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ORIGINAL PAPER

Cryptococcus cerealis sp. nov. a psychrophilic yeast species isolated from fermented cereals

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Abstract Two yeast strains isolated in 2007 from fermented pig feed were studied, including the analysis of sequences of the D1/D2 and ITS-regions of the rDNA-repeats, their morphology and nutritional physiology. Sequence comparison of the D1/D2 and ITS regions demonstrated that the strains do not belong to any known species. Therefore, a new species, *Cryptococcus cerealis* with the type strain CBS 10505, is proposed. The species belongs to Filobasidiales (Agaricomycetes, Basidiomycota), and has *Cryptococcus saitoi* as the closest related species. The new species is psychrophilic, showing significant growth at 4 and 10°C.

Keywords Cereal grain · *Cryptococcus* · Fermented feed · Large subunit rDNA-sequence · Psychrophilic yeasts

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Introduction

Yeasts of the genus *Cryptococcus* have been isolated from a variety of environments, including soil, water or stored agricultural products (Kurtzman and Fell 1998; Barnett et al. 2000; Olstorpe et al. 2008). In a recent study, we isolated and identified a number of yeasts belonging to this anamorphic genus from fermented pig feed, produced from a mixture of a liquid and cereal grain (Olstorpe et al. 2008). The growth conditions in the fermentation systems are characterised by relatively high concentrations of lactic and other organic acids, low pH and low oxygen concentrations (Lyberg et al. 2008). Temperatures can vary between less than 10°C and more than 30°C, dependent on the climate conditions. Under Swedish farming conditions, it can frequently happen that such fermentations run at 10°C or even lower temperatures. Due to the spontaneous character of the fermentation, different microbial interactions occur in such systems (Lyberg et al. 2008; Olstorpe et al. 2008, 2009).

While investigating such fermentation systems, we isolated yeast strains that could not be identified by sequencing their D1/D2 domains of the large subunit (LSU) of the ribosomal DNA (rDNA) because the sequences showed identities of less than 99% to those of known *Cryptococcus* species (Olstorpe et al. 2008). According to Fell et al. (2000), basidiomycetous strains that differ in more than two nucleotides in their D1/D2 regions may represent different taxa. Thus, the isolates did not belong to a known species,

but they can probably be classified as a member of the genus *Cryptococcus*, as the most closely related species belong to this genus. However, the genus *Cryptococcus* is highly polyphyletic and species of this genus have been assigned to the four orders Tremellales, Trichosporonales, Filobasidiales and Cystofilobasidiales (Fell et al. 2000). It goes beyond the scope of this investigation to resolve the taxonomic structure of the genus *Cryptococcus* and, hence, in the present study the phylogenetic relationships, morphological and physiological characters of two isolated strains are investigated and a new *Cryptococcus* species, belonging to the lineage Filobasidiales is proposed.

Materials and methods

Yeast strains

Two yeast strains were isolated from feed fermentations at 10°C in an experiment running in 2007 and preliminary identified as belonging to the genus *Cryptococcus*. The strains have been deposited in the strain collection of the CBS Fungal Diversity Centre, Utrecht as CBS 10505 and 10507. Strain CBS 10505 (numbered SLU J595 in the strain collection of the Department of Microbiology, Swedish University of Agricultural Sciences) was isolated from a mixture of cereal grain and water after 3 days of fermentation and CBS 10507 (SLU J597) from a mixture of cereal grain and whey after 7 days of fermentation (Olstorpe et al. 2008). The strains were conserved in glycerol stocks as described (Olstorpe et al. 2008). For comparison of the growth at 4 and 10°C, strains CBS 1975 (*Cryptococcus saitoi*), CBS 7160 (*Cryptococcus friedmannii*), CBS 10160 (‘*Cryptococcus randhawii*’), and CBS 10162 (*Cryptococcus festucosus*) were all obtained from the CBS strain collection.

