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Diversity and enzyme properties of protease-producing bacteria isolated from sub-Antarctic sediments of Isla de Los Estados, Argentina

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Abstract Protease-producing bacteria isolated from sub-Antarctic marine sediments of Isla de Los Estados (Argentina) were characterized, and the thermal inactivation kinetics of their extracellular proteases compared. Isolates were affiliated with the genera *Pseudoalteromonas*, *Shewanella*, *Colwellia, Planococcus*, and a strain to the family *Flavobacteriaceae*. *Colwellia* strains were moderate psychrophiles (optimal growth at about 15°C, maximum growth temperature at around 25°C). 16S rRNA phylogenetic analysis revealed that these strains and *Colwellia aestuarii* form a distinct lineage within the genus. The remaining isolates were psychrotolerant and grew optimally between 20 and 25°C; two of them represent potentially novel species or genus (16S rRNA < 97% sequence similarity). The thermostability of the extracellular proteases produced by the isolates was analysed, and the inactivation rate constant (k_{in}), the activation energy (Ea_{in}) and the activation Gibbs free energy of thermal inactivation (ΔG^*_{in}) determined. ΔG^*_{in}, calculated at 30°C, varied between 97 and 124 kJ/mol. *Colwellia* enzyme extracts presented the highest thermosensitivity, while the most thermostable protease activity was shown by *Shewanella* spp. These results demonstrated that the stability to temperature of these enzymes varies considerably among the isolates, suggesting important variations in the thermal properties of the proteases that can coexist in this environment.

Keywords Sub-Antarctic · Cold-active proteases · Thermostability · Psychrophiles · Psychrotolerants

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Introduction

Microorganisms adapted to cold environments have been classified in two different physiological groups: psychrophiles and psychrotrophs (Morita 1975). This definition considers that psychrophilic microorganisms have an optimal temperature for growth ≤15°C, a maximal temperature for growth ≤20°C, and a minimal growth temperature at 0°C or below. On the other hand, psychrotrophic microorganisms, also called psychrotolerants, can grow at 0°C but overcome the optimal and maximal temperature growth limits specified for psychrophiles. The ability of these microorganisms to grow and carry out their metabolic processes at low temperature depends on adaptative changes in proteins (particularly enzymes), translation systems and in cellular lipids to maintain membrane fluidity and permeability (Russell 1990). Cold-active enzymes characteristically posses high catalytic efficiency at low temperatures, this is related to an increase in the flexibility of the active site and therefore a lower thermal stability than their mesophilic counterparts (Feller and Gerday 2003). Because of their unique properties, these enzymes have also gained much attention regarding their potential for biotechnological applications. High enzymatic activity at low temperature

is an important feature for energy saving in processes that would be performed at room or tap water temperature. Moreover, the use of thermosensible enzymes allows their selective inactivation in complex mixtures (Margesin and Schinner 1994; Gerday et al. 2000; Moran et al. 2001).

Numerous studies have shown that cold-adapted microorganisms are characteristic inhabitants of permanently cold ecosystems, typically with subzero temperatures, such as deep sea (DeLong et al. 1997; Bowman et al. 2003), polar seas (Connelly et al. 2006), sea ice (Bowman et al. 1997a; Groudieva et al. 2004), glaciers (Gounot 1976) and permafrost soils (Bai et al. 2006, Steven et al. 2006). However, psychrotolerant and even psychrophilic bacteria have also been found in temperate and non-permanently cold environments (Helmke and Weyland 2004).

The Atlantic coast of Tierra del Fuego Province, located in the southernmost part of South America, is an environment with constant moderately cold climate. Surface seawater temperature measured near its capital city, Ushuaia, ranged from a medium value of 9.7°C in January to 4.5°C in July (Servicio de Hidrografía Naval, Armada Argentina, http://www.hidro.gov.ar/ceado/ceado.asp). Microbiological studies performed in this sub-Antarctic area are limited in comparison to the extensive literature about microorganisms that colonize extreme cold environments, such as those mentioned above. As these coasts are exposed to oil pollution derived from petroleum exploitation and transport (Esteves et al. 2006), microbiological research was mainly focused on hydrocarbon-degrading communities. Recently, Prabagaran et al. (2006) analysed the bacterial diversity in seawater and seawater polluted with crude oil, collected off Ushuaia, by construction of 16S rRNA gene libraries and subsequent identification of bacteria. *Alphaproteobacteria*, *Gammaproteobacteria*, the Cytophaga–Flavobacterium–Bacteroides (CFB) group and unculturable bacteria were found to be the dominant clones. Moreover, they identified several clones associated only with hydrocarbon-contaminated seawater.

