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

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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 $FeCl_3$ 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.

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

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[★] *This work is dedicated to Prof. I.P. Babjeva in recognition of her contribution to yeast ecology.*

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, 0.3% malt 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 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 Kurtzman and Robnett [15]. The 18S rDNA was amplified using the polymerase chain reaction (PCR) with Taq DNA polymerase (Qiagen) and two primers, P1 (5′-ATCTGGTTGATCCTGCCAGT-3′) and NS8 (5′-TCC-GCAGGTTCACCTACGGA-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′-ATCTGGTTGATCCTGCCAGT-3′), F2 (5′-GCTACCA-CATCCAAGGAAGG-3′), F3 (5′-CTGCGAAAGCATT-TGCCAAGG-3′), F4 (5′-TCTGGGCCGCACGCGCGC-TACACTG-3′), R1 (5′-TGGAATTACCGCGGCTGC-TGGCACC-3′), R2 (5′-TCCTTGGCAAATGCTTTCG-CAG-3′), R3 (5′-AAGGTCTCGTTCGTTATCG-3′), and NS8 (5′-TCCGCAGGTTCACCTACGGA-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].
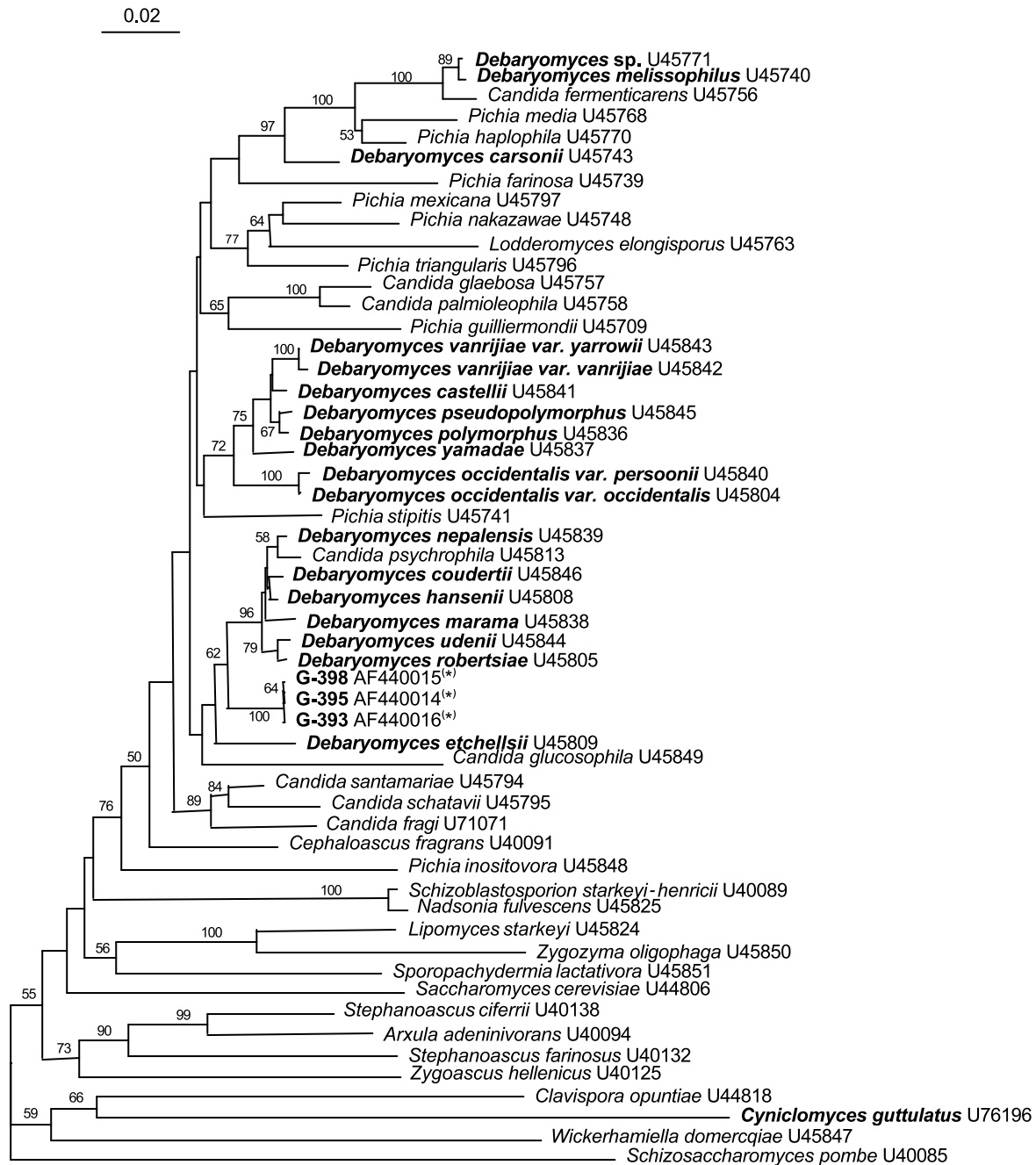
0.02

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 5′ end of the 26S 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.