Physiology and morphology

Physiology and morphology were investigated according to Yarrow (1998). Growth tests were performed in liquid medium. Test tubes (18 × 150 mm) with 10 ml liquid medium were inoculated with 100 µl cell suspension (about 25×10^6 cells ml^{-1}) prepared from overnight cultures at 25°C on YMA (yeast extract 3 g l^{-1}, malt extract 3 g l^{-1}, bacteriological peptone 5 g l^{-1}, glucose 10 g l^{-1} and agar technical 20 g l^{-1}). CBS 7160 was pre-grown at 15°C. The tubes were placed on an angle (40°), and incubated on a shaking table (147 rpm). Besides the temperatures stated in Yarrow (1998), growth was also tested at 4 and 10°C. Temperature dependent growth (cell density increase in liquid medium within 27 days) was tested in YNBG (Bacto yeast nitrogen base 6.7 g l^{-1}, glucose 1 g l^{-1}) (Yarrow 1998).

Mating experiments

Cells of the two strains to be mated were mixed and incubated at room temperature on different media including malt extract agar, yeast extract-malt agar, potato dextrose agar, corn meal agar and the cultures were regularly investigated for formation of basidiospores (Yarrow 1998).

DNA extraction and rDNA sequencing

Genomic DNA of CBS 10505 and 10507 was extracted according to Liberal et al. (2005) as described previously (Passoth et al. 2007; Olstorpe et al. 2008). PCR amplifications of the D1/D2 and ITS 1 + 2 regions were performed in separate reactions with the primer pairs NL1–NL4 (D1/D2-region) (Kurtzman and Robnett 1998) and ITS4 and ITS5 (ITS-region) (Valente et al. 1999). All oligonucleotides were purchased from Metabion International AG, Munich, Germany. The PCR was set up as described and the PCR-products were purified (Passoth et al. 2007; Olstorpe et al. 2008). Sequence reactions were mixed using the generated PCR fragments as templates and the primers NL1, NL2A, NL3 and NL4 (D1/D2-region) (Kurtzman and Robnett 1998) and ITS 4 and 5 (ITS-region) (Valente et al. 1999). Sequencing was performed at the Molecular Cloning Laboratories (San Francisco, USA). The sequences were deposited in GenBank (accession numbers: CBS 10505, FJ473376 (D1/D2-region) and FJ473371 (ITS-region); CBS 10507, FJ473375 and FJ473370).

DNA sequence analysis

The partial nucleotide sequences obtained from each DNA fragment were composed using the EMBOSS program needle (http://www.ebi.ac.uk/emboss/align/), manually adjusted and trimmed. The sequences

generated were compared to the available data in the database of NCBI with the Basic Local Alignment Search Tool (BLASTn) (Altschul et al. 1990).

A multiple alignment using ClustalW was made of all the D1/D2 and ITS domain sequences and 30 other sequences collected from the best matches of strains CBS 10505 and CBS 10507 in the BLAST search. The type strain of *Cryptococcus albidus* CBS142 was also included since strains CBS 10505 and 10507 were preliminary identified as *C. albidus* in an ID32 C test (A.-C. Andersson, unpublished results). The alignment was inspected and all sequences manually trimmed so that they would all begin and end at the same place. ClustalW was run again on the trimmed sequences with the following changes to default settings; gap opening: 10, gap extension: 0.5, end gap: 20, output format: phylip, output order: aligned. The ClustalW alignment was then used in programs from the Phylogeny Inference Package (PHYLIP) 3.66 package (J. Felsenstein, 2006, Department of Genome Sciences, University of Washington, Seattle). Two different methods were tested to investigate relationships between sequences, maximum parsimony and neighbour joining. Both methods were run on multiple datasets (1,000 pseudoreplicates) created by the resampling program Seqboot. Bootstrapping was performed to test the reliability of the trees created. Maximum parsimony was calculated using the program Dnapars and Mega 4 (Tamura et al. 2007). For neighbour joining analysis the Kimura 2-parameter model was used for the calculation. The program Consense was run to obtain majority rule consensus trees from the resulting multiple trees of the two methods. The consensus trees created were visualized in MEGA 3.1 (Kumar et al. 2004). Separate phylogenetic trees of the D1/D2 domains of the LSU rRNA gene and the ITS 1 + 2 regions were constructed for each lineage using maximum parsimony implemented in Mega4 (Tamura et al. 2007) with 1,000 bootstrap replicates (seed = 24,054) and search option CNI (level = 1) with initial tree by Random addition (10 reps). Gaps were not included in this analysis.

