RESEARCH ARTICLE

Molecular analysis of the diversity of genus *Psychrobacter* present within a temperate estuary

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Abstract

Many members of the genus *Psychrobacter* are endemic in extremely cold and saline environments and the genus has been described as only marginally successful in warmer habitats. In a previous study the *Psychrobacter* genus was, unexpectedly, the most frequently isolated bacterial genus from the sea-surface microlayer (SML) and the underlying water (UW) of a temperate estuary (Ria de Aveiro, Portugal). Here we analysed the diversity in *Psychrobacter* populations inhabiting this estuary. Samples were collected at three dates and three locations from sea-SML and UW. Isolated *Psychrobacter* strains were well-adapted to temperatures and salt concentrations above the ones described as optimal for most members of this genus. Hydrocarbon-degrading potential was not confirmed for these strains. We developed and optimized a reliable and specific denaturing gradient gel electrophoresis (DGGE)-based method for the analysis of *Psychrobacter* populations in aquatic systems. DGGE profiles inferred that *Psychrobacter* populations were very stable in the estuary, a strong indication for the presence of well-adapted phylotypes. The analysis of genus-specific clone libraries revealed a surprisingly high diversity among *Psychrobacter* in Ria de Aveiro. Results indicated that novel species were probably cultivated. Significant differences between sea-SML and UW *Psychrobacter* communities were revealed. Observed diversity trends may be related to environmental factors such as salinity and/or anthropogenic pressures such as contamination with hydrocarbons.

Introduction

The occurrence of cold-adapted bacteria outside extremely cold regions has been only sporadically reported (Martiny *et al.*, 2006). However, 'Omics' technologies have revealed that psychrophilic bacteria harbor adaptive traits that could promote their dispersion beyond low-temperature locations (Methé *et al.*, 2005; Cacace *et al.*, 2010). For instance, the study of the transcriptome of the cold-adapted genus *Psychrobacter* revealed adaptations to significant temperature variations (Rodrigues *et al.*, 2008; Bergholz *et al.*, 2009). Even so, based on quantitative PCR and 16S rRNA gene libraries, *Psychrobacter* populations from extremely cold habitats were considerably more abundant and diverse than *Psychrobacter* populations retrieved from warmer regions (Rodrigues *et al.*, 2009).

Global warming is reducing the extent of cold habitats thus affecting the evolution of cold-adapted bacteria. The extreme biotechnological relevance of cold-adapted bacteria as well as their fundamental role in biogeochemical cycles (Feller & Gerday, 2003) makes it important to understand to what extent these bacteria can adapt to environmental warming. Studying the diversity of cold-adapted bacteria in temperate habitats will add to the knowledge about this topic.

The genus *Psychrobacter* includes Gram-negative coccobacilli that are non-pigmented, oxidase-positive, non-motile, psychrophilic or psychrotolerant, and halotolerant (Juni & Heym, 1986). At the time of writing, this genus included 34 species supported by valid reports. Most of the species described so far have been isolated from cold environments, including Arctic and Antarctic sea ice, water, soils and sediments (Bowman *et al.*, 1996, 1997;

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Yumoto *et al.*, 2003; Romanenko *et al.*, 2004; Shivaji *et al.*, 2004; Bakermans *et al.*, 2006). Less frequently, new species have been isolated from temperate marine environments (Yoon *et al.*, 2005b; Yumoto *et al.*, 2010) and other sources such as pigeon feces, food products, lung tissue and human blood (Vela *et al.*, 2003; Yoon *et al.*, 2003, 2005a; Yassin & Busse, 2009; Wirth *et al.*, 2012).

Besides temperature, the occurrence of *Psychrobacter* was significantly associated with other environmental factors such as pH close to neutrality, high salinity and higher concentrations of potassium and magnesium (Rodrigues *et al.*, 2009). Anthropogenic-related factors may also influence the distribution of *Psychrobacter*. For example, *Psychrobacter* enrichment has been noted in aquatic environments contaminated with hydrocarbons (Harwati *et al.*, 2007; Prabagaran *et al.*, 2007; Lo Giudice *et al.*, 2010).

During a previous study based on culture-dependent methods, the genus *Psychrobacter* was, unexpectedly, the most frequently retrieved genus from the sea-surface microlayer (SML) and the underlying water (UW) of a temperate estuary (Ria de Aveiro, Portugal) during a warm season (May–July; Azevedo *et al.*, 2012). SML refers to the thin (1–1000 μm) film that forms between the hydrosphere and atmosphere (Azevedo *et al.*, 2012).

