World J Microbiol Biotechnol (2013) 29:183–189
DOI 10.1007/s11274-012-1159-2

SHORT COMMUNICATION

Cultivable psychrotolerant yeasts associated with Antarctic marine sponges

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Received: 13 June 2012 / Accepted: 20 August 2012 / Published online: 28 August 2012

Abstract Unlike filamentous fungi and bacteria, very little is known about cultivable yeasts associated with marine sponges, especially those from Antarctic seas. During an expedition to King George Island, in the Antarctica, samples of 11 marine sponges were collected by scuba-diving. From these sponges, 20 psychrotolerant yeast isolates were obtained. Phylogenetic analyses of D1/D2 and ITS rRNA gene sequences revealed that the marine ascomycetous yeast *Metschnikowia australis* is the predominant organism associated with these invertebrates. Other species found belonged to the *Basidiomycota* phylum: *Cystofilobasidium infirmominiatum*, *Rhodotorula pinicola*, *Leucosporidiella creatinivora* and a new yeast from the *Leucosporidiella* genus. None of these yeasts have been previously associated with marine sponges. A screening to estimate the ability of these yeasts as producers of extracellular enzymatic activities at several pH and temperature conditions was performed. Several yeast isolates demonstrated amylolytic, proteolytic, lipolytic or cellulolytic activity, but none of them showed xylanolytic activity under the conditions assayed. To our knowledge, this work is the first description of cultivable yeasts associated with marine sponges from the Antarctic sea.

Keywords Psychrotolerant yeasts · Antarctic marine sponges · Extracellular enzymes

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Introduction

Marine sponges (metazoans; phylum Porifera) are commonly known to harbor diverse microbes. Microbes can represent up to 50 % of the sponge tissue volume, exceeding microorganisms in seawater by 2–4 orders of magnitude (Wang 2006). The available information about the diversity of the sponge-associated microbes is still fragmentary. Numerous studies have shown a high diversity and abundance of bacteria, actinomycetes, and filamentous fungi in sponges (Olson and Kellogg 2010). On the contrary, information about sponge-associated yeasts is very scarce. Kutty and Philip (2008) pointed to only one study performed in 1962 which found that *Debaryomyces hansenii*, *Candida saitoana* (formerly *Torulopsis candida*) and *Trichosporon cutaneum* were associated with several invertebrates in the North Atlantic Ocean, including sponges. In recent times only three additional records of identification of yeasts associated with sponges have been published. *Pichia membranifaciens* was isolated from sponges from Japanese seas (Sugiyama et al. 2009). On the other hand, DNA sequences that phylogenetically are close to *Malassezia* were detected by molecular techniques in Hawaiian marine sponges (Gao et al. 2008). Finally, Burgaud et al. (2010) found three yeasts (*Meyerozyma guilliermondii* (formerly *Pichia guilliermondii*), *Rhodosporidium diobovatum* and an unidentified *Candida* species) associated with two sponges from deep-sea hydrothermal vent. Hence, although yeasts characterization in sponges would help to define the

wide spectrum of microorganisms associated with member of the phylum Porifera, their occurrence associated with these invertebrates remain as an almost unexplored topic.

As filter feeders, sponges swirl in a large volume of seawater containing organic particles. Hence, it is reasonable to expect that some microbes associated with sponges may produce hydrolytic enzymes to convert these organic matters into nutrients. Studies on enzymatic activities of microbes associated with sponges are rare and have been focused mainly on bacteria and filamentous fungi (Mohapatra et al. 2003; Baker et al. 2010). However, similar screenings in yeasts associated with marine sponges have not yet been performed.

Few studies have investigated the microorganisms that inhabit the Antarctic marine environment, and some of these environments, such sponges, remain largely unexplored (Webster et al. 2004). It is known that Antarctic sponges provide important habitat for bacteria, archaei, diatoms and dinoflagellates (Webster et al. 2004), but until now, most of the eukaryotic microorganisms inhabiting Antarctic sponges, especially yeasts, remain virtually ignored.

Here we report the first description of cultivable psychrotolerant yeasts associated with Antarctic marine sponges. In addition, a screening to estimate the ability of these yeasts as producers of enzymatic activities was performed.

