



## Detection of proteases from *Sporosarcina aquimarina* and *Algoriphagus antarcticus* isolated from Antarctic soil

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### ABSTRACT

Two psychrophilic bacterial samples were isolated from King George Island soil, in Antarctica. The phylogenetic analysis based on the 16S rRNA (*rrs*) gene led to the correlation with the closest related isolates as *Sporosarcina aquimarina* (99%) and *Algoriphagus antarcticus* (99%), with query coverage of 99% and 98%, respectively. The spent culture media from both isolates displayed proteolytic activities detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis containing gelatin as protein substrate. Under the employed conditions, *S. aquimarina* showed a 55 kDa protease with the best activity detected at pH 7.0 and at 27°C. *A. antarcticus* also showed a single extracellular protease, however its molecular mass was around 90kDa and its best activity was detected at pH 9.0 and at 37°C. The proteases from both isolates were inhibited by 1,10-phenanthroline and EDTA, two metalloprotease inhibitors. This is the first record of protease detection in both species, and our results may contribute to broaden the basic knowledge of proteases from the Antarctica environment and may help prospecting future biotechnological applications of these enzymes.

**Key words:** *Algoriphagus antarcticus*, Antarctica, *Sporosarcina aquimarina*, Protease.

### INTRODUCTION

The Earth has a considerable proportion of extremely cold environments, represented by the Antarctic and Arctic regions, as well as cold regions, including the oceans (usually 4–5°C below a depth of 1,000m), which cover around  $\frac{3}{4}$  of the Earth's

surface (Feller and Gerday 2003). In this context, Antarctica is the coldest, driest, highest, windiest and most isolated and unknown continent on Earth (Olson 2002). The microorganisms that survive under these inhospitable conditions play a major role in the Polar ecosystem, as they are the major components in the Antarctic ecosystem biomass (Wynn-Williams 1996, Pointing et al. 2009).

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Due to the low complexity of the food chain and the low number of individuals and diversity, any change in the physicochemical conditions of this environment is quickly noticeable in the various trophic levels (Yergeau and Kowalchuk 2008).

The great selective pressure exerted by the Antarctic environmental conditions determines either the survival, the death or the efficient adaptation of the organisms, such as that of extremophile microorganisms known as psychrophiles (Morita 1975). Adaptations developed in the cell constituents include changes that are only found in microorganisms from this region of our planet, such as changes in the membrane structure, energy-generating systems, protein synthesis machinery, biodegradable enzymes, components responsible for the nutrient incorporation and in the structure of enzymes' active sites, among others (Gerday et al. 1997, 2000, Russel 1998, Cavicchioli and Thomas 2000).

Psychrophilic enzymes are characterized by high catalytic efficiency in low temperatures, high level of thermolability and an increased structural flexibility in order to provide better access to the substrate; in this sense, the protein flexibility plays a critical role bestowing adaptability to cold (Thomas and Dieckmann 2002). Despite their usefulness in industrial and domestic processes (Feller and Gerday 2003), psychrophiles and their products are underutilized in biotechnology due to the thermolability of their enzymes and the cost of production and processing at low temperatures (Margesin and Feller 2010). However, advances in the biotechnology enzymatic area, especially with psychrophilic enzymes, have led to a rapid growth in this field (Cavicchioli et al. 2002), hence the study of proteolytic enzymes from Antarctica is an interesting theme for biotechnology (Feller and Gerday 2003).