Results and discussion

Sequence comparison

The sequences of the D1/D2 domains of the LSU rRNA-gene and ITS regions of CBS 10505 and 10507 were determined to investigate the relatedness of these strains to each other and to other yeasts. An alignment of the D1/D2 and ITS region sequences of CBS 10505 and 10507 showed that they were identical. Comparing the D1/D2 sequences of these strains with the closest described species shows a 98.5% similarity with *Cryptococcus saitoi* (nine different base pairs). However, the closest match to the D1/D2 sequence of both strains was that from four yeast strains (GenBank accession numbers EF068183, EF068190, EF068195, and EF068200) previously isolated from a ginseng cultivation field (Hong et al. 2006), whose D1/D2-sequences differed by only two nucleotides. Unfortunately, no more information about these strains is currently available, neither regarding sequence data of the ITS region nor their physiological characteristics. For the ITS region, 96.3% similarity (18 different base pairs) was found with the not yet formally described '*Cryptococcus randhawii*', whereas similarity with *C. saitoi* was only 93.9% (30 different base pairs; Table 1). Basidiomycetous yeasts that differ by two or more nucleotides in the D1/D2 region usually represent different species and differences in four base pairs in the ITS region have been regarded as adequate to separate species (Fell et al. 2000). Thus the strains CBS 10505 and 10507 are well separated from both *C. saitoi* and '*C. randhawii*' and can be considered to belong to a distinct species. Taking into account the two base pairs difference in the D1/D2 sequence of the above-mentioned four strains isolated from a ginseng field (Hong et al. 2006), it is likely that also these strains belong to a closely related but different species.

Phylogeny

Separate phylogenetic analyses made using maximum parsimony analysis of the D1/D2 domains of the LSU rRNA gene and the ITS regions placed strains CBS 10505 and 10507 in the Filobasidiales (Fonseca et al. 2000) (Fig. 1). The trees include the type strain of *Cryptococcus albidus* and related species (Fell et al. 2000). CBS 10505 and 10507 clustered in the same lineages with *Cryptococcus friedmannii*, *C. saitoi* and '*C. randhawii*' in all analyses performed with 85 and 53% bootstrap support for the D1/D2 and ITS, respectively. Similar results were obtained from the neighbour joining

Table 1 Comparison of the ITS and D1/D2 sequences between *Cryptococcus cerealis* and its closest relatives

Closest relative	*Cryptococcus cerealis*	
	ITS-identity (%), number of differing base pairs in parenthesis[a]	D1/D2-identity (%), number of differing base pairs in parenthesis[a]
'*Cryptococcus randhawii*' J11	96.3 (18)	97.8 (13)
Uncultured fungus CH33	93.9 (30)	–
Cryptococcus saitoi CBS 1975	93.9 (30)	98.5 (9)
Cryptococcus friedmannii CBS 7160	93.3 (33)	98.3 (10)
Cryptococcus sp. BC24	93.3 (33)	–
Cryptococcus sp. SG02-024	–	99.7 (2)

[a] In several cases only partial sequences of the comparison strains were available. Sequences were compared within the region that was available for all comparison strains. The lengths of the compared regions were 492 bp (ITS *C. cerealis*), and 601 bp (D1/D2 *C. cerealis*), respectively. Note that '*C. randhawii*' is not yet formally described

analyses (results not shown). These results confirm our provisional determination of the strains as *Cryptococcus* species (Olstorpe et al. 2008). However, the genus *Cryptococcus* is polyphyletic and a taxonomic revision appears to be necessary. Such a revision would require a detailed re-investigation of all species belonging to the present four orders to which species of this genus belong to, which is beyond the scope of this study.

