Cystobasidiomycetes yeasts from Patagonia (Argentina): description of *Rhodotorula meli* sp. nov. from glacial meltwater

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A basidiomycetous yeast, strain CRUB 1032^T, which formed salmon-pink colonies, was isolated from glacial meltwater in Patagonia, Argentina. Morphological, physiological and biochemical characterization indicated that this strain belonged to the genus *Rhodotorula*. Molecular taxonomic analysis based on the 26S rDNA D1/D2 domain and internal transcribed spacer region sequences showed that strain CRUB 1032^T represents an undescribed yeast species, for which the name *Rhodotorula meli* sp. nov. is proposed (type strain is CRUB 1032^T=CBS 10797^T=JCM 15319^T). Phylogenetic analysis showed that *Rhodotorula lamellibrachii* was the closest known species, which, together with *R. meli*, formed a separate cluster related to the *Sakaguchia* clade within the Cystobasidiomycetes. Additional Patagonian yeast isolates of the class Cystobasidiomycetes are also investigated in the present work.

INTRODUCTION

During a survey of carotenogenic yeasts inhabiting aquatic environments of glacier origin in Patagonia (Argentina), four novel yeast species were detected (Libkind *et al.*, 2003). Three of these species have already been described as *Sporobolomyces patagonicus*, *Sporidiobolus longiusculus* (Libkind *et al.*, 2005a) and *Cystofilobasidium lacus-mascardii* (Libkind *et al.*, 2009a). The fourth species was assigned to the basidiomycetous genus *Rhodotorula* based on phenotypic and molecular studies (Libkind *et al.*, 2003). This species clustered phylogenetically within the class Cystobasidiomycetes (subphylum Pucciniomycotina), with a close relationship to the *Sakaguchia* clade. This group is related to the order Erythrobasidiales (which includes *Rhodotorula minuta*) and currently comprises three species: *Sakaguchia dacryoidea* and two species of the genus *Rhodotorula*, *Rhodotorula lamellibrachii* and *Rhodotorula oryzae*. Here, a novel yeast species, strain CRUB 1032^T, isolated from freshwater and related to the *Sakaguchia* group is described and the name *Rhodotorula meli* sp. nov. is proposed. Physiological and molecular characterization of additional freshwater yeast isolates of the class Cystobasidiomycetes from Patagonia and of collection strains of closely related species is also included in this work.

Abbreviations: ITS, internal transcribed spacer; MSP-PCR, microsatellite-primed PCR; PSC, starch-like compounds.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are FJ807684 (*Rhodotorula slooffiae* PYCC 4761 26S rRNA gene), and FJ807683, FJ807685, FJ807686 and FJ807687 (complete ITS region, 5.8S, 18S and 26S rRNA genes of *R. meli* CRUB 1032^T, *R. slooffiae* PYCC 4761, *R. pinicola* CRUB 1028 and *R. slooffiae* CRUB 1029, respectively).

METHODS

Yeast isolation. Yeast strains of the genus *Rhodotorula* used in this report were isolated from freshwater environments of glacial origin in north-western Patagonia by filtering subsurface water samples, as described by Libkind *et al.* (2003), or were obtained from international culture collections (see Table 1). Isolate CRUB 1032^T was recovered in a 2002 summer survey. Further attempts to obtain additional strains of isolate CRUB 1032^T in subsequent years (2003 and 2004) were carried out in the Ventisquero Negro glacier using conventional culture media (Libkind *et al.*, 2003) or specifically designed medium: yeast nitrogen base (YNB) D-glucuronate agar (YNB, 67 g^{-1}; D-glucuronate, 5 g^{-1}; agar, 15 g^{-1}) containing 0.02 % chloramphenicol, pH 5. For the latter culture medium, larger volumes of water were filtered (up to 1 l).