To our knowledge, broad studies on the diversity of *Psychrobacter* within temperate aquatic environments have never been conducted. To provide a comprehensive picture of the diversity of *Psychrobacter* populations inhabiting SML and UW in Ria de Aveiro, culture-independent methods, e.g. denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone libraries, specifically targeting this genus were applied. In addition, a collection of *Psychrobacter* isolates was analysed by molecular typing and 16S rRNA gene-based phylogenetic analysis. The temperature and salt growth ranges of these isolates as well as their hydrocarbon-degrading potential were also evaluated.

Materials and methods

Sampling

Ria de Aveiro is a shallow estuary on the northwest coast of Portugal (40°38′N, 8″45′W), about 45 km long and 8.5 km wide (Fig. 1). This study was conducted during the warm season in three campaigns in May (C1), June (C2) and July (C3). Samples were collected at three sites: Cais do Chegado (CC), where the main contamination sources are industrial effluents; Costa Nova (CN), mainly impacted by urban effluents, aquacultures and run-off from agriculture fields; and Cais do Sporting (CS), subjected to anthropogenic pressure mainly due to the presence of harbour facilities. Water samples were retrieved as previously described (Azevedo *et al.*, 2012). Briefly, samples from SML were collected by adherence to glass and acrylic plates, and UW samples were collected by submerging a sterilized brown glass bottle and opening it at a depth of *c.* 0.4 m. Samples were kept in cold and dark conditions during transport and were processed within 1 h after sampling. Salinity was determined with a WTW Conductivity Meter Model LF 196 (WTW, Weinheim, Germany) and measured using the practical salinity scale.

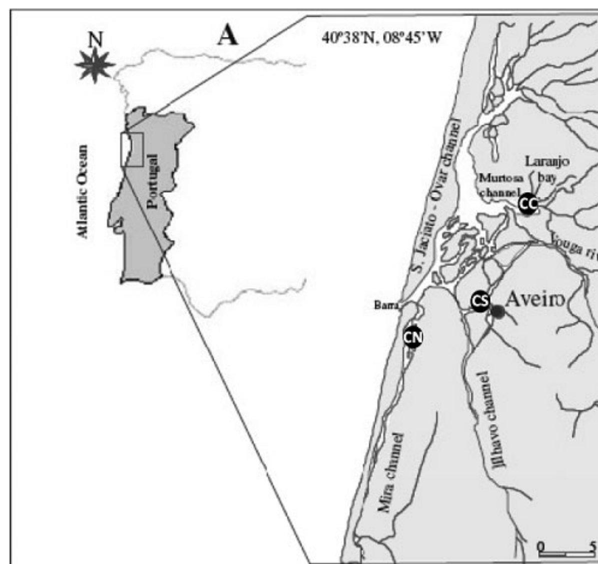

Fig. 1. Map of Ria de Aveiro showing the location of the sampling sites CN, CS and CC.

Culture-dependent analysis of *Psychrobacter* populations

Psychrobacter isolates

Isolates were obtained as described by Azevedo *et al.* (2012). In summary, isolates were retrieved from estuarine agar (EA; Weiner *et al.*, 1980) and glutamate starch phenol red agar (GSP; *Pseudomonas*/*Aeromonas* selective agar) plates. DNA was purified as previously described (Azevedo *et al.*, 2012). Eighty-one isolates (42 from SML and 39 from UW samples) were affiliated to *Psychrobacter* by sequencing the 16S rRNA gene. The pure cultures were maintained on EA at 4 °C (after growing at the same culture media for 2 days at 30 °C) and as 20% (v/v) glycerol suspensions at −80 °C.

