

Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard?

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Abstract

The biodegradation of aromatic hydrocarbons by fungi has traditionally been considered to be of a cometabolic nature. Recently, however, an increasing number of fungi isolated from air biofilters exposed to hydrocarbon-polluted gas streams have been shown to assimilate volatile aromatic hydrocarbons as the sole source of carbon and energy. The biosystematics, ecology, and metabolism of such fungi are reviewed here, based in part on re-evaluation of a collection of published hydrocarbon-degrading isolates obtained from authors around the world. Incorrect or outdated identifications in original publications are corrected by ribosomal DNA sequence analysis. The data show that many volatile-hydrocarbon-degrading strains are closely related to, or in some cases clearly conspecific with, the very restricted number of human-pathogenic fungal species causing severe mycoses, especially neurological infections, in immunocompetent individuals. Neurochemistry features a distinctive array of phenolic and aliphatic compounds that are related to molecules involved in the metabolism of aromatic hydrocarbons. Hence, there may be physiological connections between hydrocarbon assimilation and certain patterns of mammalian infection.

Introduction

Hydrocarbons are ubiquitously found in the environment, where they originate from biogenic and geological processes. Their chemical nature is extremely diverse, encompassing simple forms such as small alkanes and monoaromatic hydrocarbons as well as complex forms such as polycyclic aromatic hydrocarbons (Fig. 1). The lighter monoaromatic fraction is highly mobile in the environment owing to its relatively high volatility and water solubility (Swoboda-Colberg, 1995). Such materials frequently leak from underground fuel storage tanks and spills at petroleum production wells, refineries, pipelines, and distribution terminals, resulting in major contamination incidents. The monoaromatics involved are commonly termed BTEX, an acronym derived from the initial letters of the names of the four most common molecular types involved (inclusive of different isomers in some cases): benzene, toluene, ethylbenzene, and xylene (Fig. 1) (Swoboda-Colberg, 1995). Pollution with volatile aromatic hydrocarbons also arises from the chemical industry, particularly where containment is inadequate in

factories manufacturing polystyrene, which is based on styrene monomers (Fig. 1).

A wide biodiversity of microorganisms have adapted to metabolize aromatic hydrocarbons by means of diverse degradation pathways. These organisms have become a central interest for researchers involved in the engineered clean-up of environmental pollution (Atlas & Cerniglia, 1995; van Hamme *et al.*, 2003). In bacteria, the metabolic pathways involved in the biodegradation of monoaromatic hydrocarbons are fairly well known (<http://umbbd.ahc.um-n.edu/>), and a number of reviews on the physiological, genetic, and applied aspects of this topic have been published (Diaz & Prieto, 2000; Gibson & Harwood, 2002; Jindrova *et al.*, 2002; O'Leary *et al.*, 2002; van Hamme *et al.*, 2003). Fungi also metabolize aromatic hydrocarbons, but detailed study of fungal aromatic hydrocarbon metabolism has primarily focused on the polycyclic fraction (Cerniglia *et al.*, 1992; Muncnerova & Augustin, 1994; Cerniglia, 1997). Relatively little work has been done on degradation of monoaromatics.

In general, fungi are involved in three major modes of hydrocarbon metabolism, each involving its own distinctive