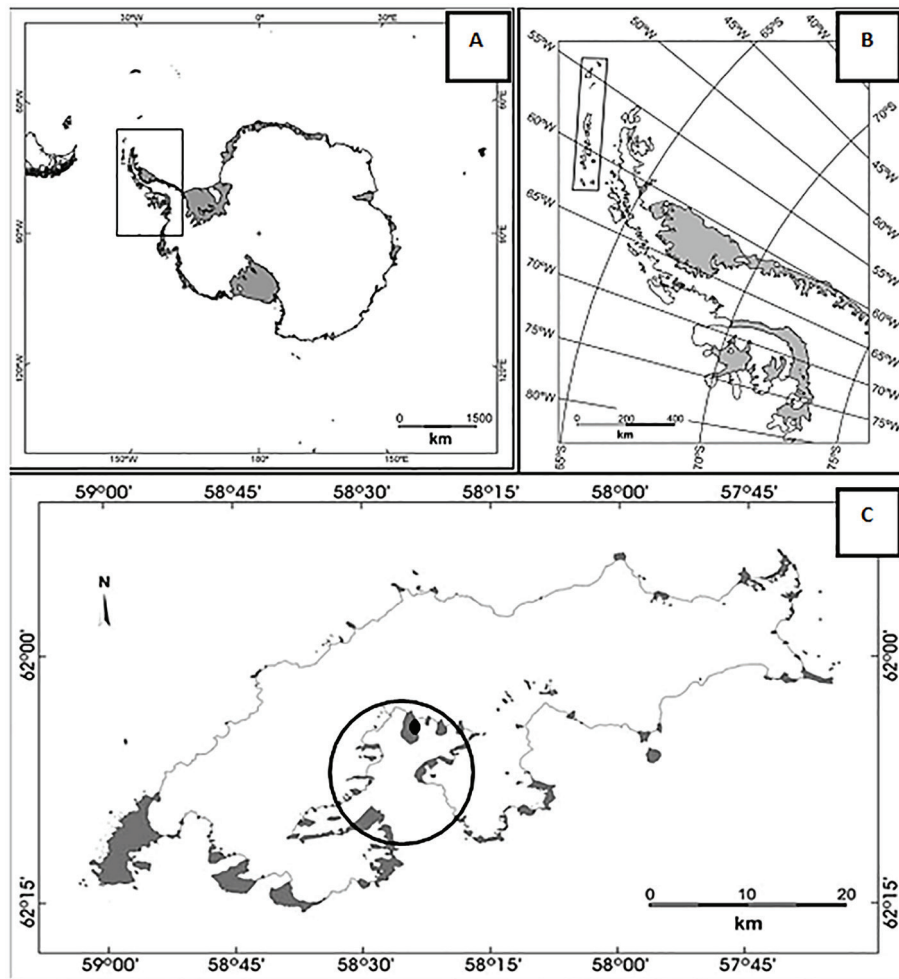
The world market for industrial enzymes is a 2-billion-dollar per year business. Proteases are among the most commercialized enzymes in the world and account for over 65% of the world's enzyme market (Rao et al. 1998). Some of their

applications include their use in the detergent industry, textile industry, food industry, bioremediation, and biocatalysts in low water conditions (Gerday et al. 2000). Further knowledge of the mechanisms involved in the molecular adaptation of enzymes to low temperatures is of great interest to biotechnology. In view of this, the scope of this work was to identify two bacteria isolated from King George Island soil, in Antarctica, and characterize the extracellular proteases produced by both isolates, in order to assess their possible biotechnological potential.

## MATERIALS AND METHODS

### ISOLATION AND IDENTIFICATION OF BACTERIAL ISOLATES

The bacterial isolates in this study were grown up from the soil samples collected from different locations at Admiralty Bay, located in the center of King George Island in the Southern Shetland Islands, which lies 130 km northwest off the Antarctic Peninsula between latitudes 61°00'S and 63°30'S and longitudes 53°55'W and 62°50' (Teixeira et al. 2010) (Fig. 1). The collection took place in the 2006/2007 austral summer and it received logistic support from the Brazilian Navy. Bacterial enumeration and isolation were performed in the Brazilian Antarctic Station laboratory (Comandante Ferraz). After homogenization of the soil sample in plastic bags, an aliquot of 5 g was transferred to Erlenmeyer flasks containing 45 ml of sterile saline solution (0.85% NaCl) and manually agitated during 10 min. The soil suspensions were then serially diluted (1:10) in conical tubes containing 9 ml of sterile saline solution before plating in duplicate on Petri dishes containing Luria-Bertani (LB) solid medium. The counting was performed after four weeks of incubation at 4°C. Pure isolates were transferred to 15-ml conical tubes containing 10ml of slanted Luria Bertani (LB) agar medium (Bertani 1951) and transported to Brazil at 4°C. Both the isolates used in this study, named initially Samples 1 and 2, were maintained in Luria-Bertani (LB) solid medium.



**Fig. 1** - Location of collection of bacterial samples: **(A)** The Antarctic continent, with the Antarctic Peninsula highlighted. **(B)** Antarctic Peninsula and archipelago of the South Shetland Islands. **(C)** The Admiralty Bay highlighted in King George Island. The circle indicates the location of the Comandante Ferraz Antarctic Station (EACF), from Brazil. Adapted from Simões et al. (2004).

For the identification of the isolates, the primers used in the analysis were 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-AAGGAGGTGATCCAGCGCA-3') due to the fact that both flank the coding region for the 16S rRNA (*rrs*) gene (Massol-Deya et al. 1995). The amplicons generated by PCR were purified with the PCR Purification System (Qiagen) kit for further automatic sequencing in the MEGABACE DNA Analysis System 500 (GE Healthcare). The sequencing was performed using the external primers

27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GTTTACCTTGTTACGACTT- 3') (Dojka et al. 2000), and the intermediate primers 532F (5'-CGTGCCAGCAGCCGCGGTAA-3') and 907R (5'- CCGTCAATTCMTTGTGAGTTT-3') (Ishii and Fukui 2001). The quality of the sequences was checked using the Phred program (Ewing and Green 1998). The sequences obtained were analyzed in the BLASTn program (<http://www.ncbi.nlm.nih.gov/>) and Genbank to align them to the sequences stored in the database.

