3e). For this enzyme, constitute an important group among industrial enzymes, and their global sales amount on the order of 60 % of total enzyme market (Sarethy et al. 2011). In addition, commercial applications have been developed by Novozymes (Kannase® and Properase®) and Kao Corporation include the use of cold-active proteases in the production of detergents that are capable of efficiently removing stains in wash at 10–20 °C (Wang et al. 2008). The chitinase play a role in the recycle to maintain carbon-nitrogen balance in the environment (Sarethy et al. 2011), for our activity assay was observed in 35 isolates by colloidal chitin degradation and hydrolysis halo formation (Fig. 3f). Some types of enzymes, e.g., lipases and chitinases, isolated from environments with biological value, are increasingly in demand for use as more efficient and less costly alternatives in several industrial processes (Kennedy et al. 2008).

Data of genetic fingerprints from both analyses can be seen in the two dendrograms generated from γ-Proteobacteria isolates. It is clear that *gyrB*-RFLP fingerprints were more discriminative than 16S-ARDRA ones (Fig. 4). Actually, the *gyrB*-RFLP dendrogram retrieved and clearly grouped most of the genera isolated separately, while *Serratia*, *Shewanella*, and *Halomonas* were grouped together when fingerprints from 16S-ARDRA fingerprints were analyzed. These results agree with previous studies, which have demonstrated the discriminative power of *gyrB* gene sequence, even when closely related organisms are analyzed (Albuquerque et al. 2006; Yin et al. 2008). This shows the close relationship between organisms of different genera, as well as the high level of genetic heterogeneity among members of the phylum Proteobacteria reported by several authors (Hagström et al. 2002; Junge et al. 2002; Cristóbal et al. 2011a). In addition, marine bacteria isolated from the Ross Sea (Pini et al. 2007), Isla de los Estados (Sanchez et al. 2008), and Beagle Channel (Cristóbal et al. 2008, 2011b) were then characterized by ARDRA and *gyrB*-RFLP analyses, establishing the degree of intra- or inter-relationship between the species studied.

The overall phylogenetic distribution of the strains isolated in this study concurs with several earlier reports obtained by analysis of the Arctic sea ice, seawater, and Antarctic research (Junge et al. 2002; Sanchez et al. 2008; Vázquez et al. 2008; Yu et al. 2009; Mwirichia et al. 2010; Trincone 2011). In terms of phylogenetic diversity, most of the selected isolates on the basis of their enzymatic activity belonged to the γ-Proteobacteria class of the proposed phylum Proteobacteria,


