Rhodotorula portillonensis sp. nov., a basidiomycetous yeast isolated from Antarctic shallow-water marine sediment

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During the characterization of the mycobiota associated with shallow-water marine environments from Antarctic sea, a novel pink yeast species was isolated. Sequence analysis of the D1/D2 domain of the LSU rDNA gene and 5.8S-ITS regions revealed that the isolated yeast was closely related to Rhodotorula pallida CBS 320 T and Rhodotorula benthica CBS 9124 T. On the basis of morphological, biochemical and physiological characterization and phylogenetic analyses, a novel basidiomycetous yeast species, Rhodotorula portillonensis sp. nov., is proposed. The type strain is Pi2 T (=CBS 12733 T =CECT 13081 T) which was isolated from shallow-water marine sediment in Fildes Bay, King George Island, Antarctica.

Introduction

It has been largely shown that yeasts inhabit several marine environments, such as coastal, estuarial, offshore and deep-sea habitats, among others (Gadanho et al., 2003; Nagahama, 2006; Fell, 2012; Nagano & Nagahama, 2012). However, the Antarctic marine ecosystem remains largely unexplored and few studies have investigated the yeasts that inhabit the Antarctic seas. To date, yeasts from the genera Candida, Cryptococcus, Cystoflobasidium, Guehomyces, Leucosporidium, Metschnikowia, Mrakia, Rhodosporidium, Rhodotorula, Synpodomyces, Sakaguchia and Sporopachydermia have been isolated from seawater, marine sediments, invertebrate organisms and algae, among other Antarctic marine environments (Nagahama, 2006; Kutty & Philip, 2008; Shivaji & Prasad, 2009; Loque et al., 2010; Hua et al., 2010; Song et al., 2010; Buzzini et al., 2012; Vaca et al., 2013). The importance of the study of Antarctic marine yeasts has been pointed by genus-wise distribution studies, which showed that marine yeasts from Antarctic oceans have the least similarity compared with the yeast biodiversity from the other oceans (Kutty & Philip, 2008). Hence, Antarctic oceans are interesting environments for the search of new yeasts.

The archipelago of the South Shetland Islands lies near the northern tip of the Antarctic Peninsula. This archipelago is separated from the peninsula by the Bransfield Strait. King George Island is the largest island among South Shetland Islands. The north side of the King George Island limits with the stormy Drake Passage, but at the south side, in front of the Bransfield Strait, there are three protected bays, one of them is Fildes Bay. Differing from other Antarctic regions, which are mostly cold deserts, with dry continental air, strong winds, and a sparse flora and fauna, the Fildes Bay area represents an example of a periglacial landscape, characterized by a cool, moist climate and a well-developed plant and animal life (Simonov, 1977). In summer, the average temperature usually does not exceed 0 °C, while in winter season average temperature is around −12 °C, and although snow falls throughout the year, it also can rain during the summer (annual rainfall is around 1250 mm). In Fildes Bay, the surface water temperature varies between 0.5 and 2 °C during the summer, and the water does not freeze even in winter in some years (Sakurai et al., 1996).

Several studies on yeast diversity of King George Island have been performed, but most of them have been mainly focused on the diversity present in soil samples (see
Carrasco et al., 2012 for a recent example). Thus, in the specific case of Fildes Bay, to the best of our knowledge there is only one report about diversity of yeasts associated with any marine environment at this location. Vaca et al. (2013) showed the presence of cultivable yeasts associated with Antarctic marine sponges from Fildes Bay. Yeasts found by these authors belonged to four genera, including the genus Rhodotorula Harrison.

The genus Rhodotorula comprises anamorphic basidiomycetous yeasts isolated from different origins including aquatic, hypersaline and psychrophilic environments (Nagahama et al., 2001, 2003; Libkind et al., 2003; Butinar et al., 2005; Margesin et al., 2007; Sampaio, 2011; de Garcia et al., 2012). Most species of this genus belong to the classes Microbotryomycetes and Cystobasidiomycetes of the subphylum Pucciniomycotina. Furthermore, a few species are classified in the classes Exobasidiomycetes and Ustilaginomycetes, subphylum Ustilaginomycotina (Bauer et al., 2006; Sampaio, 2011).

