RESEARCH ARTICLE

Organic-matter degradative potential of *Halomonas glaciei* isolated from frazil ice in the Ross Sea (Antarctica)

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Halomonas glaciei; frazil ice; Ross Sea; bacterial growth; ectoenzyme; enzyme kinetics.

Abstract

Halomonas glaciei isolated from frazil ice in the Ross Sea (Antarctica) during austral summer 2003 was phenotypically characterized and its capability of degrading organic matter was tested. We evaluated specific bacterial growth rates (μ) to understand at which temperatures bacterial growth shows a linear and direct relationship with the available substrate (4–22 °C) and afterwards we tested *H. glaciei* growth curves and degradative potential at 0, 10 and 37 °C using two different media (one enriched and one depleted in PO_4). The strain grew exponentially only at 10 °C. The fastest hydrolysis rates were expressed by enzymes aimed at polysaccharide degradation (α-D-glucosidase, β-D-glucosidase and β-D-galactosidase) while alkaline phosphatase and aminopeptidase activities were rather low. Our data suggest a preferential demand for carbon derived from carbohydrates rather than from proteins: ectoenzyme activities transformed into carbon mobilization from organic polymers, showed that the total carbon potentially released from polysaccharides can be almost one order of magnitude higher than the protein carbon mobilization. Principal component analysis of the enzyme affinity separated the six experimental conditions, highlighting how different physical (temperature) and chemical (PO_4 enrichment or depletion) features actively lead to a differentiation in the efficiency of the ectoenzymes produced, resulting in preferential degradation of diverse kinds of organic substrates.

Introduction

Sea ice is one of the most important components of both the polar regions covering millions of square kilometres throughout almost the whole year (Horner *et al.*, 1992). It is one of the coldest environments present in the earth with temperatures < -30 °C near the upper surface during winter (Vincent, 1988; Thomas *et al.*, 1995). It also critically influences the productivity of the Southern Ocean, global energy budgets and atmosphere–ocean interactions in the Antarctic zone (Legendre *et al.*, 1992; Zwally *et al.*, 2002). Nevertheless, living organisms can be found in sea ice and distinct communities concentrate in the most porous part of it either near the top, bottom or the sea surface level (Arrigo *et al.*, 1998). Most sea ice is formed annually and enclosed bacterial populations undergo a process of selection, linked to their ability to uptake substrates at increasingly lower temperatures (Helmke & Weyland, 1995). Newly formed ice contains largely psychrotolerant bacteria, while winter ice contains mainly obligate psychrophiles (Grossmann, 1994; Grossmann & Dieckmann, 1994; Helmke & Weyland, 1995).

Because of the porosity of sea ice, living organisms can live as it forms and melts and can accumulate nitrogen, phosphorus and organic carbon (Cota *et al.*, 1987). The few measurements of dissolved and particulate organic carbon in sea ice suggest that their content varies widely, depending on the type of ice, layering, manner of formation, current state of freezing and thawing and the development of living communities (Thomas *et al.*, 1995).

In the microniche constituted by brine channels within the newly formed Antarctic ice, heterotrophic bacteria, which are the most abundant community, play the essential

role of mineralizing and uptake organic carbon actively participating in modifications of the nutrient and/or biopolymers' budget (Del Negro & Bergamasco, 2008).

Although bacteria can grow over part of the range of temperature of sea ice, they do not seem to be able to efficiently utilize substrates, despite the high concentrations of dissolved organic matter sometimes present in the ice (Pomeroy & Wiebe, 2001). Deming & Huston (2000) suggest that bacteria in sea ice can produce copious amount of ectoenzymes to compensate for the suboptimal temperature. As most of the chemical reactions are catalysed by enzymes, it turns out that the enzymes produced by psychrophilic microorganisms are also adapted to cold. Their molecular structure is such that they efficiently catalyse chemical reactions at a low temperature in what Gerday *et al.* (1997) call 'a thermodynamic challenge'. Cold-adapted enzymes are also characterized by a thermal instability, which is regarded as a consequence of their conformational flexibility (Feller *et al.*, 1997). Many studies have already dealt with molecular features of enzymes produced by psychrophilic bacteria (i.e. Hoyoux *et al.*, 2001; Coker *et al.*, 2003), highlighting their possible implications in biotechnology and industry.

The main aims of this study were to characterize a bacterial strain isolated from frazil ice collected in the Ross Sea (Antarctica) and to better understand its relationship with substrates at different temperatures and cultured in different media.

