Cryptococcus tepidarius sp. nov., a thermotolerant yeast species isolated from a stream from a hot-spring area in Japan

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Anamorphic basidiomycetous yeast strains M9962^T and M9963 were isolated from water samples collected from a small stream in Ohwakudani, Hakone, a hot-spring area in Japan. These belonged to a single species and were phylogenetically closely related to *Bullera lagerstroemiae*, although the ability to form ballistoconidia was not observed. Based on sequence analyses of the D1/D2 domain of the LSU rDNA and ITS regions and differences in G + C content, the name *Cryptococcus tepidarius* sp. nov. Takashima, Sugita, Toriumi et Nakase (Trichosporonales, Tremellomycetes, Basidiomycota) is proposed for these isolates, with strain M9962^T (=SP-5^T =JCM 11965^T =CBS 9427^T) as the type strain. The strains grew in YM broth at 47 °C and in YM broth with the pH adjusted to 1.5 by HCl, indicating that the species is thermotolerant and acid-tolerant.

INTRODUCTION

Thermophilic fungi are defined as those that grow above 48 °C and do not grow below 20 °C (Watson, 1987). Strains that can grow at temperatures approaching 48 °C and also below 20 °C are called methophilic (Watson, 1987), although the term ‘thermotolerant’ is commonly used for yeasts that can grow at such high temperatures. Thermotolerant yeasts have the advantage that they can be cultured under conditions where other micro-organisms cannot grow, which reduces the risk of contamination. Proteins made by thermotolerant yeasts are also expected to have thermotolerant characteristics. Thermotolerant enzymes made by yeasts may possess special advantages because of their eukaryotic nature, compared with enzymes from thermotolerant bacteria or archaea. The mechanisms by which they are able to grow in more extreme environments, especially as they affect membranes and other lipid systems, are also of interest (Deak, 2006).

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the ITS region and LSU rDNA D1/D2 domain sequences of strain M9962^T are AB094045 and AB094046, respectively.

Results of growth of strain M9962^T at different temperatures and pH are available as supplementary material with the online version of this paper.

In the course of a study on the diversity of yeast species in various environmental samples, we isolated two thermotolerant, anamorphic, hymenomycetous yeasts from water samples taken from a small stream at Ohwakudani, Hakone, a hot-spring area in Japan. Based on analyses of internal transcribed spacer (ITS) and large-subunit (LSU) rDNA D1/D2 domain sequences and G + C content, these strains were found to represent a novel species phylogenetically related to *Bullera lagerstroemiae* Fungsin *et al.* (2006) in the Trichosporonales, Tremellomycetes, Basidiomycota.

METHODS

Strains M9962^T and M9963 were isolated on 18 March 2001 from water samples collected from different locations in the stream. Steam is released continuously from the ground in this area, and the atmosphere is sulfurous. Although the pH and temperature were not measured in the samples, the water in this area is acidic as a result of the presence of sulfuric and/or sulfurous ions (Itoh *et al.*, 2002). Approximately 100 µl water was spread on Sabouraud agar plates containing 50 µg chloramphenicol ml⁻¹ (Sankyo) and incubated at 27 °C and colonies were isolated and examined. Most morphological, physiological and biochemical characteristics were examined according to standard methods (Yarrow, 1998). Assimilation of nitrogen compounds was investigated on solid media using a starved inoculum. Vitamin requirements were determined by the method of Komagata & Nakase (1967).

Growth temperature. The growth temperature was determined using a temperature-gradient shaking incubator (TVS 126MA; Advantec Toyo). Growth at 12 temperatures between 24 and 60 °C was determined in YM broth, and optical density at 660 nm was monitored with the apparatus.

Growth pH range. The growth pH range was determined using YM broth after adjusting the pH to values ranging from 1.15 to 8.61 using HCl or NaOH and sterilization by filtration. Culture tubes were incubated with shaking and the optical density at 660 nm was monitored in the Advantec apparatus at a constant temperature of 37 °C.

Major ubiquinone. Cells were grown in 500 ml Erlenmeyer flasks containing 250 ml YM broth on a rotary shaker at 150 r.p.m. at 25 °C and were harvested in the early stationary growth phase and then washed with distilled water. Extraction, purification and identification of ubiquinones were carried out according to the method of Nakase & Suzuki (1986).

