Debaryomyces mycophilus sp. nov., a siderophore-dependent yeast isolated from woodlice

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Received 9 November 2001; received in revised form 28 May 2002; accepted 30 May 2002
First published online 16 July 2002

Abstract

Four strains of an ascogenous yeast were isolated from the guts of the woodlice species Armadillidium vulgare (Latreille). This yeast differed from all known yeasts by its inability to grow in culture without the presence of a metabolite produced by some common soil fungi such as Cladosporium cladosporioides, Aspergillus alliaceus, and Penicillium spp. Phylogenetic analysis based on 18S rDNA and 26S rDNA (domain D1/D2) sequences indicated that the yeast represents a new taxon in the genus Debaryomyces. The new species Debaryomyces mycophilus is thus proposed. It was, furthermore, shown that the fungal metabolite necessary for growth of D. mycophilus did not provide the yeast with carbon, nitrogen or vitamins. The active compound was partially purified and it was shown that it is a siderophore used by the yeast as a source of iron. The addition of ferrichrome or high concentrations of FeCl₃ to growth media replaced the obligate dependence on a fungal metabolite. Symbiosis among fungi, based on the availability and utilization of iron, is an aspect of mycology that has not previously been recognized. The addition of chelated iron to isolation media could lead to the discovery of many unknown yeasts and fungi. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Yeast taxonomy; Invertebrate; Soil fungus; Symbiosis; Iron metabolism; Siderophore

1. Introduction

Woodlice belonging to the species Armadillidium vulgare (Latreille) are saprotrophic invertebrates that feed on hardwood leaf litter. These small animals have active mycolytic activity. Like some other soil saprophages, one of the major nutrient sources available to woodlice is thought to be the mycelial fungi associated with their food [1–3].

One of the major difficulties encountered by researchers working with symbiotic microbes is the inability to cultivate the microbes separately from the organisms with which they co-exist in nature [4]. Difficulties are commonly experienced with the cultivation of symbionts from animal guts or tissues. Problems in isolating protozoa from the guts of termites [4], yeast-like fungi associated with gut epithelia of insects [5], and bacteria from gutless worms [6] are examples.

We have succeeded in cultivating a yeast symbiont from the guts of woodlice. In order to grow this yeast, which we refer to as a woodlice symbiont, in culture, it was necessary to supplement media with culture filtrates from a number of common soil fungi. In this report we characterize the nature of this requirement. Furthermore, the yeast associated with the guts of woodlice is described as a new taxon based on characteristics of its physiology and phylogenetic position determined by DNA sequence data analysis.

2. Materials and methods

2.1. Isolation of yeasts

Woodlice A. vulgare were collected from leaf litter in a mixed oak–pine forest near the Nikitin botanical garden, Jalta, Crimea, Ukraine. Isolation of yeasts from oak
leaves, guts, and excrement of the woodlice was achieved by using the technique of Byzov et al. [1], with some modification of incubation conditions. Half of the Petri dishes containing isolation medium and inoculum were incubated for 3 days at 20°C followed by a week at 4°C and, thereafter, 3 days at 20°C. The remaining dishes were incubated only at 20°C.

2.2. Taxonomic study

2.2.1. Physiology and morphology

The yeast isolates were identified on the basis of morphology and physiological properties using standard methods [7] and identification keys [8–10]. Carbon, nitrogen assimilation, and vitamin requirement tests were carried out in test tubes containing 5 ml of the test media. Carbon and nitrogen sources were from Sigma, yeast carbon base, yeast nitrogen base, vitamin-free medium and other components (malt extract, yeast extract, peptone) were from Difco. In the case of the symbiotic yeast from woodlice guts, the media were enriched with ferrichrome (Sigma) at a concentration of 0.73 µM. Complete physiological tests were performed specifically for strain G-398. Test tubes were incubated for 21 days at 25°C on a roller drum with angle 10° at 40 rph. Results were scored after 7, 14, and 21 days. If growth occurred only after 21 days, it was scored as delayed (D).

Morphology of vegetative cells and ascospore formation were studied using a light microscope (Nikon). The sizes given are based on 30 independent measurements. Photographs were taken using the differential interference contrast (DIC) option. Ascospore morphology was studied with transmission electron microscopy (TEM) by using the technique of Benade et al. [11] and based on material from a culture grown on YMA (0.3% yeast extract, 0.5% peptone, 1% glucose, 2% agar) containing 0.73 µM ferrichrome.

2.2.2. Mol% G+C

In order to determine the DNA base composition, strain G-398 was grown to stationary phase in YM broth (1% glucose, 2% malt extract, 1% peptone, 0.3% yeast extract) containing 0.73 µM ferrichrome. The cells were centrifuged and the washed three times with sterile, distilled water. The procedure of Johnson [12] was used for DNA extraction and purification. Determination of DNA base composition was done using the high-performance liquid chromatography (HPLC) method described by Nakase et al. [13].