During a study of the mycobiotic community associated with shallow-water marine floor in Fildes Bay, a novel pink yeast strain was detected, and conventional taxonomic tests and rDNA sequence analysis were used for identification. Analysis of D1/D2 domains of the large subunit (LSU) and the 5.8-internal transcribed spacer (ITS) rDNA sequences indicated that this strain clustered phylogenetically within the order Cystobasidiales (class Cystobasidiomycetes), with a close relationship to the clade formed by Rhodotorula pallida, Rhodotorula benthica, Rhodotorula pinicola and Rhodotorula laryngis. On the basis of phenotypic and phylogenetic analysis, a novel species of the genus Rhodotorula is proposed.

Methods

Sample collection and yeast isolation. Samples were collected from Fildes Bay (62° 12' 0" S 58° 57' 51" W) at the southwest side of King George Island, Antarctica. In this location, four samples of starfish specimens (class Asteroidea), four samples of algae (two Desmarestia sp., one Ascoceira sp. and one unidentified sample), two samples of sea squirts (class Ascidacea), one sample of limpet (class Gastropoda) and one sample of rock were collected from Antarctic shallow-water marine sediment (Table S1, available in IJSEM Online). Six of these isolates were obtained from the rock sample, and the rest of isolates were obtained from algae (three isolates) and limpet (two isolates). No yeasts were obtained from the sea squirt or starfish samples. Most of yeasts (eight isolates) were isolated from PDA plates, and the rest (three yeasts) from DGPY plates (see Table S1 for details). No yeasts were obtained from PDAMM or DGPYMM plates. In particular, one pink-pigmented colony identified as strain Pi2T (CBS 127333T) was isolated from a PDA plate seeded with a rock sample.

Yeast characterization and identification. Morphological, physiological and biochemical tests were carried out as described by Yarrow (1998). All assimilation and fermentation tests were performed at 18 and 25 °C in triplicate and results were recorded after 1, 2 and 3 weeks. The effect of temperature was examined at 15–35 °C (at 5 °C intervals) on YPD (5 g l−1 yeast extract, 3 g l−1 peptone, 20 g l−1 glucose) and GPY (40 g l−1 glucose, 5 g l−1 peptone, 5 g l−1 yeast extract) agar plates for 15 days. Conventional taxonomic tests and rDNA sequence analysis were used for identification.

DNA sequencing and phylogenetic analysis. Total DNA was extracted by the CTAB method (cetyltrimethylammonium bromide) described by Kurtzman & Robnett (1998). The D1/D2 domain of the LSU rDNA gene was amplified and sequenced using primers NLI (5'-GCATACATAAAGCGAGAAAAG-3') and NL4 (5'-GGTCTC-GTGTTCAGACCG-3'), while the 5.8S-ITS region was amplified and sequenced using primers ITS1 (5'-TCCGATAGTAGACCT-GCCG-3') and ITS4 (5'-TCCCTCGGCITATTGATATGC-3') according to methods described by Kurtzman & Robnett (1998) and White et al. (1990). All amplification reactions were carried out on a Swift Thermal Cycler (Eisco GB Ltd), and the products were detected by electrophoresis on 1.5 % (w/v) agarose gels. The pattern band lengths were determined by comparison with DNA Step Ladder, 50 bp (57025; Sigma-Aldrich). Amplicons were sequenced on an ABI PRISM 310 genetic analyzer using a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as recommended by the manufacturer. Sequences were deposited in the GenBank database. Comparisons with sequences from the GenBank database were done using BLASTN (Altschul et al., 1997). Sequences were initially aligned using the multiple alignment program CLUSTAL W version 2.0 (Larkin et al., 2007). The phylogenetic trees were reconstructed using the neighbour-joining method (based on 1000 bootstrap iterations) with the MEGA version 5 software program (Tamura et al., 2011). Evolutionary distances data were calculated using the Kimura two parameter model (Kimura, 1980).

Results and Discussion

Eleven yeast isolates were obtained from the samples collected from Antarctic shallow-water marine sediment (Table S1, available in IJSEM Online). Six of these isolates were obtained from the rock sample, and the rest of isolates were obtained from algae (three isolates) and limpet (two isolates). No yeasts were obtained from the sea squirt or starfish samples. Most of yeasts (eight isolates) were isolated from PDA plates, and the rest (three yeasts) from DGPY plates (see Table S1 for details). No yeasts were obtained from PDAMM or DGPYMM plates. In particular, one pink-pigmented colony identified as strain Pi2T (CBS 127333T) was isolated from a PDA plate seeded with a rock sample. A MEGABLAST similarity search in the NCBI
database using the sequence of the 5.8-ITS rDNA region identified the novel strain as a member of the genus Rhodotorula, included within the order Cystobasidiales according to Sampaio classification (Sampaio, 2011).

