Antonie van Leeuwenhoek **86:** 287–294, 2004.
© 2004 *Kluwer Academic Publishers. Printed in the Netherlands.*

Hortaea acidophila, a new acid-tolerant black yeast from lignite

Udo Hölker[1,*], Jutta Bend[1], Rolf Pracht[1], Larissa Tetsch[1], Tobias Müller[1], Milan Höfer[1] and G. Sybren de Hoog[2,3]

[1]Botanisches Institut der Universität Bonn, Kirschallee 1, D-53115 Bonn, Germany; [2]Centraalbureau voor Schimmelcultures, P.O. Box 85167, NL-3508 AD Utrecht, The Netherlands; [3]Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands; [*]Author for correspondence (email: u.hoelker@uni-bonn.de)

Received 10 September 2003; accepted in revised form 10 November 2003

Key words: Acid tolerance, Black yeasts, Extremophilic fungi, Fulvic acid, *Hortaea* new species, Humic acid, Lignite

Abstract

A hitherto undescribed black yeast was isolated from an extract of brown coal containing humic and fulvic acids at pH 0.6. The fungus showed morphological similarity to some members of the genus *Exophiala* (Chaetothyriales) and of *Hortaea* (Dothideales). Based on SSU rDNA sequence similarity to meristematic members of the Dothideales, the new species was accommodated in *Hortaea*, which presently contains only a single, halophilic species, *H. werneckii.*

Introduction

Black yeasts are melanised fungi which have double anamorph life cycles, reproducing either by preponderantly unicellular or by filamentous growth (de Hoog 1993). Their cell wall is stabilized by incrustation of melanin protecting them against UV light, cytotoxic radicals, enzymatic attack, high temperatures, dryness and high heavy metal concentrations (Wheeler and Bell 1988; Butler and Day 1998; Caesar-Tonthat et al. 1995). The thickness of the cell wall contributes to the high resistance of black yeast to environmental stress. A number of species are known to proliferate under extreme conditions e.g., on and in desert rocks or in cavities in marble surfaces (Sterflinger et al. 1999), at very high (Sterflinger 1998) as well as ultra-low temperatures (Onofri et al. 1999).

The taxonomy of black yeasts is still in a state of flux, new species regularly being described (de Hoog 1999, de Hoog et al. 2003). Species delimitation is now largely based on sequences of Internal Transcribed Spacer (ITS) of ribosomal DNA (Hershkowitz et al. 1996; Vitale and de Hoog 2003). Main phylogenetic traits and attribution to orders of the fungal kingdom, either Chaetothyriales or Dothideales, is based on SSU rDNA sequences (Sterflinger et al. 1999). Genera within the Chaetothyriales are circumscribed by morphology and are acknowledged to remain phylogenetically heterogeneous (Haase et al. 1999). Also in Dothideales, morphologically similar ecotypes can be observed in different clades. In this report we describe the isolation, physiology and morphology of a new extremophilic black yeast found in a suspension at pH 0.6 containing humic and fulvic acids, extracted from lignite as the sole carbon source. The strain had preponderantly annellidic conidiogenesis, similar to representatives of the black yeast genera *Exophiala* (Chaetothyriales) and *Hortaea* (Dothideales). In order to attribute the species to any one of these genera, a phylogenetic study involving the ribosomal operon was undertaken.

Material and methods

Enrichment medium

Five hundred g brown coal particles with a diameter of 4-10 mm (Lithotype A, Bergheim supplied by Rheinbraun AG, Cologne) were washed twice with distilled water. Subsequently the particles were stirred for 1 h at room temperature in 2 litre 1N NaOH-solution to remove humic and fulvic acids. The resulting slurry was centrifuged for 10 min at 10,000 rpm. 300 ml of supernatant, containing 30 g humic and 6 g fulvic acids were adjusted to pH 0.5 with concentrated HCl, placed in a 500 ml separating funnel and closed with cotton. Due to the low pH, the humic acids sedimented (black pellet) and the fulvic acids remained in the supernatant (yellowish supernatant).

Isolation and cultivation

The humic/fulvic acid suspension in separating funnel (see above) was kept at room temperature (20 °C). After 2 months several black fungal colonies were observed. The colonies were transferred to a medium containing 1.2% glucose, 0.6% yeast extract, 0.4% peptone, 0.12% K_2HPO_4, 0.06% $MgSO_4$, 0.04% NaH_2PO_4 and 0.1% NH_4NO_3, pH 4.8. The medium was used both in solid culture plates with 1.2% agar and for liquid cultures in 50 ml Erlenmeyer flasks. The latter were shaken on a rotatory shaker at 120 rpm. Each plate or flask was inoculated with single colony picked from the acidic enrichment medium. A well-grown culture from a glucose containing medium was used for further characterisation.