Physiology of the strains

Results from the assimilation and other growth tests performed are presented in Table 2. For all tested strains no fermentation was observed on any of the tested standard sugars (Kurtzman and Fell 1998; Barnett et al. 2000). Replicate data obtained at SLU, Uppsala and CBS, Utrecht were largely comparable. In growth tests run at CBS, no growth was observed on raffinose, but at SLU weak growth was observed. Additional assimilation tests at CBS for both strains showed growth on saccharate, but no growth was found on quinic acid, galactonic acid and L-malic acid. Additionally, we tested the ability of the strains to grow at lower temperatures and compared the increase in cell density to that of the closely related strains CBS 1975 (*C. saitoi*), CBS 7160 (*C. friedmannii*), and CBS 10160 ('*C. randhawii*') and the psychrophilic yeast *C. festucosus* (CBS 10162), belonging to the *Holtermannia* clade of Tremellales (Golubev et al. 2004). Strains CBS 10505 and 10507 displayed similar growth patterns at 10 and 4°C, with a slightly higher growth of CBS 10505 at 4°C (Figs. 2, 3). At both cultivation temperatures, CBS 10505 and CBS 10507 had the fastest initial increase in cell density. At 10°C, both strains reached the stationary phase after less than 10 days compared to about 15 for CBS 1975 and CBS 7160. The final OD of CBS 10505 and 10507 was more than double of that of the *C. saitoi* and *C. friedmannii* strains. *C. festucosus* had a longer lag phase at 10°C, but then grew at similar rates as CBS 10505 and 10507 to a final OD that was 1–2 units below that of these two strains, but still almost twice as high as CBS 1975 and CBS 7160 (Fig. 2). At 4°C, CBS 10505 and 10507 had a four to five times higher OD after 27 days compared to CBS 1975 and CBS 7160. However, these higher values were due instead to a shorter lag phase (Fig. 3). Later measurements showed that CBS 10505 and 10507 had reached the stationary phase after 27 days. The other two strains continued growing, reaching an OD similar to that of CBS 10505 and 10507 after 41 days (results not shown). CBS 10162 reached the highest OD at 4°C. Again, after a longer lag-phase than CBS 10505 and 10507, the strain grew fastest compared to the other tested strains and reached an OD of 4.3 after 27 days (Fig. 3) and 6.9 after 41 days. No substantial increase in OD was seen for CBS 10160 under the tested conditions in the investigated time interval (results not shown).

Morphology

After growth in YM broth for 3 days at 25°C the cells of CBS 10505 and 10507 (Fig. 4a) were globose to

