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Phylogenetic position and physiology of *Cerinosterus cyanescens*

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Abstract

Partial 25S rRNA sequencing of *Cerinosterus cyanescens* showed it to be a close relative of *Microstroma juglandis*, a member of the basidiomycetous order *Microstromatales*. It is unrelated to the generic type species, *C. luteoalba*, which is a member of the order *Dacrymycetales*. The clinical occurrence of *C. cyanescens* is possibly explained by its thermotolerance and lipolytic activity. The species' nutritional profile is established. Growth on n-hexadecane is rapid; it grows well on typical plant constituents like gallic, tannic, vanillic, quinic and p-coumaric acids, but not on 3-hydroxybenzoic acid, phenol and hydroquinone. The failure to assimilate D-galactose, L-sorbose and ethylamine, the presence of urease and sensitivity to cycloheximide are diagnostic for the species.

Introduction

The genus *Cerinosterus* was erected by Moore (1987) for basidiomycetous hyphomycetes previously classified in the genus *Sporothrix*. This transfer was necessary because the type species of *Sporothrix*, *S. schenckii*, had been shown to be a member of the ascomycete order *Ophiostomatales* (Weijman & de Hoog 1985).

Two species were accepted in *Cerinosterus*, with *C. luteoalba* (de Hoog) R.T. Moore as the type of the genus (Moore 1987). This species is the anamorph of *Femsjonia luteoalba* Fr., now known as *Ditiola pezizaeformis* (Lév.) Reid (Reid 1974), which belongs to the *Dacrymycetales* (Reid 1974). The second species, *C. cyanescens* (de Hoog & de Vries) R.T. Moore, was shown to have a different septal pore structure (Smith & Batenburg-van der Vegte 1985, 1986). In addition, cell wall hydrolyzates of *C. luteoalba* contain xylose whereas those of *C. cyanescens* do not (Weijman & de Hoog 1985). Consequently *C. cyanescens* seems remote from the generic type species and its precise taxonomic position remains uncertain. In the present paper we applied partial 25S rRNA sequencing in order to establish the phylogenetic affinities of this fungus.

Cerinosterus cyanescens has occasionally been found in the clinical environment (de Hoog & de Vries 1973; Jackson et al. 1990). It has low pathogenicity (Sigler et al. 1990; Flores et al. 1991). Tambini et al. (1996) suggested *C. cyanescens* as the probable etiologic agent of a pulmonary lesion in a heart transplant patient and mentioned some further, unproven cases. Several more clinical cases have come to our attention in which the fungus played a hitherto unclarified role. Timely diagnosis of the organism is necessary to clarify its potential significance as an opportunistic invader.

Current diagnosis of *C. cyanescens* is primarily based on morphology and cultural characteristics. The fungus has hyaline, easily disarticulating conidiogenous cells which produce conidia sympodially on denticles, singly or in short chains. This kind of morphology is hardly characteristic as it occurs not only in *Ophiostomatales* and *Dacrymycetales*, but also in *Saccharomycetales*, viz. in *Stephanoascus ciferrii* (de Hoog & Guarro 1995). Superficially similar morphology is also found in other basidiomycetous

Table 1. Strains of *Cerinosterus cyanescens* studied

Number	Other ref	Source	Locality	Depositor
CBS 357.73T	MUCL 19329	Human skin	Netherlands	T.F. Visser
CBS 358.73		Human skin	Finland	A. Kahanpää
CBS 549.93		Human blood culture	Netherlands	H. Mulder
CBS 604.84		Pomegranate product	Israel	B.J. Juven
CBS 876.73	IMI 178848	*Eucalyptus pauciflora*	Australia	V.F. Brown
CBS 360.73		Bedroom air	Netherlands	J. Quarles van Ufford
ATCC 201678		*Cuscuta approximata*	Gran Canaria	W.J. Middelhoven
IP-2317.95		Human lung	Italy	E. Guého

Abbreviations used: ATCC=American Type Culture Collection, Manassas, U.S.A.; CBS=Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; IP=Institut Pasteur, Paris, France; T=Type culture.

anamorphs, such as *Hyalodendron* (de Hoog 1979) and *Pseudozyma* (Boekhout 1995). In an attempt to find additional diagnostic criteria the nutritional profile of *C. cyanescens* was determined in the present study.