Materials and methods

Bacterial isolation

In February 2003, frazil ice samples were collected in the Terra Nova Bay polynya (Ross Sea) in the framework of the XVII Italian Expedition in Antarctica. Surface water and ice were collected by means of polycarbonate beakers (5 L) prewashed with HCl (1 N). Frazil ice samples were physically separated by water, melted at 2–4 °C, spread onto ZoBell 2216 agar plates (100 μL of thawed sea ice per plate; 1.5% w/v agar, Difco) and incubated at 4 °C. ZoBell 2216 medium is highly rich in organic compounds (5 g peptone, 1 g yeast extract in 1 L of 0.2 μm filtered seawater, autoclaved at 121 °C for 15 min) and allows the culturing of several marine bacterial strains, without specific selection. Colonies showing a definite morphology and colour were serially plated on the same medium at 4 °C. After serially plating the colonies of interest, one single colony was transferred to liquid ZoBell Marine Broth (ZoBell, 1934) in order to maintain the strain before its characterization.

Identification

DNA extraction was carried out by means of the DNeasy Tissue Kit (Qiagen) according to the supplier's instructions.

A *c.* 1460-bp segment of the 16S rRNA gene was amplified by modification of the touchdown protocol (Don *et al.*, 1991) consisting mainly of decreasing the annealing temperature by 0.5 °C per cycle instead of 1 °C every second cycle. PCR was performed using the universal primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the Eubacterial-specific primer 1492R (5′-GGYTACCTTGTTACGACTT-3′) (Lane, 1991). An initial 94 °C denaturing step for 5 min was followed by 30 cycles of amplification (3-min denaturation at 94 °C; 1-min annealing starting at 65 °C for the first cycle reduced 0.5 °C per cycle to 50 °C; and 3-min extension at 72 °C) and a final extension of 10 min at 72 °C. The PCR products were visualized on 0.8% (w/v) agarose gel stained with ethidium bromide using an ultraviolet transilluminator to confirm that the *c.* 1460-bp product was present.

Samples were sequenced using ABI Prism Big Dye (Applied Biosystems) using M13R primer and an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequence was aligned to known sequences in the GenBank database using BLAST (Altschul *et al.*, 1997). Multiple sequence alignment was performed using the CLUSTALX 1.81 program (Thompson *et al.*, 1997); the phylogenetic relationships were inferred by the neighbour-joining method and a phylogenetic tree was constructed with the program NJPLOT and finally edited for better graphics with CLC FREE WORKBENCH PACKAGE version 4.0.1.

Phenotypic characterization

In order to obtain a phenotypic characterization of the isolated strain, we used the following physiological tests: tolerance to several NaCl concentrations in tryptone water (0–8% NaCl), anaerobic growth, growth at pH 5–9 and motility test (Solomon, 1998). Then, the following biochemical features were tested: Gram staining, oxidase production, reduction of nitrate and arginine decarboxylation (Solomon, 1998). API 20 E, API 20 NE (BioMerieux) were used for detection of metabolic properties, incubating the strips at 20 °C for 48 h.

Characterization of the degradative potential

As suggested by Pomeroy & Wiebe (2001), in order to understand at which temperatures bacterial growth shows a linear and direct relationship with the available substrate, we evaluated the specific bacterial growth rates (μ = Bacterial Production/Bacterial Biomass) at −2, 0, 4, 10, 22 and 37 °C. In this case, the isolates were grown in Artificial Sea Water (ASW – 24.7 g NaCl, 0.7 g KCl, 6.3 g $MgSO_4$, 4.6 g $MgCl_2$, 1.3 g $CaCl_2$ and 0.2 g $NaHCO_3$ in 1 L of MilliQ) enriched in glucose and yeast extract (0.15 mg L^{-1}). The bacterial production was estimated by incorporation of ^{3}H[Leucine] (Kirchman *et al.*, 1985). Triplicate 1.7-mL aliquots and two controls killed by the addition of 90-μL 100% trichloracetic

acid (TCA) were amended with 20 nM radiotracer and incubated in the dark at the experimental temperature. Incubations were stopped with 90-μL 100% TCA after 1 h. The extraction with 5% (v/v) TCA and 80% (v/v) ethanol was carried out using the microcentrifugation method (Smith & Azam, 1992). Activity in the samples was determined by a β-counter (Packard Tri-Carb 300) after the addition of 1-mL scintillation cocktail (Ultima Gold MV; Packard). Incorporation of ^{3}H[Leucine] was converted into carbon produced via bacterial protein production according to Simon & Azam (1989), assuming a twofold isotope dilution for ^{3}H[Leucine]. Bacterial counts were performed by epifluorescence microscopy after the cell staining with 4,6-diamino-2-phenyl indole (DAPI) (Porter & Feig, 1980). Samples were filtered through black polycarbonate filters (0.2 μm porosity) in 2-mL triplicate aliquots and microscopically analysed (Olympus BX 60 F5). Bacterial density was converted into carbon equivalents using a conversion factor of 20 fg C per cell (Lee & Fuhrman, 1987).