#### PHYLOGENY

For the assembly of the phylogenetic tree, we used the DNA sequences coding for the 16S rRNA of the two isolates studied, their respective species obtained in the reference database GenBank and other bacterium species of different phylum. All sequences were converted into FASTA format using BioEdit. MEGA 4.0 software was used to assemble the tree. The trees were generated by the neighbor-joining method (Gascuel and Steel 2006), while the model used was Jukes Cantor (Chor et al. 2006), bootstrap with 10,000 repeats.

#### GROWTH CONDITIONS AND PREPARATION OF EXTRACELLULAR EXTRACTS

Bacterial isolates were grown in LB medium for 48 h at 4°C. Cultures were passed over a 0.22- $\mu$ m filtration unit (Millipore), and each spent culture medium was then concentrated 10 times using an Amicon system (Stirred Cell Model 8200) at 4°C (cut-off 10,000 MW). The protein concentration was determined according to Lowry et al. (1951), using bovine serum albumin as standard.

#### EXTRACELLULAR PROTEASE DETECTION

The extracellular proteolytic activity (50  $\mu$ g protein/slot) was determined using 10% SDS-PAGE with 0.1% copolymerized gelatin as substrate (Heussen and Dowdle 1980). Electrophoresis was performed at 120V, 4°C for 90 min, and the gels were then incubated in 2.5% Triton X-100 for 1 h, washed in distilled water and incubated for 24 h in one of the following pH buffers: 10 mM sodium citrate (pH 3.0), 50 mM sodium phosphate (pH 5.0 and pH 7.0) and 20 mM glycine-NaOH (pH 9.0 and pH 11.0). The effect of the temperature upon the enzyme activity was assessed by incubating the gels at 4°C, 27°C, 37°C and 56°C. The detection of proteolytic bands was performed by staining the gels for 2 h with 0.2% Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (50:10:40, v/v/v) and destaining in the same solvent. The molecular mass of the

proteases was calculated by comparison with the mobility of molecular mass standards (Fermentas). The gels were dried, scanned and the densitometric analysis was digitally processed using Image J public domain software.