2.2.3. Coenzyme Q system

Cells were grown in the same manner as for determination of the DNA base composition. Extraction and purification of the coenzyme Q system were performed according to Yamada and Kondo [14]. Coenzyme Q was identified using HPLC by comparing retention times with those of CoQ standards obtained from Sigma. Ubiquinone isoprenologues were analyzed on a Zorbax ODS column (Du Pont Chromatography Products) eluted with methanol–isopropanol (2:1, v/v) at a rate of 2 ml min−1. Elution of CoQs was monitored by following UV absorption at 275 nm.

2.2.4. Sequencing and phylogenetic analysis

For molecular characterization of the yeast from woodlouse gut, the D1/D2 domains of the 26S rDNA were sequenced for 3 strains (G-393, G-395, and G-398). A nearly complete sequence of 18S rDNA was also obtained for strain G-398. All sequences obtained in this study have been deposited in GenBank. The accession numbers are indicated in the phylogenetic trees along with other reference sequences (Figs. 1 and 2).

For sequencing the D1/D2 domain of the 26S rDNA the same primers and PCR conditions were used as described by Kurztman and Robnett [15]. The 18S rDNA was amplified using the polymerase chain reaction (PCR) with Taq DNA polymerase (Qiagen) and two primers, P1 (5′-ATCTGTGTTGATCTGCAAGT-3′) and NS8 (5′-TCCGAGGTTCACTACCGGA-3′). The PCR conditions for amplification of the 18S rDNA were as follows: an initial denaturation step of 95°C for 4 min followed by 30 cycles of 94°C for 1 min, 55°C for 2.5 min and 72°C for 2.5 min. The final extension step was 72°C for 7 min. The primers used for sequencing 18S rDNA were P1 (5′-ATCTGTGTTGATCTGCAAGT-3′), F2 (5′-GCTACCACTCCAAGGAAAGG-3′), F3 (5′-CTGCCGAAGACATTTGCAAGG-3′), F4 (5′-TCTGGGCCGGCGCCGCCTGACTCTG-3′), R1 (5′-TGGAATTACCGCGGCTGCGTGGCAC-3′), R2 (5′-TCTCCGGCAAATGTCTTCCGAG-3′), R3 (5′-AAAGTCTCTGTTGGATCTGCA-3′), and NS8 (5′-TCCGCAGGTTCACTACCCGGA-3′). PCRs were performed on a GeneAmp® PCR System 9700 (PE Applied Biosystems). The PCR products were purified by using QIAEX II agarose gel extraction kit (Qiagen). The DNAs were sequenced by the dideoxy chain termination method using a Thermo Sequenase Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) according to manufacturer’s instructions. A model 373 automated DNA sequencer (Applied Biosystems) was used. Sequences were initially compared with the GenBank sequences using Blast search. The reference sequences were downloaded, and then edited using the program BioEdit [16]. Sequences were aligned using CLUSTAL W [17]. The fixed gap and floating gap penalty values used were 10. The phylogenetic tree was constructed from the evolutionary distance data according to Kimura [18] using the neighbor-joining method [19] in the CLUSTAL W computer program. Bootstrap analyses [20] were performed from 1000 random re-samplings. The phylogenetic trees were generated using TreeView [21].
2.3. Characterization of the growth-promoting factor

2.3.1. Screening fungi for growth factor

Thirty-seven strains of fungi belonging to various taxonomically distinct groups (Fig. 3) were screened for the production of the factor necessary for the growth of the yeast from woodlice guts. The majority of these strains (with CMW prefix) are stored in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. The remaining isolates are housed in the culture collection of Department of Microbiology and Biochemistry, University of the Free State, Bloemfontein, South Africa. Inoculum of these fungi was prepared from stock cultures maintained on 2% malt extract agar slants. Fungi were grown in 100-ml conical flasks containing 20 ml of YM broth for 5 days at 25°C with a shaker speed of 150 rpm. The cell suspensions were centrifuged twice at 6000 rpm for 10 min to remove all cells. The supernatants were filtered through Pasteur pipets fitted with small plugs of absorbent cotton wool. Dif-

Fig. 1. Phylogenetic tree depicting the relationship between D. mycophilus (G-393, G-395, G-398), Debaryomyces spp., and other reference taxa. The tree was constructed based on nucleotide divergence in the 5S-28S rDNA (positions 63-680) using program CLUSTAL W. Bootstrapping was done in 1000 replications. Values for frequencies less than 50% are not given. (*) Sequences obtained in this study.
ferent volumes of the filtrates (0.1, 0.2, and 0.5 ml) were added to test tubes containing 5 ml of YM broth. The media were autoclaved at 121°C for 20 min.