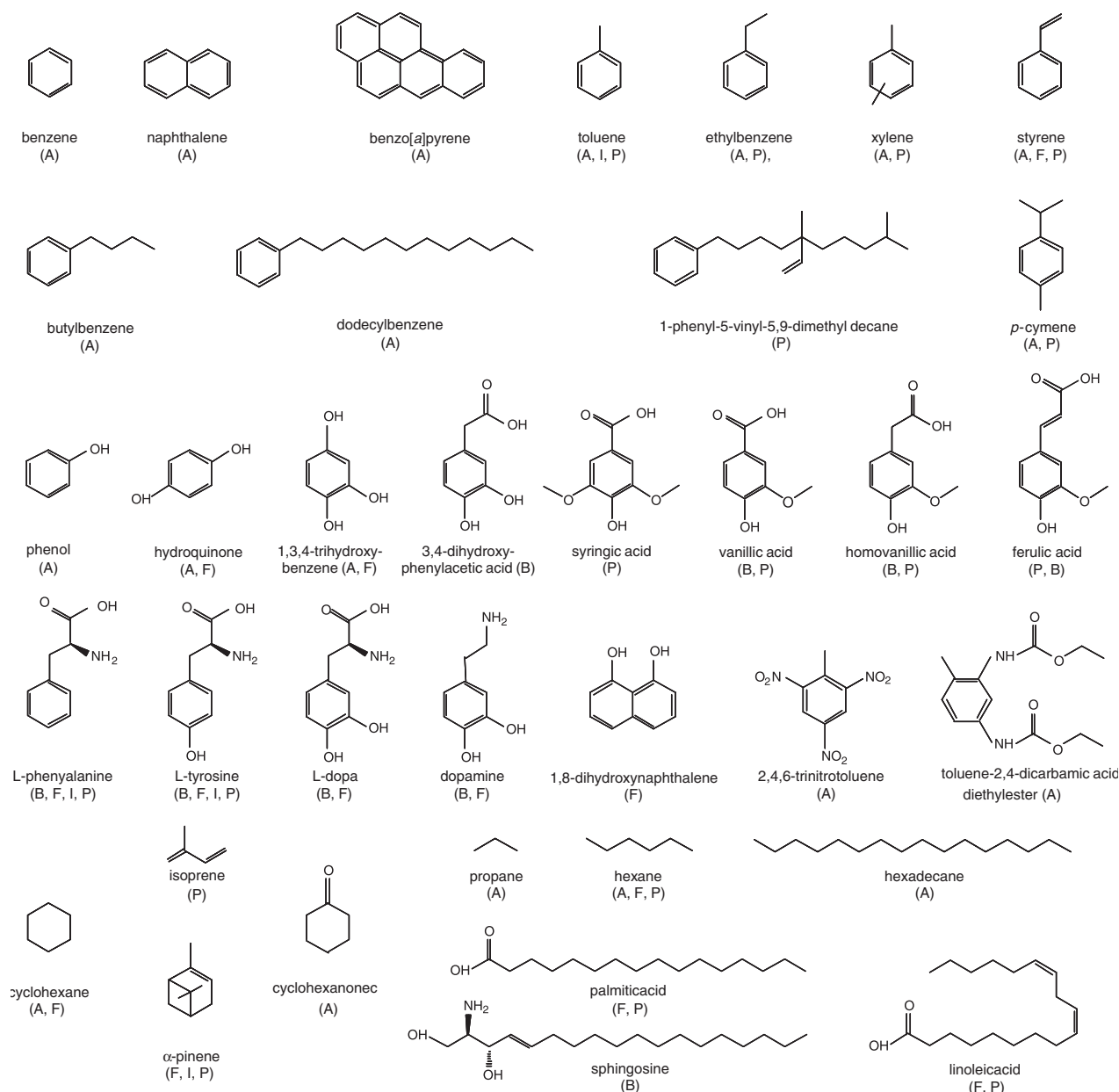


Fig. 1. Chemical structure of representative hydrocarbons, and related phenolic and aliphatic compounds mentioned in this study. Known sources of these chemicals are summarized as abiotic (A), and as biotic constituents found in the mammal brain (B), fungi (F), insects (I) and plants (P).

enzymatic mechanisms: (1) partial transformation reactions; (2) complete degradation of hydrocarbons in the presence of a second compatible substrate; and (3) independent utilization of hydrocarbons as a sole carbon source for growth.

Partial transformation processes seen in fungi commonly involve the detoxification of xenobiotics via the cytochrome-P450 monooxygenase enzyme system (Cerniglia *et al.*, 1992; van den Brink *et al.*, 1998). The initial reaction involves the activation of dioxygen in a way that results in the insertion of one oxygen atom into the substrate and the reduction of the second oxygen atom to a water molecule.

This primary substrate oxidation is usually followed by wide variety of oxidation and conjugation reactions that lead to an increase in solubility. Ultimately, metabolically inaccessible residues are excreted. In some cases, these processes are of little interest in pollution control, as the excreted products may be more toxic than the parent substrate, as occurs with the oxidation of benzo(*a*)pyrene (Fig. 1) to carcinogenic intermediates (Sutherland, 1992). This biodegradation mechanism is characteristic in eukaryotic organisms and it is widespread among fungal taxa (Cerniglia *et al.*, 1978). Detailed study of fungal aromatic hydrocarbon oxidation

has focused on zygomycetes of the genus *Cunninghamella* (Holland *et al.*, 1993; Muncnerova & Augustin, 1994; Zhang *et al.*, 1996), which have been proposed as models for the metabolism of xenobiotics in humans (Smith & Rosazza, 1974).

The degradation of hydrocarbons via cometabolic conversion to carbon dioxide and water is typically performed by the lignin-degrading white-rot fungi. Members of this specialized ecological group are phylogenetically heterogeneous, comprising ascomycetes in the order *Xylariales* and basidiomycetes in the order *Aphylliphorales* (Rayner & Boddy, 1988). Lignin is an aromatic polymer found in higher woody plants, and it is commonly deposited in plant cells in patterns that make it effective as a barrier against microbial attack of woody tissue. By degrading this compound, fungi gain improved access to the main growth-supporting substrates: cellulose and hemicellulose. The biodegradation of wood components is relatively well understood, and detailed reviews on the mechanisms of lignin degradation are available in the literature (de Jong *et al.*, 1994; Reddy & D'Souza, 1994; Leonowicz *et al.*, 1999). Briefly, lignin is degraded by extracellular peroxidases working simultaneously with a complex array of secondary enzymes, such as laccases and P-450 monooxygenases, that further metabolize the aromatic breakdown products. Even though lignin is ultimately mineralized by white-rot fungi, this compound does not serve these fungi as a sole growth substrate. Concomitant use of cellulose or other relatively accessible materials is always needed. Because of the complex nature of the lignin polymer, with a more or less random distribution of various substituent structures, lignin-degrading peroxidases function in a very nonspecific way in the degradation of a broad range of other recalcitrant chemicals, including aromatic hydrocarbons (Pointing, 2001). As with lignin, these aromatic hydrocarbons do not themselves serve as sole carbon sources for the fungi involved, but they are often mineralized by cometabolism (Cerniglia *et al.*, 1992; Yadav & Reddy, 1993).