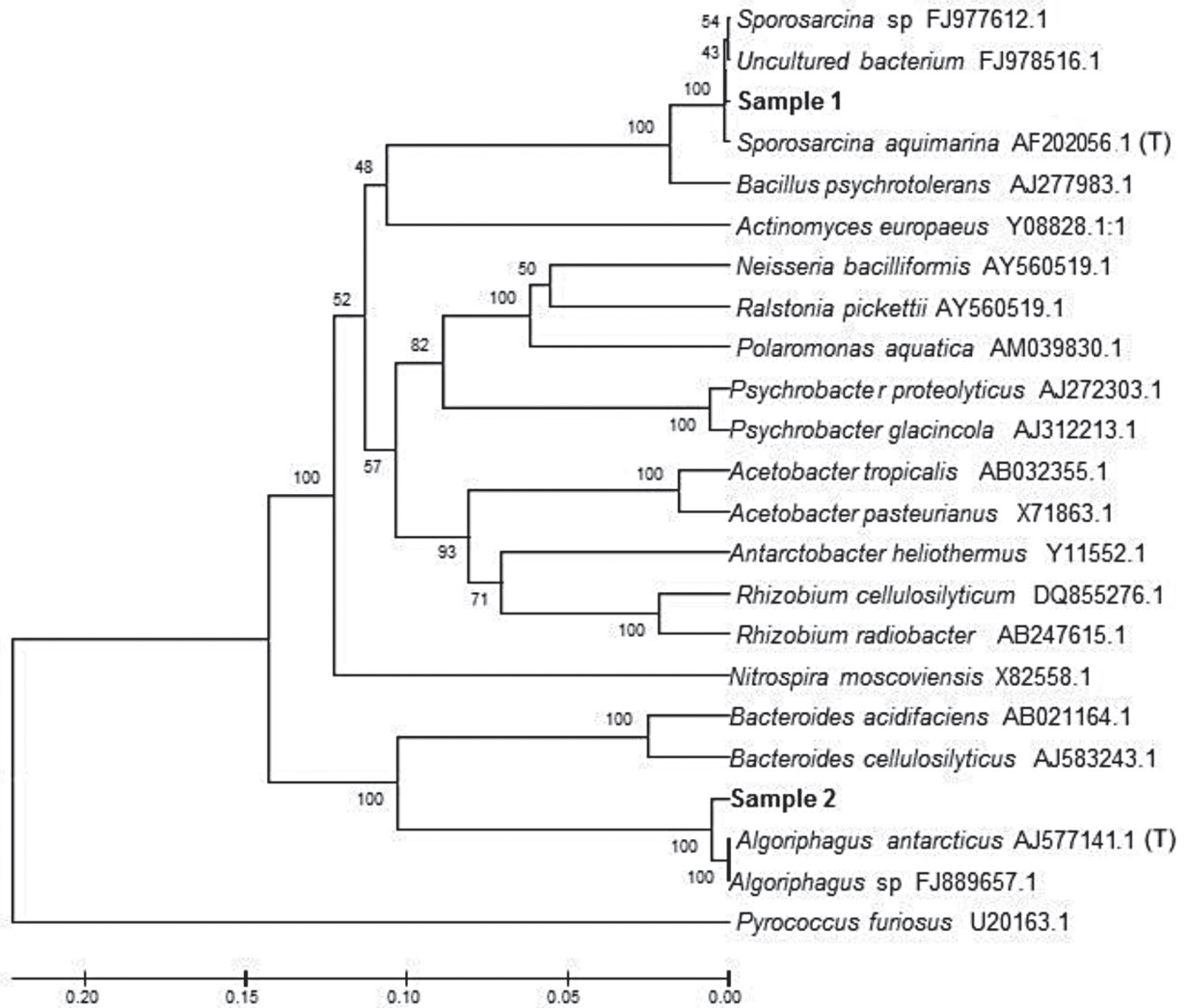
#### ENZYME CLASS DETERMINATION

In order to determine the protease class, the gels were incubated for 24 h at pH and temperature conditions that better resolved the proteolytic profile containing the following proteolytic inhibitors: 10 mM phenylmethylsulfonyl fluoride (PMSF), 100  $\mu$ M tosyl-L-lysine chloromethyl ketone (TLCK) (serine protease inhibitors), 10 mM 1,10-phenanthroline, 10 mM ethylenediaminetetraacetic acid (EDTA) (metalloprotease inhibitors), 10  $\mu$ M L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64) (cysteine protease inhibitor) and 1  $\mu$ M pepstatin A (aspartic protease inhibitor). The results were compared with a control group that had been incubated in the absence of the proteolytic inhibitors.

#### RESULTS

The results obtained in the sequencing of Samples 1 and 2 showed 99% similarity with the isolate *Sporosarcina aquimarina* – AF202056 (query coverage of 99%) and *Algoriphagus antarcticus* – AJ577141 (query coverage of 98%). The phylogenetic tree confirmed the grouping of the isolates with the reference species obtained in GenBank, in addition to revealing the evolutionary distance between these two genera with sequences from other bacterial phyla (Fig. 2).

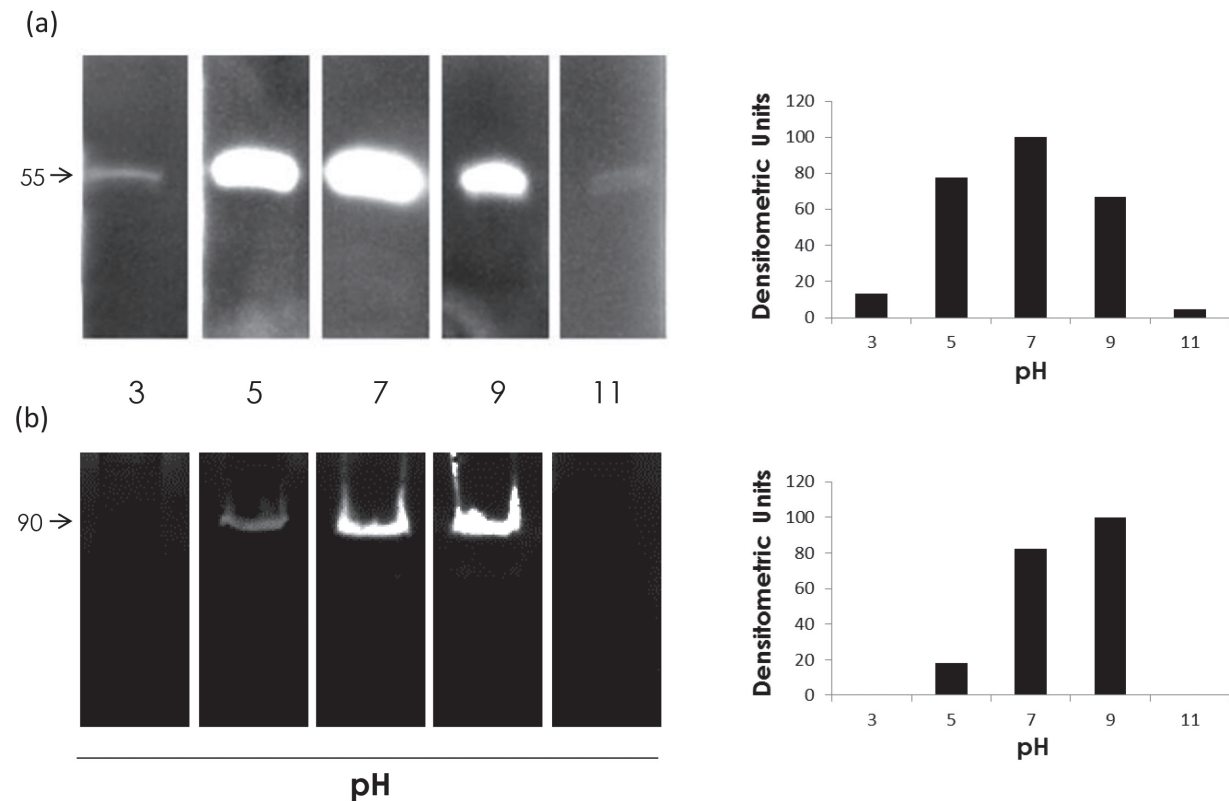
The assembly of the phylogenetic tree for both samples was followed by the detection of the extracellular protease activity from *S. aquimarina* and *A. antarcticus* by gelatin-SDS-PAGE. The spent culture media of both species tested displayed a single protease band each, representing distinct proteolytic activities. Under the employed experimental setting, the best conditions for the hydrolytic activity detected at 55 kDa in *S.*