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-
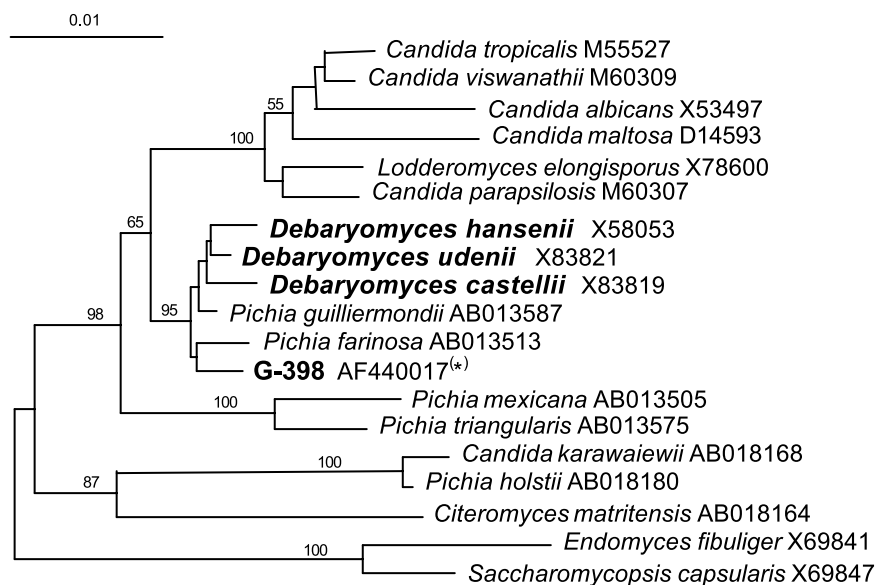
0.01

Candida tropicalis M55527
Candida viswanathii M60309
55
Candida albicans X53497
Candida maltosa D14593
100
Lodderomyces elongisporus X78600
Candida parapsilosis M60307
65
Debaryomyces hansenii X58053
Debaryomyces udenii X83821
98
95
Debaryomyces castellii X83819
Pichia guilliermondii AB013587
Pichia farinosa AB013513
G-398 AF440017(*)
100
Pichia mexicana AB013505
Pichia triangularis AB013575
100
Candida karawaiewii AB018168
87
Pichia holstii AB018180
Citeromyces matritensis AB018164
100
Endomyces fibuliger X69841
Saccharomycopsis capsularis X69847

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.

