Trichosporon xylopinii sp. nov., a hemicellulose-degrading yeast isolated from the wood-inhabiting beetle *Xylopinus saperdioides*

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Four arthroconidium-producing yeasts were isolated from the gut of wood-inhabiting tenebrionid and passalid beetles. The rRNA genes of these yeast strains were sequenced, compared and analysed. The sequence results and other taxonomic characterizations placed two of the strains into *Trichosporon porosum*, and the remaining strains, EH024^T and EH026 which were isolated from *Xylopinus saperdioides* (Coleoptera: Tenebrionidae), into a novel species of the genus *Trichosporon* in the Porosum clade. Strain EN6S23 was independently isolated from forest soil in Taiwan and was identified as the same novel species based on identical sequences in the internal transcribed spacers (ITS) and the D1/D2 region of the LSU rRNA gene and similar physiological characteristics to those of strains EH024^T and EH026. The three strains can assimilate cellulose and xylan as sole carbon source, and are clearly distinguished from their closest taxon, *T. porosum*, by 14 nt differences in the ITS and D1/D2 region. These strains did not reproduce sexually under the laboratory conditions tested. The novel species is proposed as *Trichosporon xylopinii* sp. nov. (type strain EH024^T = ATCC MYA-4670^T = CBS 11841^T).

INTRODUCTION

Yeasts in the genus *Trichosporon* are characterized morphologically by their production of arthroconidia with septate mycelia, and are often associated with soil and water although some species are commonly recognized as opportunistic pathogens (Guého *et al.*, 1998). Some species of the genus *Trichosporon* are also known to be associated with insects. For example, strains of *Trichosporon mycotoxinivorans* and *Trichosporon scarabaeorum* were isolated from the gut of termites and scarab beetles, respectively (Middelhoven *et al.*, 2004; Molnar *et al.*, 2004). *Trichosporon insectorum*, a killer yeast isolated from scarab and passalid beetles in Panama, was also reported by Fuentesfria *et al.* (2008). Members of the *Trichosporon* clade were identified as frequently collected yeasts from the digestive tract of beetles, many of which have not been described previously (Suh *et al.*, 2005).

Interestingly, a few species of the genus *Trichosporon*, such as *Trichosporon porosum*, *Trichosporon sporotrichoides* and *Trichosporon wieringae*, are able to assimilate hemicelluloses and phenolic compounds which are mainly found in

plant tissues in nature (Middelhoven, 2004; Middelhoven *et al.*, 2001). During a survey of microbial flora in the gut of wood-inhabiting insects, we found several basidiomycetous anamorphic yeasts which produce arthroconidia, and some of these yeasts were identified as an undescribed species of the genus *Trichosporon*. Here we describe the novel *Trichosporon* species isolated in this study and discuss its phylogeny and ecology.

METHODS

Yeast isolation and characterization. The wood-inhabiting beetles, *Xylopinus saperdioides* (Coleoptera: Tenebrionidae) and *Odontotaenius disjunctus* (Coleoptera: Passalidae), were collected from a rotten oak tree (*Quercus* sp.) on Bull Run Mountain (38° 49' 42" N 77° 42' 58" W) in Broad Run, Virginia, USA, on 12 June 2008. The methods for isolating yeasts from insects were described in detail in previously published papers (Suh & Blackwell, 2004; Suh *et al.*, 2004). Strain EN6S23 was isolated from a humus soil sample under bamboo forest in Meishan (23° 34' 42" N 120° 38' 57" E), Chiayi, Taiwan, on 3 November 2007 by the method previously described (Liu *et al.*, 2008; Lee *et al.*, 2009). The morphological observations and metabolic tests that constitute the yeast standard description were performed according to established methods (Yarrow, 1998; Barnett *et al.*, 2000). Assimilation tests for carbon and nitrogen sources were done in liquid media. Starved inocula were used in the nitrogen and vitamin assimilation tests. The yeasts were observed individually or in mixed cultures for basidiospore formation

Abbreviation: ITS, internal transcribed spacers.