Physiology

The metabolic capacity of the isolate was screened by using the Micronaut-RC and the Micronaut-C-System (Merlin Diagostika, Bornheim-Hersel, Germany). Tests were done following the instructions of the manufacturer, except that incubation periods were prolonged by 24 hours (up to 48 and 72 h for Micronaut-RC and Micronaut-C, respectively) due to the slow growth of the fungus. Tests with ambiguous results were repeated with longer incubation times (up to 30 days) on solid media. All experiments were done in triplicate.

Transmission electron microscopy (TEM)

Cultures were harvested after 10 days of cultivation in a glucose medium (see above) by centrifugation at 4000 g. The pellet was suspended and pre-fixed in 3% glutaraldehyd, 50 mM pipes buffer and 5 mM $CaCl_2$, pH 7, for 30 min at 22 °C. The suspension was centrifuged again at 4000 g and the pellet was washed in 200 mM pipes buffer, 5 mM $CaCl_2$ for 30 min three times and again centrifuged. The pellet was mixed with a two times higher volume of 2.5% agarose solution (35 °C) and solidified in small droplets at 4 °C on a piece of parafilm. The solid droplets were collected and suspended in 1.5% agar, 100 mM sodiumcacodylate-buffer (NCP) and 5mM $CaCl_2$ to increase the immobilisation of the probe. Finally the probe was washed 3 times for 5 min in NCP to get rid of the pipes-buffer. The final fixation was done in 2% OsO_4 solution for 2 h. The sample was repeatedly dehydrated in a graded acetone series (30%, 50%, 75% for 20 min, 90% for 30 min und three times in 100% for 20 min). The probes were infiltrated overnight in a mixture of acetone:epoxy resins (ERL) (3:1) according to a method of Spurr (1969), following 3 h incubation in acetone:ERL (1:1), 4 h incubation in acetone:ERL (1:3) and overnight incubation in pure ERL. Finally the pure ERL was polymerised for 8 h at 70 °C. Ultra-thin sections were performed on a Reichert ultramicrotome, poststained with uranyl-acetate and − citrate and observed in a Zeiss EM 10A microscope.

Molecular analysis

About 1 cm^2 of mycelium was added to a 2:1 (w/w) mixture of silicagel and Celite (silica gel H, Merck 7736 / Kieselguhr Celite 545, Machery) and 300 µl CTAB buffer [Tris·HCl, 200 mM, pH 7.5, 200 mM Na-EDTA (ethylenediaminetetraacetic acid), 8.2% NaCl w/v, 2% CTAB (cetyltrimethylammoniumbromide) w/v]. The mycelium was ground with a micropestle (Eppendorf, Hamburg, Germany) for 1-2 min. Volume was adjusted by adding 200 µl CTAB buffer. After shaking vigorously the sample was incubated at 65 °C for 10 min. One volume (~500 µl) of chloroform was added and vortexed for 1-2 s. The samples were centrifuged at 14,000 rpm for 5 min. After transferring the aqueous supernatant to a new Eppendorf tube, 2 volumes (~800 µl) ethanol 96% − 20 °C were added and mixed gently. Samples were incubated at − 20 °C during 30 min or overnight,