DNA G+C content. Cells were grown in 500 ml Erlenmeyer flasks containing 250 ml YM broth on a rotary shaker at 150 r.p.m. at 25 °C and were harvested in the exponential growth phase and then washed with distilled water and freeze-dried. Isolation and purification of nuclear DNA were done following Takashima & Nakase (2000). The DNA base composition was determined by HPLC after enzymic digestion of DNA to deoxyribonucleosides (Tamaoka & Komagata, 1984). A DNA-GC kit (Seikagaku Corp.) was used as a quantitative standard.

Sequencing and phylogenetic analysis. Nuclear DNA was extracted by the method of Makimura *et al.* (1994). The ITS regions, including the 5.8S rDNA, were amplified by PCR according to Sugita & Nakase (1999). The D1/D2 domain of the LSU rDNA was amplified following Kurtzman & Robnett (1997). PCR products were sequenced directly using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and analysed with an Applied Biosystems sequencer model 310 according to the manufacturer's instructions. Reference sequences used for the phylogenetic study were obtained from the DDBJ/GenBank/EMBL databases. The sequences were aligned with those of related species using CLUSTAL w version 1.83 (Thompson *et al.*, 1994) and the alignment was adjusted manually. The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). Evolutionary distances were calculated according to Kimura (1980). Sites where gaps existed in any sequences were excluded. Bootstrap analyses (Felsenstein, 1985) for the neighbour-joining method were performed from 100 random resamplings.

RESULTS AND DISCUSSION

Taxonomic position

Because strains M9962^T and M9963 showed identical sequences in the LSU rDNA D1/D2 domain and ITS/5.8S rDNA, these isolates are thought to represent a single species. The phylogenetic tree shown in Fig. 1 is based on

Fig. 1. Neighbour-joining tree of strain M9962^T (*Cryptococcus tepidarius* sp. nov.) and related species based on LSU rDNA D1/D2 domain sequences. Evolutionary distance was calculated according to Kimura (1980). Numerals represent values from 100 replicate bootstrap samplings (values less than 50 not shown). Sequences were retrieved from the DDBJ/GenBank/EMBL databases under the accession numbers indicated.

sequences of the LSU rDNA D1/D2 domain. Strain M9962^T formed a clade with *Bullera formosensis*, *Bullera koratensis* and *B. lagerstroemiae* (labelled the *B. formosensis* clade) in the Trichosporonales, Tremellomycetes, Basidiomycota. Sequence similarities to the type strain of the closest species *B. lagerstroemiae* were 99.8 % in the LSU rDNA D1/D2 domain (one substitution) and 93.6 % in the overall ITS region (ITS1, 6 substitutions and 2 indels; ITS2, 7 substitutions and 3 indels), indicating that strain M9962^T is closely related to *B. lagerstroemiae*, but should be regarded as a separate species based on the generalization of Sugita *et al.* (1999). The DNA G + C content of strain M9962^T (42.0 mol%) was 4 mol% lower than that of *B. lagerstroemiae*, also suggesting separate species status (Kurtzman & Phaff, 1987). Based on these results, we concluded that our isolates represent a distinct species. The higher growth temperature of this species also supported this separation (Van Uden, 1984). The physiological and biochemical properties of this species were similar to those of *B. formosensis*, *B. koratensis* and *B. lagerstroemiae*, but the latter species differ by their ability to assimilate sodium nitrite and their inability to grow at 37 °C (Table 1).

The Trichosporonales includes species assigned to the genera *Trichosporon*, *Cryptotrichosporon*, *Asterotremella*, *Cryptococcus* and *Bullera* (Fig. 1). *Cryptococcus* and *Bullera* are polyphyletic and their nomenclatural types belong to the clade Tremellales (Takashima & Nakase, 1999; Fell *et al.*, 2000; Fell *et al.*, 2001; Scorzetti *et al.*, 2002). According to the phylogenetic analysis of Okoli *et al.* (2007), *Cryptotrichosporon anacardii* occurred at a basal position with respect to the clade designated the *B. formosensis* clade in this paper. Strain M9962^T belongs to this clade (bootstrap value 96 %), but whether *Cryptotrichosporon anacardii* is also a member is not so

clear (bootstrap value 56 %). Another new genus, *Asterotremella*, was proposed for *Cryptococcus humicola*, *Cryptococcus longus*, *Cryptococcus musci* and *Cryptococcus pseudolongus*, in the Trichosporonales (Prillinger *et al.*, 2007). Consequently, phylogenetically poorly defined *Cryptococcus* species (*Cryptococcus curvatus*, *Cryptococcus daszewskae* and *Cryptococcus fragicola*) and the species in the *B. formosensis* clade remain in the Trichosporonales (Fig. 1). This situation will remain until a robust multigene study is conducted on this group of basidiomycetous yeasts. Species in the *B. formosensis* clade appear to be monophyletic.