**Fig. 2** - Phylogenetic tree of 16S rRNA gene sequences (around 1500 bp) showing the relationship between two Antarctic bacterial isolates (Samples 1 and 2) and related species identified using BLASTn searches. The tree was constructed based on a neighbor-joining method. Bootstrap analyses were performed with 10,000 repetitions, and results are represented at the branch points. The GenBank accession number of each species is cited in parentheses (*S. aquimarina* JX45902 and *A. antarcticus* JX45901). Type species are referred to as (T).

*aquimarina* were found to be at pH 7.0 and at 27 °C (Fig. 3 and 4). Nevertheless, the proteolytic band was consistently observed at pH values ranging from 5.0 to 9.0, displaying approximately 80% of its total activity at pH 5.0 and more than 60% at pH 9.0, as well as at 37°C (approximately 80% of the maximum activity detected at 27°C). A drastic reduction (more than 80%) of the activity was observed at pH values 3.0 and 11.0 as well as at 4°C and at 56°C. For *A. antarcticus*, in contrast,

the single protease detected at 90 kDa yielded a distinct profile, in which the best conditions were pH 9.0 and 37°C (Fig. 3 and 4). Hydrolysis was also observed at pH 7.0 (more than 80% of the total activity) and at pH 5.0 (approximately 20% of the total activity) as well as in the temperature range 27-56°C (approximately 40% and 10%, respectively, of the activity detected at 37°C). No proteolytic activity was detected at pH 3.0 and at pH 11.0 nor at 4°C (Fig. 3 and 4).