Materials and methods

Strains and culture conditions. Strains studied are listed in Table 1. Stock cultures were maintained on oatmeal agar slants at 22 °C. All were tested physiologically. Three of the strains (CBS 357.73, IP-2317.95 and ATCC 201678) were sequenced and found to be identical; only CBS 357.73 is shown in the tree.

RNA extraction and sequencing. Bulk rRNA was extracted from lyophilized pellets using the micromethod described by Guadet et al. (1989). rRNA was purified by a final precipitation in 4 M LiCl to eliminate double-stranded DNAs. Purity of rRNA samples was estimated from spectrophotometric absorbance ratios 260/280=2.00–2.15 and 230/260$\leq$0.5, and their integrity verified by non-denaturating agarose gel electrophoresis. Sequences of 577 nucleotides were obtained by the dideoxynucleotide chain-termination method (Guillot & Guého 1995), using bulk rRNA as a template with reverse transcriptase and subsequently modified by incorporation of [^{35}S] labelled-dATPαS in a short preliminary elongation step. Sequences were extended by chaining up partial sequences targeted in the bulk rRNA by three oligonucleotide primers complementary to evolutionary conserved regions, 5′-gCATTCCCAAACAACTCgACTC-3′ (266), 5′-TCCCTTTCAACAATTTCACg-3′ (401) and 5′-ggTC CgTgTTTCAAgACgg-3′ (636).

Sequence alignment and phylogenetic analysis. Alignment was performed on 42 LS rRNA partial sequences of 577 bases, starting at position 57 of the 5′ end, up to position 624, with reference to *Saccharomyces cerevisiae* (Georgiev et al. 1981). Ambiguously aligned positions were excluded from analyses. Phylogenetic trees were constructed using Clustal X (Jeanmougin et al. 1998). One hundred bootstrap replications were compared. Sequences used were presented by one of the authors (EG) in earlier publications; sequences with GenBank accession numbers have been published elsewhere (Begerow et al. 1997; Boekhout et al. 1995; Kurtzman & Robnett 1998).

Assimilation studies. Carbon assimilation was established using the ID32C identification strip (bioMérieux, Marcy-l'Etoile, France) following the manufacturer's instructions. Compounds not present in this kit, and those giving ambiguous results, were administered at 5 g.l^{-1} in 2.5 ml Yeast Nitrogen Base (YNB, Difco). These cultures were inoculated with a drop of a preculture in 0.5% glucose YNB and were shaken. The pH of the growth media was adjusted to pH 5.5, except with galacturonic and quinic acids and potassium *hemi*-saccharate (Middelhoven 1997a). The assimilation of unusual and potentially toxic compounds like n-hexadecane and several benzene compounds was studied by the slant culture method (Middelhoven et al. 1991). Growth on butylamine, putrescine and uric acid was demonstrated according to Middelhoven et al. (1985). The assimilation of nitrogenous compounds was studied in Yeast Carbon Base (YCB, Difco), that of nitrite, creatine and creatinine by the auxanographic technique. All assimilation studies were carried out at 25 °C.

Remaining physiological tests. Resistance to 0.01% cycloheximide was tested in 0.5% glucose YNB. Halotolerance (10% sodium chloride or anhydrous magnesium chloride) was tested in YNB containing 5% glucose per litre. Lipolytic activity