The GenBank/EMBL/DBJ accession numbers for the rRNA gene sequences from this study are FJ527221 and HQ005752–HQ005762.

on YM agar, 2 % malt extract agar or cornmeal agar at 25 °C for up to 6 weeks (Yarrow, 1998). The degradation of cellulose and xylan was tested by the dye diffusion method using cellulose azure agar and RBB-xylan agar, respectively, as described by Pointing (1999). Assessment of killer activity was performed on modified YM agar (YM broth containing 15 % glycerol, 2 % agar, and 0.003 % methylene blue) as described by Fuentesfria *et al.* (2008). The test medium was buffered to pH 3.0–6.0 at intervals of 0.5 pH units with 100 mM citrate–phosphate buffer. The following strains were used as sensitive strains for the test: *Candida albicans* ATCC 10231 and ATCC 14053; *Candida lusitanae* ATCC 42720; *Candida glabrata* ATCC 15545; *Candida parapsilosis* ATCC 22019^T; *Cryptococcus neoformans* ATCC 14115, ATCC 18365 and ATCC 24064; and *Filobasidium uniguttulatum* ATCC 24227. *T. insectorum* ATCC MYA-4361^T was used as a positive control for killer activity (Fuentesfria *et al.*, 2008).

DNA sequencing and molecular phylogenetic analyses. The methods for nucleic acid extraction, PCR amplification and sequencing of rRNA genes were those discussed by Suh & Zhou (2010). The complete sequences of the SSU rRNA gene, the internal transcribed spacers (ITS) including the 5.8S rRNA gene, and the D1/D2 region of the LSU rRNA gene were sequenced with primers NS1, NS2, NS4, NS8, ITS1, ITS4, LR0R and LR3 using an ABI 3130xl automated DNA sequencer. Sequences from new isolates were compared to those of other yeasts in GenBank using the BLAST search program (Altschul *et al.*, 1990). Selected DNA sequences were aligned with the multialignment program CLUSTAL X (Thompson *et al.*, 1997) and adjusted visually. GenBank accession numbers for the DNA sequences generated in this study are: HQ005761/HQ005752/HQ005757 (SSU rRNA gene/ITS including 5.8S rRNA gene/D1/D2 region of LSU rRNA gene) for strain EH024^T; –/HQ005753/HQ005758 for strain EH026; –/HQ005754/FJ527221 for strain EN6S23; –/HQ005756/HQ005760 for strain EH157; HQ005762/HQ005755/HQ005759 for strain EH158 (–, sequence not determined). Maximum-parsimony analyses were performed using PAUP 4.0b10 (Swofford, 2002). Heuristic tree searches were executed using the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. Bootstrap values of the most parsimonious tree were obtained from 1000 replications. Base-pair differences were counted using BLAST2 sequences (Tatusova & Madden, 1999) or from a manually aligned sequence database.

RESULTS AND DISCUSSION

Characterization and molecular phylogeny of the novel species

Among the yeasts isolated from wood-inhabiting insects and woody substrates, four strains were identified as species of the genus *Trichosporon* on the basis of DNA sequences and other taxonomic characteristics. The strains and sources are as follows: EH024^T (=ATCC MYA-4670^T) and EH026 (=ATCC MYA-4671) from the gut of *Xylopinus saperdioides*; EH157 and EH158 (=ATCC MYA-4673), from the gut of *Odontotaenius disjunctus*. Another strain, EN6S23 (=ATCC MYA-4672), which is taxonomically very similar to the above strains, was independently isolated from humus forest soil in Taiwan. Strains EH157 and EH158 were identified as *Trichosporon porosum* based on identical ITS and D1/D2 region sequences to those of the type strain CBS 2040^T (Middelhoven *et al.*, 2001). On the other hand, the remaining three strains were identified as a novel species

of the genus *Trichosporon* which is phylogenetically close to *T. porosum*. Therefore, here we propose a novel species, *Trichosporon xylopinii* sp. nov., to accommodate strains EH024^T, EH026 and EN6S23.

