***Candida bituminiphila*, a novel anamorphic species of yeast**

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A novel anamorphic species of yeast belonging to the genus *Candida* was isolated from tar in Canada. Morphological and physiological observations, as well as phylogenetic analyses, were performed. Conidiophores were produced, were usually short and had sympodial growth, numerous bud scars and a rachis-like structure. They bore one or more conidia. Pseudomycelium was scarcely produced and true mycelium was sparse. No sexual reproduction was observed on corn meal, malt, Gorodkova, Dextrose Yeast Peptone or V8 agars. *Zygoascus hellenicus* was physiologically the most closely related species, but it differed from the novel species by its ability to assimilate D-galacturonate and L-rhamnose, ferment sucrose and grow at 37 °C. From sequence analysis of the 26S rDNA D1/D2 region, *Z. hellenicus* and *Candida bertae* var. *bertae* were the closest species with 54 and 56 bp substitutions, respectively. Similar results have been obtained from analysis of the 18S rDNA. All these data support the hypothesis that the yeast, named *Candida bituminiphila*, is a novel species closely related to *Z. hellenicus*. The holotype and only isolate of *C. bituminiphila* is strain CBS 8813^T (= MUCL 41424^T).

Keywords: *Candida bituminiphila*, 26S and 18S rDNA gene sequences, phylogenetics, phenetic study

INTRODUCTION

During the study of a series of strains for a scientific identification service, a novel, interesting ascomycetous yeast was found. Details about the origin of the strain remained vague since the only information available about the yeast was the country of origin, Canada, and the substrate of isolation, bitumen. The strain was recognized as potentially novel by physiological identification methods (Robert *et al.*, 1994, 1997). Sequencing of the D1/D2 domain of the large-subunit (26S) rDNA as well as the complete sequence of the small-subunit (18S) rDNA was performed and the technique confirmed that the strain was unique. The extent of the divergence was large enough to describe the strain as a novel yeast species. Although from both the physiological and phylogenetic (26S and 18S rDNA) point of view, the closest previously described species was *Zygoascus hellenicus*, the novel

species was placed in the genus *Candida*. This was because ascospore formation was not observed and therefore it was not possible to place it in the teleomorphic genus *Zygoascus*. A complete description of the species is given and the name *Candida bituminiphila* is proposed.

METHODS

Organism studied. The strain was collected from tar in Canada. It was deposited at the Centraalbureau voor Schimmelcultures (CBS 8813^T) as a lyophilized and cryopreserved (–130 °C) culture. Duplicates of the strain have also been deposited at the Mycothèque de l'Université Catholique de Louvain (MUCL 41424^T). No additional information was available for the type strain (isolator, date and place of isolation, precise description of the substrate). No other isolate of the same species was available.

Physiology and morphology. Physiological and morphological characterization of the strain was performed as described by Robert *et al.* (1994, 1997) using the BioloMICS Automated Identification system (Robert & Moenaert, 2000). This system employs conventional taxonomic criteria with 96 tests to assess growth, fermentative ability and morphology (Barnett *et al.*, 2000; Yarrow, 1998) and

The GenBank accession number for the 26S rRNA sequence of strain CBS 8813^T is AF294910. The GenBank accession numbers for the 18S rRNA sequences of strains CBS 8813^T and CBS 6736^T are AF294911 and AF294751, respectively.

Table 1. List of strains included in the physiological/morphological analysis and the 26S rDNA data analysis

Culture collections are abbreviated as follows: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; NRRL, ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA. ^T, Type strain; ^{IT}, isotype strain.