Materials and methods

Sponge sampling

Fildes Bay (62°12′0″S 58°57′51″W) is located at the southwest side of King George Island, the largest island among the South Shetland Islands in the Antarctica. In this location, weather conditions allowed us to do two submersions. In these submersions, fragments of 11 marine sponges specimens belonging to genera *Dendrilla* sp., *Tedania* sp., *Hymeniacidon* sp., and 3 unidentified sponges belonging to the order Poecilosclerida (probably belonging to genera *Microciona* (*Clathria*) sp. and/or *Crella* sp.) were collected by hand around 6 m deep using scuba diving. In accordance with the Protocol on Environmental Protection to the Antarctic Treaty and regulations of Scientific Committee on Antarctic Research (SCAR), sponge fragments were restricted to a minimum size. Once collected, sponge fragments were transferred directly to sterile plastic bags (thus avoiding contact with air), kept cool, and transported to the laboratory facilities located by Fildes Bay.

Isolation of cultivable yeasts

For the isolation of yeasts, two parallel procedures for the processing of the sponges were used. First, samples of the inner tissues from each sponge were excised under sterile conditions with a scalpel and forceps, and directly spread onto Petri plates containing different culture media (see below). Alternatively, other samples of the inner tissues were homogenized with a minimal volume of 0.9 % NaCl. The resulting homogenate was spread onto Petri plates.

Culture media used were potato dextrose agar (PDA, Difco), PDAMM (PDA plus 2 g/L NaCl), GPY (1.0 g/L glucose, 0.1 g/L yeast extract, 0.5 g/L peptone, 15 g/L agar), and GPYMM (GPY plus 3 g/L NaCl). To prevent bacterial growth, these media contained benzyl penicillin and streptomycin (100 μg/mL each). Plates were initially incubated at 4 °C during 16 days, but not evidence of growth was detected after this time. Then, temperature was increased at 20 °C and after 1 week, growth of yeasts was observed. Temperature increase has been successfully used before to obtain yeasts from Antarctic samples (D'Ellia et al. 2009). Each yeast colony obtained was individually picked, spread onto fresh PDA plates by streaking, and incubated again at 20° C for 5 days. The above procedure was repeated until the obtainment of axenic cultures.

Yeast identifications

DNA from each yeast was isolated by means of the Bust n' Grab method (Harju et al. 2004) and used for the amplification of the D1/D2 and ITS regions. Amplifications of the D1/D2 region of 26S rRNA gene were carried out by PCR using conditions and primers described by Burgaud et al. (2010). The internal transcribed spacers segment of the 5.8 rRNA genes (ITS) of yeasts was amplified by PCR using conditions and primers previously described (Esteve-Zarzoso et al. 1999). Once amplified, PCR products were directly purified using Wizard SV Gel and PCR Clean-Up System and sequenced. D1/D2 and ITS regions sequences obtained in this work have been deposited in Genbank under the accession nos. JN181007 to JN181026, and JN197587 to JN197606, respectively.

Sequences obtained were submitted to the BlastN, thus obtaining matching with GenBank database sequences. Multiple alignments were carried out using ClustalX. Data from ClustalX were exported to the Mega 5 package to build phylogenetic trees using the Neighbor-joining method. The quality of the tree was examined by bootstrap re-sampling of the data sets with 1,000 replications.

Screening for enzymatic activities at different pHs and temperatures

Semi quantitative tests for amylase, protease, lipase, cellulase and xylanase were performed. Activities were assayed upon inoculation of yeast isolates on agar plates containing the suitable substrate for each activity. Amylase and protease activities were assayed according to Ganga and Martinez (2004) in media containing 1 % starch and 1.5 % defatted milk, respectively. Lipase activity was assayed using media containing 1 % tributyrine according to Paskevicius (2001). Cellulase activity was assayed using media containing 1 % carboxymethyl cellulose according to Pérez-González et al. (1993). Finally, xylanase activity was assayed using media containing 1 % xylan according to Chávez et al. (2002). Detailed assay conditions are described in Table 2.

Results and discussion

Cultivable yeasts from sponge samples

Yeasts were found associated with almost all the sponge samples studied (Table 1). In total, 20 isolates of yeasts were obtained, mostly from two sponge genera (*Tedania* sp. and *Hymeniacidon* sp.). All the yeasts described were obtained at 20 °C, and were able to grow in a relatively wide temperature range (being 4–23 °C the range of temperatures tested, data not shown), which suggests that they are psychrotolerant organisms. It has been described that the majority of isolates obtained from Antarctic environments appear to be psychrotolerant, rather than psychrophiles, which may be caused by local microclimate conditions, season of sampling (in Antarctica usually is in summer), ability of isolates to tolerate large variations in temperature, and laboratory conditions used in isolation (Robinson 2001; Loperena et al. 2012).