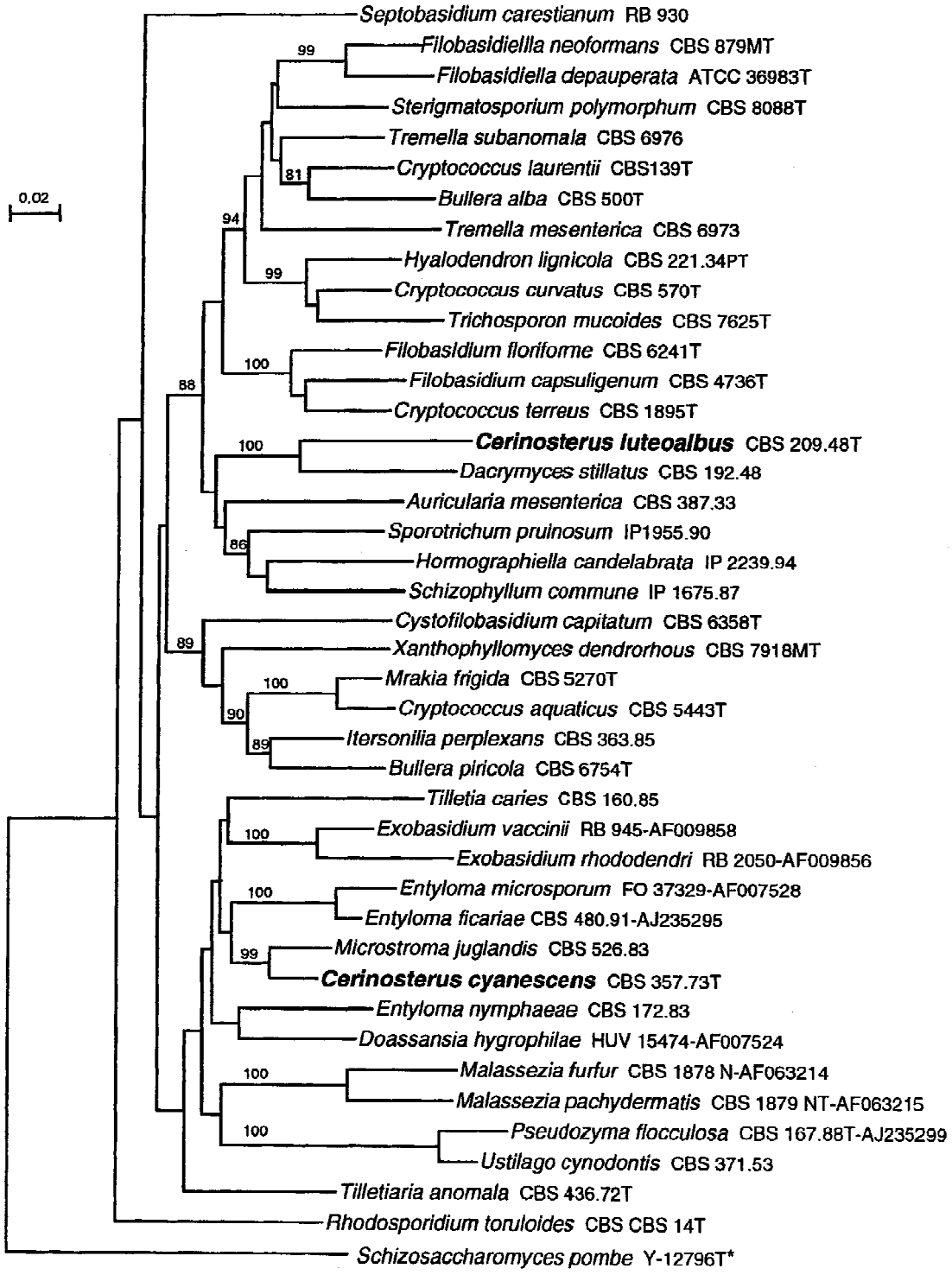

Figure 1. Phylogenetic tree based on partial LSU sequences of selected basidiomycetes. The tree was constructed using a neighbor-joining algorithm, with *Schizosaccharomyces pombe*, Y-12796 as outgroup. Tree robustness was assessed by bootstrap (100 replications), values being given at the nodes when percentages of trees supporting the branch had frequencies of more than 80%. With PAUP the same topology was found.

was demonstrated by hydrolysis of Tween 80 (Sierra 1957).