REP-PCR genomic fingerprinting

All isolates were typed by using a Repetitive Extragenic Palindromic PCR (REP-PCR) method with primers REP-1R

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and REP-2I as described previously (Versalovic *et al.*, 1991). PCR reactions were carried out with Taq polymerase, nucleotides and buffers from Promega. The PCR reaction mixtures (25 μL) contained 1× PCR buffer, 200 μM of each nucleotide, 3 mM $MgCl_2$, 5% dimethylsulfoxide, 7.5 pmol of each primer, 1 U of Taq polymerase and 50–100 ng purified DNA. Amplification was carried out as follows: initial denaturation for 7 min at 95 °C, followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 40 °C and extension for 8 min at 65 °C and a final extension of 16 min at 65 °C. The products were electrophoresed for 90 min under a constant voltage of 80 V on a 1.5% (w/v) agarose gel containing 0.5× TAE (20 mM Tris–acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2EDTA) and DNA markers purchased from MBI Fermentas (Vilnius, Lithuania). The gel images were acquired using a Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA, USA) and analysed using the software package GELCOMPAR 4.0 (Applied Maths, Sint-Martens-Latem, Belgium).

Growth temperature and salt ranges

Temperature requirements were determined by spotting 10 μL of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} culture dilutions onto marine agar plates. Inoculated plates were incubated for 14 days at 37, 28 and 4 °C. The ability of the isolates to grow in marine agar supplemented with 5%, 10% and 12% of NaCl and in trypticase soy agar without NaCl was also determined using the same inoculation protocol. Incubation was for 14 days at 28 °C. Three replicates per isolate were included for both experiments and growth was considered possible only if the highest dilution resulted in colony formation for all three replicates.

Utilization of hydrocarbons for growth

Suspensions of bacterial cells in 0.9% NaCl were used to inoculate mineral medium agar plates (Ma *et al.*, 2006) supplemented with 1% (v/v) diesel oil as the sole source of carbon. Triplicate plates were incubated at 28 °C and results were registered after 14 days.

PCR amplification of alkB and PAH-RHDα genes

All isolates were tested by PCR for the presence of the *alkB* and PAH-RHDα genes encoding the alkane hydroxylase and the ring-hydroxylating-dioxygenases, respectively, which are both involved in the degradation of hydrocarbons. Primers and PCR conditions were as previously reported (Cébron *et al.*, 2008; Paisse *et al.*, 2011).

Additionally, we searched for homologous genes in the *Psychrobacter* genomes available in the GenBank database. Based on the sequence of an alkane hydroxylase gene annotated in the genome of *Psychrobacter* PRwf-1 (accession no. NC_009524.1) primers PsyalkB_For (5′-GGCAG TTTTTACCACGTAC-3′) and PsyalkB_Rev (5′-CAGCGC CATTCTGATCAC-3′) were designed and tested against *Psychrobacter* isolates. The PCR reaction mixtures (25 μL) contained NZYTaq 2× Green Master Mix (2.5 mM $MgCl_2$; 200 μM dNTPs; 0.2 U μL^{-1} DNA polymerase; NZYtech, Portugal), 10 pmol of each primer and 50–100 ng purified DNA. Amplification was performed as follows: initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 53 °C and extension for 45 s at 72 °C, and a final extension of 10 min at 72 °C.

Culture-independent analysis

DNA extraction and 16S rRNA gene amplification

For DNA extraction, 200-mL water samples from SML and UW were filtered through 0.2-μm pore polycarbonate filters (GE Water & Process Technologies). DNA purification was performed using the Genomic DNA Extraction Kit (MBI Fermentas) as described previously (Henriques *et al.*, 2004).

Approximately 400 bp of the 16S rRNA gene was amplified from water samples using the *Psychrobacter*-specific primers 432-F/823-R and PCR conditions as described by Rodrigues *et al.* (2009). These and subsequent PCR reactions were carried out in a Bio-Rad MyCycler Thermal Cycler (Bio-Rad) using Taq polymerase, nucleotides and buffers purchased from MBI Fermentas.

Denaturing gradient gel electrophoresis

Psychrobacter-specific molecular fingerprints of each sample were obtained using a DGGE approach. PCR was as described with the following changes: initial denaturation (94 °C for 5 min); 30 cycles of denaturation (92 °C for 30 s); annealing (59 °C for 30 s) and extension (72 °C for 30 s); and a final extension (72 °C for 30 min). Also, a GC clamp was attached to the 5′ end of the forward primer to prevent complete melting of the PCR products during subsequent DGGE analysis (Muyzer *et al.*, 1993).