Physiological and biochemical characteristics. The colour of cultures was assessed using the mycological colour chart of Rayner (1970). Complete physiological characterization of *R. meli* sp. nov. CRUB 1032^T, *Rhodotorula slooffiae* CBS 5706^T, *Rhodotorula laryngis* CBS 2221^T, *R. lamellibrachii* CBS 9598^T, *R. slooffiae* CRUB 1029 and *Rhodotorula pinicola* CRUB 1028 was performed. Tests were carried out in liquid media according to Yarrow (1998). Additional assimilation tests using aldaric acids and aromatic compounds were performed as described by Fonseca (1992) and Sampaio (1999),

Table 1. List of strains studied and their origin

R., *Rhodotorula*, *O.*, *Occultifur*, *S.*, *Sakaguchia.*

Species	Strain no.	Origin
O. externus	CBS 8732^T	Plant litter, Sintra, Portugal
O. externus	PYCC 4557	Plant litter, Sesimbra, Portugal
O. externus	PYCC 4823	Shore seawater, Portugal
R. lactosa	CBS 5826^T	Air
R. lamellibrachii	CBS 9598^T	Tubeworm, deep-sea floor, Japan
R. laryngis	CRUB 1105	Ilón lake, Patagonia
R. laryngis	CRUB 1183	Ventisquero Negro glacier, Patagonia
R. laryngis	CBS 2221^T	Laryngeal swab, human male, Norway
R. laryngis	CBS 5695	Child's faeces, Hungary
R. laryngis	CBS 8020 (=JCM 3778)	Seawater, sea off Sweden
R. marina	CBS 2365^T	Shrimp, Gulf of Mexico
R. meli	CRUB 1032^T (=CBS 10797^T=JCM 15319^T)	Ventisquero Negro glacier, Patagonia
R. minuta	CBS 319^T	Atmosphere, Tokyo, Japan
R. minuta	CBS 4407	Atmosphere, Shinjuku, Japan
R. minuta	CBS 2177	Shrimp, Gulf of Mexico Aransas Bay
R. minuta	CBS 4478	Seawater, USA, off Florida
R. minuta	CRUB 25	Mascardi lake, Patagonia
R. minuta	CRUB 76	Mascardi lake, Patagonia
R. minuta	CRUB 1136	Negra lake, Patagonia
R. pinicola	CRUB 1028	Nahuel Huapi lake, Patagonia
R. slooffiae	CBS 5706^T	Throat swab, human male, Hungary
R. slooffiae	PYCC 4887 (=JCM 3779)	Unknown
*R. slooffiae**	CBS 8019	Seawater, sea off Sweden
R. slooffiae	PYCC 4761	Paper mill effluent, Setúbal, Portugal
R. slooffiae	PYCC 4762	Plant litter, Sesimbra, Portugal
R. slooffiae	PYCC 4763	Pine cone, Oeiras, Portugal
R. slooffiae	CRUB 1029	Nahuel Huapi lake, Patagonia
R. pallida	CBS 320^T	Mycotic nodule in white rat
S. dacryoidea	CBS 6353^T	Seawater, Antarctic
S. dacryoidea	CBS 6356	Seawater, Antarctic
S. dacryoidea	A41	Seawater, Faro, Portugal

*Deposited as *R. minuta* in CBS.

respectively. Mycosporine synthesis in strain CRUB 1032^T was studied previously by Libkind *et al.* (2005b).