Results

Phylogeny. In a phylogeny based on partial LSU sequencing data, *Cerinosterus luteoalba* and *C. cyanescens* were both found among the heterobasidiomycetes, although remote from each other (Figure 1). The nearest neighbour of *C. luteoalba* was *Dacrymyces stillatus (Dacrymycetales)*, CBS 192.48, whereas that of *C. cyanescens* was *Microstroma juglandis* (*Microstromatales*), CBS 526.83. Other taxa with hyaline hyphae and more or less sympodial and/or catenulate conidia are *Hyalodendron* and *Pseudozyma*. *Hyalodendron lignicola* was recovered amid the *Tremellales/Filobasidiales*, in a monophyletic branch with members of the genera *Trichosporon* and *Cryptococcus*. *Pseudozyma* was placed among the *Ustilaginales*, with *Ustilago cynodontis* as its nearest neighbour (Figure 1).

Physiology. Nutritional profiles of 8 strains of *C. cyanescens* were identical on most carbon sources (Table 2). However, growth responses to melibiose, inulin, glucono-δ-lactone, citrate and D-glucosamine (N source) varied. In addition, CBS 876.73 failed to grow with arbutin, but assimilated 2-ketogluconate. Strain IP 2317.95 did not assimilate quinic acid, but grew well with xylose, cellobiose and salicin. ATCC 201678 did not assimilate ribitol and arbutin. All strains assimilated nitrate and nitrite, but not ethylamine (Table 2).

Growth responses observed in slant cultures supplied with n-hexadecane or various benzene compounds are shown in Table 3. All strains behaved similarly, with a few exceptions. Strains CBS 357.73 and 358.73 are notable for their delayed and weak assimilation of ferulic acid (3-methoxy-4-hydroxycinnamic acid).

None of the strains required external vitamins for growth or showed resistance to cycloheximide. All grew in the presence of 5% and 10% sodium chloride and 5% magnesium chloride, but 10% magnesium chloride delayed or weakened growth in most strains, except CBS 604.84. All strains hydrolyzed urea and Tween 80 and were able to grow at 37 °C. At 41 °C good growth was obtained with CBS 357.73 and 358.73, and weak growth with CBS 604.84 and 876.73.

Senescent slant cultures on complex media (i.e. YM agar) developed a characteristic blue violet pigment at the back side of the mycelial mat, which partly diffused into the agar. Strains CBS 360.73 and ATCC 201678 did not produce this pigment. The colour of the pigment is pH-dependent; slant cultures on mineral medium with n-hexadecane developed a brick-red pigment. This was occasionally seen in liquid cultures in YNB with various carbon sources. The physiology of *M. juglandis* was very similar to that of *C. cyanescens*, but this fungus was not salt-tolerant and did not assimilate n-hexadecane, quinic, vanillic, p. coumaric and ferulic acids.

Discussion

The hyphomycete genus *Sporothrix* was introduced one hundred years ago to accommodate hyaline hyphomycetes with unicellular conidia produced on denticles. Such structures may occur in quite unrelated fungi, and thus the genus was acknowledged as being an artificial, heterogeneous assemblage of superficially similar fungi (De Hoog 1974). The type species, *S. schenckii* is known to be close to the teleomorph species *Ophiostoma stenoceras* (Ascomycota, *Ophiostomatales*; Mariat 1971; Mendonça-Hagler et al. 1974; Berbee & Taylor 1992) and hence *Sporothrix* is an ascomycetous anamorph. In the seventies, markers of main relationships were introduced which were applicable in absence of a teleomorph, viz. septal pore ultrastructure (Smith & Batenburg-v.d. Vegte 1985, 1986), cell wall carbohydrate profiles (Weijman & de Hoog 1985) and coenzyme Q data (Suzuki & Nakase 1986). The divergent affinities were acknowledged by erecting sections within the genus (Weijman & de Hoog 1985). Members of the section *Sporothrix* are *Ophiostomatales*, the type species of the section *Farinosa* is a member of *Saccharomycetales*, and species of the section *Luteoalba* are of basidiomycetous affinity. The basidiomycetous members were later given a formal generic status by the erection of the genus *Cerinosterus* R.T. Moore (1987).