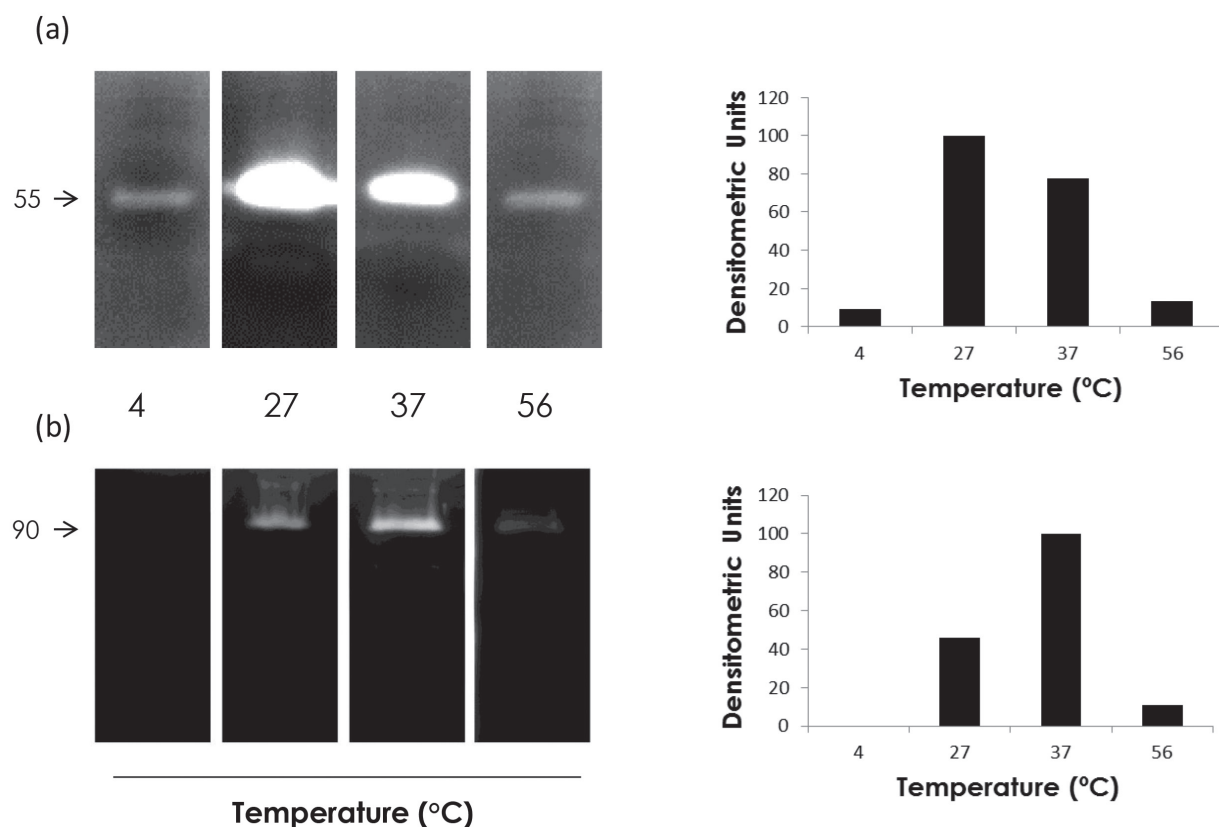
**Fig. 3** - Gelatin-SDS-PAGE showing the detection of extracellular proteases by *Sporosarcina aquimarina* (a) and *Algoriphagus antarcticus* (b) at distinct pH values and optimal temperature conditions, as described in Materials and Methods. Molecular masses of the proteases, expressed in kiloDaltons, are represented on the left. The densitometric analysis of the proteolytic halos, expressed as the percentage of the remaining activity with respect to the optimal pH conditions, is shown on the right.

The proteolytic zymograms indicated that the proteases secreted by *S. aquimarina* and *A. antarcticus* were both sensitive to 1,10-phenanthroline and EDTA (Fig. 5) at temperature and pH conditions that better resolved the proteolytic profile, which indicates the presence of metalloproteases in the culture supernatant from both isolates. In addition, incubation of gels with cysteine protease and aspartic protease inhibitors, such as E-64 and pepstatin A, did not alter the proteolytic activity in either of the samples (data not shown). Interestingly, the proteolytic activity was markedly enhanced in the presence of the serine protease inhibitor PMSF, which affected the activity in both species, while the serine protease inhibitor TLCK led to higher levels of activity in *S. aquimarina* (Fig. 5). It is likely that PMSF and

TLCK induced some conformational change in the enzyme structure so that the active site could better perform gelatin hydrolysis.

#### DISCUSSION

The diversity of microorganisms inhabiting cold environments has been extensively investigated over the past few years with a focus on culture-independent techniques, but the information obtained by such methods may be complemented by the cultivation of such microorganisms (Casanueva et al. 2010). In this sense, Gratia et al. (2009) demonstrated that, after the initial screening to ascertain exoenzyme production (proteases, lipases, amylases, cellulases and xylanases) in more than 1000 isolates collected from several cold environments (Antarctica, Kerguelen Island, Spitzberg, Siberia, Canada, Lapland and other