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 woodlice-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 woodlice-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 twice at 6000 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). The suspension passed through filter paper (Whatman) and then through a bacterial filter (Nalgene, 0.45 µm). 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$\times$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 autoclaving, 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 FeCl$_3$ added to the
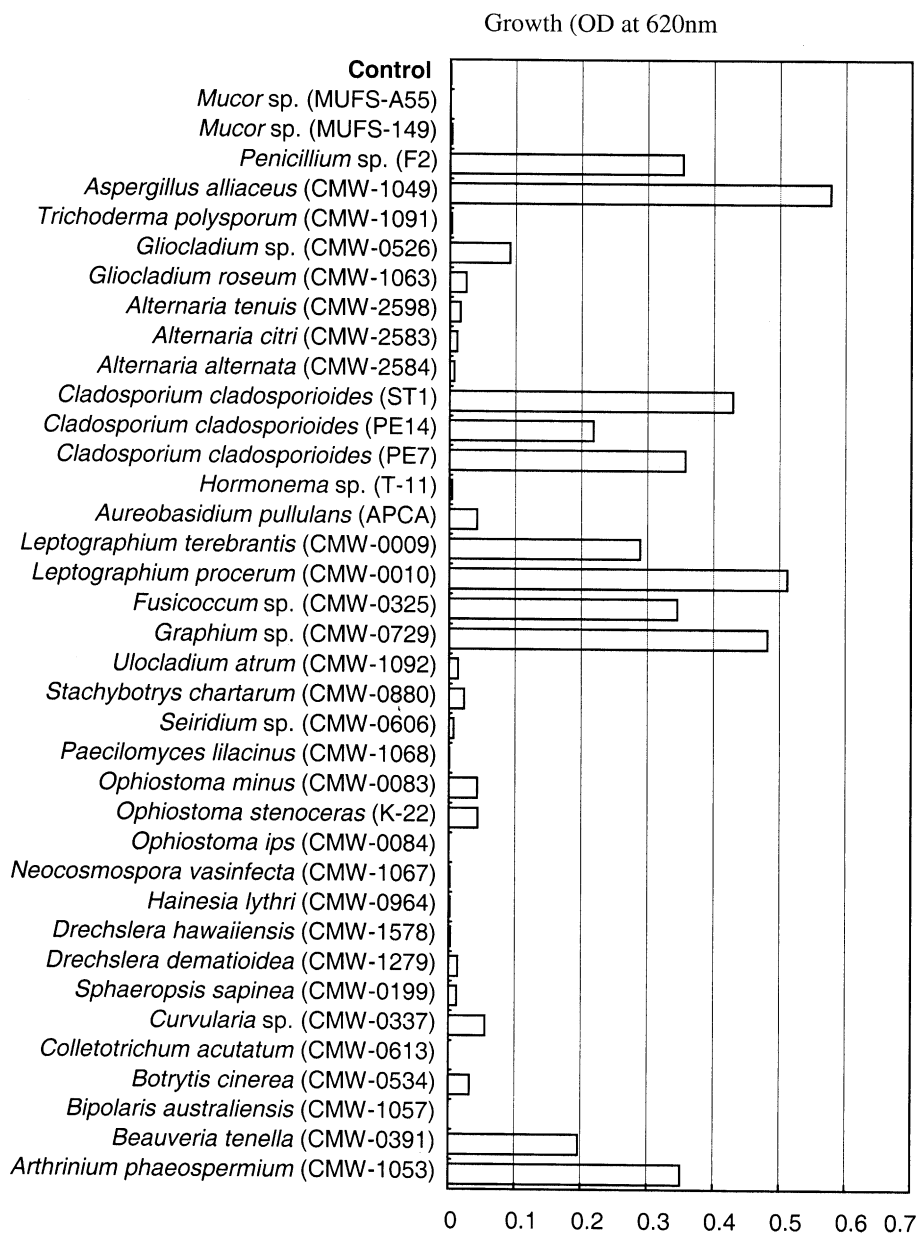
Fig. 3. Effect of culture filtrates of different fungi on the growth of *D. mycophilus* (G-398). Culture filtrate was added to the medium at ratio 1:10 (v/v).

media and the second parameter the amount of the purified growth factor added. FeCl$_3$ 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-che-

lated form, to a second series with the same ferrichrome concentrations FeCl$_3$ 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. FeCl$_3$ 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
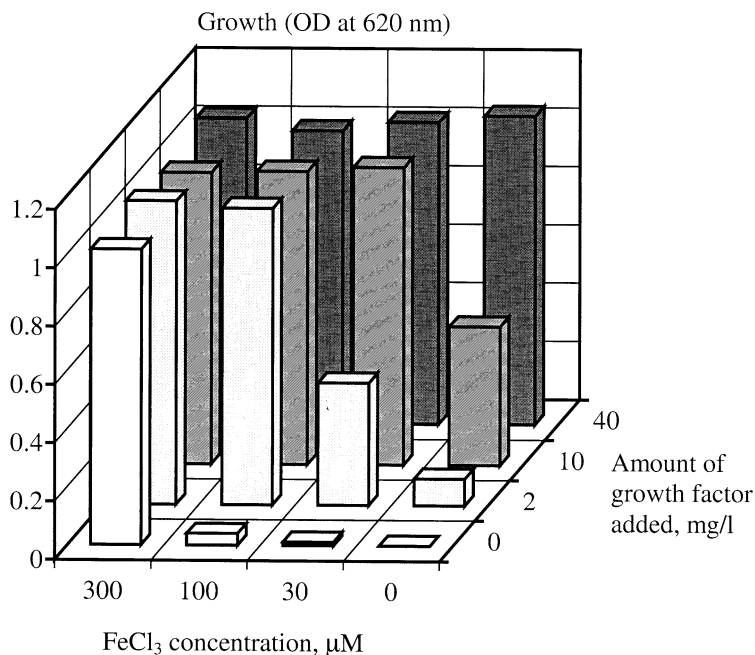
Fig. 4. Combined effect of iron and the purified growth factor produced by *Fusicoccum* sp. CMW-325 on the growth of *D. mycophilus* (G-398).