Our results suggest that cultivable yeasts are scarce in Antarctic marine sponges. According to Vishniac (2006), low temperatures inhibit primary productivity in Antarctica. Consequently, biodiversity of saprophytic yeasts in the Antarctic should be expected to be low. On the other hand, different to the seas at vicinity of heavily polluted areas where high quantities of yeasts are obtained, in non polluted seawater (such Antarctic seas) the yeast populations are normally low (Kutty and Philip 2008). In any case, the presence of many non-cultivable yeasts in our sponge samples cannot be ruled out. However, it must be emphasized that when culture-independent methods have been used to directly estimate diversity of fungi and yeasts inhabiting sponges, they usually failed and just revealed the presence of diatoms and dinoflagellates, in addition to sequences from the sponges themselves (Webster et al. 2004; Gao et al. 2008).

Identification of yeast isolates

Thirteen yeast isolates (representing 65 % of total) were identified as the ascomycetous yeast *Metschnikowia*

Table 1 Identity of yeasts obtained from Antarctic marine sponges samples

Sample	Isolate code	Yeast identity
Dendrilla sp. 1	131209-E3-C1-(GPY)-lev	*M. australis*
Tedania sp. 1	071209-E3-C1-II-lev	*M. australis*
	071209-E3-C2-II-lev	*M. australis*
Tedania sp. 2	071209-E5-C1-III-lev	*M. australis*
	071209-E5-C1-II-lev	*M. australis*
Tedania sp. 3	071209-E8-C3-II-lev	*M. australis*
	071209-E8-C1-II-lev	*M. australis*
	071209-E8-C1-IIb-lev	*C. infirmominiatum*
	071209-E8-C1-IIa-lev	*C. infirmominiatum*
	071209-E8-C4-II-lev	*L. creatinivora*
Hymeniacidon sp.1	071209-E4-C9-lev	*R. pinicola*
Hymeniacidon sp.2	131209-E2A-C2-II-lev	*M. australis*
	131209-E2A-C4-II-lev	*M. australis*
	131209-E2A-C5-II-lev	*C. infirmominiatum*
	131209-E2A-C1-II-lev	*C. infirmominiatum*
	131209-E2A-C3-II-lev	*Leucosporidiella* sp.
Poecilosclerida 1	071209-E2-C1-II-lev	*M. australis*
	071209-E2-C2-II-lev	*M. australis*
Poecilosclerida 2	131209-E1-C1-II-lev	*M. australis*
	131209-E1-C2-II-lev	*M. australis*

Three other sponge samples analyzed did not yield yeasts colonies

For each isolate, identification was obtained by D1/D2 and ITS sequencing

australis. So far, *M. australis* has been found only in Antarctic marine environments. This yeast was the most abundant and the only from the *Ascomycota* phylum found. Our result is the first description of *M. australis* inhabiting Antarctic marine sponges. Taken together, our results and the previous data (Kutty and Philip 2008; Loque et al. 2010) suggest that *M. australis* would be the most prevalent ascomycete found in almost all the Antarctic marine environments analyzed to date: seawater, sediments, several kind of marine invertebrates and several species of Antarctic macroalgae.

The seven remaining isolates belong to the phylum *Basiodiomycota*. Six of them were assigned unambiguously to three species identified previously: *Cystofilobasidium infirmominiatum* (four isolates), *Rhodotorula pinicola* (one isolate) and *Leucosporidiella creatinivora* (one isolate). However, one isolate presented 6 mismatches on 594 bp with respect to the closer D1/D2 sequences from two unidentified *Leucosporidiales* sp. strains isolated from Alaskan soils samples. In yeasts, it has been suggested as indication that strains that differ from the closest related type strain by three or more nucleotides in the D1/D2 sequence of the 26S rRNA gene could be considered to be different species (Fell et al. 2000). Thus, according the criteria described above, this isolate belongs to an entirely new and non-identified species of the *Leucosporidiella* genus. The phylogenic placement of *Leucosporidiella* sp. confirmed that this isolate is a new species (Fig. 1). Currently, its formal taxonomic description is in progress.

Current literature indicates that basidiomycetous yeasts (particularly *Cryptococcus* spp. strains) predominate in Antarctic habitats. However, *Cryptococcus* strains were not found in this study. This result can be due to a low population density and scarcity of cultivable yeasts in sponges. On the other hand, this result also has an ecological explanation. It has been described that yeasts in the class Ascomycetes are common in shallow waters. On the contrary, *Cryptococcus* and other yeasts in the class Basidiomycetes are common in deep waters (Kutty and Philip 2008). Our sponges were collected at 6 m deep, which could explain why the ascomycetous yeast *M. australis* was predominant, and why *Cryptococcus* isolates were not found.

