Cryptococcus thermophilus sp. nov., isolated from cassava sourdough

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A novel anamorphic yeast, strain LTH 6662T, was isolated from cassava sourdough. The isolate supposedly originated from cassava flour or was a contaminant thereof. Sequencing of the D1/D2 domain of the 26S rRNA gene indicated that strain LTH 6662T represents a novel species. Its closest relatives were members of the Cryptococcus humicola complex. The novel strain had several physiological characteristics that differed from those of related species: the ability to assimilate raffinose and cadaverine; the inability to assimilate soluble starch, xylitol, galactitol, butane-2,3-diol, sodium nitrite and lysine; the ability to grow without vitamins and at 42 °C; and the inability to produce starch-like substances. Its major ubiquinone was Q-10. In addition, buds were formed on small neck-like structures. In liquid medium, green or blue fluorescent substances were produced. The name Cryptococcus thermophilus sp. nov. is proposed, with LTH 6662T (=DSM 19443T—CBS 10687T) as the type strain.

The genus Cryptococcus (C.) Vuillemin includes a great variety of asexually reproducing species that are heterogeneous in their morphological and physiological characteristics. Despite common properties such as the absence of fermentative ability, the assimilation of D-glucuronate, the presence of xylose in cell hydrolysates, as well as positive urease and diazoniun blue B reactions, Cryptococcus species differ in cell shape, colony form and colour, for nutritional capabilities and habitat (Fonseca et al., 2011). They occur ubiquitously, especially on plant material and in soil, and have also been reported as human pathogens (Ahearn, 1998; Barnett et al., 2000).

The taxonomy of the basidiomycetous yeasts is still in discussion. The current status is presented by Boekhout et al. (2011) in the most recent edition of ‘The Yeasts, a Taxonomic Study’. In the subphylum Agaricomycotina, the class Tremellomycetes consists of the four orders: Cystofilobasidiales, Tremellales, Trichosporonales and Filobasidiales. Hibbett et al. (2007) proposed a different classification system with the Trichosporonales being part of the Tremellales. This point still needs to be clarified (Boekhout et al., 2011). The genus Cryptococcus is polyphyletic; thus, species are represented throughout all four (or three) orders. The following Cryptococcus species belong to the Trichosporonales clade: Cryptococcus humicola, C. musci, C. longus, C. pseudolongus, C. ramirezgonzezianus, C. fragicola, C. curvatus, C. haglerorum, C. arboriformis and C. dasewskiae (Boekhout et al., 2011). Recently, Prillinger et al. (2007) proposed the new genus Asterotremella, comprising the species of the Cryptococcus humicola complex (Takashima et al., 2001), which differ from other closely related species of the genus Cryptococcus by having Q-9 as major ubiquinone. However, there is an earlier name, Vanrijia (Okoli et al., 2007), which has priority over Asterotremella. As the nomenclature of these yeasts is still not clear, original nomenclature proposed by Takashima et al. (2001), as also used by Boekhout et al. (2011), Fonseca et al. (2011) and Sugita (2011) was used.

The microbiota of a cassava sourdough, which was made from cassava flour and water inoculated with lactic acid bacteria and fermented for 24 h at 30 °C, was investigated previously using bacteriological culture techniques (Vogelmann et al., 2009). The ripe sourdough was serially diluted in saline-tryptone-diluent and aliquots of the dilutions were plated on YGC agar which contained (lactic acid, yeast extract, 20 g glucose, 0.1 g chloramphenicol and 25 mg bromoresol green. Colonies with different morphologies were picked and species were determined by sequence analysis of the D1/D2 region of the 26S rRNA gene. Identification of the yeast microbiota revealed the presence of two yeasts belonging to the Torulaspora
delbrueckii/globosa/pretoriensis group and to the species Issatchenkia orientalis, as well as an unknown basidiomycetous yeast (strain LTH 6662T). As no yeasts were inoculated into the starter preparation, the yeasts either originated from the cassava flour or were contaminants.

DNA was isolated using a GenElute Bacterial Genomic DNA kit (Sigma) according to the supplier’s instructions with the following modification. A combination of lysing enzymes from Trichoderma harzianum (0.24 mg ml⁻¹) and lyticase (0.12 mg ml⁻¹) was used as lysing enzyme. Partial amplification of the 26S rRNA gene (799 bp of the D1/D2 region) was carried out using the primers P1 and P2 described by Sandhu et al. (1995). PCR was conducted as described previously (Meroth et al., 2003). Sequencing was performed using a capillary sequencer (Beckman Coulter). The partial 26S rRNA gene sequences were compared with corresponding sequences present in GenBank. Phylogenetic trees were computed with PAUP version 4.0 using maximum-parsimony analysis (heuristic search, stepwise addition, random addition sequence, nearest neighbour interchange, 100 maximum trees). Bootstrap analysis was based on 100 replicates.