subsequently centrifuged for 5 min at 14,000 rpm and the pellets were rinsed twice with 500 µl ice-cold ethanol 70%. After drying at room temperature pellets were resuspended in 48.5 µl TE-buffer (10 mM Tris, 10 mM Na-EDTA, pH 8.0) and 1.5 µl RNAse solution (10 mg pancreatic RNAse in 1 ml 0.01 M Na-acetate, pH 5.2) was added. The pH was adjusted by adding 100 µl 1 M Tris·HCl (pH 7.4). Samples were incubated 5-30 min at 37 °C and refrigerated. PCR was performed in 50 µl volumes of a reaction mixture containing 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, 0.01% gelatine, 200 µM of each deoxynucleotide triphosphate, 50 pmol of each primer, 10-100 ng rDNA and 0.5 U Taq DNA polymerase (Amplitherm, ITK Diagnostics, Maarssen, The Netherlands). Primers used were NS1, NS24, V9G and LS266. Forty amplification cycles: denaturing 94 °C, 30 s; annealing 58 °C, 1 min; elongation 72 °C, 30 s (with an initial delay of 1 min and a terminal delay of 2 min) were performed in a GeneAmp 9600 thermocycler (Applied Biosystems, Nieuwerkerk a.d. IJssel, The Netherlands). Amplicons were cleaned using Microspin S-300 HR columns (Amersham Pharmacia, Roosendaal, The Netherlands). PCR sequencing conditions were as follows: 25 cycles (denaturing 96 ºC, 10 s; annealing 50 ºC, 5 s; elongation 60 ºC, 4 s) carried out with primers ITS1 or 5 and ITS 4. For SSU sequencing, primers NS1, Oli 1, 2, 7, 10, 11, 13, 14 and NS24 were used. Base compositions of all primers used are given by De Hoog et al. (2000). DNA was precipitated with ethanol and sequenced using an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems). Sequences obtained were adjusted using SeqMan II of Lasergene software (DNAStar, Inc., Madison, Wisconsin, USA). ITS sequences were aligned iteratively using Ward's averaging in the BioNumerics package (Applied Maths, Kortrijk, Belgium). Nearest neighbours were found by local Blast searches. Distance trees were based on re-aligned files using the DCSE program and calculated with the Neighbor-joining algorithm implemented in the Treecon package (Van de Peer and de Wachter 1994) with Kimura-2 correction. Bootstrap values >90 of 100 resampled data sets are shown. Partial SSU sequences were aligned to an aligned data base containing about 2500 fungal sequences in the ARB package developed by W. Ludwig (www.mikro.biologie.tu-muenchen.de/pub/ARB). Trees were made with the Parsimony/ML algorithm with 100 bootstrap replications, taking the positions 169 to 1286 into account, as well as with the Neighbor joining algorithm in Treecon.

Results

After 2 months storage at room temperature (20 °C) up to 50 fungal colonies forming distinct mycelial pellets with diameters of about 5 mm were observed in the enrichment culture containing humic and fulvic acids. The colonies settled at the surface of the suspension as well as at the surface of the humic acid sediment. The pH of the suspension increased from 0.5 to 0.6 during incubation. Microscopic analysis showed predominantly single cells with a length of 5-10 µm, which in part germinated forming hyphae Figure 1. On identification media (OA, PDA; De Hoog et al. 2000) the fungus was found to have annellidic conidiogenesis.

Optimal growth rates were obtained in a liquid medium containing 1.2% glucose, 0.4% tryptone, 0.12% KH_2PO_4, 0.08% $MgSO_4$ and 0.04% NaH_2PO_4, resulting in maximum cell numbers up to 8×10^8/mL corresponding to a dry weight of 3.8 mg/mL after 30 days of cultivation. In the above medium the fungus was able to grow in a broad pH range with an optimum at pH 3.0. In a pH-range between 2 and 6 predominantly yeast cells were observed, whereas below pH 2 and above pH 6 filamentous growth dominated. The optimum temperature for growth was 22 °C; no growth was observed above 30 °C. The fungus did not tolerate an NaCl concentration of 5% (Figure 2).

Further physiological characterisation concerning the metabolic capacity (Table 1) often yielded ambiguous results. Absence of growth was checked in solid and liquid cultures containing the questionable carbon source. This was the case for melibiose and cellobiose. The isolate was not able to ferment either glucose or saccharose. On solid media the isolate grew slowly with KNO_3 and gelatine as sole nitrogen sources. Gelatine was liquefied after 2 months. L-prolinaminopeptidase and L-phenylalaninaminopeptidase but not β-cellobiosidase activity were detectable.

Judging from SSU rDNA data the species should be a member of the ascomycete order Dothideales, but it did not show identity to any of the members of this order (Figure 3). It clustered in a poorly resolved group with *Coccodinium bartschii, Hortaea werneckii, Ramichloridium apiculatum, Trimmatostroma abietis* and *T. salinum* as relatively close neighbours.

