Journal of Industrial Microbiology & Biotechnology (1999) 22, 127–132

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Cryptococcus nodaensis sp nov, a yeast isolated from soil in Japan that produces a salt-tolerant and thermostable glutaminase

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An anamorphic basidiomycetous yeast, which produced a salt-tolerant and thermostable glutaminase, was isolated from soil in Japan and classified in the genus *Cryptococcus*. Its substrate specificity suggests that this enzyme is an L-glutaminase asparaginase (EC 3.5.1.38). The strain, G60, resembles *Cryptococcus laurentii* in the taxonomic criteria traditionally employed for yeasts, however it can be distinguished as a separate species based on DNA–DNA reassociation experiments and sequence analysis of the large sub-unit rDNA. Phenotypically, the isolate can be differentiated from *C. laurentii* by the inability to utilize arbutin as a sole source of carbon. Based on sequence analysis, the strain is related to a group of hymenomycetous yeasts including *Bulleromyces albus, Bullera unica, C. laurentii* and *C. skinneri*. The strain, which is formally described as *Cryptococcus nodaensis*, is industrially important for the formation of the umami taste during production of proteolytic seasonings.

Keywords: umami; L-glutamic acid, glutaminase; *Cryptococcus*

Introduction

Proteins are digested by proteolytic enzymes such as proteases and peptidases resulting in the formation of peptides and amino acids. A component of hydrolyzed protein, l-glutamic acid, 'umami', is one of the fundamental tastes of Japanese food [17]. l-Glutamic acid can be formed from l-glutamine by the action of glutaminases such as l-glutamine amidohydrolase (EC 3. 5. 1. 2) and l-glutaminase asparaginase (EC 3. 5. 1. 38). In the absence of glutaminases, most of the l-glutamine liberated from peptides or proteins is chemically and irreversibly converted to pyroglutamic acid, which has no taste. Therefore, glutaminases are useful for enhancing the umami taste, increasing the amount of l-glutamic acid in hydrolyzed protein.

A few yeast species, such as *Cryptococcus albidus* [10] and *Bulleromyces albus* [13], produce salt-tolerant and heat-resistant glutaminases. Isolate G60, which we isolated from soil, produced more salt-tolerant and heat resistant glutaminase than other organisms. Consequently, the strain has industrial importance. The following report describes the new species, *Cryptococcus nodaensis*, and presents some characteristics of the enzyme.

Materials and methods

Strains examined

Cryptococcus G60 was isolated from soil in Noda City, Chiba Pref, Japan by the NBT method. Soil samples were suspended in saline and 100 μl of the suspension were spread on plates containing 0.2% of propionate, 25 μg ml^{-1} of tetracycline and 10 U ml^{-1} of penicillin G and incubated at 25°C for 5 days. Yeast colonies were picked, transferred to YM agar plates (glucose 1%, peptone 0.5%, yeast extract 0.3%, malt extract 0.3%, agar 1.5%, pH 6.2) and incubated at 25°C. When colonies appeared they were covered with glutaminase reaction agar containing 2% l-glutamine, 0.1 M phosphate buffer (pH 8.0) and 1.5% agar. The plates were incubated at 37°C for 30 min and then 0.5 ml of color reaction mixture containing 0.1 M of phosphate buffer pH 8.0, 3 mM of Nitro-blue tetrazolium (NBT), 3 mM of N-methyl-phenazonium methosulfate (PMS), 14 mM of NAD^+ (Oriental Yeast Co, Tokyo, Japan) and 50 U ml^{-1} of glutamate dehydrogenase from beef liver (Oriental Yeast Co) was spread on the plates. The plates were incubated at 37°C for 10 min. Yeast colonies which produced glutaminase turned dark blue.

Candida famata AHU3377 was obtained from the culture collection of the Faculty of Agriculture, The University of Hokkaido (Sapporo, Japan). Properties of the glutaminase from *C. famata* AHU3377 were determined and compared with those reported previously [10,13,18,25] in other microorganisms. The type strains of *C. laurentii* (JCM 9066^T), *C. luteolus* (JCM 3689^T) and *C. flavus* (JCM 8332^T) were used for DNA–DNA reassociation experiments.