Molecular characterization. For PCR fingerprinting, the microsatellite-primed (MSP)-PCR technique was employed. The DNA extraction protocol, primers, PCR and electrophoresis conditions and gel image analysis procedures were those reported in Sampaio *et al.* (2001). For DNA sequence analysis, DNA extraction, PCR amplification, purification and cycle sequencing followed the protocol of Sampaio *et al.* (2001). DNA was amplified using primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) and LR6 (5′-CGCCAGTTCTGCTTACC). Cycle sequencing of the 600–650 bp region at the 5′ end of the 26S rDNA (D1/D2 domains) employed forward primer NL1 (5′-GCATATCAATAAGCGGAGGAAAAG) and reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG). The internal transcribed spacer (ITS) region was sequenced using the forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG) and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC). Sequences were obtained using an Amersham Pharmacia ALF express II automated sequencer using standard protocols. Alignments were made with BioEdit V7.0.9 and corrected visually (Hall, 1999). Phylogenetic analysis was carried out using the neighbour-joining method (Saitou & Nei, 1987). Evolutionary distances were computed using the Kimura two-parameter method (Kimura, 1980) and bootstrap analysis was inferred from 1000 replicates (Felsenstein, 1985). All positions containing gaps or missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted with MEGA4 (Tamura *et al.*, 2007).

RESULTS AND DISCUSSION

Phenotypic and molecular investigations

During our investigations on the diversity of carotenogenic yeasts in Patagonian freshwater habitats, eight strains producing light pink colonies were isolated. A notable characteristic shared by the eight strains was that colonies in malt extract-yeast extract-peptone agar were highly mucous and sticky; this characteristic was less evident in richer culture media (i.e. yeast extract-malt extract agar or yeast extract-peptone-glucose). All strains showed

physiological characteristics that resembled those of the Cystobasidiomycetes yeasts (Bauer *et al.*, 2006), namely, the absence of inositol and nitrate assimilation, and production of starch-like compounds (PSC), and the presence of D-glucuronate assimilation and mycosporine synthesis. Libkind *et al.* (2005b) showed that the ability to synthesize mycosporines and the lack of PSC are sufficient criteria to assign a red-pigmented yeast isolate to the class Cystobasidiomycetes.

MSP-PCR fingerprinting studies were performed on the eight Patagonian strains and on several type and collection strains of yeast species belonging to the class Cystobasidiomycetes and related taxonomic groups. For *R. minuta*, *R. slooffiae*, *R. laryngis* and *Sakaguchia dacryoidea*, more or less conserved MSP-PCR banding patterns were obtained, given that most strains of each species had similar profiles (Fig. 1). This result highlights the usefulness of the MSP-PCR technique (with the M13 primer) for the rapid grouping and identification of relevant yeast species of the class Cystobasidiomycetes. Strain CBS 8019, deposited as *R. minuta*, had a similar DNA profile to *R. slooffiae* strains, suggesting that it might have been misidentified. Among the Patagonian strains, five different groups of DNA banding patterns were revealed (MSP-PCR groups). A comparison of the MSP-PCR profiles of type strains with those of the Patagonian strains showed that the MSP-PCR group that included isolates CRUB 1105 and CRUB 1183 had DNA patterns similar to *R. laryngis*, whereas the MSP-PCR group that included isolates CRUB 25, CRUB 76 and CRUB 1136 shared identical DNA patterns with *R. minuta*. These results were confirmed by sequence analysis of the D1/D2 region of the 26S rDNA (Fig. 2) and the ITS region (data not shown). Each of the remaining three Patagonian strains had a unique DNA profile, which differed from the collection strains tested, suggesting a different taxonomic position for each strain. Sequencing of the D1/D2 region indicated that these three strains belonged to *Rhodotorula pinicola* (100 % similarity to CRUB 1028), *R. slooffiae* (one mismatch with CRUB 1029) and to a novel yeast species closely related to *R. lamellibrachii* (six mismatches with CRUB 1032^T), for which the name *Rhodotorula meli* sp. nov. is proposed in this study. Further confirmation was obtained by ITS sequence analysis, which showed that *R. meli* sp. nov. CRUB 1032^T (GenBank accession no. FJ807683) and *R. lamellibrachii* JCM 10907^T (AB025999) differed by 22 nt. As depicted in Table 2, *R. meli* sp. nov. could be distinguished easily, on the basis of phenotypic characteristics, from *R. lamellibrachii* and other closely related species. *R. meli* differed from *R. lamellibrachii* in the assimilation of D-ribose, salicin, erythritol, D-mannitol, D-glucuronate, DL-lactate, succinate and citrate (positive for the former); and in the assimilation of maltose (weak), melezitose and growth at 30 °C (positive for the latter).