PCR products were directly applied onto 8% polyacrylamide gels (37.5 : 1, acrylamide/bisacrylamide) in 0.5× TAE buffer (20 mM Tris–acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2EDTA) with urea and formamide as denaturants. Linear denaturing gradient ranged from 35% to 62.5%. Electrophoresis was performed on a D-Code Universal Mutation Detection System (Bio-Rad) at 60 °C; initially a constant voltage of 20 V was applied for 15 min, followed by 75 V for 16 h. After electrophoresis, the gels

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were stained for 5 min with ethidium bromide and then rinsed for 20 min in distilled water. Gel images were acquired using the Gel Doc™ XR+ System (Bio-Rad).

DGGE profiles were analysed using GELCOMPAR II Software (Applied Maths). Cluster analysis of DGGE profiles was performed using the UPGMA method (group average method) applying Pearson correlation measures.

16S rRNA gene libraries

To construct *Psychrobacter*-specific 16S rRNA gene libraries, samples from the C3 campaign (July) were chosen. PCR products from SML and UW samples were mixed separately. The SML and UW mixed products were cloned using the TA cloning kit following the manufacturer's instructions (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA). Libraries will be subsequently designated *Psysml* (obtained from SML samples) and *Psyuw* (from UW samples).

Inserts were amplified using vector-specific primers. PCR products with the expected size were purified with the Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany). Sequencing reactions were carried out using the same primers from Stab-Vida (Oeiras, Portugal).

Psychrobacter population analysis

Taxonomic affiliation, alignment and clustering analysis were processed using the pipeline available at Ribosomal Database Project (RDP-II) website (http://rdp.cme.msu.edu). Operational taxonomic units (OTUs) were defined based on a 99% cut-off value.

Classical indices to estimate richness (Chao's richness estimator) and diversity (Shannon index) were obtained from the RDP-II pipeline for both clone libraries and also for the *Psychrobacter* culture collection. Distance matrixes were constructed and ∫-LIBSHUFF analysis was performed through MOTHUR (Schloss *et al.*, 2009). The genetic variation within and among samples was estimated with an analysis of molecular variance (AMOVA) by using the program ARLEQUIN version 3.1 (Excoffier *et al.*, 2005).

One representative of each defined OTU was chosen to construct the phylogenetic tree within MEGA 5.0 (Tamura *et al.*, 2011) using the Kimura 2-parameter and neighbor-joining clustering method.

Nucleotide sequence accession numbers

Sequences were deposited in GenBank under the following accession numbers: JX897712–JX897817 (for 16S rRNA gene clones) and JX897818–JX897897 and JQ072080 (for *Psychrobacter* isolates).

Results and discussion

Occurrence and characterization of *Psychrobacter* isolates from Ria de Aveiro

Psychrobacter isolates represented almost 25% of a culture collection of heterotrophic bacteria previously obtained from Ria de Aveiro (Azevedo *et al.*, 2012). In the present study, these isolates (n = 81) were categorized into nine OTUs based on an identity criteria of 99% of the 16S rRNA gene sequence (Table 1). Almost 83% of the isolates were included in the three dominant OTUs. All isolates were successfully typed by REP-PCR and 51 distinct profiles were obtained. Both the total number of OTUs and the number of REP profiles suggest the

Table 1. Diversity of *Psychrobacter* isolates retrieved from Ria de Aveiro

OTU*	No. of isolates (no. of REP types)	Closest relatives (accession number)	Distribution by site†			Distribution by water layer	
			CC	CN	CS	SML	UW
OTU1	17 (17)	100% *P. piscatorii* (AB688097.1)	2	1	14	10	7
OTU2	39 (20)	100% *P. faecalis* (JX293325.1)	11	7	21	17	22
OTU3	11 (2)	99% *P. piscatorii* (AB688097.1)	1	4	6	9	2
OTU4	3 (3)	100% *P. glacincola* (HM584295.1)	1	1	1	2	1
OTU5	5 (4)	100% *P. aquimaris* (EF101545.1)	0	1	4	1	4
OTU7	2 (2)	99% *P. faecalis* (JF710999.1)	0	0	2	0	2
OTU9	2 (2)	99% *P. celer* (EF101550.1)	0	0	2	2	0
OTU26	1 (1)	100% *P. alimentarius* (HM032857.1)	0	0	1	1	0
OTU27	1 (1)	98% *P. maritimus* (NR_027225.1)	1	0	0	1	0
Total			16	16	49	42	39
Shannon index			1.04	1.49	1.47	1.54	1.36

SML, surface microlayer; UW, underlying water.

*OTU, operational taxonomic unit, based on 99% similarity of the 16S rRNA gene sequence.