Based on the above results, we conclude that the species represented by strains M9962^T and M9963 should provisionally be assigned to the polyphyletic genus *Cryptococcus*, in agreement with the genus concept of Fell & Stetzell-Tallman (1998), as the ability to form ballisticonidia has not been observed in strains M9962^T and M9963. However, it should be transferred to an appropriate genus when a robust phylogeny of the hymenomycetous yeasts is available and the phenotypic characteristics of each genus are better defined. The name *Cryptococcus tepidarius* sp. nov. is proposed for strains M9962^T and M9963.

Thermotolerant and acid-tolerant characteristics

Our study of the temperature dependence of growth showed that *Cryptococcus tepidarius* M9962^T had almost the same growth profile between 24 and 39 °C (Supplementary Fig. S1, available in IJSEM Online). The OD₆₆₀ reached 1.0 within 24 h. At 41.8 °C, growth was delayed slightly compared with 24–39 °C, but good growth was shown within 100 h; growth was also observed at 45.6 and 47.7 °C, although it was further delayed. At 54.9 and 60 °C, no increase in OD₆₆₀ was detected after 700 h of incubation. Many ascomycetous yeasts can grow at 40 °C (Barnett *et al.*, 2000) and *Ogataea thermophila*, reported to grow at 50 °C (Shin *et al.*, 2001; Péter *et al.* 2007), has the highest maximum growth temperature known to date. In the basidiomycetous yeasts, some pathogenic yeasts such as *Filobasidiella neoformans* are capable of growth at 40 °C. Nagahama *et al.* (2003) reported that the maximum growth temperature of *Rhodotorula benthica* and *Rhodotorula calyptogenae*, isolated from a deep-sea habitat, was 41–44 °C, but neither grew at 45 °C. We therefore conclude that *Cryptococcus tepidarius* has the highest maximum growth temperature reported so far for a basidiomycetous yeast.

Our study of pH-dependent growth of *Cryptococcus tepidarius* M9962^T showed that good growth occurs at pH 1.5–8.6, with delayed and weak growth at pH 1.2 (Supplementary Table S1). The general pH range for growth for yeasts is said to be pH 2–7, with an optimum at pH 4.0–4.5 (Deak, 2006). Walker (1977) reported that a few yeasts involved in food spoilage could grow at lower pH, as low as pH 1.5. We conclude that *Cryptococcus*

Table 1. Distinctive characteristics among *Cryptococcus tepidarius* sp. nov. and related species

Species/strains: 1, *Cryptococcus tepidarius* M9962^T; 2, *B. formosensis* JCM 11142^T; 3, *B. koratensis*; 4, *B. lagerstroemiae*. Data for *B. koratensis* and *B. lagerstroemiae* were taken from Fungsin *et al.* (2006). +, Positive; –, negative; L, latent; W, weak; LW, latent and weak; ND, not determined.

Characteristic	1	2	3	4
Assimilation of carbon compounds				
L-Sorbose	–	L	L	L, LW
D-Ribose	L/+	L	L	L/–
Erythritol	–	L	L	LW/–
Saccharate	–/LW	–	L	–
Assimilation of sodium nitrite	–	+	+	+
DNA G + C content (mol%)	42.0	57.8	49.4	46.0
Maximum growth temperature (°C)	ND (>47)	29–30	29–30	29–30

tepidarius is acid tolerant and that both thermotolerance and acid tolerance might be the result of adaptation to the environment from which the yeasts were isolated (Raspor & Zupan, 2006).

Latin diagnosis of *Cryptococcus tepidarius* sp. nov. Takashima, Sugita, Toriumi et Nakase