Crude enzyme preparation

Isolate G60 was cultured in a 500-ml Erlenmeyer flask containing 100 ml of liquid medium (3.0% glucose, 0.5% yeast extract, 0.1% $MgSO_4$ and 0.1% KH_2PO_4, pH 6.0) at 28°C for 40 h with shaking. This seed culture was inoculated into 15 liters of the same medium in a 30-liter jar fermentor and cultured aerobically at 28°C for 40 h with agitation at 300 rpm. The cells were harvested by centrifugation and suspended in 2 L of a lytic mixture consisting of 0.8% cellulase Onozuka R-10 (Yakult Co, Tokyo, Japan) and 0.1 M

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Received 28 July 1998; accepted 4 February 1999

sodium acetate buffer (pH 5.0). This mixture was incubated for 14 h at 42°C, followed by centrifugation. The supernatant contained the crude enzyme.

Enzyme purification

The crude enzyme was heated at 60°C for 1 h and then immediately cooled on ice. The pH of the enzyme solution was adjusted to 7.0 with 0.1 N NaOH. The preparation was heated at 60°C for 1 h and then cooled on ice. The solution was then centrifuged to remove precipitate. All further operations were carried out at 4°C, except for HPLC.

Acetone fractionation: Two volumes of cold acetone were added to the supernatant, and it was allowed to stand overnight, then it was decanted and centrifuged. The precipitate was dissolved in 0.02 M sodium acetate buffer (pH 6.0) and dialyzed against the same buffer. Insoluble precipitate was removed by centrifugation.

DEAE-Sepharose CL-6B chromatography: The supernatant was applied to a DEAE-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) column (9×30 cm) equilibrated with 0.02 M sodium acetate buffer (pH 6.0). The column was washed with the same buffer, and the adsorbed enzyme was eluted with a linear gradient of sodium chloride from 0 to 0.5 M. Active fractions were combined and concentrated by ultrafiltration.

Phenyl-Sepharose CL-4B chromatography: The concentrated enzyme solution was applied to phenyl-sepharose (Pharmacia) column (5×20 cm) equilibrated with 0.1 M sodium acetate buffer (pH 6.0) containing 0.5 M ammonium sulfate and 20% (v/v) ethylene glycol. The column was washed with the same buffer and the enzyme was eluted with a linear gradient of 0.5–0 M ammonium sulfate and 20–60% (v/v) ethylene glycol. The active fractions were collected, concentrated by ultrafiltration and dialyzed against 0.02 M potassium phosphate buffer (pH 6.0).

Hydroxyapatite chromatography: The dialyzed enzyme solution was applied to a hydroxyapatite (Bio-Rad Laboratories, Hercules, CA, USA) column (3×15 cm), which had been equilibrated with 0.02 M potassium phosphate buffer (pH 6.0). The column was washed with the same buffer and the enzyme was eluted with a linear gradient of 0.02–0.5 M potassium phosphate buffer (pH 6.0). The active fractions were combined and concentrated by ultrafiltration.

Gel filtration on Sephacryl S-300: The enzyme from the hydroxyapatite step was chromatographed on a Sephacryl S-300 (Pharmacia) column (2.5×90 cm). The enzyme was eluted with 0.05 M sodium acetate buffer (pH 6.0) containing 0.3 M NaCl. The active fractions were combined and concentrated to approximately 2 ml by ultrafiltration.

High performance liquid chromatography with TSK-gel G3000SW: The concentrated enzyme was applied on TSK-gel G3000SW (Tosoh Co, Tokyo, Japan) column (0.75×60 cm $\times 2$) equilibrated with 0.05 M sodium acetate buffer (pH 6.0) containing 0.3 M NaCl, and the enzyme was eluted with the same buffer. In the peak fractions of the eluted enzyme, fractions which were homogeneous on polyacrylamide gel electrophoresis were collected. This enzyme preparation was used to determine substrate specificity.