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 *Cystofilobasidium 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 woodlice 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. marama*, *D. robertsiae*, and *D. udenii*. The woodlice symbiont showed rather high base differences with these closest yeasts. Thus strain G-398 had 17, 18, 22, 17, 17, 19 and 19 base differences with *D. robertsiae*, *D. udenii*, *D. marama*, *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 pedogamic 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 collicutate) 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)

Fermentation:					
D-glucose	−	α,α-trehalose	−	raffinose	−
D-galactose	−	melibiose	−	inulin	−
Maltose	−	lactose	−	starch	−
Me-α-D-glucoside	−	cellobiose	−	D-xylose	−
Sucrose	−	melezitose	−		
Utilization of carbon sources:					
D-glucose	+	arbutin	−	*myo*-inositol	−
D-galactose	+	melibiose	−	D-glucono-1,5-lactone	−
L-sorbose	−	lactose	−	2-keto-D-gluconate	−
D-glucosamine	−	raffinose	−	5-keto-D-gluconate	−
D-ribose	−	melezitose	−	D-gluconate	−
D-xylose	+	inulin	−	D-glucuronate	−
L-arabinose	+	starch	−	D-galacturonate	−
D-arabinose	−	glycerol	−	DL-lactate	−
L-rhamnose	−	*m*-erythritol	−	succinate	−
Sucrose	−	ribitol	+	citrate	−
Maltose	−	xylitol	+	methanol	−
α,α-Trehalose	DW	L-arabinitol	+	ethanol	−
Me-α-D-glucoside	−	D-glucitol	+	propane-1,2-diol	−
Cellobiose	−	D-mannitol	−	butane-2,3-diol	−
Salicin	−	galactitol	−		
Utilization of nitrogen sources:					
KNO₃	−	cadaverine	−	L-lysine	−
NaNO₂	−	creatine	−	D-glucosamine	−
Imidazole	−	creatinine	−		
Other properties:					
Growth in vitamin-free medium	−	formation of starch-like material	−		
Growth at 25°C	+	growth in 50% D-glucose	−		
Growth at 30°C	W	hydrolysis of urea	−		
Growth at 35°C	−	diazonium blue B reaction	−		
0.01% Cycloheximide tolerance	−	coenzyme Q type	9		
2% Acetic acid tolerance	−	G+C content (mol%)	38.5		

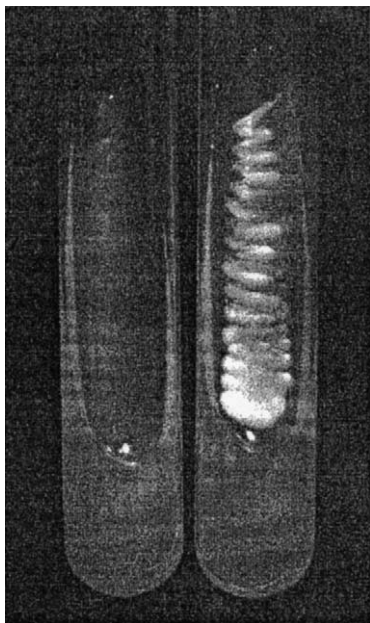(+) Growth occurred or positive reaction; (−) no growth or negative reaction; (D) delay; (W) weak.

Fig. 5. Stimulating effect of culture filtrate of *Cladosporium clado-sporioides* on the growth of *D. mycophilus*. Left: 2 weeks culture of *D. mycophilus* (G-398) on YMA, almost no visible growth; right: *D. mycophilus* (G-398) on YMA supplemented with culture filtrate of *C. cladosporioides*.

uid YM post dies 5 ad 25°C, cellulae vegetativae ovoideae, elongatae, cylindricae, 5–7×10–15 μm in fasciculis per gemmationem multipolarem reproducentes. Post unum mensem sedimentum formatur. Cultura in agaro YM, cremeo-alba vel cremea, butyrosa, haud lucida, margo pauce lobata. Post unum mensem in agaro YM asci formantur ex conjugatione inter cellulam maternam et gemmam. Ascosporae ovoideae, allantoideae 3–3.5×4–7 μm; 1–4 in asco. Mycelium non formantur. Pseudomycelium formantur. Fermentatio nulla. D-glucosum, D-galactosum, D-xylosum, L-arabinosum, α,α-trehalosum (lente et exiguum), ribitolum, xylitolum, L-arabinitolum, et D-glucitolum assimilantur, at non L-sorbosum, D-glucosaminum, D-ribosum, D-arabinosum, L-rhamnosum, sucrosum, maltosum, Me-α-D-glucosidum, cellobiosum, salicinum, arbutinum, melibiosum, lactosum, raffinosum, melezitosum, inulinum, amylum solubile, glycerolum, *m*-erythritolum, D-mannitolum, galactitolum, myo-inositolum, D-glucono-1,5-lactonum, 2-keto-D-gluconatum, 5-keto-D-gluconatum, D-gluconatum, D-glucuronatum, D-galacturonatum, DL-lactatum, succinatum, citratum, methanolum, ethanolum, propanum-1,2-diolum, nec butanum-2,3-diolum. Kalium nitricum, natrium nitrosum, imidazolum, cadaverinum, creatinum, creatininum, L-lysinum, et D-glucosaminum non assimilantur. Ad crescentiam vitaminae externae necessariae sunt. Non crescit in 50% glucoso addito. Materia amyloidea non formatur. Proportio molaris guanini+cytosini in acido deoxyribonucleico: 38.5 mol% (par HPLC). Ubiquinonum majus: Q-9. Holotypus: Isolatus ex *A. vulgare* (Latreille), Jalta, Crimea, Ukraine, CBS 8300 (originaliter ut G-398) in collectione zymotica Centraalbureau

voor Schimmecultures Rheni Trajectum, viva et exsiccata praeservata.