Knowledge about psychrophilic and psychrotolerant bacteria from these coasts and the properties of their enzymes is even more limited. Isla de Los Estados is a provincial natural protected reserve formed by a relatively pristine group of islands, with scarce human activity, which is approximately 30 km away from Tierra del Fuego coast and about 1,000 km from the Antarctic Peninsula. The aim of this study was to analyse the diversity of culturable protease-producing bacteria in marine sediments from this location, and to describe the characteristics of their extracellular proteases in terms of their thermal inactivation kinetics. In this report, we also compared thermal inactivation behaviours by analysing the thermodynamic activation apparent parameters of the proteases under study. In marine habitats, there is a relatively high proportion of proteolytic bacteria as compared to freshwater or soil habitats (Atlas and Bartha 1981). The enzymatic studies reported in this paper allowed to assess some properties of extracellular proteases that potentially coexist in such sub-Antarctic sediments. This is important not only for a future technological application of these enzymes but also because of the ecological role of proteases in the bacterial acquisition of nitrogen-rich organic compounds in the marine environment.

Materials and methods

Sampling

Marine sediments were collected from five stations in Isla de los Estados (IE) Reserve (54° 38′–54° 54′ S and 63° 47′–65° 46′ W), Argentina, in March 2003. These islands have an oceanic climate, with an annual average air temperature between 8 and 4°C. The sample stations were Crossley Bay (IE1), San Juan de Salvamento Bay (IE3), Vancouver Bay (IE4), Isla Observatorio (IE7) and Franklin Bay (IE9). Surficial sediments were obtained at a depth of 0–3 cm in the intertidal area. The water temperature measured in situ ranged from 8.1 to 10.7°C (Esteves J. L., personal communication). Samples were immediately transported to the laboratory at 4°C for further processing.

Isolation, cultivation and characterization of extracellular protease-producing bacteria

Suspensions of the sediment samples in sterile natural seawater were used to spread natural seawater agar plates containing 10 g/l skim milk and 3 g/l yeast extract. The plates were incubated at 4°C and observed daily for signs of clearing of the agar around the colonies during 15 days. The strains were purified by repeated streaking in the same medium, and named with the symbol key code of the sample station followed by the number of isolate. The bacteria were maintained at 4°C on solid medium, with plating every 2 months. They were also stored at –80°C using glycerol (20% v/v) as cryoprotectant. Protease-producing isolates were subcultured in liquid medium with the same composition. For each isolate, three 125 ml

Erlenmeyer flasks containing 40 ml of medium were inoculated with a 24-h-old culture at 1% (v/v) ratio and incubated at 12°C and 200 rpm in an orbital shaker for 72 h. The cultures were centrifuged (4°C and 12,000*g* for 20 min) and the supernatants used as proteolytic activity sources. To determine the growth temperature range of the microorganisms, plates with the isolation medium were incubated at 4, 8, 12, 15, 20, 25, 30 and 33°C. The formation of protein hydrolysis halos around the colonies was checked for each plate.

16S rDNA amplification and phylogenetic analyses

DNA from isolates was extracted using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). In a first step, almost 500 bp of the 16S rRNA gene of all the isolates were sequenced using the primers 27f and 518r. For phylogenetic analyses, the 16S rRNA sequence of some strains was amplified by PCR as described in Olivera et al. (2005). Sequencing was performed on both strands of PCR-amplified fragments using the dideoxy chain-termination method by the commercial services of Macrogen Inc. (Seoul, Korea). 16S rRNA gene sequence similarity searches against the NCBI database were carried out using BLAST (Altschul et al. 1990). Sequences showing a relevant degree of similarity were imported into CLUSTAL W program (Thompson et al. 1994), aligned and corrected manually. The percentages of similarity were calculated with BioEdit program version 7.0.5.3 (Hall 1999). Phylogenetic analyses were done according to two different methods: maximum-likelihood (ML) and maximum-parsimony (MP) with PAUP program version 4.0b10 (Swofford 2001). Sites involving gaps were treated as missing characters. Heuristic Search algorithm (1,000 replicates) was used for Parsimony analysis. The results were evaluated with 1,000-replication bootstrap analysis, and the length, the consistency (CI) and retention (RI) indices of the trees were calculated. The general-time reversible model with equal base frequencies was used to create ML trees. Strains numbers and their corresponding accession numbers are indicated on the phylogenetic trees (Fig. 1).