Enzyme assays

Glutaminase activities were determined by the method described previously [21]. Isolate G60 produced membrane-bound glutaminase and activities of glutaminase from the strain were determined in intact cells. Cells were grown at 25°C for 4 days in YM broth (Difco, Detroit, MI, USA). Reaction mixture (AG buffer, final volume 3.0 ml) containing 100 mM sodium acetate buffer (pH 6.0), 20 mM l-glutamine and cells (13.6 mg dry wt) was incubated for 30 min at 37°C. The reaction was stopped by immersing the test tube in boiling water for 3 min. One hundred microliters of sample were added to 2 ml of a glutamate dehydrogenase solution consisting of 10 mM EDTA-2Na, 4 mM NAD^+, 400 mM hydroxylamine-HCl and 30 U of glutamate dehydrogenase from beef liver (Oriental Yeast Co) in 100 mM potassium phosphate buffer (pH 7.2), and the reaction mixture was incubated at 37°C for 60 min. Glutamate was measured by the production of NADH ($OD_{340\ nm}$). One unit of glutaminase activity was defined as 1 μmol of l-glutamate per min from l-glutamine under the above conditions.

Activity at 18% NaCl: The enzyme assay was carried out by the same method except for the presence of 18% NaCl in AG buffer.

Thermal stability: After incubation of cells in their culture broth at various temperatures for 30 min, the glutaminase activities of the cells were determined.

Optimum temperature: Glutaminase activity in intact cells was determined at various temperatures for 30 min.

Optimum pH: Glutaminase activity in intact cells was determined in McIlvaine buffer (pH 2–8) and borate-KCl-NaOH buffer (pH 8–10) at 37°C.

Substrate specificity: The enzymatic reaction was carried out in AG buffer to which were added 100 μl of purified enzyme preparation. Activities were determined by following the formation of ammonia by TC urea/ammonia (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's instructions.

Characterization of isolate G60

Most of the methods adopted for the examination of morphological, physiological and biochemical characteristics were described by van der Walt and Yarrow [22] and Yarrow [24]. Other techniques included assimilation of nitrogen compounds [15], vitamin requirements [11], extraction, purification and identification of ubiquinone by high-performance liquid chromatography (HPLC) [16] and presence of xylose in whole cell hydrolysates by TLC [20].

Nucleic acid analysis

DNA was isolated and purified as described by Hamamoto and Nakase [8]. The DNA base composition was analyzed by HPLC after hydrolysis of DNA with P1 nuclease and alkaline phosphatase [8]. DNA–DNA reassociation experi-

ments used the microplate technique [2]. Hybridization experiments with isolate G60 were tested with *C.laurentii* and two negative controls, *C. flavus* and *C. luteolus*. DNA base composition analysis and DNA–DNA reassociation were done in triplicate and the standard deviation was determined.

PCR, cloning, and sequencing of large sub-unit rDNA

D1/D2 variable regions of large sub-unit rDNA were amplified by the polymerase chain reaction (PCR) using standard primers (forward primer 5′-GCATATCAATAAGCGGAGGAAAAG-3′; reverse primer 5′-GGTCCGTGTTTCAAGACGG-3′) [5]. PCR amplification was performed with Ex-Taq (TaKaRa Shuzo Co, Otsu, Japan) in a TaKaRa Thermal Cycler MP. Amplification conditions consisted of an initial cycle of denaturation of 3 min at 94°C followed by 30 cycles consisting of 30 s at 94°C, 15 s at 52°C, 1 min at 72°C and a final cycle of 5 min at 72°C. The PCR products were cloned directly in the plasmid pCR2.1 vector (Invitrogen Co, Carlsbad, CA, USA: Original TA cloning kit), and transformed into INV αF′ cell of the TA Cloning Kit. The plasmids were extracted and purified with QIAprep kit (Qiagen Co, Funakoshi Co, Tokyo, Japan), and used for sequencing. Sequencing employed the cyclic reaction method using fluorescence labeled dideoxyribonucleotide triphosphates. The sequence reaction and the processing of the reaction products for electrophoresis were performed following instructions for the sequencing kit (PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kit, Perkin-Elmer Co, Norwalk, CT, USA). Electrophoresis and data collection used an ABI Model 373A DNA Sequencer (Perkin-Elmer Co).