In present-day taxonomy, phylogenetic relationships are mainly inferred by rDNA sequencing. In our phylogenetic tree based on the LSU ribosomal gene (Figure 1), all earlier suppositions on taxonomic relationships were confirmed. *Sporothrix schenckii* (section *Sporothrix*) was confirmed to be a member of the *Ophiostomatales* (data not shown). The variety *luriei*, distinguished by absence of creatine and creatinine as-

Table 2. Nutritional profile of 8 strains of *Cerinosterus cyanescens* compared with *Microstroma juglandis*

Carbon source	357.73	358.73	549.93	604.84	876.73	360.73	201678	2317-95	*M. juglandis*
D-Glucose	3+	3+	3+	3+	3+	3+	3+	3+	3+
D-Galactose	–	–	–	–	–	–	–	–	–
L-Sorbose	–	–	–	–	–	–	–	D	–
D-Glucosamine	–	–	–	–	–	–	–	–	D
D-Ribose	W	W	W	W	W	–	W	W	D
D-Xylose	–	–	+	W	W	–	W	3+	–
L-Arabinose	3+	3+	3+	3+	3+	3+	3+	3+	2+
D-Arabinose	DW	DW	DW	DW	DW	DW	–	–	–
L-Rhamnose	–	–	–	–	–	–	–	–	–
Sucrose	3+	3+	3+	3+	3+	3+	3+	3+	3+
Maltose	3+	3+	3+	3+	3+	3+	3+	3+	2+
α-Trehalose	3+	3+	3+	3+	3+	3+	3+	3+	3+
Methyl-α-glucoside	3+	3+	3+	3+	3+	3+	3+	3+	DW
Cellobiose	D	D	D	W	W	D	W	2+	D
Salicin	–	–	–	–	–	–	–	2+	–
Arbutin	3+	2+	2+	2+	–	2+	–	2+	W
Melibiose	–	–	–	+	+	+	+	–	–
Lactose	+	+	+	+	+	+	+	–	–
Raffinose	3+	3+	3+	3+	3+	3+	3+	V	2+
Melezitose	3+	3+	3+	3+	3+	3+	3+	3+	2+
Inulin	–	–	–	–	+	–	–	–	–
Soluble starch	D	DW	DW	DW	DW	DW	–	–	–
Glycerol	3+	3+	3+	3+	3+	3+	3+	3+	3+
meso-Erythritol	3+	3+	3+	3+	3+	3+	3+	3+	2+
Ribitol	D	+	+	+	+	DW	–	D	nd
Xylitol	–	–	–	–	–	–	–	–	–
L-Arabinitol	+	+	+	2+	3+	+	DW	D	+
D-Glucitol	3+	3+	3+	3+	3+	3+	3+	3+	+
D-Mannitol	3+	3+	3+	3+	3+	3+	3+	V	D
Galactitol	–	–	–	–	–	–	–	D	–
myo-Inositol	3+	3+	3+	3+	3+	3+	3+	3+	2+
Glucono-δ-lactone	D	D	DW	+	DW	–	DW	–	–
2-Keto-D-gluconate	–	–	–	–	+	–	–	–	–
D-Gluconate	W	+	W	+	W	W	+	–	–
D-Glucuronate	–	–	–	–	–	–	–	–	–
D-Galacturonic acid	–	–	–	–	–	–	–	–	–
DL-Lactate	–	–	W	–	W	W	W	–	–
Succinate	DW	2+	+	2+	3+	+	V	V	–
Citrate	3+	3+	DW	DW	2+	2+	DW	3+	–
Methanol	–	–	–	–	–	–	–	–	–
Ethanol	+	3+	+	2+	+	2+	2+	3+	3+
Propane-1,2-diol	D	DW	D	D	D	–	–	W	D
Butane-2,3-diol	–	–	–	–	–	–	–	–	–
Quinic acid	2+	2+	2+	2+	2+	2+	+	–	–
D-Saccharate	–	–	–	–	–	–	–	–	–
D-Galactonate	–	–	–	–	–	–	–	–	–
n-Hexadecane	3+	3+	3+	3+	3+	3+	3+	2+	–

Table 2. Continued

Nitrogen source	357.73	358.73	549.73	604.84	876.73	360.73	201678	2317-95	*M. juglandis*
Nitrate	3+	3+	3+	3+	3+	3+	3+	2+	2+
Nitrite	3+	3+	3+	3+	3+	3+	3+	nd	nd
Ethylamine	–	–	–	–	–	–	–	–	–
L-Lysine	3+	+	+	+	2+	2+	+	2+	–
Cadaverine	+	DW	DW	3+	+	2+	+	2+	–
Creatinine	–	–	–	–	–	–	–	–	–
D-Glucosamine	D	D	DW	+	+	DW	–	–	D
Imidazole	–	–	–	–	–	–	–	–	–