The strains representing *T. xylopinii* sp. nov. are identical to each other in the ITS and D1/D2 regions, but are clearly distinguished from their closest taxon *T. porosum* by 13 nt substitutions and a gap in the regions. Interestingly, between the two taxa, more variability was shown in the D1/D2 region (12 substitutions and 1 gap in 568 bp) than in the ITS region (1 substitution in 523 bp). This pattern of variability in the two regions has been shown frequently among the species in the order Trichosporonales, such as between *Trichosporon laibachii* and *Trichosporon multisporum* or between *Trichosporon montevidense* and *Trichosporon domesticum* (Scorzetti *et al.*, 2002). There is no variation in the SSU rRNA gene between *T. porosum* and the novel species.

The genus *Trichosporon* could be divided into four major clades based on the sequence analyses of the D1/D2 region of the LSU rRNA gene and the ITS region (Fell *et al.*, 2000; Scorzetti *et al.*, 2002; Sugita *et al.*, 1999), which were named Gracile, Porosum, Cutaneum and Ovoides by Middelhoven *et al.* (2004). An additional clade, Brassicae, was recognized by D1/D2 sequence analyses with expanded taxon sampling including several novel species isolated from bat guano samples (Sugita *et al.*, 2005). These major clades of *Trichosporon* correspond to the serotypes associated with the occurrence of summer-type hypersensitivity pneumonitis as well as to the ubiquinone types (Sugita *et al.*, 2001, 2005). A phylogenetic tree, reconstructed from the ITS and D1/D2 sequences (about 1.1 kb) of 14 *Trichosporon* species including the novel species from this study, showed that *T. xylopinii* sp. nov. is closely related to *T. porosum* in the Porosum clade of the genus *Trichosporon* (Fig. 1). *T. xylopinii* sp. nov. made a clade with *T. porosum* with 100 % bootstrap support, and the two species were grouped with other species in the Porosum clade, i.e. *Trichosporon dehoogii*, *T. sporotrichoides*, *Trichosporon gamsii*, *T. wieringae*, *Trichosporon lignicola* and strain JCM 12596 (known as '*Trichosporon coprophilum*'). The Porosum clade was well supported by a high statistical value among the taxa compared in the tree (Fig. 1). Several phylogenetic studies with broader taxon samplings showed that some of the species in the genera *Cryptococcus*, *Bullera* and *Cryptotrichosporon* are closely related to the yeasts in the genus *Trichosporon* (e.g. Okoli *et al.*, 2007; Middelhoven *et al.*, 2004). For example, *Cryptococcus curvatus*, *Cryptococcus humicola* and a few other cryptococci were placed into clades with species of the genus *Trichosporon* in several phylogenetic trees from single or multigene sequences (e.g. Okoli *et al.*, 2007). Taxonomic revision of those taxa may be necessary based on their phylogenetic positions within the order Trichosporonales.

In physiological tests, *T. xylopinii* sp. nov. showed a similar pattern of results to *T. porosum* as the two species were able

Fig. 1. A most-parsimonious tree obtained from the sequence data of the ITS including the 5.8S rRNA gene and the D1/D2 region of the LSU rRNA gene from *Trichosporon xylopiini* sp. nov. and related taxa. *Trichosporon insectorum* was used as the outgroup taxon. GenBank accession numbers for ITS and D1/D2 sequences, respectively, are in parentheses. Tree length=258; consistency index=0.8217; retention index=0.8796; rescaled consistency index=0.7228. Numbers on branches indicate the percentages of bootstrap samplings derived from 1000 samples that supported the internal branches by 50% or higher. Bar, 5 changes per nucleotide position.

to utilize most of the carbon sources tested. However, the novel species differed from *T. porosum* by failure to assimilate quinic acid and sodium nitrite. Strains of *T. xylopiini* sp. nov. are able to utilize xylan and cellulose for growth. Xylan is the principal hemicellulose in angiosperms and many other plants, and the ability to degrade xylan is also shown in other *Trichosporon* species of the Porosum clade, e.g. *T. gamsii*, *T. lignicola*, *T. porosum*, *T. sporotrichoides* and *T. wieringae* (Middelhoven, 2004).

Morphologically *T. xylopiini* sp. nov. shares some characteristics with *T. porosum*, such as reproducing by budding more frequently than by generating arthroconidia under general culture conditions, and producing pseudomycelia in broth (Middelhoven *et al.*, 2001). Sexual reproduction was not observed in any of the strains in the novel species individually or in mixed culture under the conditions tested for up to 6 weeks.