†Sampling sites: CC, Cais do chegado; CN, Costa Nova; CS, Cais do Sporting.

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presence of a diverse population of *Psychrobacter* in the estuary. According to Bowman *et al.* (1997) and Vishnivetskaya *et al.* (2000) NaCl concentrations up to 1.0 M in the culture media and incubation at low temperatures can strongly select for *Psychrobacter* strains. Based on these references the abundance suggested by our results could not be explained by culture conditions, since the ones applied during this study were not designed to enrich for *Psychrobacter*. Samples were therefore spread onto GSP and EA, which contain lower concentrations of NaCl, and plates were incubated at 22 °C.

Temperature values registered in the estuary during sampling were similar between sites and varied from 18.7 to 21.5 °C depending on the sampling dates. Higher salinity values were always registered for site CS (22.7–29.3) and lower values for site CC (11.8–18.7). The highest values were registered in July.

The temperature and salt growth ranges were determined for isolates obtained during this study representing each REP profile ($n = 51$). As expected all isolates were able to grow at 4 and 28 °C. However, most of them (80.4%) were also able to grow at 37 °C. From the 34 species of *Psychrobacter* currently described, only five were reported to grow at 37 °C (Bowman, 2006; Wirth *et al.*, 2012). This result indicates that the *Psychrobacter* strains inhabiting Ria de Aveiro are well-adapted to temperatures above the ones described as optimal for most members of this genus.

In regard to growth at different salt concentrations, 42% of the isolates were not able to grow in the absence of NaCl, 88% were able to grow with 10% NaCl, and 48% were able to grow with 12% NaCl. The tolerance of 10% NaCl is common among most species of *Psychrobacter*. However, only six species were reported to grow in the presence of 12% NaCl (Bowman, 2006), indicating that the *Psychrobacter* population in Ria de Aveiro is particularly adapted to high salt concentrations.

The *Psychrobacter* isolates were retrieved from both water layers and the three sampling sites in all campaigns (Table 1). Approximately the same number of isolates was retrieved from both layers. However, Shannon index values suggested higher diversity among the *Psychrobacter* isolates retrieved from the SML (Table 1). SML communities are known to be subjected to a wider range of selective pressures than communities inhabiting UW. These pressures may be imposed by the presence of several contaminants, higher levels of UV radiation or higher temperature and salinity fluctuations (Maki, 1993; Cincinelli *et al.*, 2005; Cuong *et al.*, 2008; Azevedo *et al.*, 2012). The persistence of culturable *Psychrobacter* phylotypes adapted to those pressures may account for the differences between water layers in terms of diversity. For example, hydrocarbons are known to be enriched in SML (Wurl & Obbard, 2004) and were reported to strongly impact the *Psychrobacter* communities (Lo Giudice *et al.*, 2010).

We are aware that we are inferring diversity based on the cultivable fraction and therefore extrapolations to the community must be considered carefully.

About 60% of the *Psychrobacter* isolates were retrieved from sampling site CS (Table 1). This may also be related to higher contamination with hydrocarbons in this site (Coelho *et al.*, 2010), reported to select for *Psychrobacter* spp. In fact, Prabagaran *et al.* (2007) showed that *Psychrobacter* representatives were strongly enriched due to addition of crude oil to seawater collected from off Ushuaia (Argentina), which was undetectable using classic PCR approaches in the original samples. Also, *Psychrobacter* strains have emerged as hydrocarbon-degrading bacteria during studies conducted in Antarctic, Arctic and Indonesian seawater (Gerdes *et al.*, 2005; Harwati *et al.*, 2007; Lo Giudice *et al.*, 2010).

This hypothesis was assessed by testing the isolates obtained during this study for their ability to grow on medium supplemented with diesel as the only carbon source. None of the isolates was able to grow in this medium after an incubation for 14 days at 28 °C. Also the *alkB* and PAH-RHDα genes were not detected in any of the isolates using specific or degenerated primers. Even so, our results do not exclude the presence of other genes encoding hydrocarbon-degrading enzymes. Additionally, using the specific primers designed for an alkane hydroxylase gene annotated in the genome of *Psychrobacter* PRwf-1 (accession number: NC_009524.1), a fragment with the expected size was obtained using as template the environmental DNA from Ria de Aveiro water samples (data not shown). This suggests that other *Psychrobacter* members present in the estuary may have the potential to degrade hydrocarbons.