In order to verify the degradative capability, we tested the potential of isolated *Halomonas glaciei* to produce ectoenzymes. The working cultures were prepared in two different enriched liquid media: ASW + 4 mg L^{-1} glucose (− P) and ASW + 4 mg L^{-1} Glucose + 5 mg L^{-1} $NaH_2PO_4 \times H_2O$ (+P). Glucose was added to provide an energy source for the bacterial growth; nevertheless, not to constrain bacterial growth because of phosphorus limitation, in the second medium a source of phosphorus was also supplied. Incubation temperatures were 0, 10 and 37 °C and the analyses were carried out at 12, 36, 60 and 108 h after the T_0. Ectoenzymatic activities were assayed using artificial fluorogenic substrates (Hoppe, 1983; Hoppe *et al.*, 1988) derived from 7-amino-methyl-coumarin (AMC) and 4-methyl-umbelliferone (MUF). Leucine–aminopeptidase activity (AMA) was assayed as the hydrolysis rate of leucine–AMC. The activities of α-D-glucosidase (AGLU), β-D-glucosidase (BGLU), β-D-galactosidase (BGAL) and alkaline phosphatase (APA) were assayed using MUF–α-D-glucoside, MUF–β-D-glucoside, MUF–β-D-galactoside and MUF-phosphate, respectively. Enzyme activities measured by means of fluorogenic substrates were expressed in terms of the rate of MUF or AMC production. The substrates were added to 2.5-mL samples at 200 μM final concentration (except MUF-phosphate at 50 μM) and incubated for 1 h in the dark at the experimental temperature. All samples were run in triplicate. The fluorescence of MUF and AMC hydrolysed from the model substrates was measured using a Perkin Elmer LS50B spectral fluorometer (365 nm excitation and 455 nm emission used for MUF; 380 nm excitation and 440 nm emission used for AMC). Standard solutions of MUF and AMC were used to calibrate the fluorometer. AMA was converted to protein mobilized using the conversion factor 72 μg C μmol^{-1} (mass of the six carbon atoms in leucine); for carbohydrate carbon mobilization, the conversion factor 72 μg C μmol^{-1} (mass of the six carbon atoms in glucose and galactose) was considered for each of the three glucidic enzymes (AGLU, BGLU and BGAL) and then the values were summed.

In order to evaluate the enzyme–substrate affinity, the Michaelis–Menten constant (K_m) was estimated at every sampling in both media (enzyme affinity corresponds to $1/K_m$). To each sample (2.5 mL), fluorogenic substrates were added at final concentrations of 50, 100, 200, 400 and 500 μM, except for MUF-phosphate, where final concentrations of 10, 20, 50, 100 and 200 μM were used. Hydrolysis rates were measured as described previously. At every sampling, bacterial abundance was also estimated (Porter & Feig, 1980).

Data analysis

A principal component analysis (PCA) (Massart *et al.*, 1997; Vandeginste *et al.*, 1998), performed with the software Primer 5, was used to better understand the information of our multivariate set, considering the mean values of the affinity for the substrate of each enzyme under every experimental condition throughout the whole experiment. This multivariate analysis rotates a cloud of data points such that the maximum variability is visible in order to identify the most important gradients.

Results

Identification

The closest-neighbouring strain to the 16S rRNA gene sequence determined in this study was *H. glaciei* (sequence similarity 98% – GenBank accession number DQ984277), and the phylogenetic relationships with other members of the genus *Halomonas* are reported in Fig. 1.

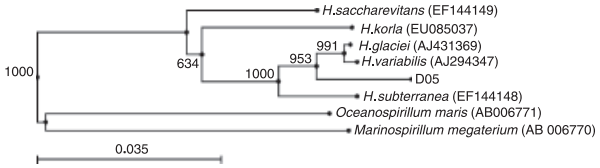

Fig. 1. Phylogenetic relationship between *Halomonas glaciei* D05 and other microorganisms of the same genus, based on the 16S rRNA gene sequence analysis using the neighbour-joining method. The tree is unrooted with two Oceanospirillales as the outgroup (all GenBank accession numbers are provided in parenthesis) and bootstrap values were attached to each branch as a measure of its confidence. The scale bar indicates 0.035 changes per nucleotide.

Phenotypic characterization

Agar colonies of the selected strain were circular, white, smooth and convex with a regular edge and were halotolerant in a range between 0% and 8% NaCl. The isolate grew from pH 5 to 9, exhibiting the optimum at pH 8. The isolate was Gram-negative, oxidase positive, able to reduce nitrate, nonmotile and aerobic but also resistant under anaerobic conditions. Acid was produced from glucose and arabinose, but lactose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, tryptophan and amygdalin were not utilized when provided as the sole carbon source. No growth occurred on Simmons citrate medium. Gelatine, urea and esculin were not hydrolysed. Arginine dihydrolase, ornithine and lysine decarboxilases were not produced. In addition, the isolate gave negative responses to the Voges–Proskauer test, indole and H_2S production.