Determination of protease activity

Protease activity was measured using Hammarsten casein as a substrate. The reaction mixture contained 1.1 ml of 1% (w/v) casein in 0.1 M Tris-HCl buffer (pH 8.0) and 0.1 ml of enzyme extract. The reaction was carried out at 25°C and stopped by the addition of 5% (w/v) trichloroacetic acid (1.8 ml); then each test tube was centrifuged at 4,000*g* for 20 min and the absorbance

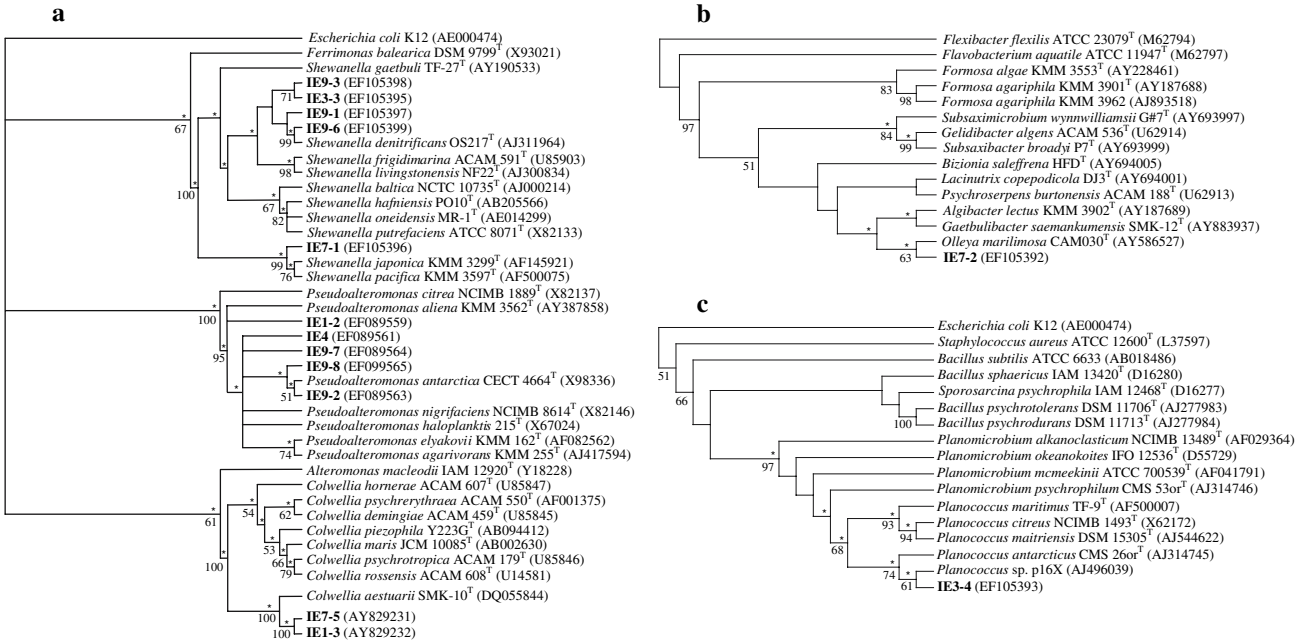

Fig. 1 Most-parsimonious phylogenetic trees, derived from 16S rRNA gene sequence data, showing the affiliation of the protease-producing isolates and (**a**) genera of the class *Gammaproteobacteria*, (**b**) the CFB group, and (**c**) gram-positive bacteria. The 16S rRNA gene sequence of *E. coli* K12 was chosen arbitrarily as the outgroup sequence for trees **a** and **c**, and *F. flexilis* for **b**. In **a** the strict phylogenetic consensus tree is shown. Numbers at internal nodes are bootstrap support values (%). Clades that were retrieved also by ML analysis are indicated by *asterisk*

of the supernatant was read at 280 nm. The results were calculated as mean values of three independent replicas (see Isolation, cultivation and characterization of extracellular protease-producing bacteria).

Thermal inactivation assay and estimation of kinetic and thermodynamic parameters

Thermal stability was investigated by incubating the enzyme extract without substrate at temperatures between 20 and 70°C from 0 to 180 min. The reaction was stopped in ice water and the residual caseinolytic activity was measured under standard assay conditions. Residual activity is a ratio of the enzyme activity after heating and the initial activity of the enzyme extract. The kinetic of thermal inactivation was predicted with a first-order irreversible deactivation model (Bailey and Ollis 1986). The thermal inactivation rate constant, k_{in}, was determined by linear regression from the slopes of the ln of residual activity versus time. Activation energy for thermal inactivation (Ea_{in}) was calculated from the slope of the Arrhenius plot of ln (k_{in}) versus $1/T$ by linear regression. The thermodynamic activation apparent parameters were computed according to the transition state theory (Lonhienne et al. 2000), using the following equation: $\Delta G^*_{in} = RT\left(\ln\frac{k_B T}{h} - \ln(k_{in})\right)$, where k_B is Boltzmann's constant; R the gas constant; T the absolute temperature; h Planck's constant and ΔG^*_{in} is the activation Gibbs free energy of the thermal inactivation process. k_{in} and ΔG^*_{in}, temperature-dependent parameters, are reported at a standard temperature of 30°C to allow comparison between isolates. Moreover, Tm values were calculated as the temperature at which the enzymes lose half of their original activity in 30 min (Kristjánsson et al. 1999).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences generated in this study were deposited in GenBank database under the accession numbers AY829231-2, EF089558-65 and EF105391-99.