Cometabolic biodegradation processes have some important restrictions in terms of their usefulness in bioremediation. The main problems encountered are low conversion rates, accumulation of toxic intermediates, the need for a cosubstrate, and unreliability caused by complex enzymatic regulation processes (Cerniglia, 1997; Kennes & Veiga, 2004). The biodegradation mode preferred by bioremediation investigators is the direct assimilation of aromatic hydrocarbons as a sole carbon and energy source, especially where this results in substantial mineralization. In relation to this topic, the amount of literature available on bacteria, in which utilization of benzene was documented as early as 1946 (ZoBell, 1946), greatly exceeds the amount of literature available on fungi. This would seem to suggest that the complete assimilative metabolism of aromatic hydrocarbons

is uncommon in fungi. It is true that some authors have claimed that the assimilation of monoaromatic hydrocarbons is widespread in the fungal kingdom (Nyns *et al.*, 1968; Rubidge, 1974; Hemida *et al.*, 1993); their conclusions, however, were based entirely on observing fungal development on agar exposed to hydrocarbon vapors. Such studies are now well known to be vulnerable to false positive results arising from microbial utilization of materials such as impurities in the agar and in the substrate, traces of volatile compounds from the atmosphere, and reserve substances stored in the initial inoculum (Randall & Hemmingsen, 1994). All these materials can be used as alternative carbon sources resulting in somewhat scanty but still significant growth. It was not until the mid 1980s that Fedorak & Westlake (1986) unequivocally demonstrated that certain fungi were able to assimilate aromatic hydrocarbons. They did this by growing axenic cultures in closed bottles and monitoring substrate depletion, transient accumulation of intermediates, and biomass formation. From oil-polluted marine water samples, they obtained four strains (identified as *Paecilomyces*, *Verticillium*, *Beauveria* and *Penicillium* species) that were able to grow on long-chained alkylbenzenes as the sole source of carbon and energy. A decade later, Cox and coworkers experimented with the biofiltration of air polluted with styrene and isolated a number of styrene-utilizing fungi, namely *Exophiala jeanselmei*, *Clonostachys rosea*, and some *Penicillium* spp., that became enriched when the biofilters were operated at low water activity levels (Cox *et al.*, 1993; Cox, 1995). Assimilation of toluene was also demonstrated with these isolates in growth experiments using closed systems. These findings prompted the development of air biofilters based on using fungi as biocatalysts for the purification of hydrocarbon-polluted air. As a result, an increasing number of fungal strains growing on volatile aromatic hydrocarbons have been isolated in the recent years (Weber *et al.*, 1995; Prenafeta Boldú *et al.*, 2001; Woertz *et al.*, 2001; Kennes & Veiga, 2004).

The pronounced difference in the levels of knowledge about hydrocarbon-metabolizing bacteria and fungi has been explained by suggesting that sealed-flask enrichment in liquid cultures, as is traditionally done for isolation of metabolically specialized organisms, tends to select for bacteria rather than for fungi (Cerniglia *et al.*, 1992; Prenafeta Boldú *et al.*, 2001). Bacteria tend to grow rapidly in aqueous media, whereas fungi, especially filamentous fungi, may be slower growing and, in general, poorly adapted for growth in liquid substrata. In air biofilters, however, organisms grow on a solid support matrix where free water is lacking and volatile substrates are supplied via the gas phase. Fungal development is particularly favored by the relatively low water activity and also by the acidification that results from the biological activity (Cox *et al.*, 1993; Prenafeta Boldú *et al.*, 2001; Kennes & Veiga, 2004).

The ability to metabolize aromatic hydrocarbons in fungi can only derive from one or more evolutionary events in which this ability was acquired. The appearance among fungi of specific degradation pathways can therefore be expected to be relatively strongly organized by inheritance – that is, by lineage. This naturally suggests that an investigation of the biosystematics of the fungal species and higher taxa associated with the degradation of aromatics may show some interesting and specific patterns.

Species identity and phylogeny of fungi degrading monoaromatic hydrocarbons

The interest in hydrocarbon-degrading fungi has so far been motivated by the prospects of biotechnological applications, rather than by interest in taxonomic or ecological relationships. This practical focus, in combination with the fact that morphological identification of some of the species involved ranges from troublesome to virtually impossible (Sterflinger *et al.*, 1999), has resulted in the incomplete or incorrect naming of many isolates. In the present study, strains that are known to assimilate aromatic hydrocarbons or related substrates were collected from authors and culture collections worldwide and their identity was reassessed. DNA sequences of the nuclear ribosomal internal transcribed spacer (ITS) region were determined for these isolates and were compared with sequences of all relevant type strains, as well as many other reference strains in our research databases. BLAST comparisons in GenBank (<http://www.ncbi.nlm.nih.gov/>) were also done. To control for any chance of misidentification of our isolates based on sequence identity with misidentified isolates in GenBank, morphological examination was performed for each received strain.