Interestingly, some strains of *T. porosum* are known to suppress growth of a variety of fungi with maximum antifungal activity at pH 3.5–4.0 (Kulakovskaya *et al.*, 2010). The suppression is due to secretion of a thermally stable fungicidal agent which is similar to the antifungal cellobiose lipids produced by *Cryptococcus humicola* (Puchkov *et al.*, 2002). Strains of *T. xylopiini* sp. nov. and *T. porosum* isolated from this study were tested for growth suppression ability against the selected yeast strains listed earlier, but none of the strains showed any antifungal activity at the pH range tested (pH 3.0–6.0). Therefore, we conclude that the antifungal activity may be a strain-variable characteristic in *T. porosum*.

Latin diagnosis of *Trichosporon xylopiini* Suh, Lee, Gujjari & Zhou sp. nov.

Cultura in liquido 'YM' post 7 dies ad 25 °C, cellulae globosae, subglobosae et ellipsoideae (3.0–5.0 × 3.0–8.0 µm), singulae

aut binae. Sedimentum formatur. Pseudohyphae fiunt. In agar 'YM', post 7 dies ad 25 °C, cultura crenea, butyracea et margo fimbriata. Cultura in agar farina Zeae maydis confecto post 11 dies ad 25 °C crenea. Arthroconidia, hyphae verae et pseudohyphae formantur. Sacchara non fermentantur. Assimilantur glucosum, galactosum, L-sorbosum, D-glucosaminum, D-ribosum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, sucrosum, maltosum, trehalosum, α-methyl D-glucosidum, cellobiosum, salicinum, arbutinum, melibiosum, lactosum, raffinose, melezitose, amyllum solubile, glycerolum, erythritolum, ribitololum, xylitololum, L-arabinitololum, D-glucitololum, D-mannitololum, galactitololum, inositololum, gluconolactolum, 2-keto D-gluconatum, D-gluconatum, D-glucuronatum, D-galacturonatum, DL-acidum lacticum, acidum succinicum, acidum citricum (lente, infirme), ethanololum et propane-1, 2-diololum. Non assimilantur inulinum, methanololum, butano-2, 3-diololum et acidum quinicum. Assimilantur ethylaminum (infirme), L-lysinum, cadaverinum et glucosaminum (lente). Non assimilantur kali nitratum, sodii nitritum, creatinum, creatininum, imidazololum et D-tryptophanum. Assimilantur cellulolum et xylanum. Amyllum non formatur. Thiaminum externum ad crescentiam necessarium est. 30 °C crescit neque 37 °C. In medio 0.01 % cycloheximido addito crescit. Typus: EH024^T (ATCC MYA-4670^T = CBS 11841^T), designat stirpem typicam. Isolata a ile coleopterorum (*Xylopius saperdioides*: Tenebrionidae), Broad Run, Virginia, USA, depositata in Collectione Culturalium American Type Culture Collection (ATCC), Manassas, Virginia, USA, et Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands.

Description of *Trichosporon xylopiini* Suh, Lee, Gujjari & Zhou sp. nov.

Trichosporon xylopiini (xy.lo.pi'ni. N.L. gen. n. *xylopiini* of *Xylopius*, referring to the genus name of the host beetle *Xylopius saperdioides* for the type strain).