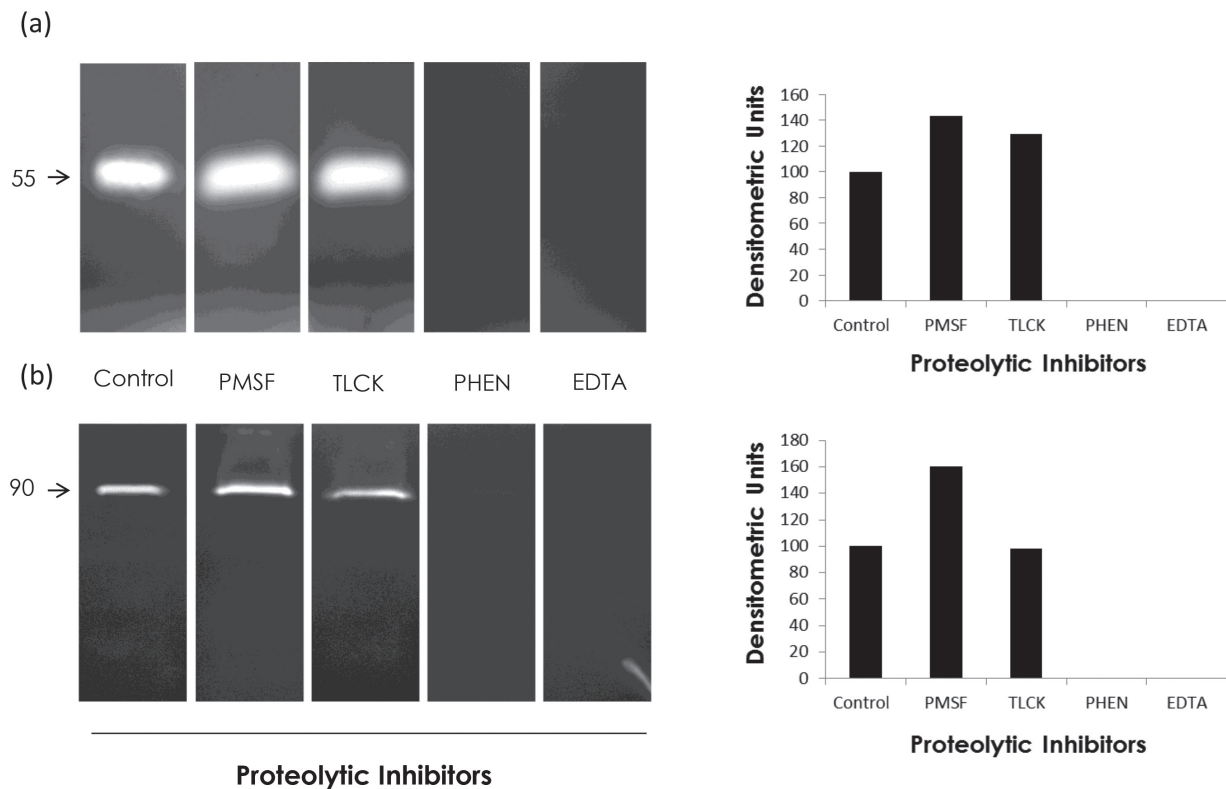
**Fig. 4** - Gelatin-SDS-PAGE showing the detection of extracellular proteases in *Sporosarcina aquimarina* (a) and *Algoriphagus antarcticus* (b) at distinct temperature values and optimal pH conditions, as described in Materials and Methods. Molecular masses of the proteases, expressed in kiloDaltons, are represented on the left. The densitometric analysis of the proteolytic halos, expressed as the percentage of the remaining activity with respect to the optimal temperature conditions, is shown on the right.

deep seawater samples), over 100 isolates showed the ability to produce at least two of the above-mentioned exoenzymes at 4°C. Taken together, these observations clearly indicate that the identification of extremophiles and their correlation with enzyme production may be linked to organismal function and provide a basis for further physiological analysis (Casanueva et al. 2010).

Through the phylogenetic analysis performed in this work, the studied isolates were more correlated to *S. aquimarina* and *A. antarcticus*, with 99% similarity. According to Stackebrandt and Ebers (2006), only similarity values below 98.7-99% are mandatory to require a deeper evaluation, such as DNA-DNA hybridization, and as such we may conclude that both the samples employed in

our analysis were properly identified. Despite the importance of the psychrophilic proteases study, our work is the first to describe proteases from *S. aquimarina*, which was originally isolated from seawater in Korea (Yoon et al. 2001), as well as from *A. antarcticus*, originally isolated from microbial mats of lakes Reid, Fryxell and Ace in Antarctica (Van Trappen et al. 2004).

In order to detect proteolytic activities in these samples, gelatin-SDS-PAGE was employed. Among the many benefits displayed by this technique, it is a simple standard method to assess the complexity of proteases in cell systems, and intensity of bands can be quantified with densitometric analysis. Although one cannot accurately estimate the molecular mass in such conditions, because of the non-reduction



**Fig. 5** - Gelatin-SDS-PAGE showing the detection of extracellular proteases in *Sporosarcina aquimarina* (a) and *Algoriphagus antarcticus* (b) at pH and temperature conditions that better resolved the proteolytic profile, as described in Materials and Methods, in the absence (Control) or in the presence of different proteolytic inhibitors: 10 mM PMSF, 100  $\mu$ M TLCK, 10 mM 1,10-phenanthroline (PHEN) and 10 mM EDTA. Molecular masses of the proteases, expressed in kiloDaltons, are represented on the left. The densitometric analysis of the proteolytic halos, expressed as the percentage of the remaining activity with respect to the control, is shown on the right.

conditions of the samples, a side-by-side comparison of the protease profiles in distinct physical conditions, such as pH and temperature, as well as in the presence of inhibitors that specifically block the enzymatic activity of proteases, provides information about the occurrence and the expression of these activities within a sample group (d'Avila-Levy et al. 2012).