The abundance of *Psychrobacter* at site CS may also be related to higher salinity values preferred by members of this genus (Romanenko *et al.*, 2004; Ponder *et al.*, 2005) and particularly by the isolates retrieved during this study. Finally the CS site is located near a harbour that receives cod-fishery boats coming from the North Sea, bringing salt-cured codfish. As previously reported, *Psychrobacter* was the dominant genus present in the cod skin mucus and survived prolonged frozen storage and concentrations of NaCl of up to 25% (w/v; Bjørkevoll *et al.*, 2003). Thus we can speculate that runoff from cod-fishery boats can also contribute to the diversity and abundance of *Psychrobacter* in the CS site.

Dynamics of *Psychrobacter* populations

We developed and optimized a DGGE-based method to assess the structure and dynamics of *Psychrobacter*

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populations. The analysis included SML and UW samples from three sampling sites and three sampling periods (May–July).

DGGE fingerprints were obtained from all samples (Fig. 2) and the method was highly reproducible (data not shown). In general, profiles shared a high degree of similarity and the number of bands per profile was rather stable, ranging from 10 to 13 bands. Clustering analysis grouped the obtained profiles in two main clusters. One of the clusters included samples collected in May from site CN and samples collected in July from site CS. An inefficient PCR amplification of two of these samples may have contributed to the formation of this separate cluster, even though other factors may account for this separation. The remaining profiles were highly similar (> 60% similarity) and grouped preferentially according to sampling date, suggesting that temporal factors (e.g. temperature) play an important role in shaping the *Psychrobacter* community. Surprisingly, profiles from most of the samples collected in July grouped with samples collected in May, although salinity and temperature values were more similar in June and July. Anthropogenic factors may account for the formation of this cluster. Grouping according to sampling sites was also frequently observed in the dendrogram, suggesting the influence of spatial-varying factors such as anthropogenic pressures or salinity. In some cases samples also grouped according to water layers (Fig. 2). In fact, differences between SML and UW profiles were detected particularly from the intensity of some bands (Fig. 2).

DGGE results indicate that *Psychrobacter* populations were rather stable in the estuary, suggesting the presence of several well-adapted phylotypes withstanding temporal and spatial environmental fluctuations.

Diversity of *Psychrobacter* populations

The diversity of the *Psychrobacter* community was further characterized by generating genus-specific *16S* rRNA gene libraries from samples collected in July. To assess differences between water layers, amplicons obtained from SML and UW samples were cloned separately.

A total of 106 clones were randomly picked and sequenced (51 from library *Psysml* and 55 from library *Psyuw*). All sequences affiliated with *Psychrobacter* members confirming the specificity of the primers. Sequences were classified into 33 OTUs (at least 99% sequence similarity; Fig. 3). The number of sequences per OTU varied from only 1 to 25. Four OTUs included 47% of the total number of sequences. OTUs represented by only one sequence accounted for 45% of the total number of OTUs. Seven of nine OTUs obtained by cultivation were