The woodlouse-associated yeast strain G-398 was used to test the fungal culture filtrates for stimulation of growth. For the preparation of the inocula, the yeast was grown in a test tube containing 5 ml of YM broth supplemented with culture filtrate of Cladosporium cladosporioides. The cell suspension was centrifuged at 3000 rpm for 10 min and washed four times with 10-ml portions of YM broth under sterile conditions. The cell pellet was resuspended in 5 ml of YM broth, from which 50 μl inocula were taken.

Incubation of the tubes containing the various fungal culture filtrates and the yeast was on a roller drum with tubes placed at an angle of 10°. Tubes were rotated at 40 rph and incubated at 25°C for 10 days. The growth of the yeast isolate G-398 in the presence of fungal culture filtrates was evaluated in terms of optical density at 620 nm. Measurements were done in a micro-titer plate reader (Biolog) with 200 μl of cell suspension in each well. Uninoculated YM broth served as a control.

2.3.2. Partial purification of growth factor

The most active producer of the factor necessary for the growth of the woodlouse-associated yeast was a strain of Fusicoccum (CMW-325). This fungus was grown for 5 days at 25°C in 1-l Erlenmeyer flasks containing 300 ml of YM broth on a rotary shaker (150 rpm). The culture liquid was centrifuged at 3000 rpm for 10 min and solids were discarded. The supernatant (2 l) was freeze-dried. The dried material was re-dissolved in distilled water (100 ml). Ethanol (500 ml) was added to the filtrate (100 ml), it was shaken well and cooled to −70°C for 2 h. The filtrate–ethanol mixture was centrifuged in the cold (4°C) at 3000 rpm for 20 min. The sediment was discarded and the supernatant evaporated at 50°C using a rotary evaporator. The dried residue was dissolved in distilled water (50 ml). Size-exclusion chromatography was performed on 5-ml portions of the dissolved residue using Biogel P4 (Bio-Rad) in a 50 cm x 2.5 cm column. The column was eluted at 4°C with distilled water (elution rate 15 ml h⁻¹) and 5-ml fractions were collected. Prior to autoclavation, one μl from each fraction was added to test tubes containing YM broth (5 ml) to test for growth stimulation using strain G-398. The active fractions were pooled and freeze-dried. The residue was washed with 50 ml of acetone, dissolved in 50 ml of methanol, and the undissolved residue discarded. The supernatant was dried, re-dissolved in water to a concentration of 10% (dry weight/volume) and stored at −20°C.

2.3.3. Possible biological function

In order to verify whether the yeast uses the fungal metabolite as carbon, nitrogen or vitamin source, a set of standard physiological tests on defined media were undertaken (Table 2). The standard complete vitamin mixture described by Barnett et al. [8] was used as a vitamin source. Purified growth factor was added to a final concentration of 20 mg l⁻¹. Incubation and scoring was done in the same manner as for assimilation tests (see Section 2.2.1).

2.3.4. Combined effect of Fe(III) and the growth factor

For studying the combined effect of Fe(III) and the growth factor, a two-dimensional matrix was constructed. The first parameter was the amount of FeCl₃ added to the

Fig. 2. Phylogenetic tree depicting the relationship between D. mycophilus (G-398), Debaryomyces spp., and other reference taxa. The tree was constructed based on nucleotide divergence in the 18S rDNA using program CLUSTAL W. Bootstrapping was done in 1000 replications. Values for frequencies less than 50% are not given. (*) Sequence obtained in this study.
media and the second parameter the amount of the purified growth factor added. FeCl₃ was added to concentrations of 0, 30, 100, 300 μM, and the growth factor was added to: 0, 2, 10, and 40 mg l⁻¹ (see Fig. 4). The test was performed in test tubes containing 5 ml of YM broth. Inoculations, incubations and evaluations of growth were done as described above (see Section 2.3.1).

2.3.5. Stimulation effect of ferrichrome

One milligram of ferrichrome (Sigma, in iron-free form, molecular mass 687.7) was dissolved in 2 ml of distilled water. Different volumes of the solution were added to YM broth (5 ml) to give the following concentrations of ferrichrome: 0, 0.022, 0.073, 0.22, 0.73, and 2.2 μM. In order to ensure the presence of ferrichrome in iron-chelated form, to a second series with the same ferrichrome concentrations FeCl₃ was added to a concentration of 10 μM. Inoculations, incubations and evaluation of growth were done as described above (see Section 2.3.1).