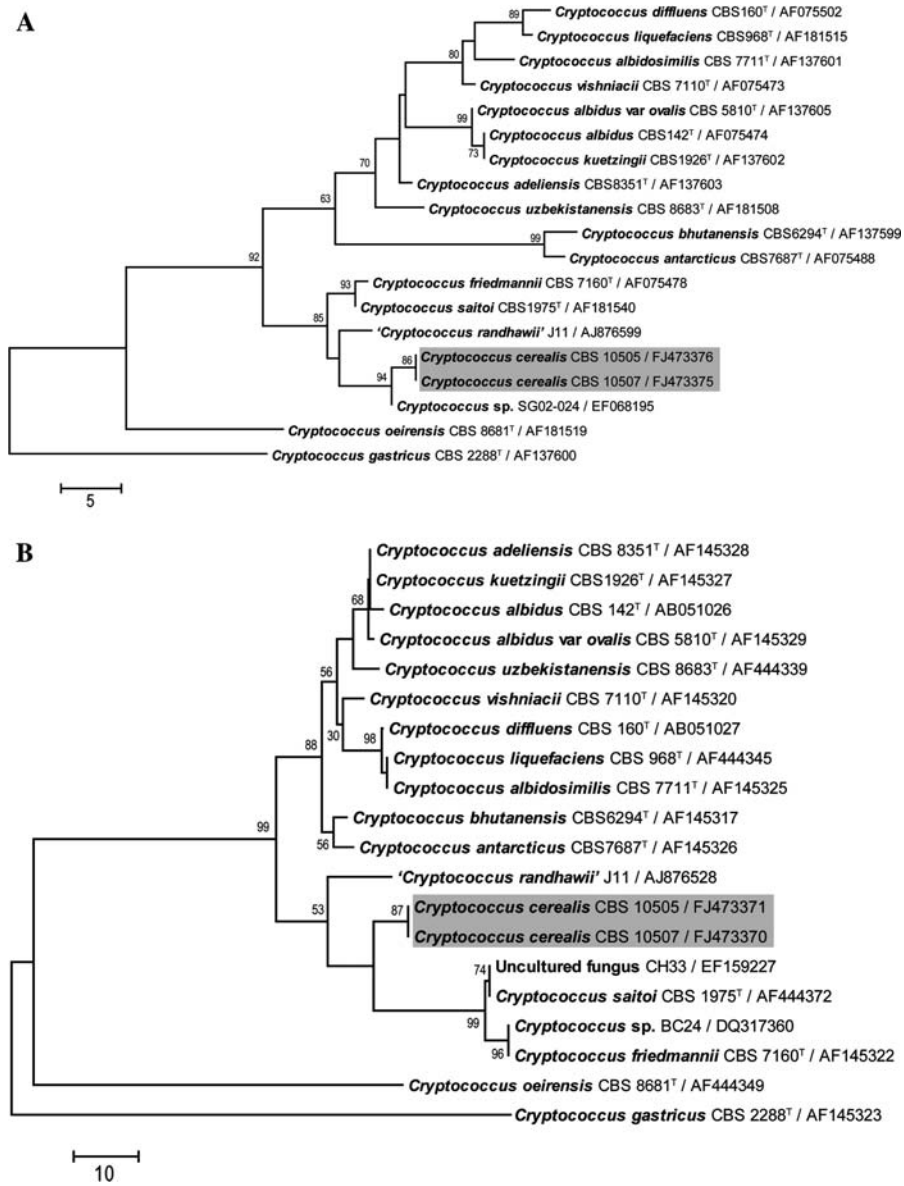

Fig. 1 Phylogeny of *C. cerealis*. maximum parsimony trees with bootstrap percentages from 1,000 replications shown on the branches. Values below 50% are not shown. **a** Tree based on the D1/D2 sequences; **b** Tree based on the ITS sequences

subglobose and approximately 6–8 μm in diameter. Vegetative reproduction occurred by polar budding. Cells of strain CBS 10505 and 10507 occurred singly or adhered into small clusters. Short filaments occurred when grown on potato-dextrose agar under microscopic cover slips (i.e., Dalmau plates). After 10 days, budding cells remained attached to each other and formed short pseudohyphal chains. Some of the cells became elongated. After 14 and 18 days cultivation on potato-dextrose agar, longer chains and more elongated cells were observed (Fig. 4b). No basidiospores were observed to be formed by strains CBS 10505 and 10507 and no mating was obtained within 2 months of cultivation.

Descriptions of new species

In addition to the identified sequence differences to known species in both the D1/D2 domains of the LSU rRNA gene and the ITS 1 + 2 regions of the rDNA, the investigated strains also showed differences in their physiological characteristics when compared to closely related species. In contrast to *C. satoi*, strains CBS 10505 and 10507 were able to assimilate