Results

Isolation, characterization and phylogenetic analyses of extracellular protease-producing bacteria

After incubation at 4°C, 19 morphologically different protease-producing bacteria were selected for further analyses. Partial 16S rDNA sequencing (almost 500 bp) allowed to assess their phylogenetic affiliation. Except for a *Planococcus* isolate (IE3-4), the microorganisms belonged to the classes *Gammaproteobacteria* and *Flavobacteria*. Eight strains corresponded to the genus *Pseudoalteromonas* (isolates IE1-1, IE1-2, IE3-1, IE4, IE7-4, IE9-2, IE9-7 and IE9-8), six to *Shewanella* (IE3-2, IE3-3, IE7-1, IE9-1, IE9-3 and IE9-6), three to *Colwellia* (IE1-3, IE7-3 and IE7-5), and one strain (IE7-2) to the family *Flavobacteriaceae*. *Colwellia* isolates showed the lowest optimal growth temperature at about 15°C, while no growth was observed at 25°C. The remaining isolates showed optimal growth between 20 and 25°C, and none of them was able to grow at 33°C (Table 1).

A comparative analysis of more than 1,400 bases of the 16S rRNA gene of 14 isolates chosen from the different genera was performed. The sequence top matches and their corresponding percentage of similarity are presented in Table 1. The closest neighbours of most of the strains still lack of taxonomic standing or belong to uncultured bacterium clones, and in many cases are from Arctic sources. The closest match of the sequences of *Colwellia* isolates (98% similarity) was to that from a protease-producing bacterium from Arctic seawater, isolate Bsw20306. The highest similarity value (97.9%) with a strain characterized to the species level was to *Colwellia aestuarii* SMK-10^T (Jung et al. 2006). Apart from strains IE7-1 and IE9-6 whose sequences were 98% similar to those of *Shewanella pacifica* KMM 3597^{T}and *Shewanella denitrificans* OS217^T, respectively, the *Shewanella* isolates shared less than 98% similarity with sequences of the type strains of the species described in this genus. On the other hand, similarity values above 98% were obtained when comparing the 16S rDNA sequences of the *Pseudoalteromonas* isolates with the type strains of the species *Pseudoalteromonas elyakovii* KMM 162^T, *Pseudoalteromonas nigrifaciens* NCIMB 8614^T, *Pseudoalteromonas antarctica* CECT 4664^T, and *Pseudoalteromona agarivorans* KMM 255^T.

The results of the phylogenetic analyses, based on the 16S rRNA gene, for each of the three datasets (*Gammaproteobacteria*, CFB group and gram-positive bacteria) are summarized in Table 2 and the trees shown in Fig. 1. The *Gammaproteobacteria* analysis resulted in 36 equally parsimonious trees (Table 2); the strict consensus tree with bootstrap values is presented in Fig. 1a. The overall topology of the tree based on MP analysis was congruent to that based on ML study, yielding three well-supported monophyletic groups (Fig. 1a). Within the *Shewanella* monophyletic group, strain IE9-6 formed a clade with *S. denitrificans*

Table 1 Comparison of phenotypic and molecular features of protease-producing bacteria from sub-Antarctic sediments