The yeast species closely related to *R. meli* sp. nov. seem to be mainly associated with aquatic habitats and, in particular, to marine environments. *S. dacryoidea* has been isolated exclusively from deep and surface seawaters of the Pacific, Indian (Fell *et al.*, 1973) and Atlantic Oceans (Gadanho *et al.*, 2003). *R. lamellibrachii* and *R. oryzae* have been isolated less frequently; only one strain of each species is known. *R. lamellibrachii* was collected from a deep-sea tubeworm in the north-west Pacific Ocean (Nagahama *et al.*, 2001), whereas *R. oryzae* was isolated from paddy rice (Bai *et al.*, 2004). Despite numerous attempts, additional isolates of *R. meli* were not obtained in subsequent years, either from the same glacier or from nearby water bodies (Libkind *et al.*, 2003, 2009b; Libkind, 2006), even when using specifically designed isolation media. Furthermore, *R. meli* sp. nov. was not even recovered during a comprehensive study on the diversity of cold-tolerant yeasts from Mount Tronador glaciers (de García *et al.*, 2007). This indicates that the occurrence of *R. meli* sp. nov. in glacial meltwater is low and suggests that this may not be the original habitat of this yeast. Several studies on the yeast diversity of soil and phylloplane of native forests in north-western Patagonia have been, and are being, carried out, but no strains of *R. meli* sp. nov. have been found so far.

Latin diagnosis of *Rhodotorula meli* Libkind, van Broock *et* Sampaio sp. nov.

Fungus Cystobasidiomycetous (*Pucciniomycotina*). *In statu unicellulari cellulae elongate ad cylindraceae*, 2–4 × 4–7 μm. *Mycelium verum non formatur. Fermentatio nulla.* D-*Glucosum*, D-*galactosum*, D-*ribosum*, L-*arabinosum*, *sucrosum*, α,α-*trehalosum*, *salicinum*, *arbutinum*, *glycerolum*, D-*mannitolum*, D-*glucuronatum*, DL-*lactatum*, *succinatum et citratum assimilantur.* L-*Sorbosum*, D-*glucosaminum*, D-*xylosum*, D-*arabinosum*, L-*rhamnosum*, *maltosum*, *cellobiosum*, *melibiosum*, *lactosum*, *raffinosum*, *melezitosum*, *inulinum*, *amylum*, *erythritolum*, *ribitolum*, *xylitolum*, D-*glucitolum*, *galactitolum*, *inositolum*, D-*glucono*-1,5-*lactonum*, *ethanolum et methanolum non assimilantur. Substrata nitrogenica assimilate*: L-*lysinum*; *neque nitratum*, *nitritum*, *ethylaminum*, *creatinum et* D-*glucosaminum. Vitamina externa at crescentiam necessaria sunt.* 25 °C *crescit neque* 30 °C. *Materia amyloidea iodophila non formantur. Urea finditur. Reactio Diazonii coerulei* B *positivum. Sequentiae acidi nucleici* ITS *et partium* D1/D2 *submonadis majoris ribosomalis typi in collectione sequentiarum acidi nucleici* NCBI (www.ncbi.nlm.nih.gov) *numeris* FJ807683 *et* AY158654 *depositae sunt. Cultura typica* CBS 10797^T *in* Centraalbureau voor Schimmelcultures, *Ultrajecti*, *Hollandia*, *et* JCM 15319^T *in* Japanese Collection of Microorganisms, Japan, *conservatur.*

Description of *Rhodotorula meli* Libkind, van Broock & Sampaio sp. nov.

Rhodotorula meli (me′li. *meli* meaning number four spelled in Mapuche aboriginal language, given that it is the fourth carotenogenic yeast species described from Patagonia).