Characterization of the degradative potential

The relationship between temperature and the specific bacterial growth rates, expressed by the strain, was evaluated in order to highlight the temperatures at which bacterial growth has a linear and direct relationship with the available substrate. Our results showed this relationship only at 22, 10 and 4 °C, while at temperatures approaching the lowest and the highest limits for growth, the relationship between substrate utilization and temperature became nonlinear, as shown in Fig. 2. For this reason, the experiment to test the degradative potential and enzyme kinetics was conducted at 10 °C (within the linear range), 0 and 37 °C (out of it).

In the −P medium, the strain expressed the highest abundances ($8.04 \pm 0.75 \times 10^9$ cells L^{-1}) at 10 °C after 108 h of incubation. A similar trend was observed for bacteria growing in the +P medium, reaching a maximum of $7.85 \pm 0.73 \times 10^9$ cells L^{-1}. In both media, cells at 10 °C increased in abundance of almost 2 orders of magnitude, as the same value at T_0 was $1.39 \pm 0.09 \times 10^8$ cells L^{-1}. At 0 and 37 °C, bacterial abundance remained substantially constant in the two media during the whole experiment (Fig. 3).

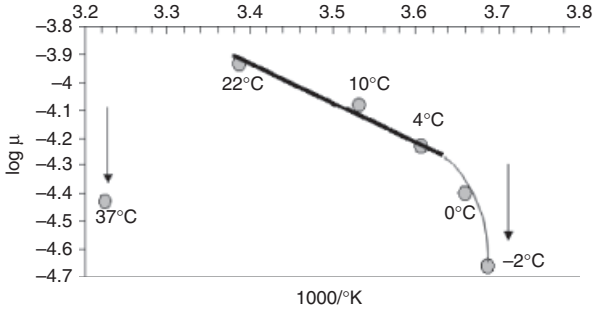

Fig. 2. Arrhenius plots of *Halomonas glaciei* grown in seawater enriched in glucose and yeast extract at 37, 22, 10, 4, 0 and −2 °C.

Ectoenzymatic activities showed very high values for glucidic substrates and relatively low rates for AMA and APA. In Table 1, the maxima, minima and mean values for each experimental condition are reported. AGLU expressed the absolute maximum value at 0 °C in the −P medium (287.9 nM h^{-1}). The enzyme production did not seem to be affected by temperature and/or by the presence of phosphorus in the media in the same way. Relative maxima for each enzyme, in fact, were found at different temperatures (BGLU, BGAL and AMA at 37 °C; APA at 10 °C; and AGLU at 0 °C) and even the phosphorus supply was not reflected in a common response in hydrolysis rates.

The amounts of carbohydrate carbon potentially released by BGAL, BGLU and AGLU were, as an average, almost 1 order of magnitude higher than the protein carbon mobilization. In the former case, an average of 4.7 μg C L^{-1} h^{-1} was transformed into monosaccharide carbon at every temperature in both media, with a sole peak of 25.2 μg C L^{-1} h^{-1} in the 0 °C −P experimental condition (Fig. 4). The carbon mobilized by AMA (Fig. 5) was as on average 20.0 ng C L^{-1} h^{-1} at 0 and 10 °C and slightly higher at 37 °C (average = 44.3 and maxima of 68.3 and 104.8 ng C L^{-1} h^{-1} in the −P and the +P medium, respectively).

Substrate affinity for APA was the highest recorded in every experimental condition ranging from 0.026 to 0.059 μM^{-1}, followed by AMA (range 0.011–0.021 μM^{-1}; Fig. 6). AGLU, BGLU and BGAL kinetics highlighted a general static or decreasing affinity with growing temperatures in both media in contrast to APA, whose values were maximum at 37 °C. Protease affinity for the substrate was higher at 10 °C both in the phosphorus-repleted and in the phosphorus-depleted medium.

The relationships among average affinity of BGAL, BGLU, AGLU, APA and AMA for the available substrate under the six experimental conditions were then analysed using a PCA. This multivariate analysis showed a helpful distribution of variables in new linear combinations (PC). Only PCs with eigenvalues > 1 were considered. Variables with the greatest absolute magnitude or loading in each PC have the greatest influence on the sample separations or projections for that PC (Meglen, 1992). PC1 and PC2 together explained 80% of the total variance (Table 2). PC1 was found to be positively related to APA and negatively to BGAL and BGLU. Conversely, PC2 was highly related to AGLU and AMA (positively and negatively, respectively). In the diagram of PC1 vs. PC2 (Fig. 7), the experimental conditions were widely distributed. At 37 °C, +P is characterized mainly by high APA affinity values and secondarily by AGLU. At the same temperature in phosphorus depletion, the major affinity for the substrate is attributable to BGLU and BGAL and weakly to AMA. The same condition was found at 10 °C −P. At 0 °C, the main affinity is expressed by APA (+P) and AMA (−P) while a high affinity for BGLU, BGAL and AMA was found at 10° +P.