3+=growth within 3 days; 2+=growth within 4–7 days; +=growth within 7–14 days; D=over 14 days required; W=weak growth; V=variable results; ND=not determined.

similation (De Hoog & Guarro 1995), differed from variety *schenckii* in two LSU rDNA positions. *Sporothrix fungorum* and *S. guttuliformis* (section *Farinosa*) were found to be members of the *Saccharomycetales*. A *Stephanoascus* teleomorph is known in a closely similar species, *S. farinosa* (de Hoog et al. 1985).

Cerinosterus luteoalba and *C. cyanescens* were compared with possibly related fungi and with some basidiomycetous anamorph genera with superficial morphological similarity (Figure 1). Judging from their nearest neighbours, the two species belong to different orders: the *Dacrymycetales* and the *Microstromatales*, respectively. The genus *Cerinosterus* is defined as being of dacrymycetalean nature, because the teleomorph of the generic type species, *C. luteoalba* is *Ditiola pezizaeformis* (Maekawa 1987). *Cerinosterus cyanescens* thus has to be accommodated in another genus. *Hyalodendron* and *Pseudozyma* are possible candidates, because they may have whitish colonies with disarticulating hyphae conidia arising in chains, sometimes produced next to each other in a more or less sympodial fashion. Guého et al. (1993) found that the type species of *Hyalodendron, H. lignicola*, was a *Trichosporon* species, which is classified in the *Tremellales/Filobasidiales*. Boekhout et al. (1995) and Boekhout (1995) found *Pseudozyma* to belong to another order, the *Ustilaginales. C. cyanescens* deviates from *Pseudozyma* in a number of essential features, such as thermotolerance (see below). The clade in Figure 1 that contains *Cerinosterus cyanescens* and *Pseudozyma flocculosa* has low bootstrap support. Indeed species united in this 'group' belong to six different orders. Begerow et al. (1997) classified the nearest neighbours of *C. cyanescens* and *P. flocculosa*, viz. *Microstroma juglandis* and *Ustilago cynodontis*, respectively, in two different subclasses. Thus it is difficult to accommodate *C. cyanescens* in *Pseudozyma*. Erecting a new genus for *C. cyanescens* would imply the necessity to erect a separate genus for the anamorphs of each basidiomycete order, despite the fact that these are often recognized with great difficulty. For this reason we prefer to maintain *C. cyanescens* in *Cerinosterus*, until better diagnostics for basidiomycete anamorphs have been developed.

Cerinosterus cyanescens is occasionally isolated from the environment (Table 1; Middelhoven 1997b). However, most strains known to date were isolated in connection with clinical cases (de Hoog & de Vries 1973; Jackson et al. 1990; Tambini et al. 1996). The etiologic role of the fungus has not been proven unambiguously. Sigler et al. (1990) found that its pathogenicity in experimental inoculation is low. The nosocomial occurrence of the species may be explained by its thermotolerance: all strains grow well at 37 °C, while growth is often still observed at 40–41 °C (de Hoog & Guarro 1995; Table 3). Thermotolerance is a rare feature in basidiomycetes. Of the DBB positive basidiomycetous yeasts listed in the monographs of Barnett et al. (1990) and Kurtzman & Fell (1998), only some *Cryptococcus* and *Trichosporon* species and the filamentous basidiomycete *Hyalodendron lignicola* (de Hoog 1979) are able to grow at 37 °C. Another feature which may be involved in the clinical occurrence of *C. cyanescens* is its assimilation of Tween 80. The combination of thermotolerance and lipolytic activity may enhance occurrence of the fungus in lipid-rich pulmonary membranes and on the scalp (Table 1).