After 7 days on MYP agar at 22 °C, colonies are light pink, glistening, mucoid and smooth with entire margins. Cells are elongated to cylindrical (2–4 × 4–7 μm) and multiply

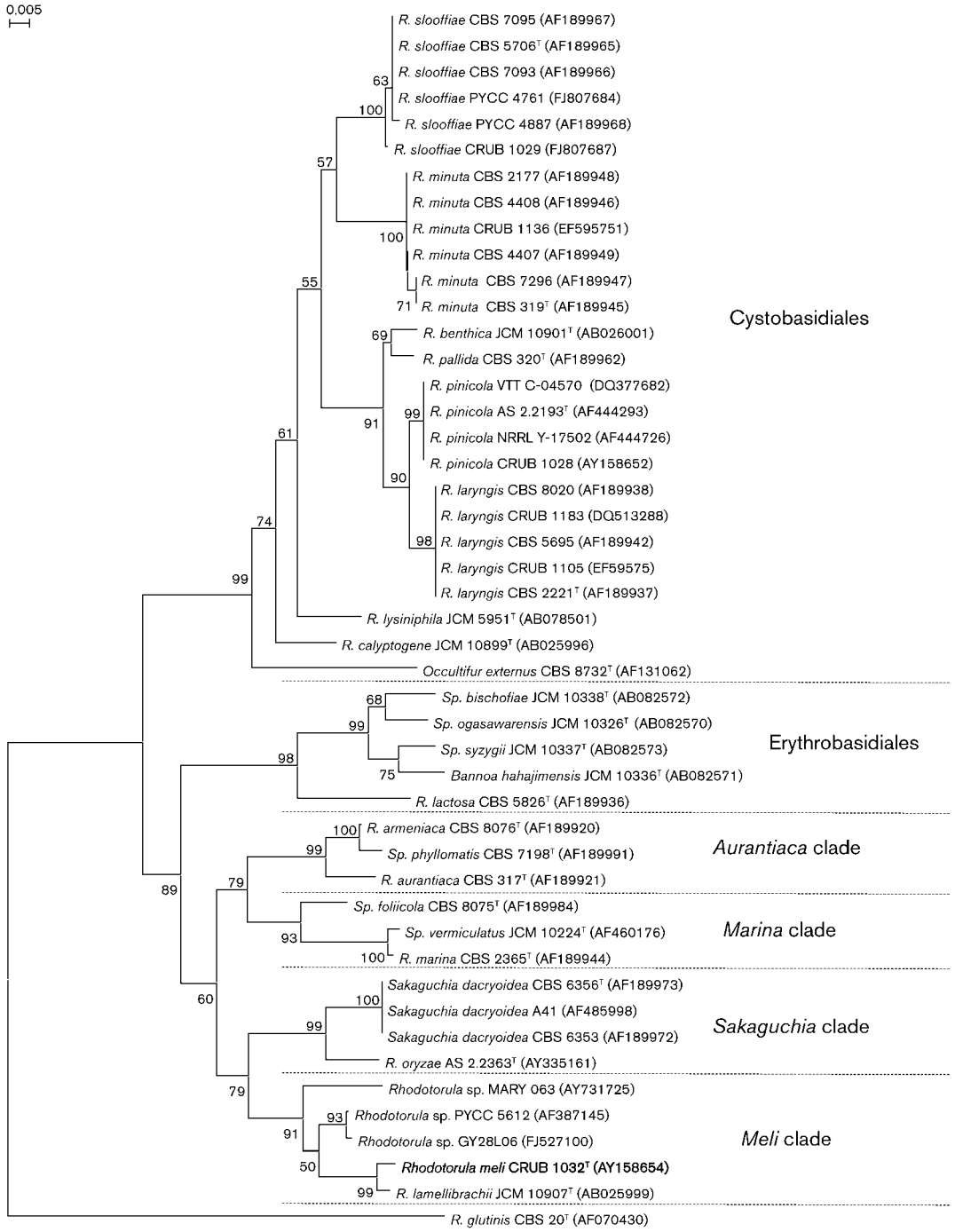

Fig. 1. Phylogenetic placement of *R. meli* sp. nov. and other Patagonian *Rhodotorula* spp. isolates (in bold) within the class Cystobasidiomycetes inferred using the neighbour-joining method based on alignment of nuclear DNA sequences of the D1/D2 region of the 26S rRNA gene. Evolutionary distances were computed using the Kimura two-parameter method. *R. glutinis* was used as the outgroup. Branch lengths are in substitutions per site. Numbers on the branches are bootstrap values (1000 replicates). GenBank accession numbers of the sequences are indicated after the species name. *Sp.