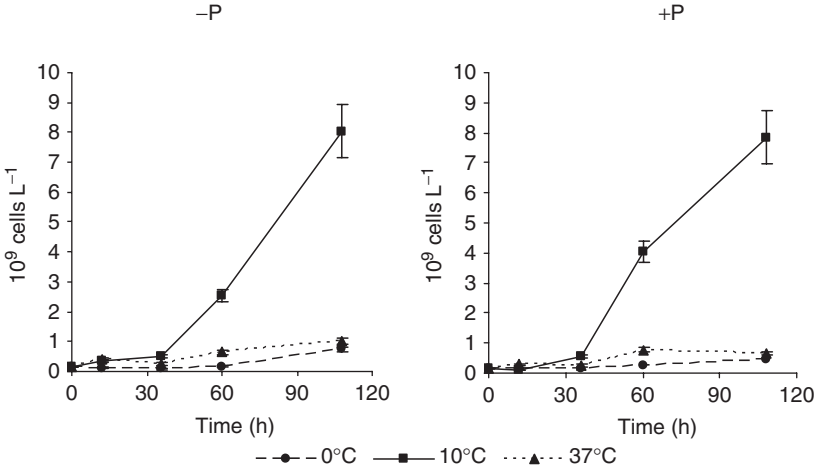

Fig. 3. Growth curves of *Halomonas glaciei* at 37, 10 and 0 °C, in the +P and −P media. Each value represents the average of three determinations ± SD.

Table 1. Maxima, minima and average values of BGAL, BGLU, AGLU, APA and AMA activity of *Halomonas glaciei* in the six experimental conditions [0, 10, 37 °C in phosphorus-enriched (P+) and phosphorus-depleted (−P) media]

	BGAL			BGLU			AGLU			APA			AMA		
	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean
0 °C −P	21.5	14.7	17.2	40.4	15.2	24.8	287.9	12.2	85.2	1.1	0.3	0.7	0.4	0.2	0.3
0 °C +P	23.5	15.5	18.5	22.3	14.9	20.0	27.5	11.9	16.6	1.3	0.3	0.8	0.3	0.2	0.3
10 °C −P	33.8	15.0	21.7	35.3	19.7	26.0	36.8	13.2	23.6	8.3	0.3	3.3	0.5	0.2	0.3
10 °C +P	24.5	14.9	19.3	33.1	17.4	23.5	31.5	9.7	18.6	1.1	0.5	0.8	0.3	0.2	0.2
37 °C −P	52.0	16.7	31.0	70.8	15.4	33.6	25.1	17.4	22.4	2.0	0.9	1.6	0.9	0.3	0.6
37 °C +P	40.5	21.5	27.8	23.9	12.0	19.0	21.4	10.3	16.3	1.5	0.5	1.1	1.5	0.2	0.6

Every value is expressed in nM h^{-1} as an average of three determinations ± an SD < 10%.

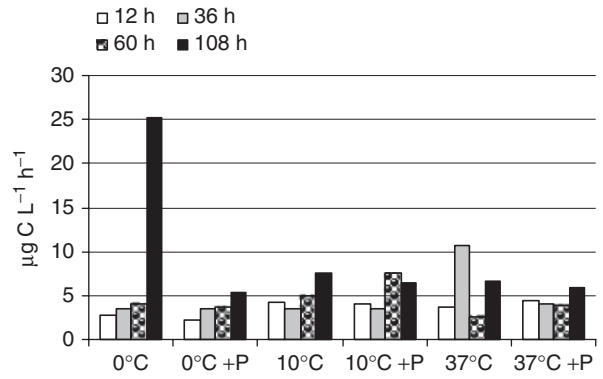

Fig. 4. Carbohydrate carbon mobilization performed by *Halomonas glaciei* at 37, 10 and 0 °C, in the +P and −P media after 12, 36, 60 and 108 h from T_0. Each value represents the average of three determinations with an SD < 10%.