Most species of the genus Rhodotorula included in the order Cystobasidiales (R. benthica, R. calyptogenae, R. laryngis, R. lysiniphila, R. minuta, R. pallida, R. pinicola and R. slooffiae) present similar morphological and physiological characteristics. Cells are subglobose to spherical, single or in pairs with predominantly polar budding. Colonies are glistening, butyrous to mucoid, and smooth with entire margins. The colony colour is typically pink, except the low-pigmented species R. pallida and some cream-coloured strains of R. slooffiae isolated in Finland.

The biochemical profile does not allow a proper discrimination between species. For this reason, until the publication of the extensive phylogenetic analysis carried out by Fell et al. (2000), over the past decades several species of the order Cystobasidiales were considered synonymous. A comparison of relevant chemotaxonomic properties of species of the genus Rhodotorula that belong to the order Cystobasidiales is shown in Table 1. Like R. pallida CBS 320T, strain Pi2T (=CBS 12733T) differed from the rest of the species of the genus Rhodotorula of the Cystobasidiales by its inability to assimilate sucrose. However, there were no other relevant biochemical differences distinguishing the novel species from the other species of this genus.

Regarding physiological characteristics, one of the most important differences between species is the ability to grow at temperatures above 35 °C. R. benthica, R. calyptogenae and some strains of R. minuta are able to grow at this temperature or higher, while the species of the order Cystobasidiales do not grow. There are also differences in cycloheximide resistance (0.01 %) between species; R. pinicola, R. pallida and some strains of R. laryngis are susceptible, while the other species are resistant. Strain Pi2T (=CBS 12733T) lacked the ability to grow at 35 °C and did not grow with 0.01 % cycloheximide (Table 1).

For accurate identification of these species, sequencing of the D1/D2 domain of the LSU rDNA gene and 5.8S-ITS regions is needed. Fell et al. (2000) suggested that basidiomycetous yeast strains that differ by two or more nucleotides in the D1/D2 domain of the LSU rDNA gene represent different taxa. Furthermore, they suggest that the taxonomy can be clarified by the analysis of the ITS region or in some cases by the intergenic spacer region. On this aspect, Scorzetti et al. (2002) conducted an extensive analysis of the ITS region sequences and could not determine a specific number of nucleotide differences to separate species. On the other hand, Bai et al. (2001) reported that to consider two species in the genus Bullera different, there should be a difference greater than 2 % in nucleotides in the 5.8S-ITS region. However, Hamamoto et al. (2002) proposed that in the genus Rhodosporidium, strains with 92 % or lower sequence similarity in the non-coding ITS regions should be considered to be different species.

The phylogenetic tree reconstructed using the neighbour-joining method, and based on D1/D2 sequences, showed that strain Pi2T (=CBS 12733T) clustered with the type strains of R. pallida CBS 320T (GenBank accession no. AF189962) and R. benthica CBS 9124T (AB026001) (Fig. 1a). Strain Pi2T (=CBS 12733T) showed five base substitutions (99.16 % sequence similarity) with respect to R. pallida CBS 320T and six base substitutions (99 % sequence similarity) with respect to R. benthica CBS 9124T. A similar analysis was performed using the 5.8S-ITS rDNA

Table 1. Comparison of phenotypic properties of Rhodotorula portillanensis sp. nov. with other recognized species of the genus Rhodotorula belonging to the order Cystobasidiales

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sequences and the results demonstrated that the 5.8S-ITS tree was congruent with the D1/D2 tree (Fig. 1b). The ITS locus of strain Pi2T showed 56 base substitutions (90.4% sequence similarity with 13 gaps) with respect to *R. pallida* CBS 320T (GenBank accession no. AF444590) and 24 base substitutions (95.9% sequence similarity with five gaps) with respect to *R. benthica* CBS 9124T (AB026001). On the other hand, BLASTN analysis of the nucleotide sequence of the D1/D2 domain of strain Pi2T (=CBS 12733T) with the closest phylogenetic relatives retrieved from the GenBank database, showed three base substitution (99.5% sequence similarity) with a uncultured fungus (clone NTS 032A 3 b12, GenBank accession no. KC557830) isolated from a desert soil sample in Nevada, USA; four base substitutions (99.33% sequence similarity) with respect to *Rhodotorula* sp. isolate YS74 (AM397866) [considered as a probable isolate of *R. pallida* by Sampaio (2011)]; and seven base substitutions plus two insertions (98.8% sequence similarity) with respect to *Rhodotorula* sp. CBS 9087 (AF444735) and CBS 9086 (AF444734), both included in the *Occultifur* clade and the *Erythrobasidium* lineage of the Cyto-basidiomycetes by Scorzetti et al. (2002) (Fig 1a). In addition, 5.8S-ITS rDNA sequences analysis revealed that strain Pi2T (=CBS 12733T) showed 32 base substitutions (94.2% sequence similarity with five gaps) with respect to *Rhodotorula* sp. CBS 9087 (AF444640) and CBS 9086 (AF444639) (Fig. 1b). Regarding the 5.8S-ITS sequence of the uncultured fungus (clone NTS 032A 3 b12) and the YS74 isolate, data were not found. Finally, the phylogenetic analysis using the combined D1/D2 and 5.8S-ITS rDNA datasets (Fig. S1) was congruent with the results obtained using the separate sequences.