When the reidentification analysis was complete, we found that strains demonstrating the degradation of aromatic hydrocarbons predominantly belonged to the ascomycetous family *Herpotrichiellaceae* of the order *Chaetothyriales*, class *Chaetothyriomycetes* (Table 1). Heretofore, this family has mainly been scientifically investigated in connection with human pathogenesis associated with certain thermotolerant members of the group (de Hoog & Guarro, 2000). A minority of isolates were distributed among four families from three more orders of ascomycetes. These were the families *Pseudeurotiaceae* and *Trichocomaceae* (order *Eurotiales*, class *Eurotiomycetes*), *Bionectriaceae* (order *Hypocreales*, class *Sordariomycetes*), and *Ophiostomataceae* (order *Ophiostomatales*, class *Sordariomycetes*). The biosystematic pattern seen suggests that assimilation of aromatic hydrocarbons has a relatively broad phylogenetic distribution consistent with either ancient or convergent evolution. A few strains reported in biofilters exposed to

toluene have been identified as members of other groups, such as *Cladosporium* spp. (order *Dothideales*), *Scopulariopsis brevicaulis* (order *Microascales*) and the *Trichosporon cutaneum* species complex (basidiomycetous order *Trichosporonales*). Utilization of toluene as sole source of carbon, however, has not been demonstrated for these strains (Veiga *et al.*, 1999; Alba *et al.*, 2003; Moe & Qi, 2004). In the review below, we present a biosystematic overview of the fungi that have a proven capacity to grow on aromatic hydrocarbons. The analysis of patterns seen is based on the verified identifications we obtained for known, preserved isolates with this capacity.

Aspergillus, *Paecilomyces*, and *Penicillium* (anamorphic *Trichocomaceae*)

The first records of fungal assimilation of aromatic hydrocarbons included an unidentified *Paecilomyces* species and an unidentified *Penicillium* species shown to grow on long-chain alkylbenzenes (Fedorak & Westlake, 1986). Assimilation of styrene has also been claimed for various *Penicillium* species including *P. fellutanum*, *P. cf. janthinellum*, *P. cf. miczynskii* and *P. minioluteum*. A similar claim was made for an isolate identified as *Aspergillus oryzae* (a name now recognized as synonymous with *Aspergillus flavus* except in the case of domesticated *A. oryzae* strains from koji fermentation; Geiser *et al.*, 2000). These fungi were all isolated from air biofilters being used to treat styrene vapors (Cox, 1995; Paca *et al.*, 2001). To our knowledge, however, these strains have not been preserved and additional studies on their taxonomy and physiology were not performed. Growth on styrene was also claimed for *Penicillium simplicissimum* CBS 170.90 (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; <http://www.cbs.knaw.nl/databases/>) isolated from the wastewater of a paper mill (de Jong *et al.*, 1990), but this result could not be reproduced in later experiments (Cox, 1995). More recently, an isolate (now accessed as CBS 113409) that was shown to be capable of growing on toluene was isolated from an air biofilter and identified as *Paecilomyces variotii* (Estevez *et al.*, 2005). Recent DNA studies evidenced that the name *P. variotii* as traditionally applied encompasses a complex of species (R. A. Samson, pers. comm.). Genbank searches on the ITS sequence of CBS 113409 yielded *Paecilomyces sinensis* as the closest relative (Table 2). This fungus was generally thought to be the anamorphic (asexual) state of the insect parasite *Cordyceps sinensis* (family *Clavicipitaceae*, order *Hypocreales*) until the molecular evidence demonstrated that it was in fact completely unrelated to that fungus, and was instead in close phylogenetic proximity to *P. variotii* in the family *Trichocomaceae* (Chen *et al.*, 2001). Toluene assimilation was also attributed to another air biofilter isolate, CBS 115145, which was initially identified as *Scedosporium apiospermum*

Table 1. List of fungi studied in this review, encompassing strains isolated from environments that contain hydrocarbons and/or phenolic compounds, strains that are known to metabolize these substrates, and strains isolated from human or animal brain infections