*, *Sporobolomyces*. Bar, 0.005 base substitutions per site.

by multilateral budding (Fig. 3). Glucose is not fermented. Mycelium or pseudo-mycelium are not produced. Glucose, D-galactose, D-ribose, L-arabinose, sucrose, α,α-trehalose, salicin, arbutin, glycerol, D-mannitol, D-glucuronate, DL-lactate, succinic acid, citric acid, L-tartaric acid (slow and weak), D-tartaric acid (slow and weak), vanillic acid (slow

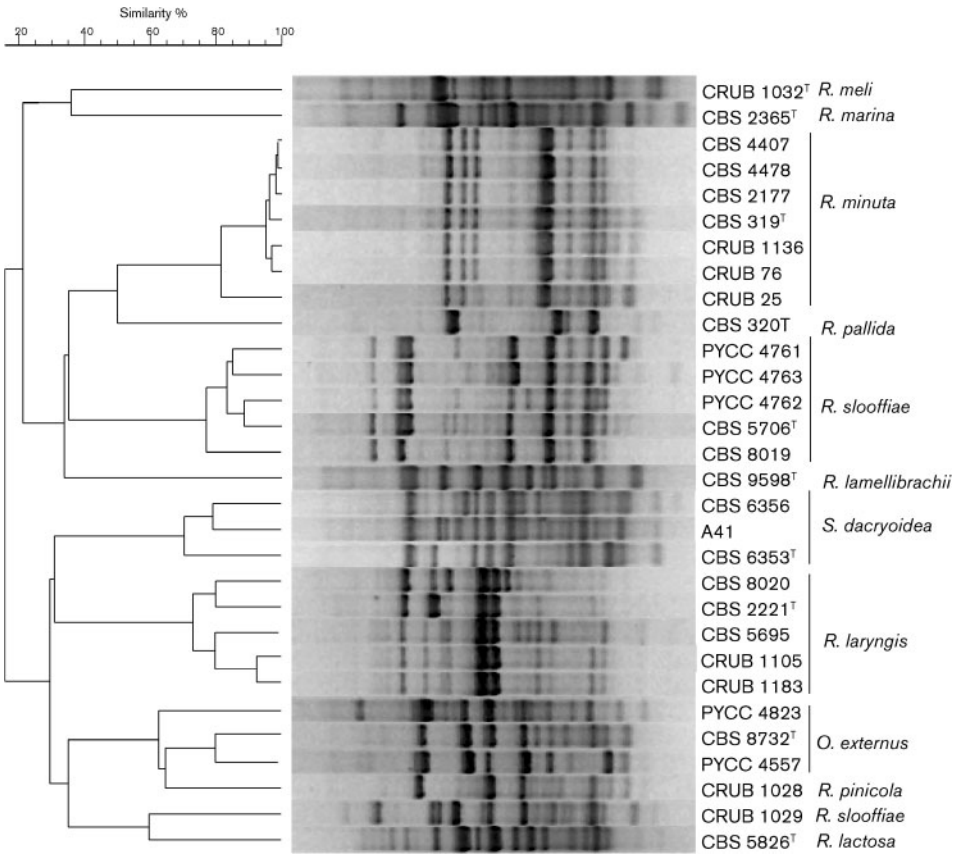

Fig. 2. MSP-PCR analysis of Patagonian isolates of the class Cystobasidiomycetes employing M13 primers. Cophenetic coefficient of 92.1.