Yeast strain LTH 6662T was characterized morphologically and physiologically using standard methods with some modifications (Barnett et al., 2000). Assimilation of carbon and nitrogen compounds was examined in liquid medium using a rotary shaker at 25 °C. Filamentous growth was investigated on agar plates and on sterile microscope slides (without a coverslip) that were coated with either cornmeal agar, yeast morphology agar or potato glucose agar according to the methods of Barnett et al. (2000). Urease activity was tested with urea agar (Christensen, 1946). All physiological and biochemical tests were performed at least twice and compared with a negative control (incubated medium without inoculation). Analysis of respiratory quinones was carried out by the Identification Service and Dr B. J. Tindall of the DSMZ, Braunschweig, Germany.

Sequence analysis of the D1/D2 region of the 26S rRNA gene of LTH 6662T revealed that C. musci was the closest relative with 25 nt substitutions (95% similarity). The remaining members of the C. humicola complex (C. ramirezgomezianus, C. humicola, C. longus and C. pseudolongus) contained 26 to 35 nt substitutions. Similarities to species of the genus Trichosporon were 94% or below. As shown in the phylogenetic tree (Fig. 1), the novel species was positioned within the C. humicola complex, but outside of the respective clade. As already shown by Takashima et al. (2001) based on 18S rRNA gene sequences, and by Prillinger et al. (2007), the type strains of C. musci, C. humicola, C. longus, C. pseudolongus and C. ramirezgomezianus form a cluster, named the C. humicola complex, within the order Trichosporonales. The tree presented in this study, which is based on the D1/D2 domain of the 26S rRNA gene sequences, revealed the same grouping. According to DNA sequence analysis, strain LTH 6662T is, however, not closely related (i.e. it has more than 25 nt substitutions) to species included in the C. humicola complex.

More and more studies address the problems of the current yeast classification system, especially the conflict between traditional phenotypic classification and modern molecular phylogeny. Okoli et al. (2007) addressed this problem for the order Trichosporonales. This order comprises several genera that are phylogenetically related, but their allocation to these different genera was based primarily on phenotypic characteristics, rather than on molecular phylogenetic relationships. Species of the genus Cryptococcus, for example, are scattered over three different orders, and often are phylogenetically unrelated. The difference to members of the genus Bullera is defined as the absence of ballistococnidia, but this is not a stable characteristic. Okoli et al. (2007) proposed a new yeast classification system based on phylogenetic differences without differentiation between anamorphic and teleomorphic species. Boekhout et al. (2011), Fonseca et al. (2011) and Takashima et al. (2009) also recommended a new reliable taxonomy that is based on molecular phylogenetic studies.

Based on comparisons of physiological characteristics, LTH 6662T is easily distinguishable from its relatives, e.g. by assimilation of some carbon and nitrogen compounds, inability to produce starch-like substances, growth without vitamins, maximum growth temperature (Table 1), and morphological characteristics such as cell shape and budding. The isolate formed buds on short neck-like structures (Fig. 2a), which is not a common characteristic for Cryptococcus species, and produced abundantly branched pseudomyceum on plates of cornmeal agar and potato glucose agar (Fig. 2b), but did not produce arthroconidia. The maximum growth temperature was 42 °C on solid medium, but in liquid medium, growth was very weak at 42 °C. Growth temperatures over 40 °C are uncommon for Cryptococcus species (Fonseca et al., 2011; our own literature research). Only Filobasiella neoformans, the teleomorphic form of Cryptococcus neoformans, is reported to grow slowly at 40 °C (Kwon-Chung, 2011). The recently described thermostolerant species C. tepidarius is able to grow at 47 °C (Takashima et al., 2009). A further special characteristic of LTH 6662T is the formation of green or blue fluorescent substances in liquid medium. Quinone analysis revealed the exclusive presence of the ubiquinone Q-10 in strain LTH 6662T. Sugita & Nakase (1998) and Takashima et al. (2001) discussed the major ubiquinone type as an important taxonomic criterion within the Trichosporonales. Species of the genus Trichosporon have Q-9 or Q-10 as major ubiquinone (Sugita & Nakase, 1998) and the ubiquinone type correlates with the clustering in the phylogenetic tree of the 26S rRNA gene partial sequences. Trichosporon species in the ‘gracile’, ‘brassicaceae’, ‘porosum’ and ‘ovoides’ clades have Q-9, whereas those in the ‘cutaneum’ clade have the Q-10 ubiquinone type (Sugita & Nakase, 1998; Sugita, 2011). According to Prillinger et al. (2007), the C. humicola complex is separated from related species of the genus Cryptococcus by having Q-9 as major
Fig. 1. Phylogenetic tree including *Cryptococcus thermophilus* sp. nov. and related species, obtained with maximum-parsimony analysis (heuristic search, random addition, nearest neighbour interchange, 100 maximum trees) of the D1/D2 region of the 26S rRNA gene. Branch lengths are proportional to the number of nucleotide differences and the numbers given on the branches are the bootstrap values (>50 %) of 100 replicates. The sequence from *Rhodotorula glutinis* VTT C-04513 was used as outgroup. GenBank accession numbers are indicated after species designations. Species names in quotation marks are those listed in the phylogenetic tree in the new edition of ‘The Yeasts’ (chapter 100 of Boekhout *et al.*, 2011), but are not validly published.
ubiquinone. *C. fragicola, C. haglerorum, C. daszewskae* and *C. curvatus* all have Q-10 as major ubiquinone.