Species name	Strain designation	GenBank no.
<i>Arxula adenivorans</i>	CBS 8244 ^T (= NRRL Y-17692 ^T)	U40094
<i>Arxula terrestris</i>	NRRL Y-17704	U40103
<i>Blastobotrys arbuscula</i>	CBS 227.83 ^T (= NRRL Y-17585 ^T)	U40108
<i>Blastobotrys aristata</i>	CBS 521.75 ^T (= NRRL Y-17579 ^T)	U40109
<i>Blastobotrys capitulata</i>	CBS 287.82 ^T (= NRRL Y-17573 ^T)	U40104
<i>Blastobotrys elegans</i>	CBS 530.83 ^T (= NRRL Y-17572 ^T)	U40095
<i>Blastobotrys nivea</i>	CBS 163.67 ^T (= NRRL Y-17581 ^T)	U40110
<i>Blastobotrys proliferans</i>	CBS 522.75 ^T (= NRRL Y-17577 ^T)	U40098
<i>Candida atlantica</i>	CBS 5263 ^T (= NRRL Y-17759 ^T)	U45799
<i>Candida atmosphaerica</i>	CBS 4547 ^T (= NRRL Y-17642 ^T)	U45779
<i>Candida bertae</i> var. <i>bertae</i>	CBS 8169 ^T (= NRRL Y-17643 ^T)	U70251
<i>Candida bertae</i> var. <i>chiloensis</i>	CBS 8168 ^T (= NRRL Y-17646 ^T)	AF017236
<i>Candida bituminiphila</i>	CBS 8813 ^T (= MUCL 41424 ^T)	AF294910
<i>Candida entomaeae</i>	CBS 6306* (= NRRL Y-7785*)	U45790
<i>Candida tammaniensis</i>	CBS 8504 ^T (= NRRL Y-8257 ^T)	AF017243
<i>Cephaloscyus albidus</i>	CBS 389.77 ^T (= NRRL Y-7343 ^T)	U39474
<i>Stephanoascus ciferrii</i>	CBS 5295 ^{IT} (= NRRL Y-10943 ^{IT})	U40138
<i>Stephanoascus farinosus</i>	CBS 140.71 ^T (= NRRL Y-17593 ^T)	U40132
<i>Stephanoascus smithiae</i>	NRRL Y-17849 ^{IT}	U76531
<i>Sympodiomyces parvus</i>	CBS 6147 ^T (= NRRL Y-10004 ^T)	U40096
<i>Zygoascus hellenicus</i>	CBS 4099† (= NRRL Y-17319†)	U40125

* Listed in GenBank as *Candida entomaeae* van der Walt *et al.* Taxonomic synonym of *Pichia mexicana* Miranda *et al.* (Kurtzman, 1998).

† Type strain of *Trichosporon hellenicum* Verona & Picci.

recognizes 719 described species of yeasts. Growth and fermentation tests were conducted in microplates. Assimilation test results were determined spectrophotometrically using an absorbance reader connected to a computer. Five replicates of the tests were performed on the same strain (CBS 8813^T) at different times. Fermentation tests were read manually. Identification of the strain was performed by computation of pairwise similarity coefficients using the BioloMICS software. A phenetic tree (UPGMA) was generated using a similarity matrix produced by the BioloMICS software. The closest species (see Table 1) were included in a phenetic analysis.

rDNA sequencing and sequence analysis. Cultures used for DNA isolations were grown in dextrose/yeast/peptone (DYP) broth for 24 h at 25 °C, harvested by centrifugation, resuspended in sterile water and transferred to 1.5 ml microfuge tubes. Total nucleic acids were extracted following the protocol of Hoffman & Winston (1987). Fragments that included approximately 600 bp positions of the 5' end of the 26S rRNA (D1/D2 region) gene were amplified by PCR using the external primers NL-1 and NL-4 (O'Donnell, 1993). Fragments that included approximately 1800 bp positions of the 5' end of the 18S rRNA gene were amplified by PCR using the external primers NS1 and NS8 (White *et al.*, 1990). Amplification reaction mixtures contained 5 µl diluted DNA template (~10 ng), 5 µl PCR buffer (100 mM Tris/HCl pH 8.3, 15 mM MgCl₂, 500 mM KCl, 1% Triton