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 YMA 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 YMA 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. Ascosporulation. Asci with ascospores are observed after one month of incubation on YMA containing ferrichrome. Asci are formed after bud-mother cell conju-
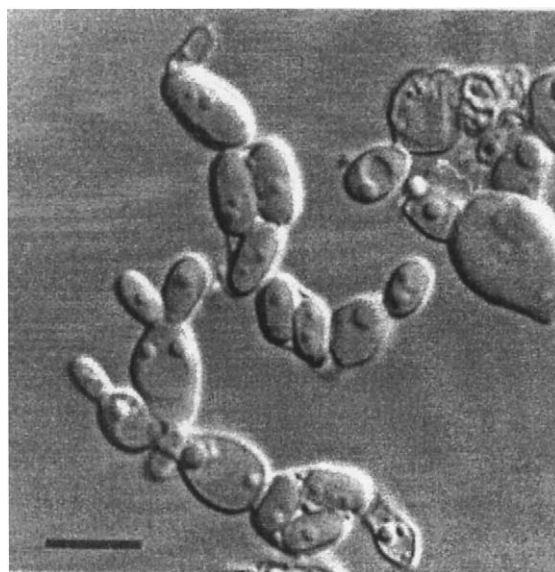

Fig. 6. Asci and ascospores of *D. mycophilus* (strain G-398) grown on YMA medium containing 0.73 μM ferrichrome. Scale bar represents 10 μm.

gation. Asci are persistent containing 1–4 oval to allantoid ascospores (3–3.5×4–7 μm) (Fig. 5). 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 Schimmecultures, 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 fea-
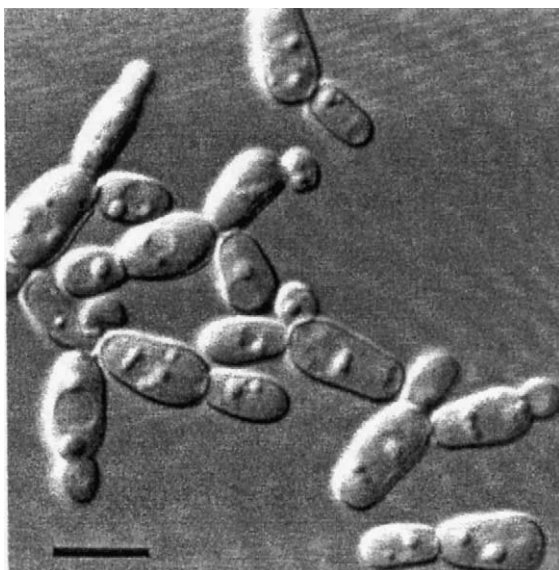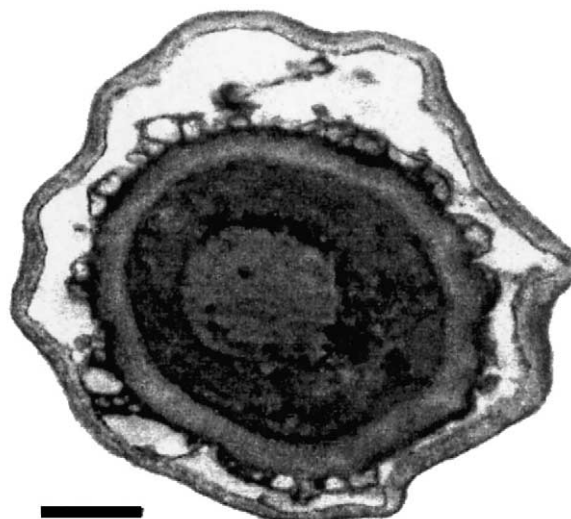

Fig. 8. TEM micrograph of ascospore of *D. mycophilus* (strain G-398) grown on YMA containing 0.73 μM ferrichrome. Scale bar represents 1 μm.

ture 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.