2.3.6. Inhibitory effect of iron on production of the growth factor

For testing the effect of iron on production of growth factor two fungi that were active producers of the putative growth factor (Graphium sp. [CMW729] and Fusicoccum sp. [CMW325]) were chosen. FeCl₃ was added to YM broth to give the following concentrations: 0, 1, 3, 10, 30, 100, and 300 μM. Fungi were grown for 5 days in YM broth at 25°C with a shaker speed of 150 rpm. The growth of fungi was evaluated in terms of dry weight for

![Fig. 3. Effect of culture filtrates of different fungi on the growth of D. mycophilus (G-398). Culture filtrate was added to the medium at ratio 1:10 (v/v).](image)
Fusicoccum sp. or optical density at 620 nm for the Graphium sp., which formed yeast-like growth in YM broth. Culture filtrates were tested for stimulation of the growth of the woodlice symbiont.

3. Results and discussion

3.1. Isolation, phylogenetic placement and description of the woodlice symbiont

The yeasts isolated from the decaying oak leaves on which woodlice feed were Aureobasidium pullulans (de Bary) Arnaud, Rhodotorula rubra, (Saito) Harrison, Cryptococcus albidus (Saito) Skinner, and Cystobasidium capitatum (Fell et al.) Oberwinkler and Bandoni. These yeasts are typical members of the yeast community associated with leaf litter. Yeasts detected using both incubation regimes were the same. The total number of yeasts did not exceed $10^6$ CFU g$^{-1}$. Only filamentous fungi were detected in the excrement of the woodlice. The total number of fungal colonies was approximately 100 times lower than that associated with the leaf litter.

Isolations from the guts of woodlice in dishes incubated at a constant temperature (20°C) yielded no yeast colonies after 1 week. These dishes were subsequently overgrown with fast-growing molds such as Penicillium, Mucor, Fusarium, and Trichoderma. When Petri dishes were initially incubated at 20°C for 3 days and then at 4°C for a week, growth of filamentous fungi was delayed. The subsequent incubation at 20°C for 3 days yielded approximately 300 yeast colonies of similar morphology for each woodlouse gut. This yeast was not detected in the leaf litter or the excrement of the woodlice. Four isolates of the yeast (G-393, G-395, G-398, G-399) were collected.

The most striking feature of the yeast strains associated with woodlouse guts was the extremely poor growth on media commonly used for the maintenance of yeasts. Thus on malt extract agar or YMA medium, growth could only be detected visually after approximately one month of incubation. Yeast colonies in the vicinity of some fungal contaminants grew faster than those farther away from these fungi. Addition of culture filtrate from fungi such as C. cladosporioides substantially enhanced growth (Fig. 5). It was subsequently shown that fungal culture filtrates could be replaced by ferrichrome.

Sequences obtained for the variable D1/D2 domain of the 26S rDNA were identical for strains G-398 and G-393 while G-395 had a base insertion not present in the other two strains. The phylogenetic analysis (Fig. 1) showed conclusively that the woodlice symbiont belongs to the family Saccharomycetaceae G. Winter (1881) and that it is loosely associated (62% bootstrap confidence) with a well-defined cluster consisting of Debaryomyces hansenii, D. coudertii, Candida psychrophila, D. nepalensis, D. manrama, D. robertsiae, and D. udenii. The woodlouse symbiont showed rather high base differences with these closest yeasts. Thus strain G-398 had 17, 18, 22, 17, 19 and 19 base differences with D. robertsiae, D. udenii, D. manrama, D. hansenii, D. coudertii, C. psychrophila and D. nepalensis, respectively. The topology of the phylogenetic tree formed by the reference yeast species agrees well with that published by Kurtzman and Robnett [15]. Small differences, i.e. the association of D. etchellsii with the D. hansenii cluster instead of a Pichia triangularis cluster [15].
were probably the result of unequal sets of species taken for the phylogenetic analysis. Strain G398 had 23 base differences with D. etchellsii in the studied region.

The 18S rDNA sequence data derived in this study gave little information regarding the phylogenetic position of G-398 amongst other Debaryomyces species. This was mainly due to a lack of reference sequence data for this group of yeasts. However, the available data provided good support for the close relationship between G-398 and other species of Debaryomyces. The woodlouse symbiont thus formed a well-defined cluster (95% bootstrap confidence) with D. hansenii, D. udenii, D. castellii, Pichia farinosa, and P. guilliermondii (Fig. 2).