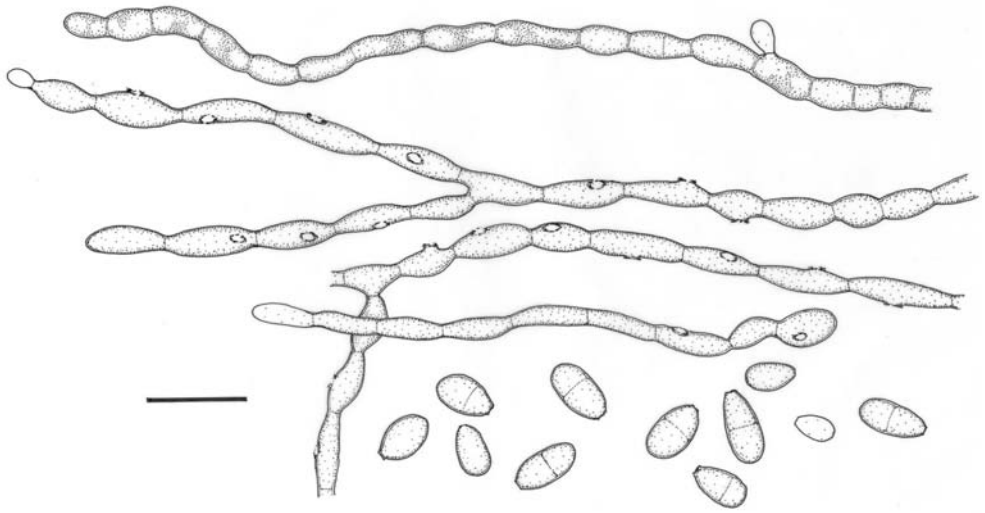

Figure 1. Microscopy of *Hortaea acidophila*, CBS 113389. Hyphae with annellated zones, and conidia. Bar represents 10μm.

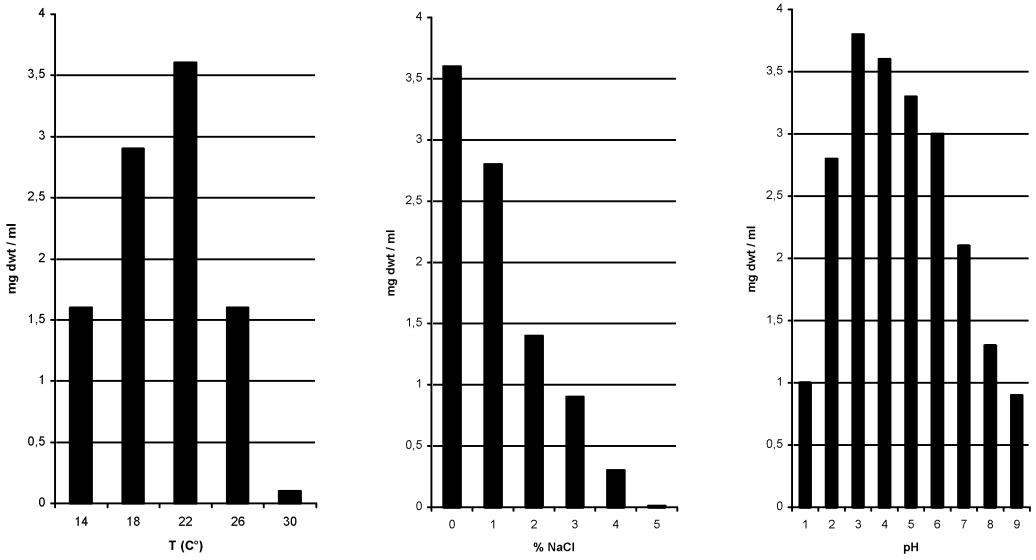

Figure 2. Fungal growth after 30 days of cultivation in dependence on temperature, sodium chloride concentration and pH.

The rDNA ITS sequences did not show close similarity to any comparable spacer deposited in Genbank. With a local Blast search in a black yeast database for research purposes maintained at CBS, *Hortaea werneckii* was found among the nearest neighbours, though at 16.0-17.8% sequence deviation. Alignment of ITS sequences was sometimes ambiguous, and the tree showed clear-cut differences between species (Figure 4). All species shown in the trees (Figure 3, Figure 4), having closest sequence similarity to the new isolated species as established by local and GenBank searches, are known for their halophilic, epiphytic, epilithic or lichenicolous ecology.

Electron microscopy of the yeast phase showed a thick osmiophilic cell wall, very likely due to melanisation. Daughter cells in budding stages lacked this layer, indicating that polymerisation of melanin occurs in a later stage of growth. Intracellular vacuoles containing osmiophilic granular substances were visible (Figure 5 a, b).

Table 1. Metabolic capacities of the isolate measured in the Micronaut C and Micronaut RC systems.