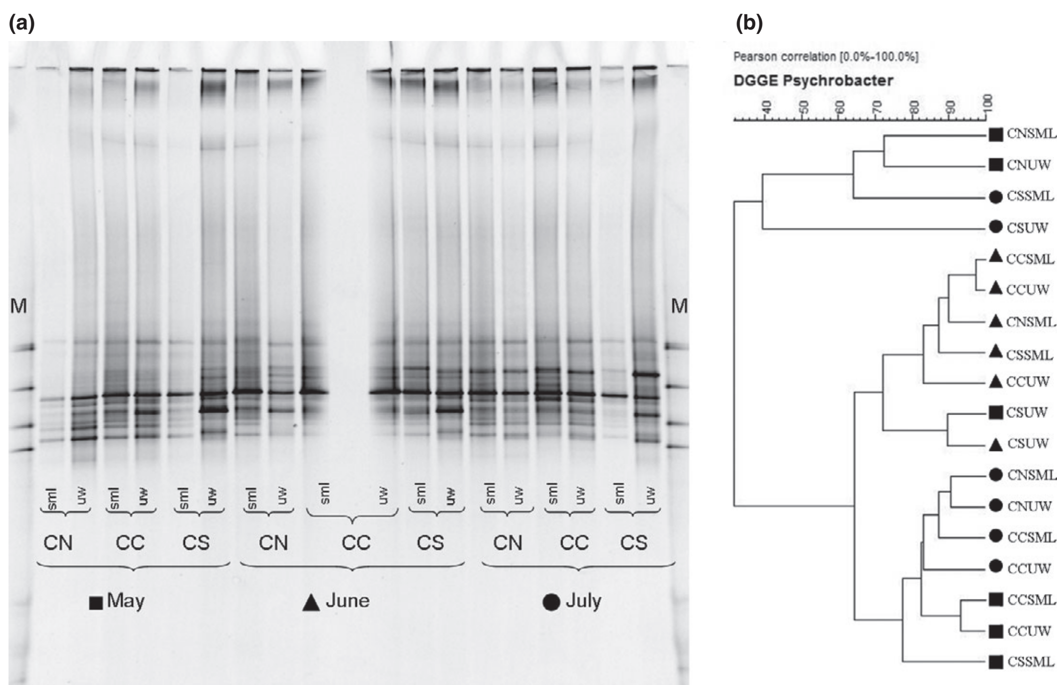

Fig. 2. (a) DGGE analysis of *Psychrobacter* populations in UW and SML samples collected in May ■, June ▲ and July ●. (b) Dendrograms of DGGE patterns created using UPGMA method (group average method) applying Pearson correlation analysis.

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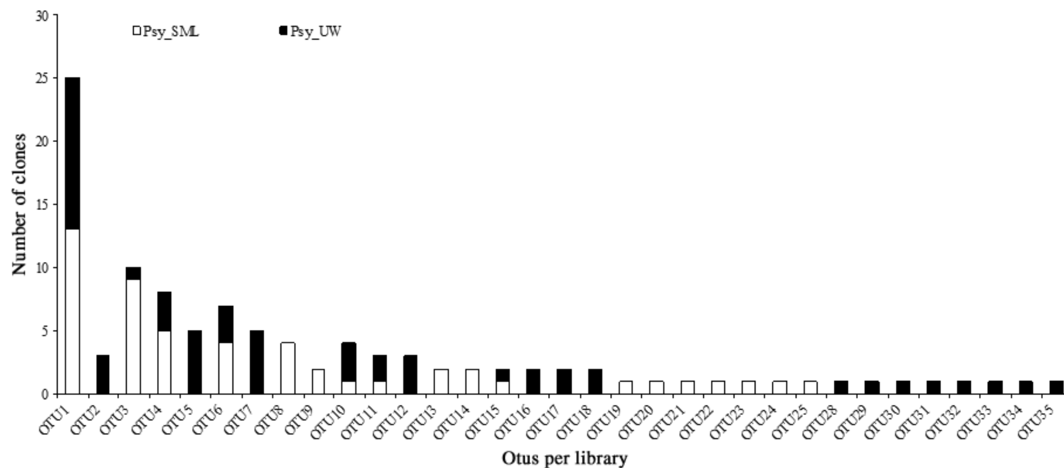

Fig. 3. Relative abundance of the observed OTUs among *Psysml* and *Psyuw* clone libraries.

represented in at least one of the gene libraries. However, whereas OTU1 was the most represented in both libraries (Fig. 3), OTU2 was by far the most frequently cultivated, constituting 48% of the total number of isolates (Table 1). Twenty-six OTUs were only detected using the culture-independent approach, and two OTUs (represented by one isolate each) were exclusively found by cultivation.

Sequences from the SML library were categorized in 18 OTUs and sequences from the UW library were distributed in 22 OTUs. Only seven OTUs included sequences from both layers. Diversity estimated by the Shannon index was higher for the UW library ($H = 2.77$) than for the SML library ($H = 2.43$). ∫-LIBSHUFF analysis indicated that *Psychrobacter* populations from SML and UW were significantly ($P < 0.05$) different. An AMOVA test was conducted to evaluate the variance in genetic diversity between *Psychrobacter* communities from SML and UW (Excoffier *et al.*, 2005). According to the AMOVA results, the *Psychrobacter* phylogenetic variance between layers was low ($F_{ST} = 7.22\%$) but significant ($P < 0.001$). Genetic variation among populations probably results from different selective pressures occurring in SML and UW (Cunliffe *et al.*, 2011). As previously reported, organic matter and of a variety of pollutants including hydrocarbons and heavy metals accumulate in SML (Cincinelli *et al.*, 2005; Cuong *et al.*, 2008). Also, stronger salinity and temperature variations are known to occur at this layer (Maki, 1993). Those factors may determine the selection of different *Psychrobacter* phylotypes.