X-100), 1 µl dNTP (10 mM), 5 µl each primer (10 µM), 0.5 µl (2.5 U) *Taq* DNA polymerase and 50 µl sterile H₂O. Each sample was overlaid with 40 µl mineral oil. Thermal cycling (DNA Thermo Cycler 480; Perkin-Elmer) parameters for amplification consisted of: an initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 5 min, annealing at 52 °C for 1 min, extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. Amplified DNA was stored at -18 °C before purification using the QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed using a Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Each reaction mixture included 4 µl Half Term (Genpack). For the large subunit (strain CBS 8813^T), sequencing primers were NL-1 and NL-4 (same as for PCR); NL2A and NL3A were used as internal primers (Kurtzman & Robnett, 1997). For the small subunit, strains CBS 8813^T and CBS 6736^T were sequenced using primers NS1-NS8 (White *et al.*, 1990). Strain CBS 6736^T is the type strain of *Candida inositoliphila* Nakase (anamorph of *Z. hellenicus*) complementary mating type of CBS 5839. The mated culture of these two strains is the type strain of *Z. hellenicus* (Smith, 1998). Excess dye terminators were removed by centrifugation using Centri-Sep columns (Princeton Separations) prior to analysis using an Applied Biosystems 373A DNA sequencer.

Sequences were edited and assembled using SEQUENCHER version 3.0 software (Gene Codes Corporation). Data were

subjected to a basic GenBank BLASTN search (Altschul *et al.*, 1990) to retrieve sequences of closely related taxa; these sequences, as well as those of physiologically and morphologically similar yeast species (see Table 1), were included in a multiple alignment generated using CLUSTAL W (gap creation penalty, 12; gap extension penalty, 2) for 26S rDNA data. No manual adjustments were done except that the ends were trimmed. Phylogenetic relationships were inferred from this alignment using the general heuristic search option in PAUP version 3.1.1 (Swofford, 1993). Support for branches was evaluated by bootstrap analysis from 1000 heuristic searches. Distance matrices were produced using PAUP version 3.1.1 (Swofford, 1993). The pairwise alignment of 18S rDNA sequences of *C. bituminiphila* and *Z. hellenicus* was done using the Smith–Waterman algorithm incorporated in the SSEARCH3 program from the FASTA package (gap creation penalty, 12; gap extension penalty, 2).

RESULTS AND DISCUSSION

Latin diagnosis of *Candida bituminiphila* sp. nov.

In extracto malti post 8 dies cultura crenea pallida, laevis, convexa, margine cellulosa. Cellulae 1.2–2.5 × 2.5–8.0 µm, ovoideae, ellipsoideae, citrifformes, leviter allantoideae vel irregulares, singulae vel binae. Conidiophora brevia, sympodialiter proliferantia, numerosas gemmarum cicatrices ferentia, rhachidis similia, conidia singula vel plura ferentia. Pseudohyphae parcae, hyphae verae non observatae. Ascosporae, ballistosporae, arthroconidia et teliosporae absentes. In culturis Dalmat, pseudohyphae et hyphae verae praesentes. In DYP medium, post 2 hebdomades, sedimentum et annulus formantur. In ‘corn meal’, maltum, ‘Gorodkova’, DYP et V8 agar ascosporae absentes. D-Glucosum, D-galactosum, trehalosum, cellobiosum fermentantur neque lactosum, maltosum, melezitolum, methyl α-D-glucosidum, raffinolum, sucrosolum, inulinum, amylum, melibiosum nec D-xylosum. D-Glucosum, D-galactosum,

L-sorbosum, D-glucosaminum, D-ribosum, D-xylosum, L-arabinosum, trehalosum, cellobiosum, salicinum, arbutinum, amylum solubile, glycerolum, D-glucitolum, D-mannitolum, D-glucuronatum, succinatolum, meso-inositolum (exigue), DL-lactatum (exigue), ethanololum (lente), D-arabinosum (lente et variabiliter), sucrosolum (lente et variabiliter), propanolum (variabiliter), maltosum (variabiliter), raffinolum (variabiliter), ribitololum (variabiliter), xylitololum (variabiliter), L-arabinitololum (variabiliter), D-glucono-1,5-lactonum (variabiliter), 5-keto-D-gluconatum (variabiliter), D-gluconatum (variabile) assimilatur at non methyl α-D-glucosidum, melibiosum, lactosum, melezitolum, inulinum, L-rhamnosum, erythritolum, galactitololum, 2-keto-D-gluconatum, D-galacturonatum, citratolum, methanololum nec butanololum. Glucosaminum (variabiliter), L-lysinum (variabiliter), cadaverinum (variabiliter) assimilantur neque natrium nitricum, natrium nitrosolum, creatinum, creatininum, ethylaminum et imidazololum. Ad crescentiam vitamina externa necessaria sunt: thiaminum, biotinolum, thiaminum, pyridoxinum. Crescit sine meso-inositolo (variabiliter), pantothenato (lente), pyridoxino (variabiliter), niacino (lente), acido aminobenzoico (variabiliter). Maxima temperatura crescentiae: 30 °C. Crescit in medio 0.01% cycloheximidum continente, 0.1% cycloheximidum (lente) neque 50% D-glucosum. Urea non finditur, diazolum coeruleo B probatio negativa. Amylum non formatur, arbutinum non finditur, acidum aceticum non formatur. Typus: stirps CBS 8813^T (= MUCL 41424^T), ex bitumine isolata. Holotypus lyophilus conservatur in collectione culturarum Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum.