None of the basidiomycetous yeasts described in this work have been previously associated with marine sponges. In addition, to our knowledge, this work is the first description of *R. pinicola* and *L. creatinivora* in any Antarctic environment. The finding of these yeasts in Antarctica extends its worldwide geographical distribution.

Screening for extracellular enzymatic activities

Enzymatic activities or the purification and characterization of some enzyme from yeast associated with marine sponges have not been previously described. Therefore, amylase, protease, lipase, cellulase and xylanase activities were screened. Results are summarized in Table 2. Eleven of the 20 isolates obtained produced amylase, lipase,

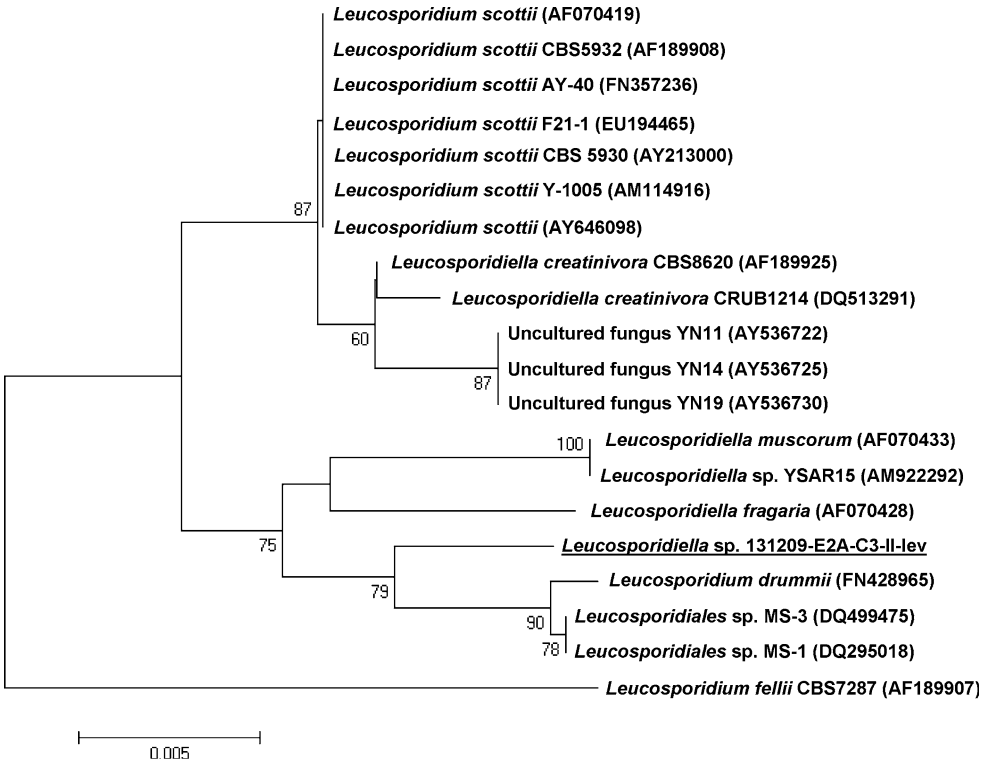

Fig. 1 Phylogenic placement of the *Leucosporidiella* sp. isolate identified in the present study (labeled as 131209-E2A-C3-II-lev and underlined) respect to the most related species previously submitted to Genbank. The tree was derived from neighbor-joining analysis using the 26S rRNA gene D1/D2 domain sequences. Numbers on branches represent bootstrap percentages from 1,000 replicates (values below 50 % are not shown). *Leucosporidium fellii* strain CBS7287 sequences was used as outgroup. GenBank accession numbers are indicated in parentheses. Topology of tree derived using ITS sequences was consistent with this result (data not shown)

Table 2 Summary of extracellular enzymatic activities detected in yeast from Antarctic marine sponges at different temperature and pH conditions