Carbon source	Assimilation
Adonitol	+
Arbutin	–
Cellobiose	w
Citrate	–
Crythriol	–
D-Glucosamine	–
D-Glucose	+
D-Lyxose	+
D-Xylose	+
D-Galactose	+
Gentobiose	–
D-Gluconate	–
Glycerin	+
Inositol	+
Lactate	–
Lactose	–
L-Arabinose	+
Maltose	+
Melibiose	w
N-Acetylglucosamine	+
Raffinose	+
Rhamnose	+
Ribose	+
Saccharose	+
Sorbitol	+
Trehalose	+

Abbreviation used: + = assimilation, w = weak assimilation, – = no assimilation

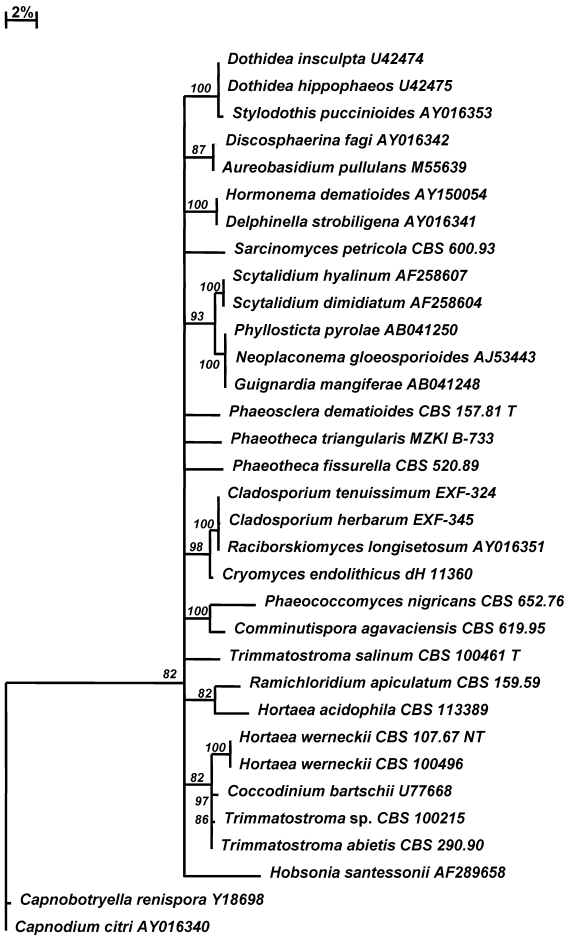

Figure 3. Neighbor joining tree constructed for 34 SSU rDNA sequences of dothidealean fungi in the Treecon package with Kimura-(2) correction taking 1117 positions into account and with 100 bootstrap resampled data sets, with consensus of >80 bootstrap values. *Capnodium citri*, AY016340 was used as outgroup.

Discussion

Despite its very low pH and its high content of poisonous aliphatic and aromatic compounds, lignite seems to be colonized by a diversity of fungi. Several yeasts (U. Hölker, unpubl.) were isolated from the lignite Hambach A, which has a high aliphatic content. *Trichoderma* sp. (Hölker et al. 1997), *Fusarium* sp. (Hölker et al. 1995) and *Penicillium* sp. (U. Hölker, unpubl.) were found particularly on Garzweiler B lignite, characterised by a higher lignocellulose content. From lignite Bergheim A, which has higher contents of saturated aromatic compounds, black yeasts were isolated, in addition to other fungi. All mentioned lignites were obtained from open cast mining regions and thus, accessible to microbial colonisation for undefined time intervals. It is possible that the isolated black yeast assimilates toxic aromatic compounds of humic and fulvic acids and thereby has a competitive advantage over more rapidly growing contaminants. Other black yeast-like fungi are known to assimilate benzene compounds (Middelhoven 1993) and kerosene (Sterflinger et al. 1999).

Initial development of the fungus was very similar to *Exophiala* in the torulose character of the hyphae and the flat annellated zones resembling those of *E. dermatitidis*. Repeated suspension plating precluded a possible mixture of two black yeasts. In a later stage of development, some seemingly mature hyphal cells formed an extra septum and became locally incrusted with melanin-like material, matching Takeo and de Hoog's (1987) criterion of indeterminate thallus maturation characterising dothidealean black yeasts. Also many yeast cells showed gradual inflation and melanisation and eventually developed a median sep-