In our study, the proteolytic zymograms showed the presence of metalloproteases as the only extracellular proteolytic activity detected in both isolates, *S. aquimarina* and *A. antarcticus*, with the best temperature conditions displayed at 27°C and 37°C, respectively. Concerning the best pH value tested, *S. aquimarina* extracellular metalloprotease presented the highest activity at pH 5.0-7.0, and *A. antarcticus* at pH 7.0-9.0. Extracellular

proteases of psychrotolerant bacteria isolated from Antarctica have already been previously classified as metalloproteases, with optimal activity at 40°C and pH 7-9 for *Pseudomonas* sp. strains (Vazquez et al. 2004) and at 45°C and pH 6-10 for *Pseudoalteromonas* sp. strain P96-47 (Vazquez et al. 2008). Proteases produced by this class of microorganisms apparently are not totally adapted to function in cold environments, even when the original environment from which these bacteria were isolated hardly reached over 10°C. Although no activity is displayed in the 0-4°C temperature range, i.e. similar to those of the collection sites, we cannot rule out the improvement in the enzymes performance in their natural environment, which could be better than the results displayed *in vitro*



(Vazquez et al. 2008). This behavior may be due to innumerable cofactors that influence the enzyme activity present in those environments, and that have not been mimicked *in vitro* yet. One of the most common methods of freeze-protection in cells is the accumulation of compatible solutes, which results in a reduction of the freezing point of the cytoplasmic aqueous phase and might also directly stabilize macromolecules, particularly enzymes. There are also more specific mechanisms for freeze-protection, such as ice-nucleating proteins and anti-freeze proteins (Casanueva et al. 2010).

Microorganisms not yet isolated, such as protease producers and isolates from the Antarctic environment, may reveal new information concerning the enzymes produced, such as different variations in temperature tolerance and other adaptive traits. In this sense, Olivera et al. (2007) isolated bacteria of different genera with proteolytic activity in the sediment samples from subantarctic areas in Argentina, characterizing their proteases and their thermokinesis. The results showed that the thermodynamic parameters of the enzymes varied considerably among the different isolates, which suggests important variations in the thermo-properties of the proteases in this environment relative to bacterial original genus. In addition, enzymes produced by psychrophilic can offer biotechnological advantages to be used in different areas, such as food processing, laundry powder for cold-water washing, leather industry as well as in the treatment of effluents and bioremediation of polluted soils and waste waters in cold environment (Demain 1999, Van den Burg 2003). Moreover, the capacity of these psychrophilic microorganisms, including *S. aquimarina* and *A. antarcticus*, to grow and produce enzymes at low temperatures is beneficial as it fosters substantial energy saving in large scale enzyme production processes, since there is no need to heat up bioreactors (Margesin and Feller 2010). Our results demonstrate that *S. aquimarina*

and *A. antarcticus* species have turned out to be promising sources of proteases, which highlights the need for further studies in order to assess the biotechnological potential of these enzymes.

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#### RESUMO

Duas amostras de bactérias psicrófilas foram isoladas do solo da Ilha do Rei Jorge, na Antártica. A análise filogenética baseada no gene do RNAr 16S (*rrs*) levou à correlação destes isolados com *Sporosarcina aquimarina* (99%) e *Algoriphagus antarcticus* (99%), com porcentagem de alinhamento de 99% e 98%, respectivamente. O sobrenadante do meio de cultura de ambos os isolados apresentou atividades proteolíticas detectadas por eletroforese em gel de poliácridamida contendo dodecil sulfato de sódio e gelatina como substrato proteico. Sob estas condições, *S. aquimarina* apresentou uma protease de 55 kDa com melhor atividade em pH 7,0 e a 27°C. Também foi detectada uma única protease extracelular em *A. antarcticus*, no entanto com massa molecular em torno de 90 kDa e com melhor atividade em pH 9,0 e a 37°C. As proteases de ambos os isolados foram inibidas por 1,10-fenantrolina e EDTA, dois inibidores de metaloproteases. Este é o primeiro registro de detecção de proteases em ambas as espécies, e nossos resultados podem contribuir para ampliar o conhecimento básico de proteases provenientes do ambiente antártico e podem ajudar na prospecção de futuras aplicações biotecnológicas dessas enzimas.

**Palavras-chave:** *Algoriphagus antarcticus*, Antártica, *Sporosarcina aquimarina*, Protease.

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