In summary, strain LTH 6662<sup>T</sup> clearly differed in some important characteristics from members of the *C. humicola* complex (Takashima *et al.*, 2001; Prillinger *et al.*, 2007), including containing ubiquinone Q-10 instead of Q-9, no production of extracellular amyloid substances, no need for thiamine for growth, growth at 42 °C, and absence of formation of true hyphae. Taking into consideration its differences to its closest relatives, strain LTH 6662<sup>T</sup> should be considered phylogenetically distinct from the species included in the *C. humicola* complex.

Therefore, the genotypic and phenotypic comparisons described above clearly indicate that strain LTH 6662<sup>T</sup>
represents a novel species, for which the name Cryptococcus thermophilus sp. nov. is proposed.

**Latin diagnosis of Cryptococcus thermophilus Vogelmann, Chaves and Hertel sp. nov.**


**Typos:** isolatus ex fermento Manihot esculentum Crantz, LTH 6662 T, depositus in collectione zymotica Centraal-bureau voor Schimmelcultures (CBS 10687 T) et German Collection of Microorganisms and Cell Cultures (DSM 19443 T).

**Description of Cryptococcus thermophilus Vogelmann, Chaves & Hertel sp. nov.**

Cryptococcus thermophilus [ther.mo’phil. us. Gr. adj. ther. mos hot; N.L. adj. philus -a-um (from Gr. adj. philos -é- on) friend, loving; N.L. masc. adj. thermophilus heat loving].

After 3 days at 25 °C in liquid YG medium, cells are ovoid to ellipsoidal (2–4 × 3–5 μm) and occur singly or in parenchyma chains of up to four cells. Buds are produced multilaterally on short neck-like structures. Sediment is formed. In liquid medium, yeast growth causes bright green or blue fluorescence of the broth. On YG agar after 3 days at 25 °C, colonies have a butyrous texture and are white-coloured and shiny in appearance with entire margins. After 4 weeks, colonies are yellowish with a corrugated surface and a fringe of pseudomycelium reaching into the agar. On yeast morphology agar, potato glucose agar and cornmeal agar after 3 days at 25 °C, masses of ovate budding cells are formed. Long, thin, branched pseudomycelium grows into the agar. Chains of ovate cells are formed terminally and laterally on hyphe. Fermentation ability is negative. The following carbon compounds are assimilated: D-glucose, D-galactose, L-sorbose (weak), sucrose, maltose, cellobiose, trehalose, lactose, raffinose, melezitose, melibiose, D-xyllose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, methyl α-D-glucoside, salicin, glycerol, erythritol, D-mannitol, myo-inositol, D-glucitol, ribitol, ethanol, propano-1,2-diol, D-glucono-1,5-lactone, citric acid (weak), DL-lactic acid (weak), succinic acid, D-galacturonic acid, D-glucuronic acid, D-glucurate, and acetylglucamine. No growth occurs on inulin, soluble starch, xylitol, galactitol, methanol or butane-2,3-diol. The nitrogen compounds ethylamine, cadaverine and D-glucosamines are assimilated, but not potassium nitrate, sodium nitrite or L-lysine. Growth in vitamin-free medium is positive. Growth occurs at 40 °C, weakly at 42 °C, but not at 45 °C. Growth on yeast extract agar with 10 % NaCl/5 % glucose is positive. Growth on 50 % glucose/0.5 % yeast extract is negative. Starch-like compounds are not produced. Growth is positive in 0.01 %, but negative in 0.1 % cycloheximide. Urea activity and Diazonium Blue B reactions are positive. The ubiquinone present is Q-10.

The type strain is LTH 6662 T (=DSM 19443 T=CBS 10687 T), which was isolated from cassava sourdough in Stuttgart, Germany.

**Acknowledgements**

The authors thank Jean Euzéby and Marc-André Lachance for assistance with the Latin diagnosis.

**References**