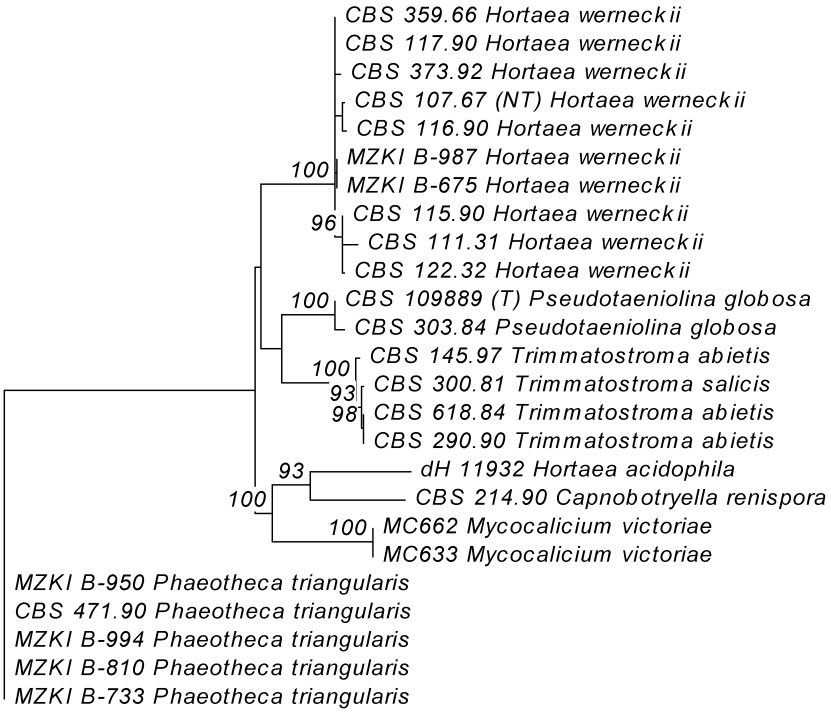

Figure 4. Consensus tree of ITS rDNA of 25 members of Dothideales, constructed with the Neighbor joining algorithm in the Treecon package with Kimura-(2) correction and 100 bootstrap replicates (values >90 are shown with the branches). *Phaeotheca triangularis*, CBS 471.90 was used as outgroup.

tum. In contrast to the bicellular yeast cells of *Hortaea werneckii*, the median septum was not remarkably thickened.

The affinity to the Dothideales was confirmed by SSU rDNA sequencing. Based on similar data (Sterflinger et al. 1999), *Hortaea* was also suggested to be an anamorph member of the order Dothideales, and it was found among the nearest neighbours of the new strain. However, the mutual distances among species were found to be relatively large and distances more or less equal, leading to very poorly resolved trees. This was the case with several algorithms used, based on parsimony, maximum likelihood as well as on distance. All species with relative sequence similarity to the new strain were either halophilic, such as *Hortaea werneckii* (Zalar et al. 1999a) and *Trimmatostroma salinum* (Zalar et al. 1999b), or epilithic, such as *Trimmatostroma abietis* (Butin et al. 1995), *Pseudotaeniolina globosa* (De Leo et al. 2003) and *Capnobotryella renispora* (Titze and De Hoog 1990), or lichenicolous, such as *Hobsonia santessonii* (Lowen et al. 1986), or grew on honeydew on leaves, such as *Coccodinium bartschii* (Eriksson 1981). Thus, they all share tolerance of growth conditions to low water activity.

Another possible category to classify the new acidophilic strain is the plant-opportunistic genus *Hormonema*, with repetitive production of conidia through the same scar (De Hoog and Yurlova 1993). It also belongs to the order Dothideales. However, the presence of unambiguously annellated zones and the slow growth of the organism exclude *Hormonema.* Some of the black fungi mentioned above are phylogenetically close to the new strain, both in ITS and SSU sequences, but lack annellated zones. Since taxonomic schedules presently are primarily based on morphology, we prefer classification of the new acidophilic strain in *Hortaea*. The remaining morphological bias is justified, given the extremely premature state of currently available sequence data of these fungi.

Hortaea is monotypic, with the halophilic species *H. werneckii* as the only species described to date (Zalar et al. 1999). The large ITS deviation of $>16\%$ and the remarkable differences in the physiology between *Hortaea werneckii* and the new isolate does not preclude its accommodation in the genus *Hortaea*. A

