NOTE

Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, PO Box 115 Yusong, Taejeon 305-600, Republic of Korea

Metschnikowia koreensis sp. nov., a novel yeast species isolated from flowers in Korea

Soon Gyu Hong, Jongsik Chun, Hyun Woo Oh and Kyung Sook Bae

Author for correspondence: Kyung Sook Bae. Tel: +82 42 860 4610. Fax: +82 42 860 4677. e-mail: ksbae@mail.kribb.re.kr

A novel ascomycetous yeast was isolated from flowers of *Lilium* sp. and *Ipomoea* sp. in Korea. The name *Metschnikowia koreensis* sp. nov. (type strain SG99-34^T = CBS 8854^T = KCTC 7998^T) is proposed for this novel species based on comparative sequence analyses of the D1/D2 domain of 26S rDNA and phenotypic characteristics.

Keywords: yeast, *Metschnikowia*, 26S rDNA, *Metschnikowia koreensis* sp. nov.

The ascomycetous yeast genus *Metschnikowia* is defined by the presence of multilateral budding of vegetative cells and by the production of one or two needle-shaped ascospores in elongated asci (Miller & Phaff, 1998). Species isolated from terrestrial habitats are typically associated with flowers or fruits and transmitted to new niches by insects; species isolated from aquatic habitats are often parasitic in invertebrates but can also be isolated as free-living forms in water (Miller & Phaff, 1998). The extent of rRNA sequence diversity among *Metschnikowia* species is surprisingly high considering the relative phenotypic homogeneity of these taxa (Mendonça-Hagler *et al.*, 1993). It has also been suggested that the genus has numerous missing taxa because inclusion of several undescribed species has considerably shortened some terminal branches (Kurtzman & Robnett, 1998).

In the course of an isolation programme of yeasts from various natural sources in Korea, five strains of yeast were isolated from *Ipomoea* sp. and *Lilium* sp. from one location. Determination of the nucleotide sequence of the D1/D2 domain of the 26S rDNA of the strains and comparison with a database of sequences from all currently recognized yeasts confirmed that five strains represent novel species. Two strains were included in the *Sporidiobolus* clade (Fell *et al.*, 2000) and three strains were included in the *Metschnikowia* clade (Kurtzman & Robnett, 1998). Among them, two strains had similar physiological, biochemical and molecular characteristics. These two strains (SG99-34^T and SG99-25) are described here as *Metschnikowia koreensis* sp. nov.

The morphological and physiological characteristics of strains SG99-34^T and SG99-25 were determined by

conventional techniques described by Yarrow (1998). The utilization of carbon sources was examined at 25 °C on a rotary shaker (120 r.p.m.) at 7 d intervals for 21 d. Carbon sources that were utilized only after more than 7 d were scored as a delayed reaction and weakly positive assimilation during 3 weeks was scored as weak. The utilization of nitrogen sources was examined by the auxanographic technique for 7 d. Urease activity was tested in Christensen's urea agar (Christensen, 1946). Determination of the coenzyme Q system was carried out as described by Yamada (1998) with an HPLC equipped with a Spherisorb S5 ODS2 column (Waters). The DNA base composition (G + C mol %) was determined by examining *T_m*. As a control, a DNA preparation from *Escherichia coli* KCTC 2443 (G + C content = 51.0 mol %) was included. DNA–DNA hybridization was performed using the DIG High Prime DNA Labelling and Detection Starter kit II (Roche) and a Bio-Dot SF slot blotting apparatus (Bio-Rad) following the manufacturers' instructions.

For transmission electron microscopy, material was fixed in a 2.5% paraformaldehyde-glutaraldehyde fixative mixture buffered with 0.1 M phosphate (pH 7.2) for 2 h at 4 °C. The sample was washed three times with 0.1 M phosphate (pH 7.2), post-fixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol and propylene oxide, and then embedded in Epon-812. Thin sections (80 nm), made using the Reichert-Jung ULTRACUT E (Leica) ultramicrotome, were stained in a saturated uranyl acetate solution in distilled water (2 h at 25 °C) followed by lead citrate (15 min at 25 °C). A CM 20 (Philips) electron microscope was used for analysis.

The D1/D2 domain of nuclear 26S rDNA was amplified and sequenced using the primer pair no. 4 (ACCCG CTGAA YTAA GCATA T) and no. 11

The GenBank accession numbers for the 26S rDNA sequence of strains SG99-34^T and SG99-25 are AF296438 and AF257272, respectively.

(CTCCT TGGTC CGTGT TTCAA GACGG) (Van der Auwera *et al.*, 1994). The amplified 26S rDNAs were purified using a Wizard PCR prep (Promega). The nucleotide sequences were determined with BigDye terminator cycle sequencing kits (PE Applied Biosystems) following the manufacturer's instructions. The gel electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems). The sequences were proofread, edited and merged into composite sequences using the PHYLIT program version 3.0 (Chun, 1995). The 26S rDNA D1/D2 sequences of the strains were aligned with other 26S rDNA sequences from the genus *Metschnikowia* (Kurtzman & Robnett, 1998; Lachance *et al.*, 1998) on the basis of similarity of the primary and secondary structures using the PHYLIT program. Tree topology was reconstructed with the Kimura's 2-parameter distance model (Kimura, 1980) and the neighbour-joining method (Saitou & Nei, 1987) using the PHYLIP 3.05 package (Felsenstein, 1993). *Saccharomyces cerevisiae* was used as the outgroup. Confidence levels for the individual branches of the resulting tree were assessed by bootstrap analysis (Felsenstein, 1985) in which 1000 bootstrapped trees were generated from the resampled data. The resultant phylogenetic trees were visualized using the TREEVIEW program (Page, 1996).