The identification of the woodlouse symbiont as a species of Debaryomyces is also supported by phenotypic characteristics such as its pedagogic mode of ascus formation (Fig. 6) and the presence of Q-9 as a major ubiquinone isoprenologue (Table 1). However, it exhibits an assimilation profile which is different and considerably more limited than the assimilation profiles of other Debaryomyces spp. (Table 1). Xerotolerance, which is a distinguishing property of Debaryomyces spp. [22], is also absent. Long oval to allantoid ascospores have also not been reported for species of Debaryomyces. To accommodate the new yeast from woodlice, we propose the following amended description of the genus Debaryomyces:

Vegetative reproduction is by multilateral budding. Pseudomycelium is absent, primitive, or occasionally well developed. Heterogamous conjugation between a cell and its bud generally precedes ascus formation. Isogamous conjugation also occurs. Ascospores are spheroidal, globose, ovoidal to allantoidal or lenticular and have smooth or warty walls (verrucose to colliculate) that may also have an equatorial ledge or spiral ridges. Ascospores are not liberated from the ascus except for three species, whose asci lyse. One to two spores are usually formed per ascus; in some species up to four spores are present in the ascus. Fermentation is absent, weak or occasionally vigorous. Nitrate is not assimilated. The major ubiquinone is Q-9. Diazonium blue B reaction is negative.

Given the loose phylogenetic association of the woodlouse symbiont with other species of Debaryomyces as well as its distinctive physiological properties, a new species name is proposed as follows.

3.1.1. Debaryomyces mycophilus Thanh, Van Dyk and Wingfield, sp. nov.

Ad crescentiam siderophorum necessarium sunt. In liq-

Table 1
List of physiological properties of Debaryomyces mycophilus (strain G-398)

<table>
<thead>
<tr>
<th>Fermentation:</th>
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</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>−</td>
<td>α-α-trehalose</td>
<td>−</td>
<td>raffinose</td>
<td>−</td>
</tr>
<tr>
<td>D-galactose</td>
<td>−</td>
<td>melibiose</td>
<td>−</td>
<td>inulin</td>
<td>−</td>
</tr>
<tr>
<td>Maltose</td>
<td>−</td>
<td>lactose</td>
<td>−</td>
<td>starch</td>
<td>−</td>
</tr>
<tr>
<td>Me-α-β-glucoside</td>
<td>−</td>
<td>cellubiose</td>
<td>−</td>
<td>D-xylitol</td>
<td>−</td>
</tr>
<tr>
<td>Sucrose</td>
<td>−</td>
<td>melezitose</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Utilization of carbon sources:</td>
<td></td>
<td></td>
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<tr>
<td>D-glucose</td>
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<td>arbutin</td>
<td>−</td>
<td>myo-inositol</td>
<td>−</td>
</tr>
<tr>
<td>D-galactose</td>
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<td>melibiose</td>
<td>−</td>
<td>2-keto-D-glucurate</td>
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<tr>
<td>L-sorbitose</td>
<td>−</td>
<td>lactose</td>
<td>−</td>
<td>5-keto-D-glucurate</td>
<td>−</td>
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<td>D-gluconamine</td>
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<td>−</td>
<td>D-glucurate</td>
<td>−</td>
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<td>melezitose</td>
<td>−</td>
<td>D-galacturionate</td>
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<td>inulin</td>
<td>−</td>
<td>D-galacturionate</td>
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<td>starch</td>
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<td>−</td>
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<td>D-lactate</td>
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<td>myo-erythritol</td>
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<td>succinate</td>
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<td>−</td>
<td>xylitol</td>
<td>+</td>
<td>methanol</td>
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<td>α-α-Trehalose</td>
<td>DW</td>
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<td>+</td>
<td>ethanol</td>
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<td>butane-2,3-diol</td>
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<td>galactitol</td>
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<tr>
<td>Utilization of nitrogen sources:</td>
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<tr>
<td>KNO3</td>
<td>−</td>
<td>cadaverine</td>
<td>−</td>
<td>L-lysine</td>
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<td>NaNO2</td>
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<td>creatine</td>
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<td>D-glucosamine</td>
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<tr>
<td>Imidazole</td>
<td>−</td>
<td>creatine</td>
<td>−</td>
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<tr>
<td>Other properties:</td>
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<tr>
<td>Growth in vitamin-free medium</td>
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<td>formation of starch-like material</td>
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<td>−</td>
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<tr>
<td>Growth at 25°C</td>
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<td>growth in 50% D-glucose</td>
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<tr>
<td>Growth at 30°C</td>
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<td>hydrolysis of urea</td>
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<tr>
<td>Growth at 35°C</td>
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<td>diazonium blue B reaction</td>
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<tr>
<td>0.01% Cycloheximide tolerance</td>
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<td>coenzyme Q type</td>
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<tr>
<td>2% Acetic acid tolerance</td>
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<td>G+C content (mol%)</td>
<td>38.5</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

(*) Growth occurred or positive reaction; (−) no growth or negative reaction; (D) delay; (W) weak.

3.1.2. Debaryomyces mycophilus Thanh, Van Dyk and Wingfield, sp. nov.

3.1.2.1. Growth in YM broth with and without ferrichrome. In YM broth without ferrichrome, growth is not detectable after 1 month at 25°C. After 5 days at 25°C in YM broth containing ferrichrome, elongated cells (5–7×10–15 μm) are formed in short clusters or branches (Fig. 7). Multilateral budding occurs in narrow or broad bases. After one month a sediment is formed.