Name*	BSL [†]	CBS [‡]	Other collections [§]	Source	Geography	GenBank ^{*†}
<i>Cladophialophora arxii</i>	H3	306.94 ^T	IFM 52022	Tracheal abscess, human	Germany	AB109181
		102461	CDC B-5887	Brain, human	USA	AY857509
<i>Cladophialophora bantiana</i>	H3	155.53	–	Brain, human	Belgium	AB091211
		173.52 ^T	IFM 46165	Brain, human	USA	AB091211
		982.96	UAMH 5195	Soil	Uruguay	AY857514
		101158	ATCC 44223; CDC B-3426	Brain, human	Japan	AY857516
		102586	–	Brain, human	Brazil	AF397182
<i>Cladophialophora boppii</i>	H2	126.86 ^T	IFM 52024	Skin lesion, human	Brazil	AB109182
<i>Cladophialophora carrionii</i>	H2	160.54 ^T	ATCC 16264; CDC A-835; MUCL 40053; IFM 4808	Man, chromoblastomycosis	Australia	AB109177
		859.96	–	Dry plant debris	Venezuela	AY857520
		101252	ATCC 58040; CDC B-3466	Brain, human	USA	AY857519
<i>Cladophialophora devriesii</i>	H3	147.84 ^T	ATCC 56280; CDC 82-030890	Disseminated mycoses, human	USA	AB091212
<i>Cladophialophora emmonsii</i>	H2	979.96	CDC B-3875; NCMH 2247; UAMH 4994	Subcutaneous lesion, human	USA	AB109184
		–	DH 13029	Brain, human	USA	AY857518
		556.83I	ATCC 52853; IMI 298056	Decaying wood	Japan	AY251087
<i>Cladophialophora sp.</i>	–	102230	–	Vegetable cover, soil	Brazil	AY857508
		110551	ATCC MYA-2336	Soil polluted with gasoline	The Netherlands	AY857510
		110553	ATCC MYA-2335	Soil polluted with gasoline	The Netherlands	AY857517
		114326	ATCC 200384	Air biofilter degrading toluene	The Netherlands	AY857507
<i>Exophiala dermatitidis</i>	H2	207.35 ^T	ATCC 28869; IMI 093967; UAMH 3967	Subcutaneous phaeoerythromycosis, human	Japan	AF050269
		109154	–	Brain, human	Korea	AY857525
		116726	–	Railway tie	Thailand	AY857526
<i>Exophiala heteromorpha</i>	H2	116.97	–	Soil polluted with petroleum	USA	AY857521
		117.97	–	Soil polluted with petroleum	USA	AY857523
		232.33 ^T	CDC B-2823; MUCL 9894; NCMH 17	Wood pulp	Sweden	AY857524
		633.69	DAOM 75853k; MUCL 15475	Railway tie	Canada	AY857522
<i>Exophiala jeanselmei</i>	H2	–	DAOM 216391	Insect galleries in pine	–	AF050267
		507.90 ^T	ATCC 34123; NCMH 123	Mycetoma, human	Uruguay	AY156963
		528.76	ATCC 10224; NIH 8724	Skin, human	–	AY857530
<i>Exophiala lecanii-corni</i>	H2	123.33 ^T	ATCC 12734; IMI 062462	Scale insect (<i>Lecanium corni</i>)	USA	AY857528
		102400	–	Air biofilter degrading toluene	USA	AY857527
<i>Exophiala oligosperma</i>	H2	579.76	–	Brain, human	Japan	AY857533
		658.76	ATCC 28180	–	–	AY857532
		680.76	ATCC 26272	Activated sludge	Canada	AY857534
		725.88 ^T	–	Tumor, human	Germany	AY163551
		814.95	–	Air biofilter degrading styrene	The Netherlands	AY163549
		113408	–	Air biofilter degrading toluene	Spain	AY857531
<i>Exophiala spinifera</i>	H2	899.68 ^T	ATCC 18218; NCMH 152	Nasal granuloma, human	USA	AY156976
<i>Exophiala sp.</i>	–	642.82	–	Soft rot in power pole	Australia	AY857537
		110555	–	Soil polluted with gasoline	Germany	AY857538
		115831	–	Browncoal	Germany	AY857539
		–	DH 11807	Railway tie	The Netherlands	AY857535
		–	DH 13236	Soil polluted with petroleum	Venezuela	AY857536
<i>Fonsecaea monophora</i>	H2	269.37	–	Chromoblastomycosis, human	Brazil	AY857511
		100430	ATCC 32280	Brain, human	Brazil	AY857513
		102225	–	Decaying wood	Brazil	AY857512
<i>Fonsecaea pedrosoi</i>	H2	271.37N	ATCC 18658; IMI 134458	Human	South Africa	AY366914
<i>Phialophora sessilis</i>	H2	238.93	–	Air biofilter degrading styrene	The Netherlands	AY857541
		243.85 ^T	–	Resin of <i>Picea abies</i>	The Netherlands	AY857542
<i>Ramichloridium mackenziei</i>	H3	650.93 ^T	MUCL 40057	Brain, human	Saudi Arabia	AY857540
<i>Rhinochlamydia atrovirens</i>	H2	317.33A	IFM 4931; MUCL 40416	Pine wood	Sweden	AB091215

Table 1. Continued.