Phylogenetic analysis

Comparative sequences of the D1/D2 region of the large sub-unit rDNA of other species of hymenomycetous yeasts were provided by Fell *et al* [4]. Alignments were made with MegAlign (DNAStar) and visually corrected. Phylogenetic trees were computed with PAUP version 4.0d61 (with permission of D Swofford) using parsimony analysis (heuristic search, stepwise addition, random addition sequence, nearest-neighbor interchange, 100 maximum trees). Bootstrap analysis [6] was based on 1000 replicates.

Results and discussion

Enzyme studies

The purification steps are summarized in Table 1. The enzyme was purified 1250-fold with a recovery of 3.3%. The purified enzyme obtained from TSK gel G3000SW, which showed a single band on polyacrylamide gel electrophoresis (data not shown), was tested for its substrate specificity. Table 2 shows the substrate specificity of the enzyme towards several glutamyl derivatives. l-Glutamine and d-glutamine were the best substrates for the enzyme. Activities for l- and d-asparagine were 45% and 23% respectively of that for l-glutamine. However peptides were not substrates of the enzyme (data not shown). These findings suggest that the enzyme from isolate G60 could be defined as glutaminase asparaginase (l-glutamine l-asparagine amidohydrolase, EC. 3. 5. 1. 38) which was reported in *Pseudomonas* [19] and *Acinetobacter* [9] species. However, the isolate G60 enzyme differed from bacterial ones in its activity for d-glutamine as well as l-glutamine.

Table 1 Purification of glutaminase asparaginase from isolate G60

Step	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Yield (%)
Crude extract	21400	2060	0.096	100
1st heating	15000	2100	0.13	102
2nd heating	15500	1970	0.13	95.6
Acetone precipitation	1020	1400	1.37	68.0
DEAE-Sepharose	322	1200	3.37	58.3
Phenyl-Sepharose	11.7	274	23.4	13.3
Hydroxyapatite	2.69	210	78.1	10.2
Sephacryl S-300	0.83	76.7	92.4	3.7
HPLC	0.55	67.6	123	3.3

Table 2 Substrate specificity of isolate G60 glutaminase asparaginase

Substrate	Relative activity (%)
l-glutamine	100
d-glutamine	96
l-asparagine	45
d-asparagine	23

Activity is expressed as a percentage of the activity toward l-glutamine.

Since the digestive reaction in production of hydrolyzed proteins is generally done in the presence of NaCl and under higher temperature conditions (>40°C), both salt-tolerance and thermostability are industrially important. Thus, we examined salt-tolerance and thermostability of crude glutaminase from isolate G60 with intact cells. The salt-tolerant and thermostable glutaminase produced by isolate G60 exhibited more salt- and heat-tolerance than those of other microorganisms (Table 3). The enzyme activity of isolate G60 in the presence of 18% NaCl was 85% of that without NaCl, and more than 90% of the activity remained after incubation at 65°C for 30 min.

These results strongly suggest that the glutaminase of isolate G60 is useful for an increase in the amount of l-glutamate in enzymatically hydrolyzed protein containing a large amount of l-glutamine in the presence of NaCl and/or under higher temperature conditions.

Characterization of isolate G60

Physiological and biochemical tests demonstrated that isolate G60 had xylose in whole cell hydrolysates, assimilated inositol, produced starch-like substances, lacked the ability to ferment alcohol, and demonstrated a positive DBB color reaction and urease activity. These characteristics are in accord with those of the genus *Cryptococcus* [23]. Identification within the genus, based on the most recent taxonomic key [3], implied that isolate G60 was a strain of *C. laurentii*. The isolate and *C. laurentii* had similar phenotypic characteristics with the exception that isolate G60 did not utilize arbutin.