Phylogenetic affiliation (phylum, class, family)	Isolate	Optimal growth temperature (°C)	Maximal temperature for growth (°C)	16S rDNA sequence length (bp)	Top matches, source, GenBank accession number	Nucleotide identity (%)
Proteobacteria, Gammaproteobacteria, Pseudoalteromonadaceae	IE1-2	25	30	1,473	Bacterium Bsw20353D, Arctic seawater, (DQ064613)	99
					Pseudoalteromonas sp. BSi20652, Arctic Sea ice, (DQ537522)	99
	IE4	25	30	1,459	*Pseudoalteromonas haloplanktis* TAC125, marine Antarctica, (CR954246)	100
					Pseudoalteromonas sp. 643A, (DQ104401)	99
	IE9-2	25	30	1,426	Bacterium Bsw20353D, Arctic seawater, (DQ064613)	99
					Pseudoalteromonas sp. BSi20538, Arctic sea ice, (DQ517873)	99
	IE9-7	25	30	1,425	Bacterium Bsw20353D, Arctic seawater, (DQ064613)	99
					Pseudoalteromonas sp. NJ62, Antarctic seawater, (DQ145735)	99
	IE9-8	25	30	1,426	*Pseudoalteromonas* sp. BSi20669, Arctic sea ice, (DQ537523)	99
					Pseudoalteromonas citrea KMM 216, (AF082563)	99
Proteobacteria, Gammaproteobacteria, Shewanellaceae	IE3-3	20	30	1,425	*Shewanella* sp. JL-56, North Pacific Ocean, (AY745827)	97
					Shewanella sp. BSi20150, Arctic Sea Ice, (DQ492723)	97
	IE7-1	20	<30	1,429	*Vibrio* sp. OS53, marine, (AB038028)	98
					Shewanella baltica KT0246, North Sea, (AF173966)	98
	IE9-1	20	<30	1,429	*Shewanella livingstonensis* clone SE21, Arctic, (AY771751)	98
					Alteromonadaceae bacterium E1, marine, (AF539787)	97
	IE9-3	20	<30	1,425	*Alteromonadaceae* bacterium E1, marine, (AF539787)	99
					Alteromonadaceae bacterium RE1-1, marine, (AF539788)	98
	IE9-6	20	<30	1,418	*Shewanella denitrificans* OS217^T, (CP000302)	98
					Shewanella denitrificans clone SE8, Arctic, (AY771743)	97
Proteobacteria, Gammaproteobacteria, Colwelliaceae	IE1-3	15	<25	1,435	Bacterium Bsw20306, Arctic seawater, (DQ064606)	98
					Uncultured bacterium clone FS140-74B-02, ridge flank crustal fluid, (DQ513017)	98
	IE7-5	15	<25	1,521	Bacterium Bsw20306, Arctic seawater, (DQ064606)	98
					Colwellia piezophila clone SE15, Arctic, (AY771749)	98
Bacteroidetes, Flavobacteria, Flavobacteriaceae	IE7-2	20	<30	1,459	*Formosa algae* clone SE60, Arctic, (AY771766)	99
					Formosa sp. AKS193, sea leed Antarctica, (DQ167237)	99
Firmicute, Bacilli, Planococcaceae	IE3-4	25	30	1,435	*Planococcus* sp. p16X, Ross Island (Antarctica), frozen soil, (AJ496039)	98
					Planococcus sp. TSBY-25, Tianshan Mountains (China), frozen soil, (DQ172994)	98

Table 2 Summary of the datasets that were used in MP phylogenetic analysis

Dataset	No. of taxa	No. of characters (bp)	No. of parsimony informative characters	No. of trees	Tree length	CI	RI
16S rRNA, *Gammaproteobacteria*	40	1,437	272	36	1,061	0.5203	0.8205
16S rRNA, CFB group	15	1,438	218	1	951	0.6540	0.4694
16S rRNA, gram-positive bacteria	17	1,436	180	1	800	0.6800	0.5477

Number of taxa refers to the total number of taxa in the dataset, including one outgroup

CI consistency index, *RI* retention index

OS217^T, with 99% recovery in bootstrap analysis. Isolates IE9-1, IE3-3 and IE9-3 were more closely related to the latter than to any of the other *Shewanella* species. However, the topology of the tree at the level of these three isolates lacked of bootstrap support. Isolate IE7-1, *Shewanella japonica* KMM 3299^T and *S. pacifica* KMM 3597^T formed a monophyletic lineage grouped with 99% bootstrap support. In the *Pseudoalteromonas* monophyletic group, only isolates IE9-2 and IE9-8 were found to be related to a validly published species, *P. antarctica* CECT 4664^T. Nevertheless, this grouping was not confirmed by strong bootstrap support. The cladogram also showed that IE1-3 and IE7-5 clade is the sister group of *C. aestuarii* SMK-10^T, with 100% recovery in bootstrap analysis (Fig. 1a).

Isolate IE7-2 belonged to the family *Flavobacteriaceae*; however, a sequence similarity value lower than 97% was found when comparing its 16S rDNA sequence with those of the different species of this family. In the most parsimonious tree presented in Fig. 1b, IE7-2 formed a clade with *Olleya marilimosa* (63% bootstrap value). Lastly, 16 rDNA sequence of strain IE3-4, the only gram-positive isolate, revealed 98% similarity with that of *Planococcus antarcticus* CMS 26or^T. The cladogram shown in Fig. 1c corresponds to the most parsimonious tree obtained. *Planococcus* species formed a monophyletic group, where strains IE3-4 and p16X formed the sister clade of *P. antarcticus* CMS 26or^T. The taxonomic integrity of this cluster was supported by a 74% bootstrap value. The topologies of the trees inferred by MP and ML analyses, for *Flavobacteriaceae* as well as for gram-positive bacteria, were congruent at the level of isolates under study.