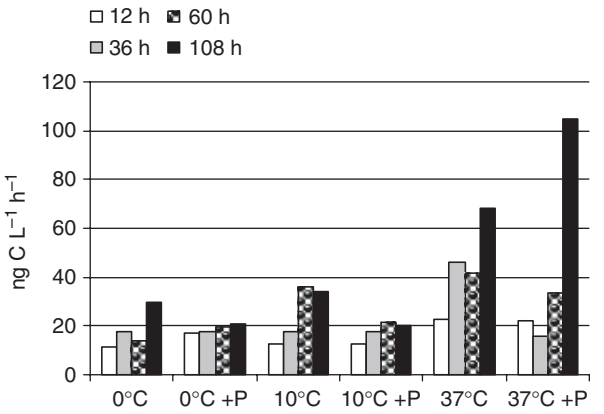

Fig. 5. Protein carbon mobilization performed by *Halomonas glaciei* at 37, 10 and 0 °C, in the +P and −P media after 12, 36, 60 and 108 h from T_0. Each value represents the average of three determinations with an SD < 10%.

Discussion

A bacterial strain isolated during the XVII Italian Expedition in Antarctica (February 2003) from frazil ice of the Ross Sea was determined as *H. glaciei* (*Gammaproteobacteria*). Studies of polar sea ice communities using cultivation-dependent and -independent techniques have previously shown that the *Gammaproteobacteria* are among the dominant taxonomic groups (Bowman *et al.*, 1997; Staley &

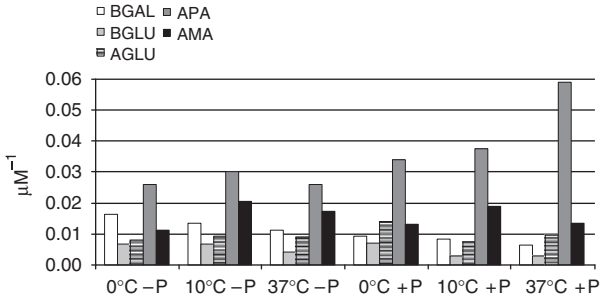

Fig. 6. Substrate affinity for BGAL, BGLU, AGLU, APA and AMA of *Halomonas glaciei* under six experimental conditions [0, 10, 37 °C in phosphorus-enriched (P+) and phosphorus-depleted (−P) media]. Each value represents the average of three determinations with an SD < 10%.

Table 2. Rotate variables (loadings), percentage variance and cumulative variance for the first and second components of the PCA, considering the average affinity of BGAL, BGLU, AGLU, APA and AMA for the available substrate, in the six experimental conditions [0, 10, 37 °C in phosphorus-enriched (P+) and phosphorus-depleted (−P) media]

	BGAL	BGLU	AGLU	APA	AMA	Variance (%)	Cumulative
PC1	−0.59	−0.57	−0.07	0.56	0.07	48.3	48.3
PC2	−0.21	0.30	0.69	0.24	−0.58	31.6	80.0

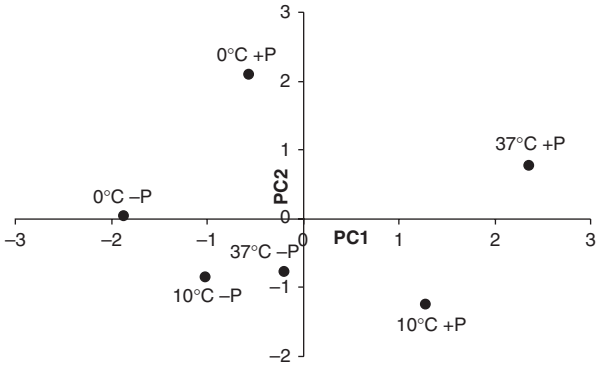

Fig. 7. Diagram of PC1 vs. PC2 obtained by the average affinity of BGAL, BGLU, AGLU, APA and AMA for the available substrate, under the six experimental conditions [0, 10, 37 °C in phosphorus-enriched (P+) and phosphorus-depleted (−P) media].

Gosink, 1999; Brown & Bowman, 2001; Brinkmeyer *et al.*, 2003) and species of the genus *Halomonas* are not uncommon (Bowman *et al.*, 1997; Brown & Bowman, 2001; Reddy *et al.*, 2003). *Halomonas glaciei* had been described for the first time by Reddy *et al.* (2003). The phenotypic characterization made in this study gave results comparable to those present in the literature (Reddy *et al.*, 2003), except for nonmotility and the capability to reduce nitrate.

Within sea ice, high bacterial activities that quickly mineralize organic matter usually occur (Vetter & Deming, 1994; Helmke & Weyland, 1995; Deming & Huston, 2000), but to the best of our knowledge, data on the Ross Sea newly formed sea ice are very scarce (Del Negro & Bergamasco, 2008). We studied *H. glaciei* with the aim of elucidating the potential relationship between the isolate and the available substrate in the sea ice.