Table 2. Salient physiological differences between *Rhodotorula meli* sp. nov. and closely related species of the Cystobasidiomycetes

Species: 1, *R. meli* sp. nov. 2, *R. lamellibrachii*; 3, *S. dacryoidea*; 4, *R. oryzae*; 5, *R. marina*. +, Growth; −, no growth; S, slow growth; W, weak growth; V, variable; ND, not determined. Physiological information for species other than *R. meli* sp. nov. was obtained from Bai *et al.* (2004), Nagahama *et al.* (2001) and the CBS database.

Characteristic	1	2	3	4	5
D-Ribose	+	−	−	−	S
Maltose	−	W	V	−	+
Salicin	+	−	−	−	+
Melezitose	−	+	−	+	W, S
Erythritol	+	−	−	−	−
D-Mannitol	+	−	+	+	+
D-Glucuronate	+	−	+	ND	−
DL-Lactate	+	−	S	−	−
Succinate	+	−	+	+	+
Citrate	+	−	+	+	W, S
Growth at 30 °C	−	+	−	+	+

and weak), *p*-hydroxybenzoic acid (slow and weak) and *m*-hydroxybenzoic acid (slow and weak) are assimilated as carbon sources. Carbon sources not assimilated are

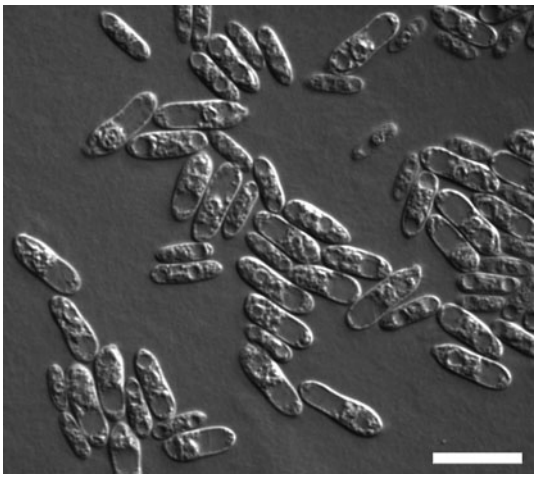

Fig. 3. Vegetative cells of *R. meli* sp. nov. CBS 10797^T after 3 days in MYP agar at 20 °C. Bar, 10 μm.

L-sorbose, D-glucosamine, D-xylose, D-arabinose, L-rhamnose, maltose, methyl α-D-glucoside, cellobiose, melibiose, lactose, raffinose, melezitose, inulin, soluble starch, erythritol, ribitol, xylitol, D-glucitol, galactitol, inositol, glucono-δ-lactone, methanol, ethanol, L-malic acid, *m*-tartaric acid, saccharic acid, mucic acid, veratric acid, ferulic acid, protocatechuic acid, catechol, gallic acid, salicylic acid, gentisic acid and phenol. L-Lysine is assimilated as a nitrogen source. No growth is observed using nitrate, nitrite, ethylamine, creatine or D-glucosamine as nitrogen sources. Growth in vitamin-free medium is negative. Mycosporine synthesis is positive. Grows at 25 °C, but not at 30 °C. PSC are not produced. Does not grow in 100 μg cycloheximide ml^{-1}. Urease activity is positive. Diazonium Blue B reaction is positive.

The type strain is CRUB 1032^T (=CBS 10797^T=JCM 15319^T), isolated by D. Libkind in February 2009 from meltwater of the Ventisquero Negro glacier of the Tronador Mount in north-western Patagonia, Argentina (41° 24′ S 71° 83′ W).

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