Table 3 Properties of glutaminases produced by various microorganisms

Species	Activity at 18% NaCl[a]	Stable temp. range (°C)	Optimum temperature	Optimum pH
Cryptococcus nodaensis[b]	85 (1.9)	≦65	70	5.8–8.0
Cryptococcus albidus[c]	50	≦60	70	5.5–8.5
Candida famata[b]	70 (1.5)	≦60	60	6.5–8.5
Bulleromyces albus[d]	80	ND	ND	6.0–9.0
Aspergillus sojae[e]	6	≦45	ND	7.5–8.5
Escherichia coli[e]	65	≦45	ND	5
Bacillus subtilis[f]	90	≦45	ND	6

ND, No data.
(), Standard deviation value ($n = 3$).
[a]Percentage relative to the activity when no NaCl was added.
[b]Data obtained in this study.
[c]Data from Reference [9].
[d]Data from Reference [12].
[e]Data from Reference [23].
[f]Data from Reference [17].

Isolate G60 demonstrated a low DNA relatedness value (7–11%) with *C laurentii* (Table 4), indicating that the strains represent separate species. Therefore, a new species, *C. nodaensis*, is proposed. Sequence analysis (Figure 1) confirmed that isolate G60 is a member of the tremellales belonging to a group of anamorphic and teleomorphic species of *Bulleromyces*, *Tremella*, *Sirobasidium*, *Cryptococcus* and *Bullera* [4]. These species are mainly plant associated and isolation of isolate G60 from soil suggests that plants may also be its natural habitat. Sexual mechanisms have been observed in two of the species: the teleomorph *Bulleromyces albus* [1] and between strains of *Cryptococcus laurentii* [12]. The latter species is genetically heterogeneous as indicated by the range of mol% G+C values of 51–59% [14] and sequence analysis [7]. The phenotypic similarity of *C. laurentii* and isolate G60 indicates that G60 represents one of the multiple species within *C. laurentii*. Formal presentation of isolate G60 as a separate species is an initial step in clarification of this species complex and future studies with the isolate should include mating studies with strains of *C. laurentii*.

Description of Cryptococcus nodaensis sp nov

In YM broth, after 3 days at 25°C, cells are lemon- to spindle-shaped, ovoid, 4–9 × 6–13 μm (Figure 2), occurring singly or in pairs. A ring, islets and sediment are formed. After 1 month at 17°C, a ring, pellicle and sediment are present. On YM agar, after 1 month at 17°C, the streak culture is cream-colored, smooth, dull-shining, soft to mucous and with an entire margin. On dalmau plate culture on corn meal agar, pseudo- and true mycelia are not observed. It does not ferment glucose. It assimilates glucose, galactose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, soluble starch, d-xylose, l-arabinose, d-arabinose (latent), l-rhamnose, d-ribose, ethanol (latent), glycerol (latent), erythritol (latent), ribitol, galactitol, d-mannitol, d-glucitol, α-methyl-d-glucoside, salicin, 2-ketogluconic acid, dl-lactic acid, succinic acid, citric acid (weak), inositol, d-psicose (latent and weak), gentiobiose, palatiose, d-turanose, xylitol (latent and weak), maltitol, d-arabitol (latent and weak), gluconic acid (latent and weak), β-methyl-d-glucoside (latent and weak), d-gluconate, fumaric acid (latent and weak) and l-malic acid (latent and weak). It does not assimilate l-sorbose, inulin, methanol, amygdalin, arbutin, N-acetyl-d-glucosamine or d-glucosamine. It assimilates sodium nitrite, ethylamine hydrochloride, l-lysine hydrochloride and cadaverine dihydrochloride. It does not assimilate potassium nitrate. Maximum growth temperature is 28–30°C. It does not require a vitamin for growth. Starch-like substances are produced. It grows weakly on 50% (w/w) glucose-yeast extract agar. There is no growth on 60% (w/w) glucose-yeast extract agar. It grows weakly in the presence of 1000 ppm of cycloheximide. Urease positive. Gelatin is