Name*	BSL [†]	CBS [‡]	Other collections [§]	Source	Geography	GenBank [¶]
<i>Rhinocladiella similis</i>	H2	111763 ^T	–	Skin, human	Brazil	AY040855
			DH 13054	Brain, human	Slovenia	AY857529
<i>Sporothrix schenckii</i>	H2	110552	–	Air biofilter treating toluene	The Netherlands	AY857546
			CMW 7617; MRC6963	Soil	South Africa	AF484471
<i>Teberdinia hygrophila</i>	H0	326.81 ^T	–	Wastewater of a potato meal factory	The Netherlands	AY129293
			110554	Soil polluted with gasoline	The Netherlands	AY857545
<i>Bionectria ochroleuca</i>	H0	102.94	–	Air biofilter treating styrene	The Netherlands	AY876924
			710.86N	Soil	The Netherlands	AF358235
<i>Paecilomyces sinensis</i>	H0	–	HMIGB Zhw02	Fruit bodies of <i>Cordyceps sinensis</i>	China	AJ243771
			113409	Air biofilter treating toluene	Spain	AY857543
			115145	Air biofilter treating toluene	Mexico	AY857544

*Identity corrected by molecular methods according to Fig. 2 and Table 1.

[†]Biosafety level (de Hoog & Guarro, 2000) H3, pathogens potentially able to cause severe deep mycoses in otherwise healthy individuals; H2, agents of cutaneous and subcutaneous mycoses, they may cause deep mycoses in immuno compromised patients; H1, infections are coincidental, superficial and noninvasive, or mild; H0, nonpathogenic.

[‡]T, type strain; N, neo-type strain; H, holotype strain; I, isotype strain; A, authentic strain.

[§]ATCC, American Type Culture Collection (Manassas, VA, USA); CDC, Centers for Disease Control and Prevention (Atlanta, GA, USA); DAOM, National Mycological Herbarium (Ottawa, Canada); HMIGD, Mycological Herbarium of Guangdong Institute of Microbiology (Guan g Zhou, China); IMI, CABI Bioscience Genetic Resource Collection (Surrey, UK); MUCL, Mycothèque de l'Université Catholique de Louvain (Louvain-la-Neuve, Belgium); NCMH, The North Carolina Memorial Hospital, University of North Carolina (Chapel Hill, NC, USA); NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, US Department of Agriculture (Peoria, IL, USA); UAMH, University of Alberta Mold Herbarium and Culture Collection (Edmonton, Canada); DH, G. S. de Hoog personal collection (only DNA available).

[¶]Accession sequence number of the ribosomal ITS1-5.8S-ITS2 genes.

^{||}Previously published sequences.

Key taxonomic reference strains such as type strains have also been included.

(anamorph of *Pseudallescheria boydii*, family *Microasaceae*) (Auria et al., 2000; Garcia Pena et al., 2001). Morphological re-examination of the culture, after it was obtained from the authors of the biodegradation studies, showed that it also appeared to have affinities with *P. variotii*. This result is consistent with a SEM picture in the original study showing a biofilter bed inoculated with the strain (Garcia Pena et al., 2001): here, the strain can be seen to form chains of variably sized ellipsoid conidia, typical of the *P. variotii* complex, rather than the dry, clumped heads of pyriform conidia characteristic of the anamorph of *P. boydii*. Molecular characterization of the strain demonstrated that it, like the strain discussed above, is highly homologous to *P. sinensis* (Table 2). The conformity of our morphological observations with the structures seen in the original study's SEM photograph shows that it is unlikely that any strain mix-up or contamination event occurred that could explain the radical change in identification of the strain involved in this case. If a true strain of *S. apiospermum* was also involved in the biodegradation study yielding CBS 113409, it may have been overgrown in biofiltration experiments by the strain photographed in SEM and received here for analysis.

***Cladophialophora* and *Exophiala*, related to teleomorphs in *Capronia* (*Herpotrichiellaceae*)**

Most of the fungal strains that are known to assimilation of aromatic hydrocarbons and that are preserved in public collections belong to the genera *Cladophialophora* and *Exophiala*. The first detailed study on the fungal assimilation of toluene identified the investigated strain (now newly accessed as CBS 114326) as *Cladosporium sphaerospermum*. This strain naturally colonized a compost biofilter that was being used to treat toluene-polluted air (Weber et al., 1995). The ITS sequence of this fungus, however, is almost identical to that of an undescribed *Cladophialophora* species, already represented in our database by strain CBS 102230 from vegetative litter on soil (Fig. 2). The genus *Cladophialophora* is morphologically very similar to *Cladosporium*; the two genera, however, are quite unrelated (de Hoog et al., 1995), with *Cladosporium* falling into the family *Mycosphaerellaceae*, class *Dothidiomycetes*. Two additional *Cladophialophora* spp. strains capable of growth on toluene have recently been obtained: CBS 110551 from a toluene-charged air biofilter and CBS 110553 from a solid state-like incubation, both inoculated with gasoline-polluted soil (Prenafeta

Table 2. Revision of the identity of nonherpotrichiellaceous fungi that assimilate aromatic hydrocarbons, based on GenBank matching of the complete ITS1-5.8S-ITS2 ITS ribosomal genes