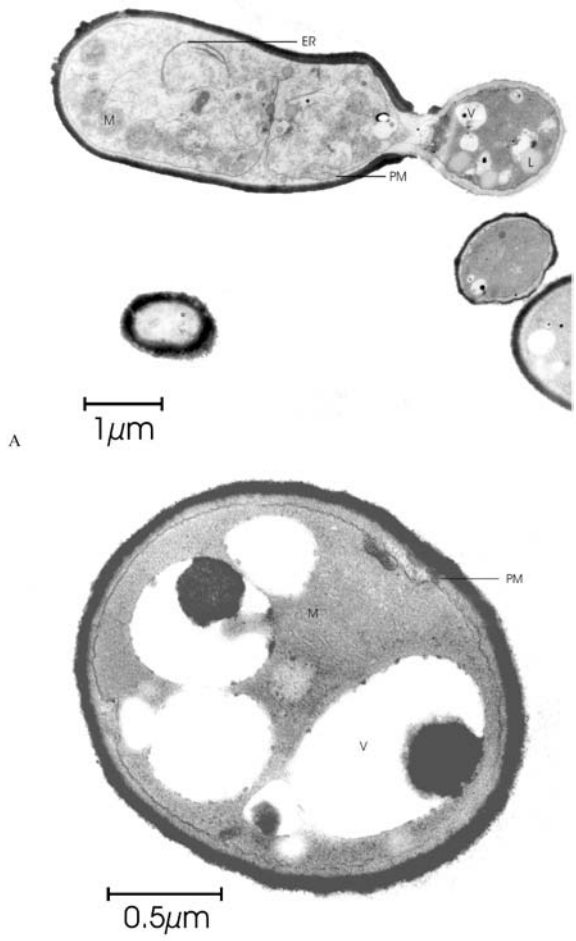

Figure 5. TEM-photography of *Hortaea acidophila*, CBS 113389. (A) budding cell; (B) cell with vacuoles containing osmiophilic granular substances.

new species is described and formally introduced below.

A comparison of the physiological properties of the isolated black yeast with members of the order of Dothideales (de Hoog et al 1995; Butin et al. 1996; Sterflinger et al. 1999) showed analogies, such as the ability to liquefy gelatine and to assimilate a variety of carbohydrates. Like several other members of the Dothideales (de Hoog et al 1997) the new species was not able to grow at temperatures above 30 °C. A major difference was its ability to grow under extremely acidic conditions and its low degree of halotolerance (Untereiner et al. 1999). The latter feature is remarkable since its nearest neighbour, *Hortaea werneckii*, is halophilic (Zalar et al. 1999) while many other dothidealean black yeasts show osmotolerance. Like the marble-inhabiting members of the genus *Coniosporium* (de Leo et al. 1999) the new black yeast can grow only in media with concentrations below 2% NaCl.

The question how the new isolate can grow at pH 0.6 with humic and fulvic acids as carbon source remains still open. Studies regarding the role of the melanised cell wall and the contribution of oxidising cell wall bound enzymes are in progress.

Hortaea Acidophila sp. nov.

Hyphis pauce ramosis, circa 2.0-2.5 μm latis; conidiophoris unicellularis intercalaris annellatis. Conidia ellipsoidea, subhyalina, uniseptata, circa 2.5-3.0 × 1.5-2.0 μm.

Holotypus (vivus et exsiccatus): CBS 113389,U. Hölker isolatus ex lignite, Bergheim, Germania, Maio 2001.

Colonies on PDA at 25 °C attaining about 4 mm in 10 days, glistening, smooth, slimy, jet black. Budding cells initially subhyaline, smooth, thin-walled, ellipsoidal, about 2.5-3.0 × 1.5-2.0 μm, later swelling, becoming darker and developing a central septum, then broadly ellipsoidal, up to about 6 × 4 μm. Hyphae initially pale olivaceous, thin-walled, often starting with a series of ellipsoidal cells (torulose hyphae), later becoming darker with thicker walls, pigmentation finally becoming somewhat irregular; hyphae about 2.0-2.5 μm wide, cells about 12-20 μm long, occasionally subdivided in a later stage. Local clumps of blackish-brown extracellular material are produced. Single annellated zones inserted laterally on each intercalary cell, about 1.0-1.5 μm wide, very short, annellations usually invisible with the light microscope. Conidia soon developing into yeast cells.

Holotype (living and dried): CBS 113389, isolated by U. Hölker from lignite, Bergheim, Germany, May 2001.

Acknowledgements

We wish to thank Markus Braun and Brigitte Buchen, University of Bonn, for TEM microscopy. We are indebted to *HöFer* Bioreact GmbH, Bonn for financial support to U.H.. Bert Gerrits van den Ende is thanked for the molecular analysis.

References

Butler M.J. and Day A.W. 1998. Fungal melanins: a review. Can. J. Microbiol. 44: 1115–1136.

Butin H., Pehl L., de Hoog G.S. and Wollenzien U 1996. *Trimmatostroma abietis* sp. nov. (hyphomycete) and related species. Antonie van Leeuwenhoek 69: 203–209.

Caesar-Tonthat T.C., van Ommen Kloeke F., Geesey G.G. and Henson J.M. 1995. Melanin production by a filamentous soil fungus in relation to copper and localization of copper sulfide by sulfide silver staining. Appl. Environm. Microbiol. 61: 1968–1975.

De Hoog G.S., Vicente V., Caligiorne R.B., Kantarcioglu S., Tintelnot K., Gerrits van den Ende A.H.G. and Haase G. 2003. Species diversity and polymorphism in the *Exophiala spinifera* clade containing opportunistic black yeast-like fungi. J. Clin. Microbiol. 41: 4767–4778.