Fig. 7. Vegetative cells of *D. mycophilus* (strain G-398) grown on YMA medium containing 0.73 μM ferrichrome. Scale bar represents 10 μm.
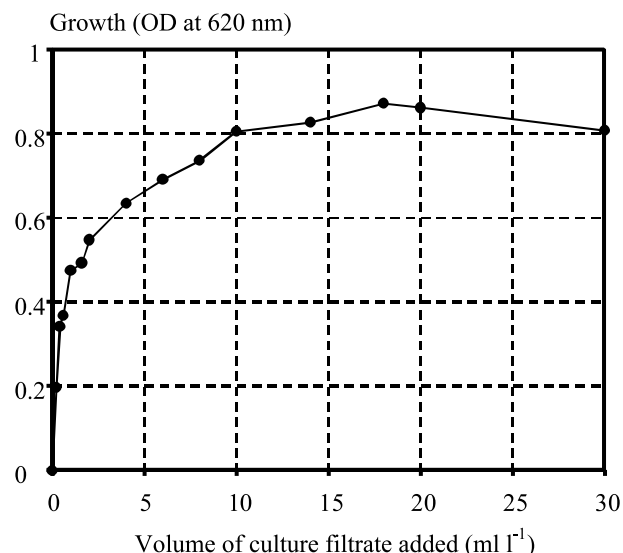

Fig. 9. Stimulating effect of culture filtrate of *Fusicoccum* sp. (CMW-325) grown in YM medium on the growth of *D. mycophilus* (G-398).

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 FeCl$_3$) without growth factor also effectively stimulated growth of *D. mycophilus* (Fig. 4). Thus, it appeared that

Table 2

Growth of *D. mycophilus* (G-398) on defined media with and without addition of purified growth factor

Media	Growth
YNB	−
YNB+glucose	−
YNB+growth factor	−
YNB+glucose+growth factor	+
YCB	−
YCB+(NH$_4$)$_2$SO$_4$	−
YCB+growth factor	−
YCB+(NH$_4$)$_2$SO$_4$+growth factor	+
VFM	−
VFM+vitamins	−
VFM+growth factor	−
VFM+vitamins+growth factor	+

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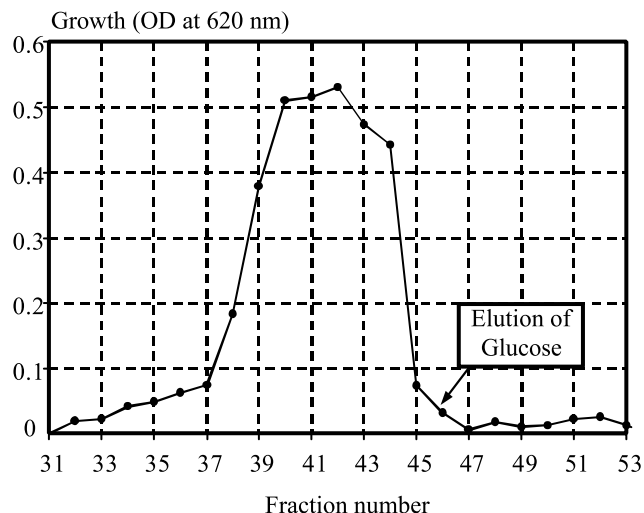

Fig. 10. Elution of the growth-promoting factor produced by *Fusicoccum* sp. CMW-325 from size-exclusion column (Biogel P4). Glucose was eluted from 46th fraction.

D. mycophilus lacks an efficient mechanism for iron uptake. Addition of ferrichrome (a commercially available siderophore, produced by the fungal species *Ustilago sphaerogena*) 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 FeCl$_3$) also slightly enhanced the effect of the ferrichrome. At concentrations of 30 μM or higher, FeCl$_3$ dramatically decreased or completely inhibited the production of growth factor by *Fusicoccum* sp. (CMW-325) (data not shown) and *Graphium* sp. (CMW-729)
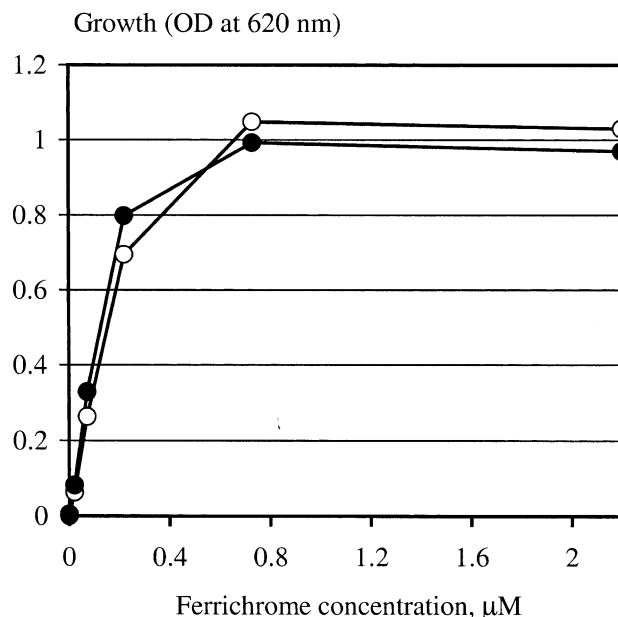