3.1.2.2. Growth on YM agar without and with ferrichrome. Growth on YM without ferrichrome is extremely poor. After a few generations thick-walled chlamydospores (10–15×10–15 μm) are formed. Oval cells (5–7×10–15 μm) with thick walls are also formed in short chains. The majority of cells autolyse. Growth on YM supplemented with ferrichrome is good. After 3 days at 25°C oval, swollen, elongated cells (5–7×10–15 μm) are formed in short clusters or short branches. Multilateral budding occurs on narrow or broad bases. Streak cultures are cream-colored, dull, butyrous, with lobate, irregular margins.

3.1.2.3. Dalmau plate cultures on corn meal agar supplemented with ferrichrome. Pseudohyphae present but no true hyphae are formed.

3.1.2.4. Ascosporation. Ascii with ascospores are observed after one month of incubation on YM containing ferrichrome. Asci are formed after bud-mother cell conju-
Ascospores contain one or two lipid droplets and have a bilayered cell wall (Fig. 8). Upon germination, the ascospores become swollen within the ascus.

3.1.2.5. Physiological tests. Results for the physiological tests commonly used in yeast taxonomy are given in Table 1.

3.1.2.6. Source of cultures and type. The type strain G-398 as well as other strains (G-393, G-395, G-399) were isolated from the gut of woodlice A. vulgare (Latreille) and have been deposited live with the Yeast Collection of the Centraalbureau voor Schimmelcultures, Utrecht. They have been assigned the numbers: CBS 8300, CBS 8298, CBS 8299, and CBS 8301, respectively.

3.1.2.7. Etymology. D. mycophilus – The species name refers to the fact that we could initially grow this yeast only in the presence of fungi or fungal metabolite. It is most likely that, in the natural environment, this species also depends on siderophores supplied by other associated microorganisms.

3.2. Characterization of the growth-promoting factor

3.2.1. Screening of fungi for production of growth factor

In order to identify an efficient producer of the putative growth factor required for the growth of D. mycophilus and for further characterization of this factor, thirty-seven strains of fungi belonging to various taxonomic groups (Fig. 3) were screened. The screening experiment showed that many filamentous fungi produced metabolites that stimulated the growth of D. mycophilus (Fig. 3). This feature was apparently restricted to certain genera and species of fungi. Culture filtrates from most of the Cladosporium strains tested stimulated growth of D. mycophilus, while those from Alternaria had no effect. The most active producers of the growth-stimulation factor were a Graphium sp. (CMW-729) and a Fusicoccum sp. (CMW-325). However, at high volume of culture filtrate added (0.5 ml/5 ml) the effect of these fungi was not the strongest (Fig. 3). Culture filtrates of these two strains showed the highest activity compared to the other fungi when they were added in smaller volumes (0.1 ml/5 ml) (data not shown). Stimulation of growth of D. mycophilus by a culture filtrate from Fusicoccum sp. (CMW-325) showed saturation with very small volumes (10 ml l⁻¹) of culture filtrate (Fig. 9).
9). This *Fusicoccum* sp. was, therefore, chosen for further characterization of the growth factor.

### 3.2.2. Chemical and biochemical properties of the growth factor

The active component from the culture filtrate of the *Fusicoccum* sp. showed the following properties. The compound was thermo-resistant and remained active after autoclaving at 121°C for 30 min. It was water-soluble and could not be extracted with low-polar organic solvents (hexane, chloroform, and ethyl acetate) at any pH (3, 7 or 9). It dissolved in methanol and 83% ethanol. This property was used for initial purification from proteins, salts and nucleic acids. Filtration and size-exclusion chromatography showed that the compound had a molecular mass between 800 and 1000 Da, since it passed through an ultra-filtration membrane with 1000 Da cut-off and eluted before glucose from a Bio-gel P4 column with a minimum selective size of 800 Da (Fig. 10). The compound has a positive charge at alkaline pH (pH = 9.3) as indicated by electrophoresis. This property suggested that the compound might be a complex with metal ion. Standard assimilation tests with and without addition of partially purified growth factor showed that it did not serve as carbon, nitrogen or vitamin source (Table 2). An alternative possibility was that *D. mycophilus* uses the fungal metabolite as a siderophore.