Species of the genus *Rhodotorula* are ubiquitous saprophytic yeasts that can be recovered from many environmental sources. Several authors describe the isolation of this yeast from different ecosystems, and as an emerging opportunistic pathogen with the ability to colonize and infect susceptible patients (Wirth & Goldani, 2012). The yeast species closely related to strain Pi2T (=CBS 12733T) are associated with a variety of habitats around the world. According to the CBS online catalogue, the type strain of *R. pallida* (CBS 320T) has been isolated from a mycotic nodule in a white rat, and the type strain of *R. laryngis* (CBS 2221T) has been isolated from a laryngeal swab of human male in Norway. On the other hand, the type strain of *R. benthica* (CBS 9124T) has been isolated from a tubeworm collected from the deep-sea floor in Sagami Bay, Japan (Nagahama et al., 2003) and the type strain of *R. pinicola* (CBS 9130T) has been isolated from the xylem of *Pinus tabulaeformis* in Beijing (Zhao et al., 2002). In the case of strain YS74 (probably another isolate of *R. pallida*), it was isolated from plant bark in India (Bhadra et al., 2008), and the strains *Rhodotorula* sp. CBS 9087 and CBS 9086 were isolated from water in Antarctica. It is interesting to note that other strains of *R. laryngis* (not included in the phylogenetic trees) were isolated from children’s faeces in Hungary (CBS 5695), seawater in Sweden (CBS 8020), grapes in Australia (Prakitchaiwattana et al., 2004), nectarine trees in USA (Janisiewicz et al., 2010), the Dead Sea (Butinar et al., 2005), glaciers and freshwater habitats in Argentina and Italy (de Garcia et al., 2007; Turchetti et al., 2008; Libkind et al., 2010), and a soil sample from King George Island in Antarctica (Carrasco et al., 2012). Furthermore, other strains of *R. pinicola* (not included in the phylogenetic trees) were isolated from freshwater habitats in Argentina (Libkind et al., 2003, 2010), wines in Greece (Nisiotou & Gibson, 2005), an industrial malting ecosystem in Finland (Laitila et al., 2006), nectarine trees in USA (Janisiewicz et al., 2010) and marine sponges from Fildes Bay in Antarctica (Vaca et al., 2013). Such dissimilar substrates and environments in different regions of the world preclude the identification of the habitat of the genus *Rhodotorula*. However, these isolation sources demonstrate the adaptability of members of the genus *Rhodotorula* to freshwater and seawater, and cold conditions.

Sequences of the D1/D2 domain of the LSU rDNA gene of strain Pi2T (=CBS 12733T) indicated that the novel species was phylogenetically related with other Antarctic yeasts. According to our phylogenetic tree reconstructed with the sequences available in GenBank and using the proposed phenetic standard by Fell et al. (2000) as mentioned above, the clone NTS 032A 3 b12 (GenBank accession no. KC557830) probably represents a second representative of the proposed novel species.