De Hoog G.S., Beguin H. and Batenburg-van de Vegte W.H. 1997. *Phaeotheca triangularis*, a new meristematic black yeast from a humidifier. Antonie van Leeuwenhoek 71: 289–295.

De Hoog G.S., Guého E., Masclaux F., Gerrits van den Ende A.H.G., Kwon Chung K.J. and McGinnis M.R. 1995. Nutritional physiology and taxonomy of human pathogenic *Cladosporium / Xylohypha* species. J. Med. Vet. Mycol. 33: 339–347.

De Leo F., Urzi C., de Hoog G.S. 1999. Two *Coniosporium* species from rock surfaces. Stud. Mycol. 43: 70–79.

Eriksson O. 1981. The families of bitunicate ascomycetes. Opera Bot. 60: 1–209.

Figueras M.J., de Hoog G.S., Takeo K. and Guarro J. 1995. Stationary phase development of *Trimmatostroma abietis*. Antonie van Leeuwenhoek 69: 217–222.

Haase G., Sonntag L., Melzer-Krick B. and de Hoog G.S. 1999. Phylogenetic inference by SSU gene analysis of members of the *Herpotrichiellaceae* with special reference to human pathogenic species. Stud. Mycol. 43: 80–97.

Hershkowitz M.A. and Lewis L.A. 1996. Deep-level diagnostic value of the rDNA-ITS region. Mol. Biol. Evol. 13: 1276–1295.

Hölker U., Fakoussa R.M. and Höfer M. 1995. Growth substrates control the ability of *Fusarium oxysporum* to solubilize low-rank coal. Appl. Microbiol. Biotechnol. 44: 351–355.

Hölker U., Mönkemann H. and Höfer M. 1997. A test system to analyze the complex physiological states of coal solubilizing fungi. Fuel Proc. Technol. 52: 65–71.

Lowen R., Brady B.L., Hawksworth D.L. and Paterson R.R.M. 1986. Two new lichenicolous species of *Hobsonia*. Mycologia 78: 842–846.

Middelhoven W.J. and de Hoog G.S. 1997. *Hormonema schizolunatum*, a new species of dothideaceous black yeast from phyllosphere. Antonie van Leeuwenhoek. 71: 297–305.

Middelhoven W.J. 1993. Catabolism of benzene compounds by ascomycetous and basidiomycetous yeasts and yeastlike fungi. Antonie van Leeuwenhoek 63: 125–144.

Onofri S., Pagano S., Zucconi L. and Tosi S. 1999. *Friedmanniomyces endolithicus* (Fungi, Hyphomycetes), anam.-gen. and sp. nov., from continental Antarctica. Nova Hedwigia 68: 175–181.

Spurr A. 1969. A low-viscositiy epoxy resin embedding medium for electron microscopy. Ultrastruct. Res. 26: 31–43.

Sterflinger K., de Hoog G.S. and Haase G. 1999. Phylogeny and ecology of meristematic ascomycetes. Stud. Mycol. 43: 5–22.

Sterflinger K. 1998. Temperature and NaCl-tolerance of rock-inhabiting meristematic fungi. Antonie van Leeuwenhoek 74: 271–281.

Swofford D.L. 2002. PAUP. Phylogenetic Analysis Using Parsimony. Version 4. Sinauer Associates, Sunderland, Massachusetts, USA.

Takeo K. and de Hoog G.S. 1991. Karyology and hyphal characters as taxonomic criteria in ascomycetous black yeasts and related fungi. Antonie van Leewenhoek 60: 35–42.

Titze A. and de Hoog G.S. 1990. *Capnobotryella renispora* on roof tile. Antonie van Leeuwenhoek 58: 265–269.

Van de Peer Y. and De Wachter R. 1994. Treecon for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Appl. Biosci. 10: 569–570.

Untereiner W.A., Gerrits van den Ende A.H.G. and de Hoog G.S. 1999. Nutritional physiology of species of *Capronia*. Stud. Mycol. 43: 98–106.

Wheeler M.H. and Bell A.A. 1988. Melanins and their importance in pathogenic fungi. Curr. Topics Med. Mycol. 2: 338–387.

Zalar P., De Hoog G.S. and Gunde-Cimerman N. 1999a. Ecology of halotolerant dothideaceous black yeasts. Stud. Mycol. 43: 38–48.

Zalar P., De Hoog G.S. and Gunde-Cimerman N. 1999b. *Trimmatostroma salinum*, a new species from hypersaline water. Stud. Mycol. 43: 57–62.