Table 4 DNA relatedness among *Cryptococcus nodaensis* and closely related species

Species	Strain	G+C content* mol%	% Relative binding of labeled DNA from			
			G60	JCM 9066^T	JCM 3689^T	JCM 8332^T
C. nodaensis	G60^T	56.9 (0.49)	100	7 (0.6)	8 (1.2)	6 (1.4)
C. laurentii	JCM 9066^T	57.0 (0.21)	11 (1.5)	100	12 (1.5)	11 (0.4)
C. luteolus	JCM 3689^T	59.5 (1.20)	8 (0.9)	9 (1.5)	100	8 (1.1)
C. flavus	JCM 8332^T	57.1 (0.35)	5 (2.5)	7 (1.0)	10 (2.8)	100

*This study.
^{T}Type strain.
(), Standard deviation value ($n = 3$).

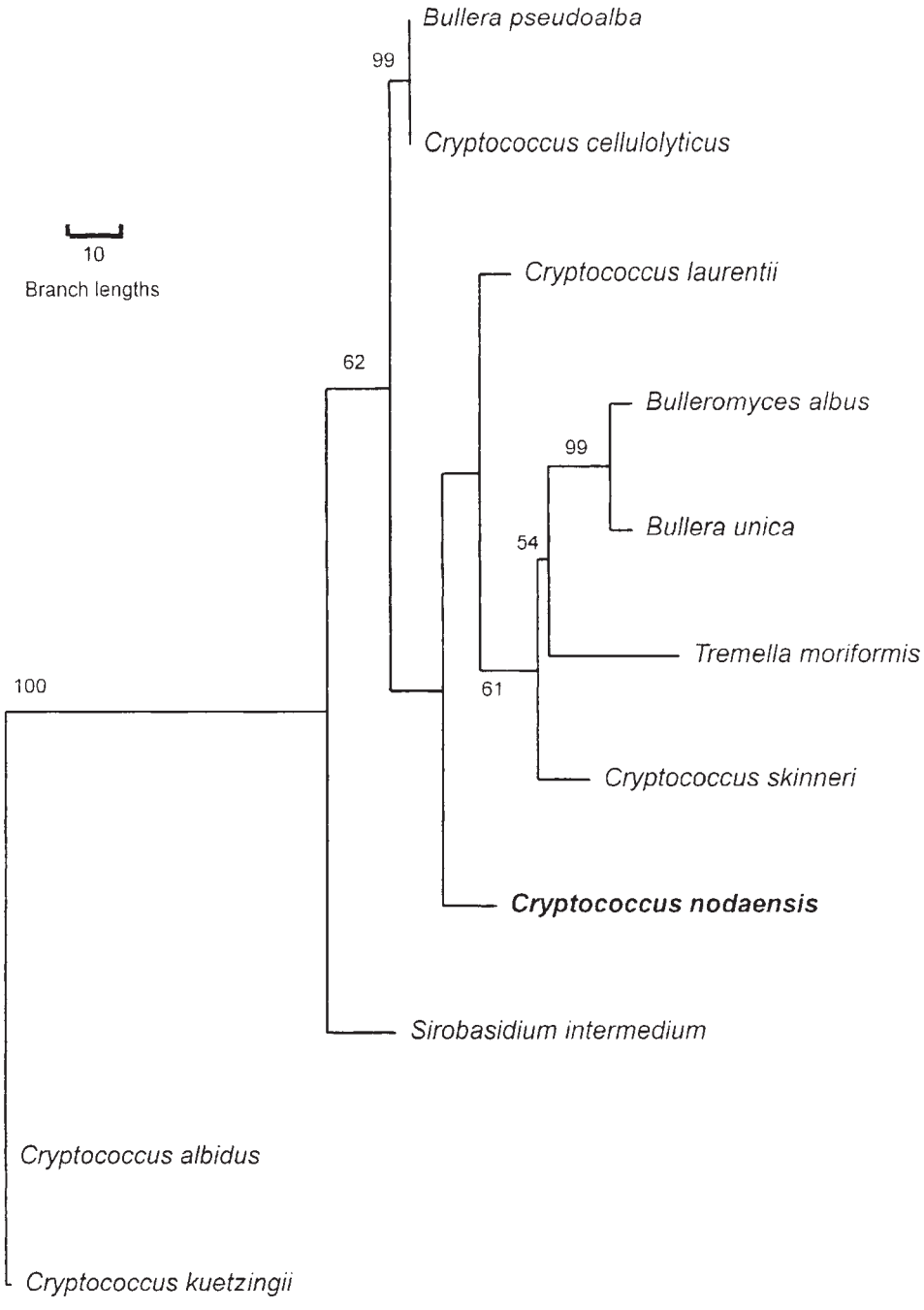

Figure 1 Phylogenetic tree of a cluster [4] of related species of tremellaceous yeasts including *Cryptococcus nodaensis*. The tree, based on a sequence analysis of the D1/D2 region of the large subunit rDNA, was derived from parsimony analysis (heuristic search, stepwise addition, random addition sequence, nearest-neighbor interchange, 100 maximum trees). Branch lengths are proportional to the number of nucleotide differences. The numbers given on branches are the frequencies (percentages) that a given branch appeared in 1000 bootstrap replicates (PAUP test version 4.0d61 with permission of D Swofford). *C. albidus* and *C. keutzingii* are used as outliers.