The phylogenetic placement of strain SG99-34^T was determined by analysis of the D1/D2 domain sequences of 26S rDNA from all currently recognized ascomycetous yeast species, as reported in the studies of Kurtzman & Robnett (1998) and Lachance *et al.* (1998). Strain SG99-34^T clustered with the core group of the genus *Metschnikowia* including *Metschnikowia bicuspidata*, the type species of the genus, and was closely related to *M. reukaufii* (Fig. 3). Morphological characteristics of strain SG99-34^T also demonstrated that it should be assigned to the genus *Metschnikowia*. It reproduced by multilateral budding and produced ellipsoidopedunculate asci containing two acerospores.

It has been proposed that yeast strains showing greater than 1% nucleotide substitution in the D1/D2 domain of 26S rDNA are likely to belong to different species (Kurtzman & Robnett, 1998). The sequence similarity of the D1/D2 domain between strain SG99-34^T and *M. reukaufii* NRRL Y-7112^T was 93.7%, which strongly implies that strain SG99-34^T represents a unique species. Strain SG99-34^T can be differentiated phenotypically from *M. reukaufii* by its ability to assimilate D-glucosamine, lack of growth on hexadecane, growth in 16% NaCl/5% glucose, production of urease and growth at 35 °C (Miller & Phaff, 1998; Pitt & Miller, 1968). Urea hydrolysis is rarely observed in ascomycetous yeast, apart from in *Schizosaccharomyces pombe*, *Yarrowia lipolytica* and *Lipomyces* sp. (Yarrow, 1998). Therefore, it can be used as a unique characteristic to distinguish *M. koreensis* from other *Metschnikowia* species. On the basis of molecular and physiological characteristics, it

is fair to describe strain SG99-34^T as a novel species in the genus *Metschnikowia*.

Strain SG99-25 differed in the following characteristics from SG99-34^T. The sequence difference of the D1/D2 domain between the two strains was 1 out of 532 sites. It produced asci in 1:29 diluted V8 agar rather than 1:9 diluted V8 agar and formation of asci was not frequent. The major ubiquinone of both strains was Q9, but SG99-25 contained considerable amounts (Q9:Q8 = 4:1) of the Q8 ubiquinone system. The assimilation of D-galactose and ethanol was negative and weak, respectively. Because of the considerable differences in physiological and biochemical characteristics between the two strains, chromosomal DNA similarity was analysed by DNA-DNA hybridization to test conspecificity. The result of 91% similarity indicated that the two strains are conspecific.

Latin diagnosis of *Metschnikowia koreensis* Hong, Chun, Oh et Bae sp. nov.

Cultura in aqua extracto malti post dies 3 ad 25 °C cellulae ellipsoideae ad cylindratae (3–6 × 4–7 µm), singulae vel catenatae. Post unum mensem sedimentum formatur. Cultura in agaro malti post dies 3 ad 25 °C albida crenea et butyrosa. Chlamydosporae subgloboseae (6–8 × 7–9 µm), refractivae. In agaro farnae Zea mays post dies 10 pseudomycelium nullum vel rudimentarium. Asci ellipsoidopedunculati (4–5 × 14–20 µm), 2-spori. Ascosporae aciculares vel acerosae (13–19 × 1 µm). Glucosum fermentantur, at non galactosum, saccharum, maltosum, lactosum, raffinoseum nec inulinum. Glucosum, galactosum (lente), L-sorboseum, saccharum, maltosum, cellobiosum, trehalosum, melezitoseum, D-xylosum, D-ribosum (lente), D-glucosaminum, N-acetylglucosaminum, ethanolum, glycerolum, ribitolum, D-mannitolum, D-glucitolum, methyl α-D-glucosidum, salicinum, acidum D-gluconicum et acidum succinicum assimilantur at non lactosum, melibiosum, raffinoseum, inulinum, amyllum solubile, L-arabinosum, D-arabinosum, L-rhamnosum, erythritolum, galactitolum, acidum DL-lacticum, acidum citricum, inositolum, hexadecanum nec acidum D-glucuronicum. Assimilatio ethylamini, cadaverini et L-lysini ad non kallii nitratis, sodii nitrosi, D-glucosaminum, creatini nec creatinini. Vitaminae externae ad crescentiam necessariae sunt. Maxima temperatura crescentiae: 35 °C. Materia amyloidea non formatur. Urea finditur. Proportio molaris guanini plus cytosini in acido deoxyribonucleico: 41.2 ± 0.8 per centum. Typus: SG99-34^T (= KCTC 7998^T = CBS 8854^T), ex flora Lilium sp. isolatus est. In collectione zymotica Korean Collection for Type Cultures et Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, praeservatus.