Description of *Candida bituminiphila* sp. nov.

Candida bituminiphila (bi.tu.mi.ni'phi.la. M.L. neut. n. bitumen, -inis asphalt, tar; Gr. adj. *philos* loving; N.L. fem. adj. *bituminiphila* tar-loving).

Fig. 1. Vegetative cells of *C. bituminiphila* CBS 8813^T with some allantoic cells (a), with a rachis-like structure, see arrow (b). Extension of conidiophore with scars, see arrows (c). Grown on GPY agar for 1 week at 25 °C. Bars, 10 µm.

Table 2. Physiological characteristics of the type strain (CBS 8813^T) of *C. bituminiphila*

–, Negative; +, positive; D, delayed positive (after 14 d or more); w, weak; –, +, variable.

Characteristic	Result
Fermentation of:	
D-Glucose	+
D-Galactose	+
Maltose	–
Methyl α -D-glucoside	–
Sucrose	–
α,α -Trehalose	+
Lactose	–
Cellobiose	+
Melezitose	–
Raffinose	–
Inulin	–
Starch	–
Assimilation of (carbon compounds):	
D-Glucose	+
D-Galactose	+
L-Sorbose	+
D-Glucosamine	+
D-Ribose	+
D-Xylose	+
L-Arabinose	+
D-Arabinose	D, –
L-Rhamnose	–
Sucrose	D, –
Maltose	–, +
α,α -Trehalose	+
Methyl α -D-glucoside	–
Cellobiose	+
Salicin	+
Arbutin	+
Melibiose	–
Lactose	–
Raffinose	–, +
Melezitose	–
Inulin	–
Soluble starch	+
Glycerol	+
Erythritol	–
Ribitol	–, +
Xylitol	–, +
L-Arabinitol	–, +
D-Glucitol	+
D-Mannitol	+
Galactitol	–
<i>myo</i> -Inositol	w
D-Glucono-1,5-lactone	–, +
2-Keto-D-gluconate	–
5-Keto-D-gluconate	–, +
D-Gluconate	–, +
D-Glucuronate	+
D-Galacturonate	–
DL-Lactate	w

Table 2 (cont.)

Characteristic	Result
Succinate	+
Citrate	–
Methanol	–
Propane 1,2-diol	–, +
Butane 2,3-diol	–
Ethanol	D, +
Assimilation of (nitrogen compounds):	
Nitrate	–
Nitrite	–
Ethylamine	–
L-Lysine	–, +
Cadaverine	–, +
Creatinine	–
Glucosamine	–, +
Creatine	–
Imidazole	–
Growth in the absence of:	
Vitamins	–
<i>myo</i> -Inositol	–, +
Pantothenate	D, +
Biotin	–
Thiamin	–
Biotin + thiamin	–
Pyridoxine	–, +
Pyridoxine + thiamin	–
Niacin	D, +
PABA	–, +