Table 2 Physiological characteristics of the two *Cryptococcus cerealis* strains

Test	Substrate	CBS 10505	CBS 10507
Carbon assimilation			
C1	D-Glucose	+	+
C2	D-Galactose	D	D
C3	L-Sorbose	D	D
C4	D-Glucosamine	−	−
C5	D-Ribose	D	−
C6	D-Xylose	+	+
C7	L-Arabinose	+	+
C8	D-Arabinose	D	D
C9	L-Rhamnose	+	+
C10	Sucrose	+	+
C11	Maltose	+	+
C12	α,α-Trehalose	+	+
C13	Me α-D-glucoside	+	+
C14	Cellobiose	+	+
C15	Salicin	+	+
C16	Arbutin	+	+
C17	Melibiose	−	−
C18	Lactose	D	D
C19	Raffinose	W	W
C20	Melezitose	+	+
C21	Inulin	W/D	−
C22	Starch	+	+
C23	Glycerol	−	−
C24	Erythritol	−	−
C25	Ribitol/Adonitol	D	D
C26	Xylitol	+	+
C27	L-Arabinitol	+	+
C28	D-Glucitol/D-sorbitol	+	+
C29	D-Mannitol	+	+
C30	Galactitiol/Dulctol	D	D
C31	*myo*-Inositol	+	+
C32	D-Glucono-1,5-lactone	D	D
C33	2-Keto-D-gluconate	+	+
C35	D-Gluconate	+	+
C36	D-Glucuronate	+	+
C37	D-Galacturonic acid	−	−
C38	DL-Lactate	−	−
C39	Succinate	D	D
C40	Citrate	−	−
C41	Methanol	−	−
C42	Ethanol	+	+
C43	Propane 1,2 diol	−	−
C44	Butane 2,3 diol	−	−
N1	Nitrate (KNO_3)	+	+
N2	Nitrite ($NaNO_2$)	+	+
N3	Ethylamine	+	+
N4	L-Lysine	+	+
N5	Cadaverine	+	+
N6	Creatine	−	−
N7	Creatinine	−	−
N8	Glucosamine	−	−
N9	Imidazole	−	−
N10	D-Tryptophane	W/D	W/D
V1	Vitamin free	+	D
O1	Cycloheximide 10 μg/ml	+	+
O2	Cycloheximide 100 μg/ml	−	−
T1	25°C	+	+
T2	30°C	+	+
T3	35°C	−	−
O4	D-Glucose 50%	−	−
O6	NaCl 10%	+	+
O7	NaCl 16%	−	−
M1	Starch formation	+	+
M3	Urea hydrolysis	+	+
M4	Diazonium Blue B	+	+

+, Growth within 7 days; D, growth after 7 days; W, weak growth; −, no growth

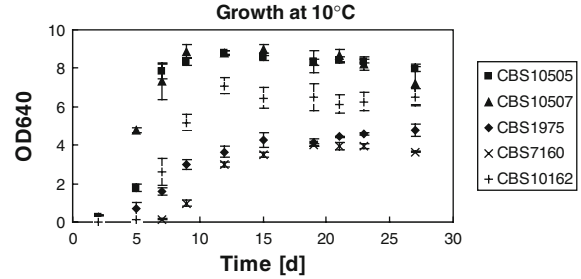

Fig. 2 Growth of CBS 10505, CBS 10507, CBS 1975 (*C. saitoi*) and CBS 7160 (*C. friedmannii*) in YNBG at 10°C as measured by optical density at 640 nm. *Points* in the diagram represent mean values out of three independent cultures; *error bars* show the standard deviation

L-sorbose, ribitol, galactitol, while they could not assimilate citrate. They were also able to grow in vitamin-free medium and in the presence of 10 μg/ml cycloheximide, which has not been observed for

C. satoi (Fonseca et al. 2000). The two strains CBS 10505 and 10507 differed only slightly from each other: on D-ribose and inulin delayed growth was observed for CBS 10505, while no growth was found

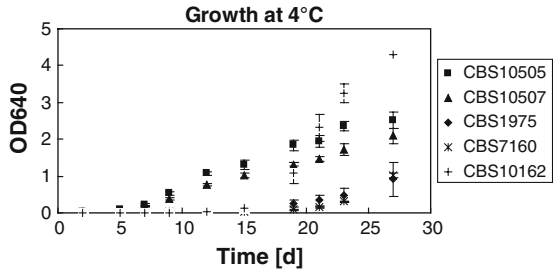

Fig. 3 Growth of CBS 10505, CBS 10507, CBS 1975 (*C. saitoi*) and CBS 7160 (*C. friedmannii*), in YNBG at 4°C as measured by optical density at 640 nm. *Points* in the diagram represent mean values out of three independent cultures; *error bars* show the standard deviation