### 3.2.3. Confirmation that the growth factor was a siderophore

The results from three experiments done to test whether the growth factor required by *D. mycophilus* is a siderophore were all positive. When the partially purified growth factor was added in concentrations lower than optimum (40 mg l⁻¹), the addition of iron enhanced the effect of the growth factor. High concentrations of iron (300 μM FeCl₃) without growth factor also effectively stimulated growth of *D. mycophilus* (Fig. 4). Thus, it appeared that *D. mycophilus* lacks an efficient mechanism for iron uptake. Addition of ferrichrome (a commercially available siderophore, produced by the fungal species *Ustilago sphaeroergena*) showed the same effect as the growth factor extracted from *Fusicoccum* sp. (CMW-325). At ferrichrome (iron-free) concentrations higher than 0.73 μM, the stimulation effect reached plateau (Fig. 11). Addition of iron (10 μM FeCl₃) also slightly enhanced the effect of the ferrichrome. At concentrations of 30 μM or higher, FeCl₃ dramatically decreased or completely inhibited the production of growth factor by *Fusicoccum* sp. (CMW-325) (data not shown) and *Graphium* sp. (CMW-729).

![Fig. 10. Elution of the growth-promoting factor produced by Fusicoccum sp. CMW-325 from size-exclusion column (Bio-gel P4). Glucose was eluted from 46th fraction.](image)

**Growth (OD at 620 nm)**

![Fig. 11. Stimulating effect of ferrichrome on the growth of D. mycophilus (G-398). Ferrichrome was supplied in iron-free form (○) and iron-chelated form (●).](image)

<table>
<thead>
<tr>
<th>Media</th>
<th>Growth</th>
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<tbody>
<tr>
<td>YNB</td>
<td>−</td>
</tr>
<tr>
<td>YNB+glucose</td>
<td>−</td>
</tr>
<tr>
<td>YNB+growth factor</td>
<td>−</td>
</tr>
<tr>
<td>YNB+glucose+growth factor</td>
<td>+</td>
</tr>
<tr>
<td>YCB</td>
<td>−</td>
</tr>
<tr>
<td>YCB+(NH₄)₂SO₄</td>
<td>−</td>
</tr>
<tr>
<td>YCB+growth factor</td>
<td>−</td>
</tr>
<tr>
<td>YCB+(NH₄)₂SO₄+growth factor</td>
<td>+</td>
</tr>
<tr>
<td>VFM</td>
<td>−</td>
</tr>
<tr>
<td>VFM+vitamins</td>
<td>−</td>
</tr>
<tr>
<td>VFM+growth factor</td>
<td>−</td>
</tr>
<tr>
<td>VFM+vitamins+growth factor</td>
<td>+</td>
</tr>
</tbody>
</table>

YNB – yeast nitrogen base (Difco); YCB – yeast carbon base (Difco); VFM – vitamin-free medium (Difco); (−) growth absent; (+) growth present.

**Table 2**

Growth of *D. mycophilus* (G-398) on defined media with and without addition of purified growth factor.
3.2.4. *D. mycophilus* has an absolute requirement for exogenous siderophores

The role of siderophores in iron acquisition by microorganisms living in aerobic and microaerobic environments, such as the guts of small animals like woodlice [23], is critical. The loss of the ability to produce siderophores should be considered as an ecological strategy rather than a result of mutational defects. Not producing its own siderophores might give an organism such as *D. mycophilus*, living in a dense microbial population, an advantage in the competition for other nutrients. The loss of some conventional properties often implies, in the case of symbionts, a high degree of adaptation to the specific environment. Such organisms are often considered to be more advanced in evolutionary terms [24]. This yeast, therefore, occupies a unique ecological position, since it is the only isolated yeast which cannot survive in isolation from other microorganisms.

The morphology of *D. mycophilus* is most similar to that of *Cyniclomyces guttulatus* (Robin) van der Walt et Scott. The latter yeast is also unusual in having complex requirements for growth such as a high concentration of CO₂ and temperatures above 30°C [25]. The reason why CO₂ stimulates the growth of *C. guttulatus* remains unknown except that it makes the media more similar to the rabbit gut environment where *C. guttulatus* occurs naturally. The recommended media for cultivation of *C. guttulatus* have low levels of oxygen as well as low pH. These conditions might improve the availability of iron. However, our attempt to replace it with addition of siderophores and/or Fe(III) yielded no success (unpublished observation). Thus, the growth requirements of *C. guttulatus* and *D. mycophilus* differ significantly. Moreover, *C. guttulatus* has Q-6 system [25] while *D. mycophilus* has Q-9 system. The two yeasts are also phylogenetically unrelated as indicated by the 26S rDNA sequences (Fig. 1).