Differences between SML and UW bacterial communities have been addressed in several aquatic systems including Ria de Aveiro (Azevedo *et al.*, 2012). Although some controversy remains, different communities are presumed to occur in each layer (Cunliffe *et al.*, 2011). However, which phylogenetic groups contribute to those differences is mainly unknown. This study adds to the existing information by assessing differences at an intra-genus level.

Phylogenetic analysis of *Psychrobacter* isolates and clones

Phylogenetic analysis was done based on the partial 16S rRNA gene sequences (≈400 bp) representing each OTU and the type strains of all *Psychrobacter* species described so far. Whenever an OTU includes isolates or clones retrieved from SML and UW, a representative of each layer was also included in the tree. Most sequences retrieved during this study fall into a large cluster that also included 13 *Psychrobacter* species mainly retrieved from marine or estuarine waters. *Psychrobacter* species from Arctic and Antarctic sediments as well as from fermented seafood, infected lung tissue, feces and human blood grouped in separate clusters.

In particular, the *Psychrobacter* species specifically from extremely cold environments (e.g. *Psychrobacter frigidicola*, *Psychrobacter okhotkensis, Psychrobacter glacincola* and *Psychrobacter cryohaloentis*) grouped in a separate cluster that was only distantly related to the sequences retrieved in the present study. Again, this result points to the presence in the estuary of specific *Psychrobacter* species adapted to broader temperature and salt ranges.

According to the phylogenetic analysis a high diversity of *Psychrobacter* phylotypes were retrieved from Ria de Aveiro (Fig. 4). Some clusters comprised only sequences from unculturable clones, indicating that new species present in the estuary are still being cultivated. On the other hand, in some cases bacteria isolated during this

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Fig. 4. Phylogenetic tree based on 16S rRNA gene sequences of *Psychrobacter* strains (*Psycult*, ♦) and clone libraries from SML (*Psysml*) and UW (*Psyuw*). One representative sequence of each *Psycult*, *Psysml* and *Psyuw* OTU was included. The phylogenetic tree was obtained by using the neighbor-joining method and rooted using 16S rRNA gene sequences from two *Acinetobacter* species. Bootstrap values higher than 50% are shown in nodes.

study were only distantly related to previously described species (e.g. OTUs represented by isolates ENNN9_III/GSDP2_III, GCDN4_II/GSNP3_II, and ENNN4_II/GNDP5_II). Thus it is highly probable that novel species of *Psychrobacter* are present in our culture collection. These results suggest the existence of phylotypes adapted to temperate estuarine or marine aquatic environments. Salinity and contamination with hydrocarbons may play a role in the selection of these phylotypes (Bowman *et al.*, 1997; Lo Giudice *et al.*, 2010). Layer-specific clusters were not identified within the tree.

Final considerations

We conducted an in-depth analysis of the structure and dynamics of the *Psychrobacter* populations in Ria de Aveiro by combining culture-dependent and culture-independent approaches. A newly developed *Psychrobacter*-specific DGGE-based method was particularly helpful for the simultaneous analysis of a large number of samples, allowing the clear identification of compositional shifts in the communities' structure. However, since DGGE only assesses the most dominant members of the community (Muyzer *et al.*, 1993), differences between SML and UW communities were more clearly observed through the analysis of 16S rRNA gene libraries, which provided a more detailed picture of the phylogenetic composition of the *Psychrobacter* populations.

As often described by other authors (Kisand & Wikner, 2003; Brightwell *et al.*, 2009), culture-dependent and culture-independent approaches did not provide totally overlapping results. Although most of the cultivated OTUs were represented in the clone libraries, a higher diversity was assessed using culture-independent methods and groups characterized by culturing were under-represented when using culture-independent methods. Thus, the combination of the approaches gave a more comprehensive picture of *Psychrobacter* communities.

Our overall results revealed the presence in Ria de Aveiro of populations of *Psychrobacter* that are composed of a large diversity of members, suggesting that this genus is well-adapted to this environment. Also, different *Psychrobacter* populations were found in the SML and UW.

Observed diversity trends may be related to environmental factors such as salinity and/or anthropogenic pressures such as the presence of hydrocarbons.

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