Protease thermal inactivation kinetics

Isolates were cultured at 12°C and 200 rpm for 72 h. Then, the supernatants were recovered and the thermal inactivation kinetic of the enzyme extracts produced by the different microorganisms was determined. Table 3 shows the values obtained for k_{in}, Ea_{in}, ΔG^*_{in} and Tm, at a reference temperature of 30°C, for each of the enzyme extracts. The activation Gibbs free energy (ΔG^*_{in}) varied between approximately 97 and 124 kJ/mol for proteases of isolates from different genera. Proteases produced by *Gammaproteobacteria* isolates showed an increasing trend in thermal stability from *Colwellia* < *Pseudoalteromonas* < *Shewanella*. Such pattern was also observed when comparing Tm values (Table 3). ΔG^*_{in} of thermal inactivation of *Planococcus* sp. IE3-4 protease activity was comparable to those of *Shewanella* strains. However, as IE3-4 was the only isolate from genus *Planococcus*, no comparison with related strains could be done.

The highest thermosensitivity was registered for *Colwellia* sp. IE1-3 enzyme extract (Fig. 2a, c). As

Table 3 Comparison of k_{in}, Ea_{in}, ΔG^*_{in} and Tm of protease extracts at a reference temperature of 30°C

Source of proteases	k_{in} (min^{-1})	Ea_{in} (kJ/mol)	ΔG^*_{in} (kJ/mol)	Tm (°C)
Colwellia sp. IE1-3	8.61 E-03	181.4	96.6	34
Colwellia sp. IE7-5	4.70 E-03	176.9	98.2	37
Colwellia sp. IE7-3	3.43 E-03	211.3	99.0	37
Pseudoalteromonas sp. IE4	1.84 E-03	140.3	100.6	44
Pseudoalteromonas sp. IE9-7	1.76 E-03	186.9	100.7	41
Pseudoalteromonas sp. IE9-2	1.68 E-03	197.9	100.8	41
Pseudoalteromonas sp. IE9-8	1.38 E-03	187.3	101.3	42
Pseudoalteromonas sp. IE1-1	1.33 E-03	185.6	101.4	42
Pseudoalteromonas sp. IE1-2	3.44 E-04	193.6	104.8	48
Shewanella sp. IE9-1	4.77 E-05	255.6	109.8	50
Shewanella sp. IE3-2	1.53 E-05	262.7	112.6	53
Shewanella sp. IE9-6	3.80 E-07	288.6	121.9	62
Planococcus sp. IE3-4	1.96 E-05	226.7	112.0	56