Description of *Metschnikowia koreensis* sp. nov.

Metschnikowia koreensis (ko.re.en'sis. L. nom. fem. adj. *koreensis* of Korea, referring to the country where the yeast was isolated).

Fig. 1. Differential interference contrast micrographs of vegetative cells (top) and chlamydospores (bottom) of *Metschnikowia koreensis* SG99-34^T. Bars, 10 µm.

In 5% malt extract broth after 3 d at 25 °C, the cells are ellipsoidal to short cylindrical shapes (3–6 × 4–7 µm). They mainly occur singly or in short chains. Vegetative reproduction proceeds by multilateral budding (Fig. 1, top). A sediment forms after 4 weeks. A pellicle is absent. On 10% malt extract agar after 3 d at 25 °C, the colonies are white to cream-coloured and butyrous. Two morphotypes become apparent after repeated subculturing: one has a somewhat rough and dull surface and the other is smooth and slightly glistening. The former consists mainly of actively reproducing vegetative cells and the latter consists mainly of refractile subglobose chlamydospores (6–8 × 7–9 µm; Fig. 1, bottom). The margins are usually smooth, but fringed in some cases. In Dalmau plate cultures on cornmeal agar after 10 d at 25 °C, two types of cells are observed in one colony: one is subglobose and glistening under the microscope and the other has cylindrical shape and dull texture. Pseudomycelium is rudimentary or absent. Sporulation of strain SG99-34^T is observed on dilute (1:9) V8 agar after 2 weeks incubation at 20 °C. Ellipsoidopedunculate asci (4–5 × 14–20 µm) containing two

Fig. 2. Asci and ascospores of *Metschnikowia koreensis* SG99-34^T. (top) Scanning electron micrograph of asci; bar, 10 µm. (middle) Transmission electron micrograph of ascus; bar, 2 µm. (bottom) Transmission electron micrograph of tail of ascus; bar, 2 µm.

ascospores arise from chlamydospores (Fig. 2). The ascospores are acerose (13–19 × 1 µm). Asci do not lyse at maturity. Strain SG99-25 produces asci on dilute (1:29) V8 agar. D-Glucose is fermented; D-

Fig. 3. Neighbour-joining tree for *Metschnikowia koreensis* and related taxa using *Saccharomyces cerevisiae* as the outgroup. The phylogram was calculated from nucleotide divergence in the D1/D2 region of the 26S rDNA. The numbers given on branches are the frequencies (> 50%) with which a given branch appeared in 1000 bootstrap replications.

galactose, sucrose, maltose, lactose, raffinose and trehalose are not fermented. Assimilation of carbon compounds is as follows: D-glucose, +; D-galactose, + (latently); L-sorbose, +; sucrose, +; maltose, +; cellobiose, +; trehalose, +; lactose, –; melibiose, –; raffinose, –; melezitose, +; inulin, –; soluble starch, –; D-xylose, +; L-arabinose, –; D-arabinose, –; D-ribose, + (latently); L-rhamnose, –; D-glucosamine, +; N-acetyl-D-glucosamine, +; methanol, –; ethanol, +; glycerol, +; erythritol, –; ribitol, +; galactitol, –; D-mannitol, +; D-glucitol, +; methyl α -D-glucoside, +; salicin, +; D-gluconate, +; DL-lactate, –; succinate, +; citrate, –; inositol, –; hexadecane, –; and glucuronate, –. Assimilation of nitrogen compounds is as follows: nitrate, –; nitrite, –; ethylamine, +; cadaverine, +; L-lysine, +; D-glucosamine, –; creatine, –; and creatinine, –. Growth or response in other tests: 10% NaCl/5% glucose, +; 16% NaCl/5% glucose, +; 0.01% cycloheximide, –; 0.1% cycloheximide, –; 1% acetic acid, –; starch formation, –; urease, +; growth at 35 °C, +; growth at 37 °C, –; vitamin-free medium, –; Diazonium Blue B, –. The major ubiquinone system of strain SG99-34^T is Q9. However, Q8 is also present in small proportions (39:1). The amount of Q8 is higher in strain SG99-25 than in strain SG99-34^T (4:1). The G + C molar ratio is 41.2 ± 0.8 mol% (mean of four determinations). The closest phylogenetic relative of SG99-34^T based on the D1/D2 domain sequences of 26S rDNA is *Metschnikowia reukaufii* NRRL Y-7112^T (Fig. 3). The similarity of the two sequences is 93.7%. The sequence difference between strains SG99-34^T and SG99-25 is 1 out of 532 sites. The type strain SG99-34^T (= KCTC 7998^T = CBS 8854^T) and strain SG99-25 (= KCTC 7828) were isolated in August 1999 from flowers of *Lilium* sp. and *Ipomoea* sp., respectively, in Kyungsang province, Korea. Strain SG99-25 differs from the type strain in the following assimilation characteristics: it does not assimilate D-galactose and weakly assimilates ethanol.

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