Original identification	Collection no.	GenBank no.	Homology*	Current identification	Best GenBank match	GenBank no.	Classification (family)
<i>Paecilomyces variotii</i>	CBS 113409	AY857543	99	<i>Paecilomyces sinensis</i>	HMIGB Zhw02	AJ243771	<i>Trichocomaceae</i>
<i>Scedosporium apiospermum</i>	CBS 115145	AY857544	99				
<i>Clonostachys rosea</i>	CBS 102.94	AY876924	100	<i>Clonostachy rosea</i> (teleomorph <i>Bionectria ochroleuca</i>)	CBS 710.86	AF358235	<i>Bionectriaceae</i>
<i>Sporothrix</i> sp.	CBS 110552	AY857546	99	<i>Sporothrix schenckii</i>	CMW 7617	AF484471	<i>Ophiostomataceae</i>
<i>Leptodontidium</i> sp. strain T5	CBS 110554	AY857545	99	<i>Pseudeurotium</i> -like anamorph (<i>Teberdinia hygrophila</i> .)	CBS 326.81	AY129293	<i>Pseudeurotiaceae</i>

*Percentage of homology to the most similar strain cited in GenBank.

Boldú *et al.*, 2001). The molecular evidence indicates that CBS 110551 is phylogenetically related to *Cladophialophora arxii* and *Cladophialophora devriesii*, whereas CBS 110553 is very close, although perhaps not identical, to the notorious human pathogen *Cladophialophora bantiana* (Fig. 2). *C. bantiana* isolates consistently contain a distinctive 558-bp intron beginning at position 1768 in the small subunit of the ribosomal DNA gene (van den Ende & de Hoog, 1999). Prior to the analysis of CBS 110553, this intron had only been seen in isolates clearly conspecific with *C. bantiana*. The intron is present in CBS 110553, but, in general, the sequence of this isolate deviates sufficiently from that of the type strain of *C. bantiana* that the conspecificity of these genotypes is called into question.

Members of the genus *Exophiala* were recognized as prominent degraders of different classes of organic xenobiotics as growth substrates very soon after systematic study of this topic began. In a survey of fungal assimilation of a wide variety of oxidized aromatic compounds, *Exophiala jeanselmei* CBS 658.76 was shown to exhibit a comparatively broad substrate specificity (Middelhoven, 1993). Isolation of *E. jeanselmei* has also been reported from hydrocarbon liquid culture enrichments. Strain CBS 680.76 (initially identified as *Phialophora jeanselmei*, an older synonymous name for *E. jeanselmei*) was isolated from raw sewage used as an inoculum for enrichments based on natural gas hydrocarbons such as ethane, propane, and butane (Fig. 1) (Davies *et al.*, 1973). Two additional strains were isolated by enriching soil samples with cyclohexanone and with *n*-tolylcarbamic acids (Fig. 1). The latter compound was also assimilated by an *E. jeanselmei* strain isolated from a human patient and deposited in our collection as CBS 528.76 (Hasegawa *et al.*, 1990; Owen *et al.*, 1996). Selective enrichment in liquid cultures at low pH yielded two fungi

growing on styrene (Hartmans *et al.*, 1990), of which one was identified and preserved as *E. jeanselmei* isolate CBS 238.93. The same species name was assigned to the strain CBS 814.95 isolated from two successively operated air biofilters treating styrene (Cox *et al.*, 1993,1996). On the basis of recent molecular data, *E. jeanselmei* has been subdivided into *E. jeanselmei sensu stricto* (*E. jeanselmei* as defined in the strict, modern sense), *Exophiala heteromorpha*, *Exophiala lecanii-corni*, and *Exophiala oligosperma* (de Hoog *et al.*, 2003). *Exophiala heteromorpha* and *E. lecanii-corni* had previously been recognized as varieties, whereas *E. oligosperma* is newly described. Our phylogenetic analysis (Fig. 2) shows that of the strains initially identified as *E. jeanselmei*, only CBS 528.76, the strain from a human source, is a true *E. jeanselmei* strain. The strains CBS 658.76, CBS 680.76, and CBS 814.95 belong to *E. oligosperma*. An additional strain of *E. oligosperma*, CBS 113408, and an *E. lecanii-corni* strain, CBS 102400, have been obtained from air biofilters fed with toluene (Woertz *et al.*, 2001; Estevez *et al.*, 2005). Microbial enrichment in liquid cultures supplemented with toluene has yielded CBS 110555, a member of an undefined *Exophiala* species (Prenafeta Boldú *et al.*, 2001). Sequence analysis indicated that this fungus belongs to a clade containing several strains isolated from substrates such creosote-treated wood, benzene water, brown coal, and from soil polluted with petroleum. This clade will soon be introduced as a new species of *Exophiala*. Liquid enrichment culture containing petroleum and polluted soil yielded two strains, CBS 116.97 and CBS 117.97, that were initially suspected to be *Exophiala dermatitidis* based on molecular fingerprinting (Kleinheinz *et al.*, 1996). Sequence analysis, however, shows that these strains belong to the closely related *E. heteromorpha* (Fig. 2). The sequence data of the styrene-associated CBS 238.93 indicated that this