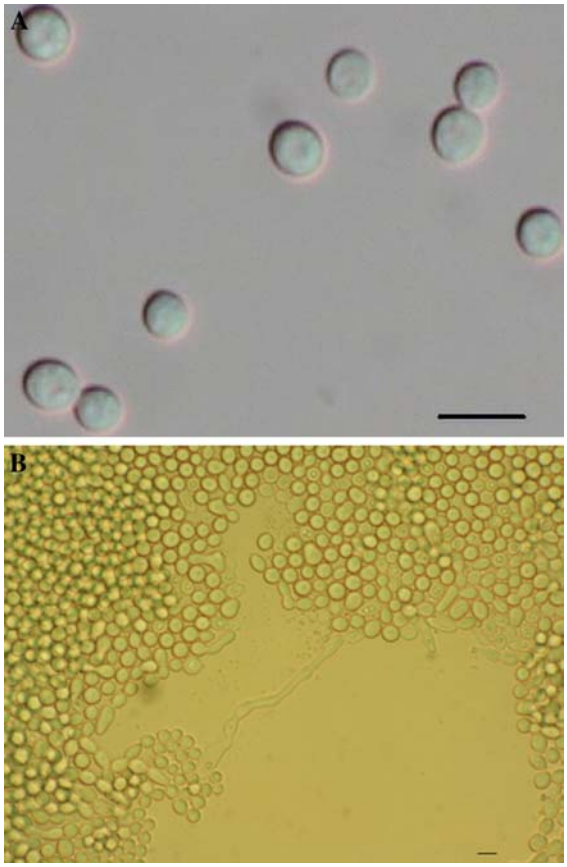

Fig. 4 Microphotographs (400 fold magnification) of cells of CBS 10505 grown for 3 days in YM broth (**a**), and 18 days on potato-dextrose agar (**b**). *Bars*, 10 μm

for CBS 10507 (Table 2). Thus, we propose to describe a new species, including strains CBS 10505 and CBS 10507, with the type strain CBS 10505.

Latin diagnosis of Cryptococcus cerealis Passoth, A.-C. Andersson, Olstorpe, Theelen, Boekhout & Schnürer sp. nov.

In medio liquido YM post dies tres ad 25°C cellulae globosae vel subglobosae, 6–8 μm diam, singulae vel binae. Reproductio vegetative gemmatione monopolari. Sedimentum adest. Cultura in agaro YM post 1 mensem ad 25°C glabra, nitida, butyracea, cremea. Basidiosporae non formatur. In agaro potato-dextroso pseudomycelium exiguum rudimentarium formatur. Fermentatio nulla. Glucosum, galactosum (lente), L-sorbosum (lente), D-ribosum (variabiliter), D-xylosum, L-arabinosum, D-arabinosum (lente), L-rhamnosum, saccharum, maltosum, trehalosum, α-methyl-D-glucosidum, cellobiosum, salicinum, arbutinum, lactosum (lente), rafinosum (exigue), melezitozum, inulinum (lente vel exique), amylum, ribitolum (lente), xylitolum, L-arabinotolum, D-glucitolum, D-mannitolum, galactitolum (lente), inositolum, glucono-δ-lactonum (lente), 2-keto-D-gluconatum, acidum gluconicum, acidum D-glucuronicum, acidum succinicum (lente) et ethanolum assimilantur neque D-glucosaminum, melibiosum, glycerolum, erythritolum, acidum D-galacturonicum, acidum DL-lacticum, acidum citricum, methanolum, propanum-1,2-diolum, nec butanum-2,3-diolum. Kalium nitricum, natrium nitrosum, ethylaminum, L-lysinum et cadaverinum assimilantur neque creatininum, D-glucosaminum nec imidazolum. Vitamina externa crescentiae non necessaria sunt. 30°C crescit neque 35°C. Materia amyloidea formatur. Ureum finditur. Reactio Diazonii caerulei B positiva. In medio cum 0.01% cycloheximido et 10% NaCl crescit neque cum 0.1% cycloheximido vel 50% glucosio.