Fig. 11. Stimulating effect of ferrichrome on the growth of *D. mycophilus* (G-398). Ferrichrome was supplied in iron-free form (○) and iron-chelated form (●).
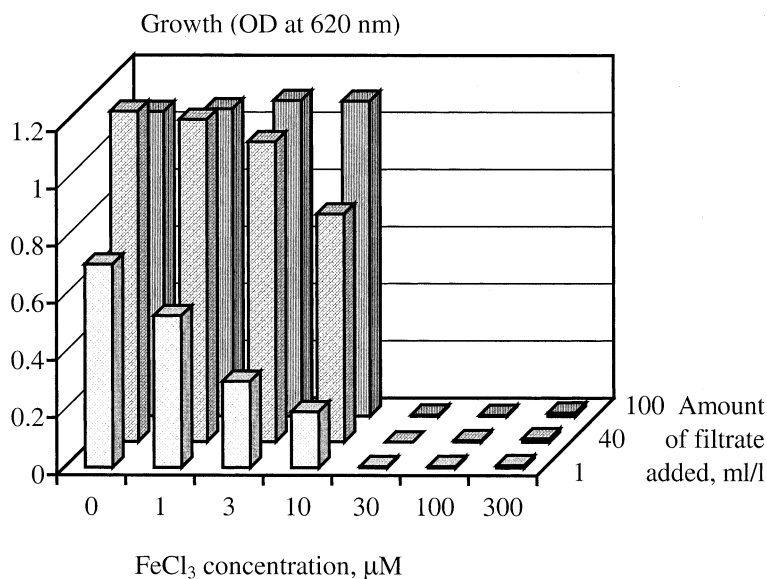
Growth (OD at 620 nm)

Fig. 12. Inhibition effect of iron on production of the growth factor by *Graphium* sp. (CMW-729). The production of the growth factor was evaluated in terms of stimulating effect on *D. mycophilus* (G-398).

(Fig. 12). Although they inhibited the effect of culture filtrates on *D. mycophilus*, these iron concentrations did not affect the growth of the fungi that produce the growth factor (data not shown).

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 CO_2 and temperatures above 30°C [25]. The reason why CO_2 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 Fe^{3+} form and eventually precipitates as an oxide–hydroxide polymer. The solubility constant of $Fe(OH)_3$ is 10^{-38}, which means that the maximum Fe^{3+} 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 Fe^{3+} 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 sidero-
phore, therefore, acts as a shuttle vector for iron [28]. The
fact that *D. mycophilus* did not require siderophore sup-
plementation when media contained high concentration of
FeCl$_3$ (Fig. 4), might have been due to diffusion of iron
through ion channels.

A natural deficiency in independent iron uptake capa-
bility 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. my-
cophilus*, 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 side-
rophores that can withhold iron from host iron-containing
proteins. Some pathogens are specialized in using host
heme, even if it is complexed directly to hemeoglobin, 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 (un-
published observation). Woodlice, like some other soil in-
vertebrates, 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 ani-
mals, might give *D. mycophilus* an advantage in the com-
petition for other nutrients.

Many mycelial fungi are visible in the natural envi-
ronments, but the presence of microorganisms such as
D. mycophilus can easily escape detection. If sidero-
phore-producing fungal contaminants had not been
present during the initial isolation from woodlice guts,
we would never have discovered this yeast. Typically, iso-
lation media for yeast and mycelial fungi are supple-
mented 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 fun-
gi. 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.

References

[1] Byzov, B.A., Thanh, V.N. and Babjeva, I.P. (1993) Interrelationships
     between yeasts and soil diplopods. Soil Biol. Biochem. 25, 1119–1126.
[2] Byzov, B.A., Thanh, V.N. and Babjeva, I.P. (1993) Yeasts associated
     with soil invertebrates. Biol. Fert. Soil 16, 183–187.
[3] Thanh, V.N., Byzov, B.A. and Babjeva, I.P. (1994) The sensitiveness
     of yeasts at the digestive fluid in the gut of soil diplopods *Pachyiulus
     flavipes*, C.L. Koch. Mikrobiologiya 63, 715–720.
[4] Margulis, L., Chase, D. and Guerrero, R. (1986) Microbial commun-
     ities invisible to the scrutiny of naturalists, most microbial commun-
     ities have escaped description. BioScience 36, 160–170.
[5] Phaff, H.J. and Starmer, W.T. (1987) Yeasts associated with plants,
     insects and soil. In: The Yeasts, 2nd edn., Vol. 1 (Rose, A.H. and
     Harrison, J.S., Eds.), pp. 123–180. Academic Press.
[6] Dubilier, N., Giere, O., Distel, D.L. and Cavanaugh, C.M. (1995)
     Characterization of chemoautotrophic bacterial symbionts in a gut-
     less marine worm (*Oligochaeta*, *Annelida*) by phylogenetic 16S rRNA
     sequence analysis and in situ hybridization. Appl. Environ. Micro-
     biol. 61, 2346–2350.
[7] Van der Walt, J.P. and Yarrow, D. (1984) Methods for the isolation,
     maitenance, classification and identification of yeasts. In: The
     Yeasts: A Taxonomic Study, 3rd edn. (Kreger-van Rij, N.J.W.,
     Ed.), pp. 45–104. Elsevier, Amsterdam.
[8] Barnett, J.A., Payne, R.W. and Yarrow, D. (1990) Yeasts: Charac-
     teristics and Identification, 2nd edn. Cambridge University Press,
     Cambridge, NY.
[9] Kreger-van Rij, N.J.W. (Ed.) (1984) The Yeasts: A Taxonomic
     Study, 3rd edn. Elsevier, Amsterdam.
[10] Kurtzman, C.P. and Fell, J.W. (Eds.) (1998) The Yeasts: A Taxo-
     nomic Study, 4th edn. Elsevier, Amsterdam.