a
H. variabilis (AJ306893)
H. taeheungii (AB354933)
Halomonas sp. En18 (EU075121)
Halomonas sp. CsQ5 (EU075119)
H. venusta (AJ306894)
H. marina (AJ306890)
Halomonas sp. A5 (AY745740)
Halomonas sp. A1 (DQ103508)
Cluster I
P. urativorans (AJ609555)
P. glacincola (AJ430830)
P. immobilis (AJ309942)
P. fozii (AJ430827)
Psychrobacter sp. C2 (EU075120)
P. namhaensis (AY722805)
Psychrobacter sp. En38 (EU075122)
Psychrobacter sp. En40 (EU075123)
Psychrobacter sp. P5 (GQ327988)
Cluster II
P. fluorescens (DQ207731)
P. antartica (NR 025586)
P. poae (AJ492829)
P. trivialis (DQ885949)
P. marginalis (AF364098)
Pseudomonas sp. 48xA (DQ103513)
Pseudomonas sp. E3 (AY745742)
Pseudomonas sp. G8 (AY745741)
P. viridiflava (AF364097)
Cluster III
A. molluscorum (AY532691)
A. bestiarum (AY987755)
A hydrophila (AB027544)
A. salmonicida smithia (AM296506)
A. hydrophila (AY987754)
Aeromonas sp. D2 (DQ103509)
Aeromonas sp. D6 (DQ103510)
Cluster IV
S. fonticola (AF286869)
S. liquefaciens (AJ306725)
Serratia sp. Lac4 (EU075113)
Serratia sp. Lac5 (EU074114)
S. proteomaculans (NC009832)
S. proteomaculans (AJ233434)
S. plymuthica (AJ233433)
S. proteomaculans (AF286867)
S. proteomaculans (AJ233435)
Serratia sp. F2 (DQ103512)
Serratia sp. A7 (DQ103507)
Serratia sp. D1 (DQ103511)
Serratia sp. D5 (EU100389)
Serratia sp. D3 (AY745744)
Cluster V
P. ar ctica (DQ787199)
P. citrea (AF529062)
P. elyakovii (AB000389)
P. agarovorans (AJ417594)
P. haloplanktis(X67024)
P. nigrifaciens (X82146)
Pseudoalteromonas sp. B4 (EU100394)
Pseudomonas sp. B2 (EU100392)
Pseudomonas sp. P2 (EU100394)
Pseudoalteromonas sp. P8 (EU100395)
Cluster VI
S. frigidimarina (U85902)
S. baltica (AJ000215)
S. baltica (AJ000214)
S. masilia (AJ006084)
S. arctica (AJ877256)
Shewanella sp. LasQ1 (EU075115)
Shewanella sp. LasQ7 (EU075117)
Shewanella sp. LasQ3 (EU075116)
Shewanella sp. CSQ2 (AY158039)
Shewanella sp. G5 (AY158039)
Shewanella sp. Lac1 (EU075115)
S. putrefaciens (AF006671)
S. putrefaciens (AF006670)
Shewanella sp. CSQ3 (GQ327989)
Cluster VII
Bordetella hinzii (AF177667)
Herbaspirillum lusitanum (AF543312)
Hydrogenophaga flava (AJ420328)
Shinella granuli (AB187585)
Maritimibacter alkaliphilus (DQ915443)
Acetobacter nitrogenifigens (AY669513)
Desulfomicrobium norvegicum (AJ277897)
Desulfobacterium phenolicum (AJ237606)
Out Goup
0.02
b
Arthrobacter sp. CSQ5 (AY158033)
A. sulfurous (X83409)
A. psychrophenolicus (AJ616763)
A. bergeri (AJ609630)
Cluster VIII
P. marginali (AF364098)
Pseudomonas sp. LnSQ1 (AY158035)
Pseudomonas sp. LnSQ5 (AY158038)
P. poae (DQ536518)
P. trivialis (DQ536514)
Pseudomonas sp. LaSQ6 (AY158028)
Pseudomonas sp. LnSQ2 (AY158027)
P. fluorescens (DQ207731)
P. antartica (R_ 025586)
P. cannabina (AJ492827)
P. congelan (AJ492828)
Cluster III
Shewanella sp. U128 (AY100676)
Shewanella sp. EQ2 (AY158029)
S. frigidimarina (U85905)
S. frigidimarina (U85902)
Shewanella sp. LaSQ8 (AY158031)
Shewanella sp. CSQ1 (AY158035)
Shewanella sp. LaQ7 (AY158039)
S. baltica (AJ000215)
S. baltica (AJ000214)
Shewanella sp. CSQ2 (AY158039)
Cluster VII
Colwellia sp. U27 (AY100674)
C. demingiae (U85844)
C. psychroerythrus (U85841)
Cluster IX
Vibrio sp. LnSQ6 (AY158028)
V. logei (X74708)
V. salmonicida (X70643)
Cluster X
Pseudoalteromonas sp. L31 (AY100680)
Pseudoalteromonas sp. G3 (AY100685)
P. agarovorans (AJ417594)
P. citrea (AF529062)
Cluster VI
Glacielocola sp. L52 (AY100683)
G. polaris (AJ293820)
G. polaris (AJ548478)
G. mesophila (AJ548479)
Cluster XI
Pseudomonas sp. L38 (AY100681)
Colwellia sp. U7 (AY100g73)
Colwellia sp. U1 (AY100672)
0.05
c
S. glacialipiscicola (AB266201)
S. putrefaciens (AF005675)
S. hafniensis (AB208056)
S. baltica OS195 (CP000891)
S. baltica OS185 (CP000753)
Shewanella sp. G5 (DQ268831)
Cluster VII
P. pulmonis (AB458223)
P. okhotskensis (DQ143920)
P. proteolyticus (DQ143915)
P. fozii (DQ143916)
P. aquimaris (AB490500)
Psychrobacter sp. F5 (GQ327999)
Cluster II
Aeromonas sp. D6 (GQ327998)
Aeromonas sp. D2 (GQ328003)
A. molluscuorum (AM179827)
A. molluscorum (AY987538)
A. eucrenophila (AM116969)
A. veronii (AF488547)
Cluster IV
Pseudomonas sp. E3 (GQ328001)
S. fonticola (AJ300539)
S. marcescens (AJ300535)
S. entomophila (AJ300543)
S. phymutica (AJ300532)
S. liquefaciens (AJ300537)
S. grimesii (AJ300538)
S. grimesii (AJ300538)
S. proteomaculans (AJ300531)
Serratia sp. A3 (GQ328005)
Serratia sp. D5 (GQ327994)
Serratia sp. D3 (GQ327995)
Serratia sp. D1 (GQ327993)
Serratia sp. F2 (GQ328002)
Serratia sp. E6 (GQ328004)
Serratia sp. A7 (GQ328000)
Cluster V
Halomonas sp. A1 (GQ327997)
H. variabilis (AB367320)
H. marina (AB053136)
H. halmophila (AB014924)
Cluster I
Pseudomonas sp. G8 (GQ327992)
P. fluorescens (AB039385)
P. fluorescens (AB039383)
P. putida (AB039481)
P. marginalis (AB039403)
P. tolaasii (AB039424)
P. putida (D86013)
Cluster III
Ralstonia solanacearum (DQ657395)
Bukholderia pyrrocinia (AY987934)
Variovorax paradoxus (AB014953)
Bordetella hinzii (AB444711)
Out group
0.05