Holotypus CBS 10505, depositus in collectione zymotica Centraalbureau voor Schimmelcultures, Utrecht, Nederlandia.

Cryptococcus cerealis Passoth, A.-C. Andersson, Olstorpe, Theelen, Boekhout & Schnürer sp. nov.

Etymology: cerealis, derived from cerealia, cereals, because the strains have been isolated from cereal-based fermented feed. After cultivation for 3 days at

25°C in YM broth, cells are globose to subglobose, 6–8 µm in diameter, and occur singly or in pairs. Budding is polar. Sediment is present. After 1 month at 25°C on YM agar the streak culture is cream to tan coloured, butyrous, smooth and glossy. After 21 days small, poorly developed pseudohyphae are formed on Dalmau plate cultures of potato-dextrose agar. No basidiospores were observed. Fermentation is absent. Additional physiological characteristics are listed in Table 2.

Holotype: CBS 10505, isolated from a fermented mixture of grain and water, Sweden. The strain was isolated during an experiment running at the Department of Microbiology, SLU, Genetic Center in Uppsala, Sweden (59°48′ N, 17°38′ E) (Lyberg et al. 2008; Olstorpe et al. 2008). The grain for the experiment was obtained from a farm east of Uppsala (about 59°50′ N, 17°49′ E). Strain CBS 10507, which also belongs to *C. cerealis* was isolated from the same place.

Ecology: The two strains were isolated from cereal based fermented feed, CBS 10505 from a mixture of grain and water, and CBS 10507 from a mixture of cereal grain and whey. The fact that the strains were found in grain-based fermentations with different liquid phases indicates that they were present on the cereal grain before fermentation was initiated. Strains of this species were only found in fermentations running at 10°C (Olstorpe et al. 2008), indicating that they are adapted to lower temperatures. This is confirmed by our results that show substantial growth at 10 and 4°C (Figs. 2, 3). The closely related species *C. saitoi* has been isolated from high Arctic glaciers (Butinar et al. 2007), indicating that also this yeast is cryophilic. The other closely related yeast, *C. friedmannii*, which has been isolated from the Antarctic cryptoendolithic community, also preferentially grows at lower temperatures (Vishniac 1985). Interestingly, like *C. cerealis* many Antarctic yeast isolates including *C. friedmannii* are able to grow without vitamins, in contrast to most other *Cryptococcus* species (Vishniac 1985; Kurtzman and Fell 1998). On the other hand, *C. saitoi* requires vitamins, so cryophilic behaviour is not necessarily connected to the ability to grow without external vitamins. Thus, *C. cerealis*, *C. friedmannii* and *C. saitoi* might be preferably present in geographical regions with a relatively low average temperature, like Scandinavia, the Arctic or Antarctic regions. Both strains of *C. cerealis* have been found in feed fermentations at 10°C. *C. cerealis* was not the major species in the feed fermentation. However, taking in mind that only 10 colonies were identified per sampling the occurrence of this yeast in two independent fermentations indicates that it has a substantial abundance on the grain. Strains of *C. festucosus* and *C. saitoi* have also been found in the feed fermentations, showing again that psychrophilic yeasts are well adapted to conditions of Swedish grain storage (Olstorpe et al. 2008). Another interesting finding was that the strains were found in feed fermentations that are usually characterised by low oxygen concentrations (Lyberg et al. 2008), although they are obviously not able to ferment (see above). This indicates that *C. cerealis* can efficiently utilise low amounts of oxygen for respiration, although it is not possible to provide a statement about the lowest tolerable oxygen concentration for the yeast because actual values of oxygen tension have not been measured (Lyberg et al. 2008). Anyway, this yeast might play an important role in feed fermentation for removing residual oxygen, providing one factor of conservation to the material.

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