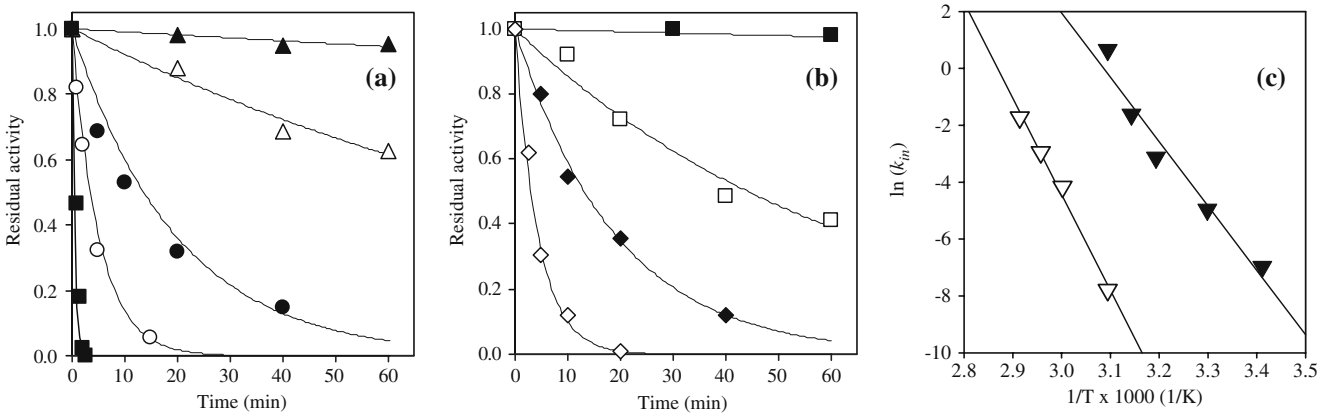

Fig. 2 Thermostability of extracellular proteases produced by **a** *Colwellia* sp. IE1-3 and **b** *Shewanella* sp. IE9-6, as a function of time and temperature. Temperatures of the inactivation assay (°C): *filled triangle* 20; *open triangle* 30; *filled circle* 40; *open circle* 45; *filled square* 50; *open square* 60; *filled diamond* 65; *open diamond* 70. **c** The Arrhenius plot for the first-order thermal inactivation rate constant (k_{in} expressed in min^{-1}) of *Colwellia* sp. IE1-3 (*filled inverted triangle*) and *Shewanella* sp. IE9-6 (*open inverted triangle*) proteases. *Symbols* stand for experimental data and *full lines* for fitted values calculated from Table 3 parameters

regards the percentages of residual activity, after incubation for 2 h at 20°C, *Colwellia* proteases retained 90% of the activity, and at 30°C 45% (data not shown). No residual activity was observed after incubation for 1 h at 40°C or 2 min at 50°C. In the other extreme, the most thermostable protease activity was shown by *Shewanella* sp. IE9-6 (Fig. 2b, c). Its residual activity was 100 and 93% after incubation for 2 h at 40°C and 50°C, respectively (data not shown). Even after a 10 min-incubation period at 70°C, its residual activity reached 12%.

Some isolates (*Pseudoalteromonas* spp. IE 3-1 and IE7-4, *Shewanella* spp. IE3-3, IE 7-1 and IE9-3 and isolate IE 7-2) produced low levels of protease activity under the culture conditions mentioned previously, whereas the thermal stability of their extracellular proteases was not analysed.

Discussion

A lot of effort has been concentrated on the characterization of cold-active proteases, particularly with the aim to understand their structural and functional adaptations to cold or to assess their technological potential (Morita et al. 1998; Kulakova et al. 1999; Irwin et al. 2001; Chen et al. 2003; Wang et al. 2005). However, few studies have dealt with the comparison of these enzymes in relation with the characteristics of the different strains, isolated from a particular habitat, which produce them. In this study, the protease-producing bacteria isolated from sub-Antarctic marine sediments fell in three phylogenetic groups: *Gammaproteobacteria*, CFB group, and low G + C gram-positive bacteria. In general, the strains were closely related to Antarctic as well as Arctic isolates of marine origin which frequently lacked of taxonomic standing or were uncultured microorganisms. *Planococcus* sp. IE3-4 was the only isolate related to strains of terrestrial sources (Table 1). When their 16S rDNA sequences were compared with those of validly published species, *Shewanella* sp. IE9-1 and the only isolate of the CFB group (IE7-2) could represent novel species or genus as the levels of similarity were lower than 97%. Levels of similarity between 16S rRNA gene sequences below 97% suggest that the strains do not correspond to the same species (Stackebrandt and Goebel 1994).

The three genera of *Gammaproteobacteria* found in this study (*Colwellia*, *Pseudoalteromonas* and *Shewanella*) were reported to be present in different habitats of the marine environment, such as sediments, seawater and sea ice (Bowman et al. 1997a; Mancuso Nichols et al. 2005). Other than psychrophilic *Colwellia* strains, the isolates were psychrotolerant. Even though the members of the genus *Colwellia* are considered as strictly psychrophilic, requiring temperatures of ≤20°C to grow on solid media (Methé et al. 2005), the latest species described *C. aestuarii* can grow up to 32°C with an optimum temperature of 25–30°C (Jung et al. 2006). Such strain was isolated from tidal flat sediments in Korea. 16S rRNA phylogenetic studies, involving all validly published *Colwellia* species, showed that *C. aestuarii* and IE *Colwellia* isolates form a distinct lineage within the genus (Fig. 1a). IE isolates neither meet Morita's definition of psychrophile accurately, because their optimal temperature for grow was around 15°C though they also grew well at 20°C. Helmke and Weyland (2004) proposed to introduce the

term ''moderate psychrophiles'' to call microorganisms with a minimum growth temperature of ≤0°C and a maximum growth temperature of ≤25°C. This is the most suitable definition for the sub-Antarctic *Colwellia* isolates characterized in this study.