3.2.5. Addition of chelated iron to growth media can lead to the discovery of new taxa

In this study, we have characterized the fungal metabolite necessary for the growth of *D. mycophilus*. All data obtained thus far support the view that this metabolite acts as an iron-chelator, providing the yeast with solubilized iron. Iron is an essential element for most forms of life [26]. Even though iron comprises 5% of the earth’s crust, there is a constant competition for it in nature. The reason for this is the limited availability of dissolved iron in aerobic and microaerobic environments. In the presence of oxygen, iron exists in Fe³⁺ form and eventually precipitates as an oxide-hydroxide polymer. The solubility constant of Fe(OH)₃ is 10⁻³⁸, which means that the maximum Fe³⁺ concentration at pH 7 is 10⁻¹⁷ M. This is much lower than the minimum concentration of 10⁻⁶ M required for optimum growth of microorganisms [26]. Most microorganisms growing in aerobic or microaerobic environments, therefore, require some relatively low-molecular-mass compounds with high Fe³⁺ affinity (siderophores) to complex and take up iron from the substrate on which they are growing. Thus, most known aerobic and facultative anaerobic microorganisms produce at least one siderophore [26,27].

In siderophore-mediated iron metabolism, Fe(III) complexed by a siderophore is transported into the cells where it is reduced to Fe(II). The latter has a relatively low affinity for the siderophore and thus can be passed to
other proteins with a higher affinity for iron. The siderophore, therefore, acts as a shuttle vector for iron [28]. The fact that D. mycophilus did not require siderophore supplementation when media contained high concentration of FeCl₃ (Fig. 4), might have been due to diffusion of iron through ion channels.

A natural deficiency in independent iron uptake capability has never before been reported in yeasts. Such a deficiency, and the need for siderophore supplementation is, as far as we are aware, only known in one filamentous fungus. This is in the genus Pilobolus [29,30], which occurs naturally on the dung of ungulates. Initially, media for the cultivation of Pilobolus spp. had to be supplemented with dung extract. Later, it was shown that dung extract could be replaced by some siderophores, like coprogen or ferri- chrome [29,30]. Ferrichrome is also known as a potent growth factor for Arthrobacter [29]. In the case of D. mycophilus, ferrichrome (U. sphaerogena siderophore) could also replace the siderophores supplied by Fusicoccum sp. and other fungi.

The most effective iron uptake systems are known to exist in animal pathogens. These organisms produce siderophores that can withhold iron from host iron-containing proteins. Some pathogens are specialized in using host heme, even if it is complexed directly to hemoglobin, as in the case of Haemophilus and Vibrio cholerae. These pathogens do not have to produce siderophores [31]. It seems unlikely that D. mycophilus uses the host’s heme or other iron-binding proteins to obtain iron, since our earlier attempt to stimulate the growth of D. mycophilus with extracts from woodlouse tissue was unsuccessful (unpublished observation). Woodlice, like some other soil invertebrates, have a mycolytic habit and thus digest fungi [1–3]. It seems most likely that D. mycophilus derives the siderophores necessary for its growth from the digested fungi in the guts of these animals.

Most organisms produce their own siderophores, but are also able to utilize ‘foreign’ siderophores. Escherichia coli, for example, can take up fungal siderophores such as ferrichrome, coprogen, and rhodotorulic acid, as well as bacterial siderophores like enterobactin and aerobactin [29]. Most fungi benefit from the addition of siderophore supplements to growth media [32]. Special adaptation to use and depend on siderophores readily available in a dense microbial population, as occurs in the gut of animals, might give D. mycophilus an advantage in the competition for other nutrients.

Many mycelial fungi are visible in the natural environments, but the presence of microorganisms such as D. mycophilus can easily escape detection. If siderophore-producing fungal contaminants had not been present during the initial isolation from woodlouse guts, we would never have discovered this yeast. Typically, isolation media for yeast and mycelial fungi are supplemented with only trace amounts of iron. The assumption is that microorganisms produce their own siderophores and can thus access iron present in low concentrations.

The discovery of D. mycophilus, which is dependent on the presence of other microorganisms for growth, has highlighted an important aspect of the physiology of fungi. It has also suggested that symbiosis among microbes, based on the availability and utilization of iron, is an aspect of microbiology that has thus far been overlooked. It is unlikely that the special requirement of D. mycophilus and Pilobolus for siderophores is unique amongst fungi. Thus, supplementation of isolation media with chelated forms of iron or high concentrations of iron could lead to the discovery of a wide range of previously unknown yeasts and mycelial fungi. Furthermore, it is hoped that it will lead to a better understanding of symbioses among microorganisms, as well as between them and animals such as woodlice.

Acknowledgements

We thank Prof. J.P. van der Walt (FABI, University of Pretoria), Prof. D. Litthauer, University of the Free State, Prof. I.P. Babjeva, Dr. B.A. Byzov (Moscow State University), Dr. Maudy T. Smith, D.G.A. Poot (CBS), and Dr. B. Gnunn (Perkin Elmer Applied Biosystems) for valuable advice and assistance. The research was made possible through financial support from the South African National Research Foundation (NRF).

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