By the application of the Arrhenius law of temperature, in order to highlight a linear relationship between temperature and substrate, we found out that *H. glaciei* responds linearly with the available organic matter in the temperature range 4–20 °C. As suggested by Pomeroy & Wiebe (2001), this range would represent the interval of thermal optima, which verifies the linear relationship described above. For this reason, we studied the strain at one temperature included in such a range (10 °C) and two extreme temperatures outside it (0 and 37 °C). At 0 and 37 °C, bacterial growth was constrained and rather constant during the whole experiment (108 h) without any expression of exponential growth, while at 10 °C, abundances increased by almost 2 orders of magnitude in both the tested media. The phosphorus supply does not seem to have produced any difference in growth rates, with the exception of an anticipation of the exponential phase according to the amount of cells recorded at 60 h (2.5 and 4.0×10^9 cells L^{-1} in −P and +P, respectively). Growth curves of another Antarctic sea ice gammaproteobacterium (*Pseudoalteromonas*) in batch cultures were recently performed by Mancuso Nichols *et al.* (2005) and their results highlighted an exponential phase at −2 °C too. These differences could be attributable to the different counting techniques used, but also to different features of the two strains, stressing how the synergic presence of different bacteria within an extreme environment could be somewhat useful for the whole community. In both cases, anyway, it clearly emerged that temperatures of 10 or 20 °C represent optimal conditions for growth and that natural populations in sea ice are commonly subjected to limiting extremes.

Ectoenzymatic activity provides important indications on the organic matter flux through bacteria. Rates of carbohydrate hydrolysis (Table 1) were in general very high, reaching a maximum of 287.9 nM h^{-1} (AGLU) at 0 °C in the phosphorus-depleted medium. Except for this result, the other rates were, on average, around 20–30 nM h^{-1}, which represents a rather fast activity for these enzymes. In the Ross Sea seawater, Misic *et al.* (2002), in fact, found extremely lower values for BGLU, but Bergamasco *et al.* (2003) estimated hydrolysis rates comparable with ours, or even faster. On the contrary, phosphatase and protease activities were very low (always < 8.5 and 1.5 nM h^{-1}, respectively) compared with studies performed in Antarctic waters (Misic *et al.*, 2002; Bergamasco *et al.*, 2003; Sala *et al.*, 2005).

Our data suggest a preferential demand for carbon derived from carbohydrates rather than from proteins: ectoenzyme activities transformed into carbon mobilization (Figs 5 and 6) from organic polymers, showed that the total carbon potentially released from polysaccharides can be almost one order of magnitude higher than the protein carbon mobilization. In fact, even excluding the peak ($25.2\,\mu g\,C\,L^{-1}\,h^{-1}$) observed in the $-P$ medium at 0 °C, the total carbon mobilization from carbohydrates is rather stable around $5\,\mu g\,C\,L^{-1}\,h^{-1}$ under every experimental condition at every sampling. The addition of glucose in the media was made after the phenotypic characterization of the strain as it was one of the monosaccharides utilized by this bacterium when provided as the sole carbon source. Notwithstanding the availability of glucose, *H. glaciei* showed a very important potential mobilization of this compound (as also of galactose) and we hypothesize that this effort in creating new carbon availability is due to an intense requirement, not satisfied by the media. In order to understand the single-cell behaviour, we calculated the percentage of every single hydrolytic activity over the total per cell (Fig. 8). What clearly emerges from our data is the absolute predominance of per cell glycolytic activities (ranging from 97% to 99%) under every experimental condition. This behaviour is in accordance with Martinez *et al.* (1996), who found that among 44 isolates, each strain expressed a defined ectoenzymatic pool in which only a few enzymes were particularly active. In their work, however, only a relatively small fraction (*c.* 25%) of isolates expressed a predominant glycolytic activity over phosphatase, protease and lipase.

On the contrary, the amount of potentially mobilized carbon from proteins by *H. glaciei* was around $20\,ng\,C\,L^{-1}\,h^{-1}$ but at 37 °C it reached 68.4 and $104.8\,ng\,C\,L^{-1}\,h^{-1}$ in the $-P$ and $+P$ medium, respectively, indicating how the hydrolysis of polypeptides is, for this strain, influenced more by temperature than by phosphorus availability. In field investigations, Christian & Karl (1995) noted a very pronounced shift from the predominance of proteolysis in temperate and polar pelagic environments to the development of glycolysis at low latitudes, suggesting that in temperate to polar waters bacteria subsist mainly on peptides and amino acids. Similarly, Misic *et al.* (2006), in a transect through the Indian Ocean, from the Mediterranean to Australia, found significantly higher BGLU within the latitudinal range from 12°N to 16°S, whereas, proceeding southward, proteolysis was considerable. However, even though this appears to confute our findings because of the predominance of proteolysis at high latitudes, several authors (i.e. Christian & Karl, 1995; Martinez *et al.*, 1996) stressed how different hydrolytic activities are expressed by different prokaryotic assemblages, and thus the predominance of some bacteria (or taxa) in the community would be