As general rule, species descriptions should be based on multiple yeast strains. However, in some cases the obtainment of multiple yeast strains is very difficult. In Antarctica, low temperatures inhibit primary productivity and consequently, biodiversity of yeasts should be expected to be low (Vishniac, 2006). In the particular case of Fildes Bay, two recent reports suggest that the obtainment of cultivable yeasts from aquatic environments of this location is very difficult (Carrasco et al., 2012; Vaca et al., 2013). Our own results also suggest the difficulty to obtain cultivable yeasts from this Antarctic marine environment: from 12 different samples obtained, eight did not yield yeast colonies (Table S1). Thus, and compared with other geographical locations, yeasts seem to be sparse in Antarctic marine environments, making the recovery of multiple strains of the same yeast difficult. On the other hand, Antarctica is hardly accessible, and the organization of an expedition to this place is a huge and expensive venture. More importantly, the sampling in this remote and hostile place is subject to weather, geographical and logistical constraints, especially in the case of underwater sampling. As a consequence, diversity of marine microorganisms from Antarctica has been poorly studied. The yeast species here described was isolated from this very uncommon environment, and our results indicate that this novel species appears well-separated from previously
described, phylogenetically related species. Accordingly, we consider the description of this species important as a significant contribution to the current knowledge of the yeast biodiversity of marine Antarctic environments.

Based on phenotypic and phylogenetic analysis, strain Pi2T (=CBS 12733T) represents a novel species of the genus *Rhodotorula*, for which the name *Rhodotorula portillonensis* sp. nov., is proposed.
Latin diagnosis of *Rhodotorula portillonensis* Laich, Vaca & Chávez sp. nov


*Typus stirpis Pi2T (=CBS 12733T=CECT 13081T) isolatus ex petram superficiem oceanic fissa in insula King George, Antarctica, Chile. Colección Española de Cultivos Tipo (CECT), Valencia, Spain, et Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, deposita est.*

Description of *Rhodotorula portillonensis* Laich, Vaca & Chávez sp. nov.

*Rhodotorula portillonensis* (por.til.lo.nen’sis. N.L. fem. adj. portillonesis of or belonging to Portillo, named after Portillo village in Castilla-León, Spain, birthplace of Inmaculada Vaca who collected the yeast).

After 7 days on YM agar at 25 °C, colonies are circular (1.9–4.8 mm in diameter) or elliptical (up to 4.8 × 6.2 mm), pink–reddish-pigmented, butyrous, low convex, with circular and entire margins, and a smooth and glistening surface (Fig. 2a). On 5 % malt extract agar (ME5 %), colonies are similar to those on YM, but the morphology is predominately circular (1.5–3.4 mm in diameter), salmon-pink-pigmented and flat or low convex (flatter than on YM). Cells in YM broth at 25 °C for 2 days are usually ellipsoidal (2.9–3.9 × 3.9–5.8 μm) (Fig. 2b). In ME5 % broth, cells are usually spherical (2.9–3.9 μm in diameter). In both media, reproduction occurs singly or in pairs by polar budding. After 1 month at 25 °C, sediment and thin ring formation is observed. Pseudohyphae and hyphae are not observed in Dalmau plate culture or corn meal agar after 21 days at 25 °C. Fermentation ability (glucose) is absent. Assimilates D-glucose, D-galactose (weak), L-sorbose (weak), D-xyllose, α,α-trehalose, cellobiose, salicin (weak), arbutin, lactose (weak), melezitose, inulin (weak), glycerol, ribitol (weak), xyitol, D-glucitol, D-mannitol, 2-keto-D-Gluconate, 5-keto-D-gluconate, D-glucate, D-glucurionate, DL-lactate (weak), succinate, L-malic acid and ethanol. No growth occurs on D-glucosamine, D-ribose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, methyl α-D-glucoside, melibiose, raffinose, D-fructose, D-xylose, D-glucitol, D-mannitol, D-xylose, L-arabinose, L-rhamnose, arbutin, sucrose, maltose, methyl α-D-glucoside, melibiose, raffinose, starch, erythritol, L-arabinose, galactitol, myo-inositol, D-glucono-γ-lactone, D-galacturonate, citrate,
palatinose or methanol. Nitrogen compound assimilation tests are negative for nitrate, nitrite, ethylamine, cadaverine, creatine, creatinine and glucosamine, but weak for L-lysine. No growth occurs on vitamin-free medium or in the presence of cycloheximide (0.01 %), acetic acid (1 %) or D-glucose (50 and 60 %). Growth on 10 % NaCl plus 5 % glucose is weak, but in 16 % NaCl is negative. Urease hydrolysis and Diazonium blue B reactions are positive. No starch-like substance is produced. Acid production is negative. The maximum temperature for growth is 30 °C.

The type strain, Pi2T (=CBS 12733T =CECT 13081T) was isolated from a rock surface collected from shallow-water marine sediment in Fildes Bay, King George Island, Chilean Antarctica, in 2009. The MycoBank number is MB 804507.

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*Rhodotorula portillonensis* sp. nov., an Antarctic yeast