In liquido YM, post dies 3 ad 25 °C cellulae ovoideae, ellipsoideae aut elongatae, 3.8–5.5 × 4.3–10 µm, singulae, binae, vel parvi-racemosae; post unum mensem ad 17 °C annullus fragilis, insulae et sedimentum formantur. In agarō YM, post unum mensem ad 17 °C cultura xanthoalba, glabra, subnitida, mollis aut butyracea, et margo glabra. Mycelium et pseudomycelium non formantur. Fermentatio nulla. Glucosum, galactosum (vel lente), sucrosum, maltosum, cellobiosum, trehalosum, lactosum (lente), raffinsum (lente vel lente et exiguum), melezitum, amyllum solubile, D-xylosum, L-arabinosum, D-arabinosum (vel lente), D-ribosum (vel lente), L-rhamnosum, D-glucosaminum (lente), N-acetyl-D-glucosaminum, glycerolum (lente et exiguum vel nullum), ribitolum (vel lente), galactitolum, D-mannitolum, D-glucitolum, methyl-α-D-glucosidum, salicinum, glucono-δ-lactonum, acidum D-gluconicum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum (lente et exiguum vel nullum), acidum succinicum, acidum citricum, inositolum, acidum saccharicum (lente et exiguum vel nullum), xylitolum, L-arabinitolum, acidum D-glucuronicum, et acidum D-galacturonicum assimilantur at non L-sorbosum, melibiosum, inulinum, methanolum, ethanolum, erythritolum, hexadecanum, propane-1,2-diolum nec butane-2,3-diolum. L-Lysinum, ethylaminum, et cadaverinum assimilantur at non kalium nitricum nec natrium nitrosum. Cultura crescens ad 45 °C. Ad crescentiam thiaminum necessarium est. Commutatio colori per diazonium caeruleum B positiva. Ubiquinonum majus: Q-10. Proportio molaris in acido deoxyribonucleico: 42.0 mol% (per HPLC). Teleomorphosis ignota. Holotypus: Isolatio M9962 ex aqua ad rivulum, Ohwakudani, Hakone, Japonia, Y. Toriumi et T. Sugita, 18/03/2001, in collectionibus culturarum ut JCM 11965 quas Japan Collection of Microorganisms (JCM), RIKEN, Wako, Saitama, Japonia conservata, item in collectionibus culturarum ut CBS 9427 quas Centraalbureau voor Schimmelcultures (CBS), Trajectum ad Rhenum, Hollandia, conservata.

Description of *Cryptococcus tepidarius* sp. nov.

Cryptococcus tepidarius (te.pi.da'ri.us. L. masc. adj. *tepidarius* of or belonging to tepid water, because the type strain was isolated from water of a tepid stream).

After 3 days in YM broth at 25 °C, cells are ovoidal, ellipsoidal or elongate, 3.8–5.5 × 4.3–10 µm, single, in pairs or in small clusters (Fig. 2). A ring and sediment are formed. After 1 month, a complete and fragile ring, islets and a heavy sediment are produced. After 1 month on YM agar at 17 °C, the streak culture is light yellow, smooth,

Fig. 2. Vegetative cells of strain M9962^T grown in YM broth for 3 days at 25 °C. Bar, 10 µm.

semi-shiny, soft to butyrous and has an entire margin. Mycelium and pseudomycelium are not formed in slide culture on cornmeal agar. Does not ferment glucose. Assimilates glucose, galactose (or latent), sucrose, maltose, cellobiose, trehalose, lactose (latent), raffinose (latent or latent and weak), melezitose, soluble starch, D-xylose, L-arabinose, D-arabinose (or latent), D-ribose (or latent), L-rhamnose, D-glucosamine (latent), N-acetyl-D-glucosamine, glycerol (latent and weak or null), ribitol (or latent), galactitol, D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, glucono-δ-lactone, D-gluconate, 2-ketogluconic acid, 5-ketogluconic acid, DL-lactic acid (latent and weak or null), succinic acid, citric acid, inositol, saccharic acid (latent and weak or null), xylitol, L-arabinitol, D-glucuronic acid and D-galacturonic acid. Does not assimilate L-sorbose, melibiose, inulin, methanol, ethanol, erythritol, n-hexadecane, propane-1,2-diol or butane-2,3-diol. Assimilates L-lysine hydrochloride, ethylamine hydrochloride and cadaverine dihydrochloride. Does not assimilate potassium nitrate or sodium nitrite. Thiamine is required for growth. Grows at 45 °C. No starch-like substances are produced. Does not liquefy gelatin, hydrolyse fat, produce acid from glucose or grow on 50% (w/w) glucose-yeast extract agar. Urease and diazonium blue B reactions are positive. Major ubiquinone is Q-10. DNA G+C content of the type strain is 42.0 mol% (by HPLC).

The type strain, M9962^T (=SP-5^T =JCM 11965^T =CBS 9427^T), was isolated from water collected from a small stream at Ohwakudani, Hakone, Japan, on 18 March 2001 by Y. T. and T. S.

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