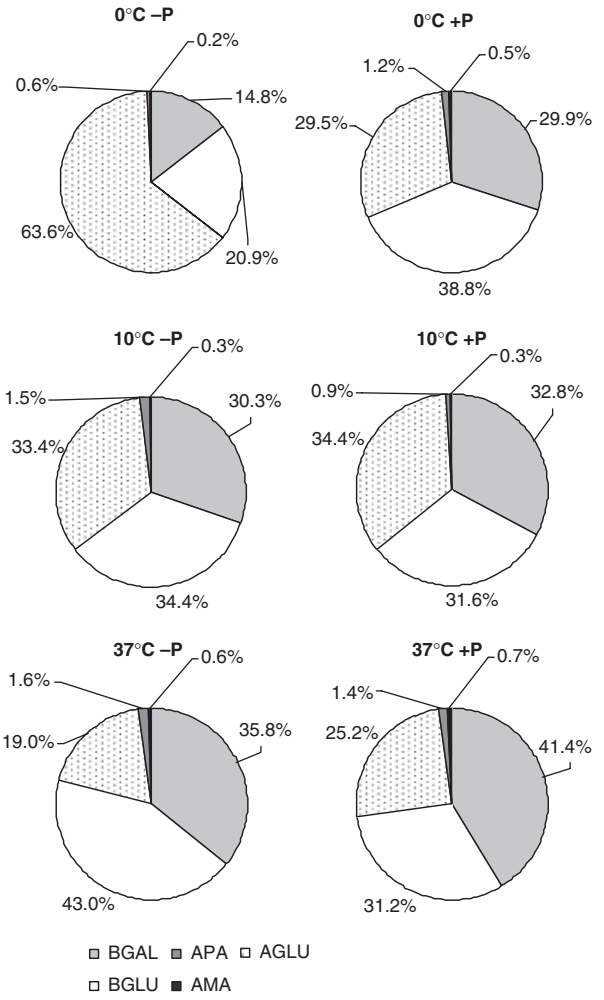

Fig. 8. Relative per cell hydrolytic activities expressed by *Halomonas glaciei* under the six experimental conditions [0, 10, 37 °C in phosphorus-enriched (P+) and phosphorus-depleted (− P)] media. For definitions of enzymes' abbreviations, see Table 1.

reflect in a given predominant hydrolytic activity. The high polysaccharide degradation by *H. glaciei* could be an adaptation for living in sea ice where copious amounts of exopolysaccharides produced by microorganisms can accumulate in its brine channels (Krembs *et al.*, 2002). Furthermore, within sea ice as in other environments, bacterial communities made up of different organisms specialized in the preferential degradation of a given (or a limited subset of) substance would hydrolyse many macromolecular components, leading to an advantage for the whole assemblage (Martinez *et al.*, 1996).

Substrate affinity for APA was the highest recorded in every experimental condition ranging from 0.026 to $0.059\,\mu M^{-1}$: the highest values were recorded in the $+P$ medium increasing towards higher temperatures while in the phosphorus-depleted medium no significant difference

occurred at different temperatures. AGLU, BGLU and BGAL kinetics showed a general static or a decreasing affinity with increasing temperatures in both media in contrast to APA. Protease affinity for the substrate was slightly higher at 10 °C both in the phosphorus-repleted and in the phosphorus-depleted medium. Because an interpretation of these results is rather complex, a PCA was performed (Table 2, Fig. 7). The average affinities of the enzymes under every experimental condition were plotted in a diagram of PC1 vs. PC2 and the location of the plots represents different features of the pool of enzymes produced by the strain. In − P, a sort of gradient led by temperature can be assumed by the PCA plot. In fact, the increasing temperatures lead to a modification in the pool of highly affine enzymes, and in particular a decrease in affinity for the substrate by glucidic enzymes and a major affinity for protease. In the other medium, no clear gradient was actually found even though at the extreme temperatures (0 and 37 °C) a major affinity was peculiar for AGLU, and at the temperature at which the bacterial growth linear relationship with the substrate was found, we found a better affinity by AMA. These results highlight how *H. glaciei* can vary the conformation and thus the efficiency of the ectoenzymes produced depending on the physical and biochemical features of the environment, which could represent a very useful adaptation to a severe ecosystem as sea ice. Moreover, even if a clear dependence of growth from temperature was found, under our experimental conditions, the carbon fluxes rates did not substantially vary, indicating how the degradative potential is not so tightly coupled with bacterial growth or cell amounts, thus leading to a probable major effort for organic carbon recovery in suboptimal environmental systems.

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