After 8 d on 5% malt extract agar at 20–22 °C, colonies are 3 mm in diameter, pale cream coloured, smooth, convex, with a glistening surface, a finely fimbriate margin and soft texture. Budding is unipolar or bipolar. Budding cells are 1.2–2.5 $\times$ 2.5–8.0 μ m, ovoid, ellipsoid, citriform, allantoid or irregular (Fig. 1a). Conidiophores are usually short, producing blastoconidia on a sympodially proliferating apex that, at maturity, shows a rachis-like structure (Fig. 1b) with numerous slightly denticulate scars left after the dehiscence of conidia (Fig. 1c). Shapes and sizes of conidia are comparable with those of the budding cells. Pseudomycelium is rare and true mycelium is sparse. Ascospores and arthroconidia are absent. On Dalmau plate with potato dextrose agar after 8 d at 20–22 °C, true mycelium is produced as well as pseudomycelium and a few budding cells. On DYP broth after 2 weeks, sedimentation and a ring pellicle are observed. No sexual reproduction has been observed on corn meal, malt, Gorodkova, DYP or V8 agar. The fermentation of carbon compounds as well as the assimilation of carbon and nitrogen compounds, and the growth properties without some vitamin compounds are given in Table 2. Growth at 25 and 30 °C is positive. Growth at 35 °C is negative. Growth on

Fig. 2. UPGMA phenogram generated by the BioMICS software based on pairwise similarity (simple matching coefficient) of physiological and morphological properties of the yeast species that are most similar to *C. bituminiphila*.

Fig. 3. Phylogenetic tree showing the placement of 21 species of yeasts most closely related to *C. bituminiphila* as inferred from 26S rDNA D1/D2 region sequence data. The tree is the most parsimonious tree of a bootstrap analysis with a heuristic search (1000; 50% majority-rule consensus). Branch lengths are proportional to the number of nucleotide differences as indicated on the bar (30 bp substitutions). Bootstrap percentages (1000 replicates) above 50% are shown above branches.

0.01% cycloheximide is positive, whereas growth on 0.1% cycloheximide is slow. No growth in presence of 50% D-glucose. No urea hydrolysis. Diazonium blue B reaction is negative. No starch formation. No acetic acid production. No arbutin hydrolysis. The type strain, CBS 8813^T (= MUCL 41424^T), has been isolated from tar in Canada; no further information about strain origin was available.

Phenotypic comparison with closely related species

Z. hellenicus was physiologically the most similar species to *C. bituminiphila* (Fig. 2), but differs by its ability to grow on D-galacturonate and L-rhamnose and at 37 °C. The fermentation of sucrose was negative for *C. bituminiphila* but variable for *Z. hellenicus*. The two species also differed in the morphology of their

vegetative cells (cells of *Z. hellenicus* are more regular in size and shape) and by the absence of ascospores in *C. bituminiphila*. *Blastobotrys arbuscula*, another physiologically similar species, differed by the fermentation of maltose and by the assimilation of erythritol, galactitol, salicin and arbutin. *Blastobotrys aristata* differed by the fermentation of maltose and by the assimilation of lactose, erythritol, galactitol and ethylamine. Although *C. bituminiphila* and *Blastobotrys* spp. shared several morphological characteristics, such as sympodial conidiophore proliferation and the ability to produce conidia on a rachis or laterally on hyphae, *C. bituminiphila* differed by failing to produce conidia in clusters. *Sympodiomyces parvus* was the most similar morphologically, but there were many physiological differences. *S. parvus* is non-fermentative and does not assimilate L-sorbose, salicin, soluble starch, DL-lactate or succinate. Also, *C. bituminiphila* can grow at 30 °C, unlike *S. parvus*.

DNA relatedness studies

A BLASTN search (26S rDNA) of GenBank and our own database allowed us to select the strains most closely related to *C. bituminiphila*. *Z. hellenicus* is the closest species to *C. bituminiphila* (54 bp substitutions in the large-subunit D1/D2 domains). *Candida bertae* var. *bertae* differed by 56 bp. The phylogenetic positioning of species compared to *C. bituminiphila* can be seen in Fig. 3. The high bootstrap value (92%) supported the hypothesis that the novel species is phylogenetically related to *Z. hellenicus*. Pairwise alignment of the 18S rDNA region revealed 39 bp substitutions in 1756 overlapping nucleotides, giving a 97.8% identity between the two species. This evidence strongly supports the hypothesis that *C. bituminiphila* CBS 8813^T is a novel species. Although strain CBS 8813^T showed enough physiological and genetic (sequence) differences with the most similar existing genera to be placed into a novel genus, we believed that, since there was only one strain (with little data about its origin) the available information was not sufficient to allow this. It was therefore decided to place the species in the very broadly defined anamorphic genus *Candida*.

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