◀ **Fig. 5** The sequences of full-length or nearly full-length genes were sequenced with an ABI Prism 3100 Genetic Analyzer System following the manufacturer's recommendations. Phylogenetic trees are based on 16S rDNA and *gyrB* sequences and constructed using neighbor-joining and maximum parsimony methods. The reproduction of each branch in 1,000 bootstrap analyses is indicated by the absence of marks at branching points. The representative isolates identified in this study are shown in bold type, and the accession number for all strains is given in parentheses. The 16S rDNA and *gyrB* sequences were submitted to the NCBI (http://www.ncbi.org.com) database. **a** Phylogenetic tree based on full-length 16S rDNA sequences (approximately 1,500 bp), members of β- and δ-Proteobacteria were included as an outgroup; *scale bar* represents 0.02 Knuc. **b** Phylogenetic tree based on partial 16S rDNA sequences (500 bp), members of β- and δ-Proteobacteria were included as an outgroup; *scale bar* represents 0.05 Knuc. **c** Phylogenetic tree based on full-length *gyrB* sequences (approximately 1,200 bp); *scale bar* represents 0.05 Knuc. The black points in the diagram represent the strains sequenced in the present study

which is consistent with previous reports (Junge et al. 2002; Lee et al. 2010; Ohnishi et al. 2011; Cristóbal et al. 2011b). In this work, numerous members of 12 genera of γ-Proteobacteria class were identified and classified as belonging to *Alteromonadales* order: *Shewanella*, *Alteromonas*, *Colwellia*, and *Pseudoalteromonas* (Fig. 5a–c). As we observed and previously reported, the prevalence in aquatic habitats, marine sediments, and intestines of the species of these genera and species of *Psychrobacter* (*Pseudomonadales* order), which are readily cultured, reflects their ability to successfully grow under heterotrophic conditions (Yu et al. 2009). The strains related closely to typical aquatic bacteria belonging to the genus *Aeromonas* are widespread in freshwater and have been implicated as pathogens in human and animal diseases. Species belonging to the family *Halomonadaceae* are ubiquitous and have been isolated from seawater and bodies of hypersaline water as moderate halophiles from the *Halomonas*/*Cobetia* groups (Mwirichia et al. 2010). Considered as psychrotolerant, species from the *Serratia* group are ubiquitous and have been isolated from marine sediments, soil, and seawater as we also previously reported (Sanchez et al. 2008). *Pseudomonas* species are also easily cultivable and widely isolated from clean and contaminated soils, in freshwater, and seawater (Olivera et al. 2007). The strain collection being studied are able to produce a variety of bioactive compounds, including metabolic cofactors, light-harvesting complexes, ether-linked lipids (Satyanarayana et al. 2005), extracellular enzymes (Cristóbal et al. 2011b), exopolysaccharides (Mwirichia et al. 2010), and compounds with antimicrobial activities (Sanchez et al. 2008) from Antarctic microorganisms. Various pharmaceutically relevant activities and members of *Halomonadaceae* family and *Alteromonadales* order have been shown to be of importance in the use in environmental bioremediation processes (Mwirichia et al. 2010).

Schmidt et al. (1991) reports that a phylogenetic tree constructed based upon only 200 bp of 16S rDNA genes was congruent with one calculated by using full sequences for the major bacterial phyla, with diverging lineages established at the level of genus. Therefore, in our study using 500 bp of 16S rDNA sequences, we identified microorganisms belonging to clusters V, VI, and VII (Fig. 5b). This region contains sequence variability in conserved secondary structure and is therefore useful for rapid comparison of sequences. Junge et al. (2002) reported close relationships with known marine psychrophiles within the genera *Alteromonas*, *Colwellia*, *Glacielocola*, *Octadecabacter*, *Pseudoalteromona*, and *Shewanella*. Yamamoto et al. (2000) reported that *rpoB* and *gyrB* genes show a greater degree of evolutionary divergence and proposed their use as markers for taxonomic analyses. The difference in the base substitution frequencies for 16S rDNA and *gyrB* genes may be due to the difference in the number of sites available for neutral base substitutions (Cristóbal et al. 2008). We observed the correct taxonomy within each genus, with the exception that the E3 strain (*Pseudomonas* sp.) was classified inside of *Serratia* genus. Phylogenetic studies based on culture collections from Antarctic regions and sea ice has been indicated close relationships with known marine psychrophiles (Cristóbal et al. 2011b; Sanchez et al. 2008).

## Conclusions

The seawater and benthonic organism samples from the Beagle Channel, Argentina, showed high levels of diversity in terms of microbes and varieties of cold-active enzymes. This work is an attempt to describe their diversity, as well as the production of biocatalyzer with potential for biotechnological processes. This study also confirms that 16S rDNA and *gyrB* genes can serve as powerful markers for elucidating phylogenetic relationships between marine bacteria. These approaches have the potential to contribute to research involving new bacterial strains that exhibit unusual properties. Further efforts are clearly needed to generate culture collections from cold habitats, by applying a wide range of culturing methods. Such efforts will provide avenues for high-throughput screening of new cold-active products and microbial industrial processes.

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