[11] Benade, E., Wingfield, M.J. and van Wyk, P.S. (1996) Conidium development in the *Hyalorhinocladiella* anamorphs of *Ceratocystiopsis minuta-bicolor* and *Ophiostoma minus*. Can. Bot. 74, 891–897.

[12] Johnson, J.L. (1985) Determination of DNA base composition. In: Methods in Microbiology, Vol. 18, pp. 1–31. Academic Press.

[13] Nakase, T., Itoh, M. and Sugiyama, J. (1989) *Bensingtonia ingoldii* sp. nov., a ballistospore-forming yeast isolated from *Knightia excelsa* collected in New Zealand. J. Gen. Appl. Microbiol. 35, 53–58.

[14] Yamada, Y. and Kondo, K. (1973) Coenzyme Q system in the classification of the yeast genera *Rhodotorula* and *Cryptococcus*, and the yeast-like genera *Sporobolomyces* and *Rhodosporidium*. J. Gen. Appl. Microbiol. 19, 59–77.

[15] Kurtzman, C.P. and Robnett, C.J. (1997) Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. 35, 1216–1223.

[16] Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. 41, 95–98.

[17] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

[18] Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

[19] Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

[20] Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39, 783–791.

[21] Page, R.D.M. (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

[22] Yamada, Y., Maeda, K., Banno, I. and van der Walt, J.P. (1992) An emendation of the genus *Debaryomyces* Lodder et Kreger-Van Rij and the proposal of two new combinations, *Debaryomyces carsonii* and *Debaryomyces etchellsii* (*Saccharomycetaceae*). J. Gen. Appl. Microbiol. 38, 623–626.

[23] Bignell, D.E. (1984) The arthropod gut as an environment for microorganisms. In: Invertebrate-microbial interactions (Anderson, J.M., Rayner, A.D.M. and Walton, D.W.H., Eds.), pp. 205–228. Cambridge University Press, Cambridge.

[24] Margulis, L. (1993) Symbiosis in Cell Evolution: Microbial Communities in the Archean and Proterozoic Eons. W.H. Freeman, New York.

[25] Phaff, H.J. and Miller, M.W. (1998) *Cyniclomyces* van der Walt and D.B. Scott. In: The Yeasts: A Taxonomic Study, 4th edn. (Kurtzman, C.P. and Fell, J.W., Eds.), pp. 154–156. Elsevier, Amsterdam.

[26] Neilands, J.B. (1995) Siderophores: Structure and function of microbial iron transport compounds. J. Biol. Chem. 45, 26723–26726.

[27] Haselwandter, K. (1995) Mycorrhizal fungi: Siderophore production. Crit. Rev. Biotech. 15, 287–291.

[28] Leong, J. (1986) Siderophores: Their biochemistry and possible role in biocontrol of plant pathogens. Annu. Rev. Phytopathol. 24, 187–209.

[29] Bagg, A. and Neilands, J.B. (1987) Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. 51, 509–518.

[30] Hesseltine, C.W. and Ellis, J.J. (1973) Chapter II: Mucorales. In: The Fungi – An Advanced Treatise, Vol. 4B (Ainsworth, G.C., Sparrow, F.K. and Sussman, A.S., Eds.). Academic Press, Orlando, FL.

[31] Payne, S.M. (1993) Iron acquisition in microbial pathogenesis. Trends Microbiol. 2, 66–69.

[32] Winkelmann, G. (1991) Importance of siderophores in fungal growth, sporulation and spore germination. In: Frontiers in Mycology (Hawksworth, D.L., Ed.), pp. 49–65. C.A.B. International.