Pseudoalteromonas and *Shewanella* strains accounted for 14 of the 19 isolates, likely the high hydrolytic activities presented by strains of these genera could explain their prevalence among the isolates. The synthesis of highly active proteolytic enzymes is a widespread characteristic in members of the genus *Pseudoalteromonas* (Mikhailov et al. 2002; Ivanova et al. 2004). Antarctic *Shewanella* strains, isolated from different sources, also showed strongly proteolytic and lipolytic activity (Bowman et al. 1997b). Arctic sea ice studies indicated that these genera made up a significant fraction of culturable protease-producing bacteria in algae-rich sea ice and seawater samples, which was associated with the ability of such bacteria to compete under heterotrophic conditions (Groudieva et al. 2004).

Even when the protease-producing strains were isolated from the same environment at the same time, the thermal stability of their extracellular proteolytic activity differed. ΔG^*_{in} is the energy barrier that must be reached by an enzyme to become thermally irreversible inactivated. Comparison of these values reflects k_{in} behaviour and in consequence, the rate of thermal inactivation at a given temperature. For the strains analysed, the protease thermostability correlated to a certain extent with the genera, following a decreasing trend in Tm, ΔG^*_{in}, and consequently in thermostability, from *Shewanella* and *Pseudoalteromonas* to *Colwellia*. Flexibility is the main adaptive character of cold adapted-enzymes (Lonhienne et al. 2000; Kristjánsson et al. 1999), exhibiting high catalytic activities at low temperatures. However, the adjustment of optimal structural flexibility for activity at low temperatures requires weakening or alteration of some intramolecular interactions in proteins, which may result in lowering their stability (Kristjánsson et al. 1999). Therefore, thermostability (ΔG^*_{in}) of the tested enzymes could be used as a screening tool for cold-active protease-producing microorganisms.

The protease thermostabilities analysed in this study corresponded to crude enzymatic extracts that could contain more than a single protease. Even though a direct comparison with purified proteases cannot be done, it is interesting to consider the results in the context of the thermostability reported for extracellular proteases produced by other bacteria of the same genera as IE isolates.

Colwellia proteases, produced by the only moderate psychrophilic microorganisms isolated, presented the highest thermosensitivity (Table 3). An extracellular protease from the Antarctic bacterium *Colwellia* sp. NJ341 retained about 50% of its maximum activity for 50 min at 40°C (Wang et al. 2005). The residual protease activities of IE *Colwellia* isolates, at the same temperature and time, were about 15%.

Most of the protease extracts produced by IE *Pseudoalteromonas* isolates became inactivated after 2 to 10 min of incubation at 60°C, while they retained 14 to 68% of residual activity after 10 min at 50°C. Protease CP1 from *Pseudoalteromonas* sp. CP76 retained almost 100 and 60% of the initial activity after 10 and 60 min of incubation at 50°C, respectively (Sánchez-Porro et al. 2003). Protease MCP-02 produced by *Pseudoalteromonas* sp. SM9913, a deep-sea psychrotrophic bacterium, was stable below 50°C, but its activity was reduced by 30.5% after incubation at 60°C for 10 min. Another protease produced by the same strain (MCP-01) lost about 90% of its activity in 20 min at 40°C (Chen et al. 2003).

Shewanella proteases were the most stable to temperature (Table 3). The cold-active serine protease produced by *Shewanella* sp. Ac10, strain which presented the same optimum and maximal growth temperatures as IE *Shewanella* isolates, was almost inactivated by incubation at 60°C for 15 min (Kulakova et al. 1999). A similar level of inactivation was found for IE9-1 and IE3-2 proteases, while the residual protease activity of IE9-6 after incubation at 60°C for 1 h was about 40%. The serine peptidase of the sub-Arctic marine psychrophile *Shewanella* sp. PA-43 lost most of its activity after 30 min at 60°C (Irwin et al. 2001).

In the present study, a set of protease-producing isolates from sub-Antarctic marine sediments was characterized. We demonstrated that these sediments harbour novel microbial strains and through phylogenetic analyses, based on 16S rRNA gene sequences, improved the understanding of their relationship with previously described psychrophilic and psychrotolerant bacteria. Our results also showed that the thermostability of the proteases produced by the isolates of different genera varied considerably, reflecting the different thermal properties of the proteases that could coexist in this environment. The intertidal area of sandy beaches is exposed to cycles of changing environmental conditions, which are probably more complex and harsher than those faced by other marine habitats (Brown and McLachlan 1990). A possible hypothesis is that, the heterogeneity in protease thermostability observed could be related with microbial adaptations to the physical variations occurring in intertidal sediments. Besides the ecological importance

of proteases in their native environment, cold-active enzymes also have interesting biotechnological properties. In this study, the kinetic analysis of thermal inactivation of different protease extracts and the subsequent estimation of ΔG^{*}_{in} resulted in a useful approach to be considered in a comparative screening for cold-active enzymes.

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