	4 °C			10 °C			15 °C			23 °C		
	pH 5	pH 7	pH 9	pH 5	pH 7	pH 9	pH 5	pH 7	pH 9	pH 5	pH 7	pH 9
C. infirmominiatum 071209-E8-C1-IIa-lev	Lipase			Amylase	Amylase	Amylase	Amylase	Amylase	Amylase	Amylase	Amylase	Amylase
				Lipase			Lipase			Lipase	Lipase	Lipase
C. infirmominiatum 131209-E2A-C1-II-lev		Amylase		Amylase	Amylase		Amylase	Amylase	Amylase	Amylase	Amylase	Amylase
				Lipase			Lipase	Lipase	Lipase	Lipase	Lipase	Lipase
C. infirmominiatum 131209-E2A-C5-II-lev	Amylase	Amylase		Amylase	Amylase	Amylase	Amylase	Amylase	Amylase	Amylase	Amylase	Amylase
C. infirmominiatum 071209-E8-C1-IIb-lev					Amylase		Cellulase	Amylase		Cellulase	Amylase	
								Cellulase			Cellulase	
M. australis 071209-E8-C3-II-lev					Amylase			Amylase			Amylase	
M. australis 071209-E8-C1-II-lev					Amylase			Amylase			Amylase	
M. australis 131209-E3-C1-(GPY)-lev								Lipase		Lipase	Lipase	
M. australis 131209-E2A-C4-II-lev	Lipase			Lipase			Lipase			Lipase		
R pinicola 071209-E4-C9-lev										Lipase	Lipase	
Leucosporidiella sp. 131209-E2A-C3-II-lev					Protease		Protease	Protease		Protease	Protease	
								Amylase			Amylase	
L. creatinivora 071209-E8-C4-II-lev					Protease		Protease	Protease		Protease	Protease	

For each yeast isolate, all the combinations of temperature and pH were analyzed in triplicate. Yeasts were grown for 7 days. Media were adjusted with MES 50 mM (pH 5.0), MOPS 50 mM (pH 7.0) or Tris–HCl 50 mM (pH 9.0)

protease and/or cellulase activities under the conditions tested. Seven isolates (two *M. australis*, *Leucosporidiella* sp. and four *C. infirmominiatum*) produce amylase activity. In addition, five isolates (two *M. australis*, two *C. infirmominiatum* and *R. pinicola*) produce lipase activity under the conditions tested. In the case of protease activity, we detected this activity in *Leucosporidiella* isolates (Table 2). It has been described that these three activities (mainly lipases) are frequently found among Antarctic yeasts obtained from maritime Antarctica (Loperena et al. 2012). Thus, our data indicates that these activities may be also broadly distributed among Antarctic yeasts inhabiting marine sponges.

Interestingly, cellulase activity was observed in one *C. infirmominiatum* isolate (Table 2). To our knowledge, there is only one recent report describing the presence of cellulase activity in yeasts obtained from maritime Antarctica (Loperena et al. 2012). In the marine Antarctic environment, the cell walls of green and red algae are source of cellulose (Collins et al. 2002). This result strongly suggests that this metabolic ability could be associated to Antarctic yeasts living in maritime environments. It is important to note that cellulose-degrading activity was not found when several yeasts isolated from European and South American cold freshwater habitats (sediments, melt water and ice samples from glaciers and temperate lakes) were analyzed (de García et al. 2007; Turchetti et al. 2008), which supports the previous suggestion. Finally, in the case of xylan degradation, none of the isolates grow or produce xylanase activity (data not shown).

Few Antarctic yeasts species have been tested by their ability to produce hydrolytic enzymes (Loperena et al. 2012; Buzzini et al. 2012). On the other hand, in recent years, several authors have suggested that yeasts from cold environments may have a potential auxiliary role as nutrient recyclers in their environments, hydrolyzing natural compounds by the secretion of extracellular hydrolytic enzymes (Turchetti et al. 2008; Loque et al. 2010; Buzzini et al. 2012). In the case of yeasts associated with Antarctic marine sponges, our work supports these suggestions, demonstrating that several of these yeasts would be able to hydrolyze some organic compounds in their environment.

Acknowledgments This work was supported by Instituto Antártico Chileno (INACH) grant G_06-10, FONDECYT grant 11090192, "Programa Bicentenario de Ciencia y Tecnología" (Chile) project PDA13, and DICYT-USACH. Fermín Acosta thanks "Programa IFARHU-MEF" (República de Panamá). We thank Antarctic collaborators Dr. José Darias, Dr. María Darias, Dr. Aurelio San Martín and Carol San Martín, and all the team of Professor Julio Escudero Base in Antarctica for allowing us to use their accommodations and laboratory facilities. We also thank to Karen Vergara for lab assistance and Dr. Jaime Eyzaguirre for critical reading of this manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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