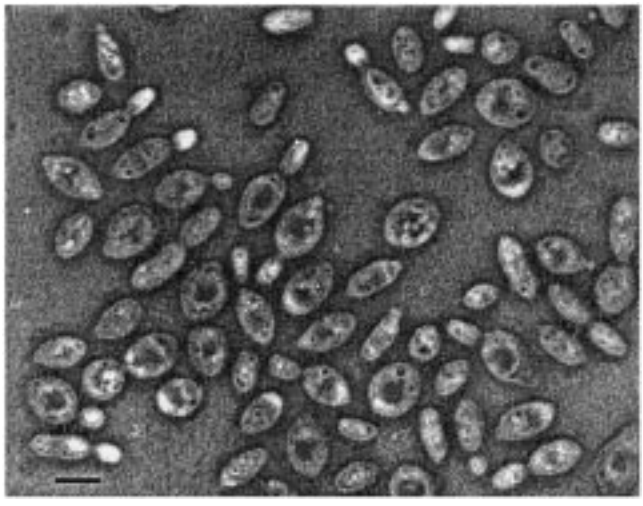

Figure 2 Vegetative cells of *Cryptococcus nodaensis* sp nov grown in YM broth for 3 days at 25°C. Scale bar indicates 10 μm.

liquefied. Fat is hydrolyzed. The diazonium blue B reaction is positive. It does not produce acid on chalk agar. It grows in the presence of 5% (w/w) NaCl. The G+C content of nuclear DNA is 56.9 mol% as determined by HPLC. The major ubiquinone is Q-10. Xylose is present in the cells. The type strain of *C. nodaensis*, G-60^T, was isolated from soil in Noda City, Chiba Pref, Japan. This strain has been deposited in the Centraalbureau voor Schimmelcultures as CBS 101036. Nucleotide sequence of the D1/D2 partial region of large sub-unit and ITS region will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB16233 and AB16234, respectively.

Acknowledgements

We are grateful to Dr T Nakase, Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN, Wako, Japan), and Dr G Kawai, Research and Development Division, Mori & Company, Kiryu, Japan, for helpful suggestions and discussion. We also thank Dr K Hyashi and Dr S Fujii for helpful discussions.

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