From the pattern of carbon compounds assimilated it appears that *C. cyanescens* is a well-defined species. Most strains responded identically when various carbon compounds were administered as the sole source

Table 3. Miscellaneous physiological test results of *Cerinosterus cyanescens*

Carbon source	357.73	358.73	549.93	604.84	876.73	360.73	201678	2317-95	*M. juglandis*
n-Hexadecane	3+	3+	3+	3+	3+	3+	3+	2+	–
p-Coumaric acid	3+	3+	3+	3+	3+	3+	3+	2+	–
3,5-Dihydroxybenzoic acid	–	–	–	–	–	–	–	–	–
Ferulic acid	DW	DW	3+	3+	3+	3+	3+	2+	–
Gallic acid	3+	3+	3+	3+	3+	3+	3+	2+	2+
Hydroquinone	–	–	–	–	–	–	–	–	–
3-Hydroxybenzoic acid	–	–	–	–	–	–	–	–	–
3-Hydroxycinnamic acid	DW	DW	DW	DW	DW	DW	DW	+	–
Protocatechiuc acid	+	+	+	+	+	–	3+	+	2+
Tannic acid	3+	3+	3+	3+	3+	3+	3+	2+	W
Vanillic acid	3+	3+	3+	3+	3+	3+	3+	2+	–
Miscellaneous tests	**357.73**	**358.73**	**549.93**	**604.84**	**876.73**	**360.73**	**201678**	**2317-95**	***M. juglandis***
Tween 80	3+	3+	3+	3+	3+	3+	3+	3+	3+
5% NaCl	3+	3+	3+	3+	3+	3+	3+	3+	D
10% NaCl	3+	3+	3+	3+	3+	3+	3+	2+	–
5% $MgCl_2$	3+	3+	3+	3+	3+	3+	3+	3+	D
10% $MgCl_2$	w	w	w	3+	w	w	w	2+	–
0.01% Cycloheximide	–	–	–	–	–	–	–	–	–
Growth at 37 °C	+	+	+	+	+	+	+	+	–
Growth at 41 °C	+	+	–	w	w	–	–	nd	nd
Starch production	–	–	–	–	–	–	–	–	–

of carbon and energy (Table 2; Table 3), with the exception of melibiose, inulin and 2-keto-D-gluconate. Strains CBS 876.73 and ATCC 201678 failed to grow with arbutin (Table 2). The species is notable for its assimilation of nitrate but not of ethylamine. Growth rates on lysine and cadaverine, if administered as the sole nitrogen source, were variable.

Cerinosterus cyanescens is physiologically characterized by the presence of urease and inability to assimilate D-galactose, L-sorbose and ethylamine. The pH-dependent, blue violet pigment which is exuded in the medium by most strains is a less reliable character, because it is not expressed on all media and may easily be lost after a few transfers.

Growth on n-hexadecane already became visible after 2–3 days. This hydrocarbon is assimilated very rapidly, compared to other yeasts. In contrast to most n-hexadecane positive yeasts (Middelhoven et al. 1985), *C. cyanescens* did not assimilate butylamine or uric acid. Variable growth on putrescine was observed with strain ATCC 201678 only. The pattern of assimilation of benzenes (Table 3) is very characteristic. Unlike many species of yeasts and yeast-like fungi (Middelhoven 1993; Sampaio & Fonseca 1995) simple benzene compounds, such as phenol, hydroquinone and 3-hydroxybenzoic acid were not assimilated, but more complex ones, such as vanillic acid (3-methoxy-4-hydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), tannic acid (glucose esterified with gallic acid), p-coumaric acid (4-hydroxycinnamic acid) and ferulic acid (3-methoxy-4-hydroxycinnamic acid) supported growth of slant cultures of most strains within a few days. Assimilation of methoxybenzoic and cinnamic acids is characteristic of red yeasts, such as *Rhodotorula* and *Sporobolomyces* (Middelhoven 1993) and *Pseudozyma* (Middelhoven 1997b). Methoxy- and trihydroxybenzene compounds are plant constituents, being building blocks and products of degradation of lignin. Ferulic acid is esterified to cell wall polysaccharides of several plants (Hartley & Jones 1977). Assimilation of these complex benzene compounds of plant origin suggests that living or decaying plant tissue must be the natural habitat of *C. cyanescens*.

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