After 7 days of growth in YM broth at 25 °C, cells are globose to ellipsoidal (3.0–5.0 × 3.0–8.0 µm), and occur singly or in pairs (Fig. 2a) with sediment formed; budding yeast cells and pseudohyphae are present. The culture on YM agar after 7 days at 25 °C is cream coloured, butyrous and slightly wrinkled on top with filamentous edge. After 11 days of growth on Dalmau plate culture on cornmeal agar at 25 °C, true hyphae are present (Fig. 2b); fragmentation was observed only under the coverslip (Fig. 2b); aerobic growth is cream coloured with filamentous margin. Sexual reproduction was not observed from individual or mixed cultures of strains on YM agar, 2 % malt extract agar or cornmeal agar at 25 °C for up to 6 weeks. Glucose, galactose, maltose, α -methyl D-glucoside, sucrose, trehalose, melibiose, lactose, cellobiose, melezitose, raffinose, inulin, starch and D-xylose are not fermented. Glucose, galactose, L-sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, trehalose, α -methyl D-glucoside, cellobiose, salicin, arbutin, melibiose, lactose, raffinose, melezitose, soluble starch, glycerol, erythritol, ribitol, xylitol, L-arabinitol, D-glucitol, D-mannitol, galactitol, *myo*-inositol, D-glucono-1,5-lactone, 2-keto-D-gluconate, D-gluconate, D-glucuronate, D-galacturonic acid, DL-lactate, succinate, citrate (delayed, weak), ethanol and propane-1,2-diol are assimilated; inulin, methanol, butane-2,3-diol and quinic acid are not assimilated. Ethylamine (weak), L-lysine, cadaverine and D-glucosamine (delayed; as nitrogen source) are assimilated; potassium nitrate, sodium nitrite, creatine, creatinine, imidazole and D-tryptophan are not assimilated. Cellulose and xylan are assimilated. Thiamine is required for growth. Growth in 0.01 % cycloheximide is positive. Growth on 1 % acetic acid, 50 % D-glucose, 60 % D-glucose, 10 % NaCl and 16 % NaCl is negative. Growth at 30 °C is positive, while growth at 37 °C is negative. Starch-like compounds are not produced. Diazonium blue B reaction is positive. Urease activity is positive.

The type strain is EH024^T (ATCC MYA-4670^T = CBS 11841^T), isolated from the gut of *Xylopinus saperdioides* (Coleoptera: Tenebrionidae) on rotten log (*Quercus* sp.), Bull Run Mountain, Broad Run, Virginia, USA.

Fig. 2. *Trichosporon xylopinii* sp. nov. strain EH24^T. Budding yeast cells (a) after 7 days on YM agar at 25 °C, and arthroconidia (b) on Dalmau plate culture on cornmeal agar after 11 days at 25 °C. Bars, 5 µm.

Ecology

The darkling beetle *Xylopinus saperdioides* (Tenebrionidae) lives mainly on oak trees or under bark, although it is occasionally found on mushrooms such as *Polyporus betulinus* and *Pleurotus ostreatus* (Majka *et al.*, 2008; Minch, 1952; Robinson, 1918; Cline & Leschen, 2005). Two of the *T. xylopinii* sp. nov. strains were isolated from this wood-inhabiting beetle found on a rotten oak tree, and the remaining strain was independently collected from humus soil. Previous studies showed that some of the gut yeasts were found not only from the digestive tract but also from the environment the host insects inhabited, such as frass of the host (e.g. *Kodamaea laetipori*; Suh & Blackwell, 2005). Therefore, although *T. xylopinii* sp. nov. strain EN6S23 was isolated from forest soil, we assume that its origin could be directly or indirectly related to insects, and the wood-inhabiting insects might play a major role in dispersing this yeast in nature.

It is interesting that strains of *T. xylopinii* sp. nov. can degrade and utilize cellulose and xylan, which are the major components of plant tissues. As shown in previous studies on the gut micro-organisms of termites (Breznak & Brune, 1994; Varma *et al.*, 1994; Schäfer *et al.*, 1996), this fact indicates that the yeast may help its host insect to digest the wood substrates taken as diet. Interestingly, the majority of *Trichosporon* species in the Porosum clade are known to utilize xylan, i.e. *T. gamsii*, *T. lignicola*, *T. porosum*, *T. sporotrichoides*, *T. wieringa* and the novel species *T. xylopinii* (Middelhoven, 2004; this study), and their isolation sources are somewhat related to plant materials. For example, the strains of *T. lignicola* and the type strain of *T. porosum* were isolated from the wood pulp and exudate of yew tree, respectively (Fell & Scorzetti, 2004; Middelhoven *et al.*, 2001). Strains of *T. gamsii*, *T. sporotrichoides* and *T. wieringa* were found from forest soil such as moist humus (Guého *et al.*, 1998; Middelhoven, 2004; Middelhoven *et al.*, 2004). This circumstantial evidence indicates that those species of the genus *Trichosporon* may play a role in the decaying process of plants in the forest.

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