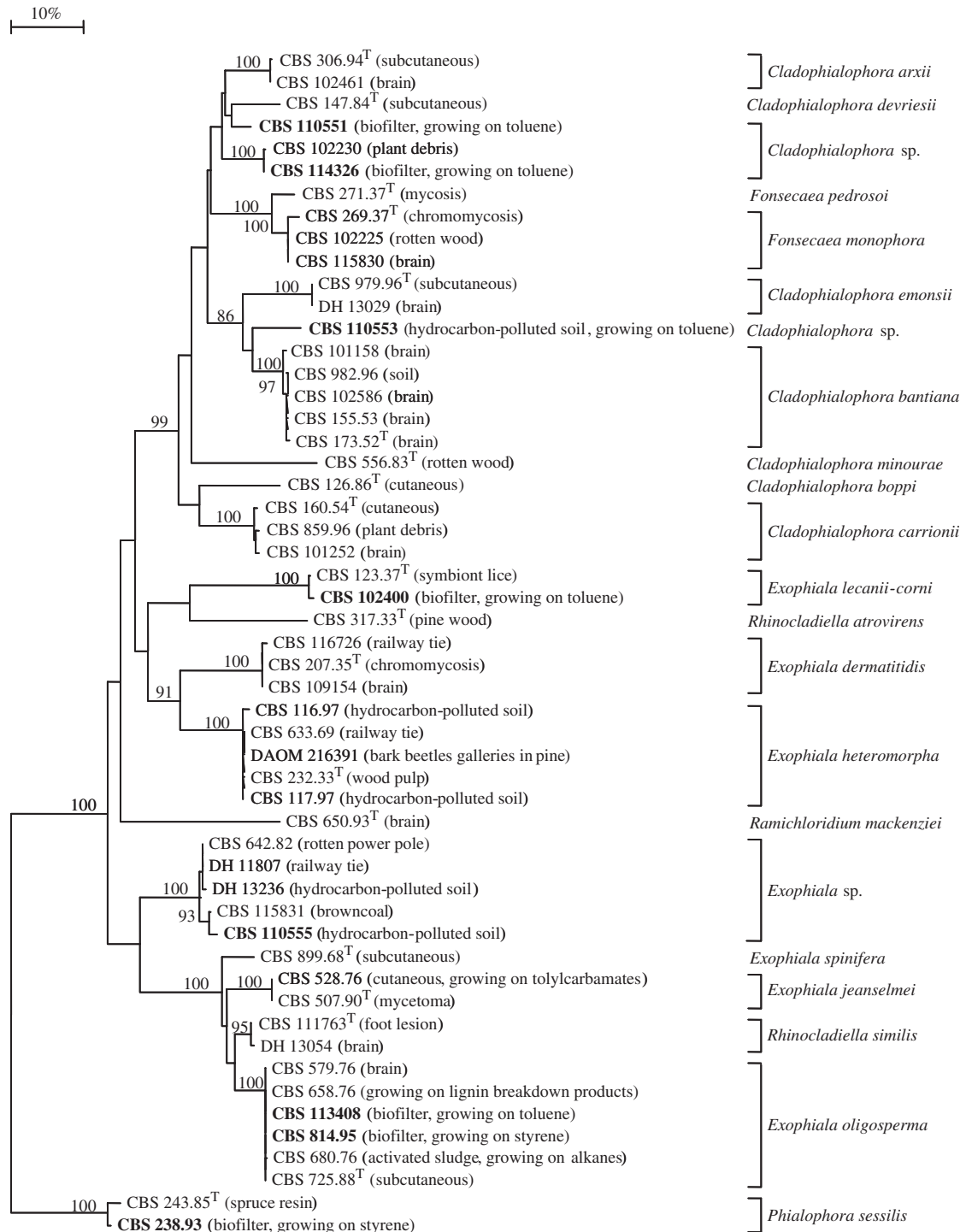


Fig. 2. Phylogenetic tree of the herpotrichiellaceae fungi presented in Table 1 based on confidently aligned ITS1-5.8S-ITS2 rDNA sequences. Reference strains and sources of isolations are indicated between brackets. Reference type strains of described species are indicated as 'T'. The isolates with a proven capacity to grow on aromatic hydrocarbons are indicated in bold. The tree was generated with the TREECON software (Department of Biochemistry, University of Antwerp, Belgium) using the Neighbor-joining algorithm and the Kimura correction. The tree was bootstrapped 100 times and values above 85% are indicated near the branches. The *Phialophora sessilis* clade was selected as an outgroup.

strain is a representative of *Phialophora sessilis*, another herpotrichiellaceous species described recently and related to members of the genus *Exophiala* (de Hoog *et al.*, 1999).

***Clonostachys rosea*, anamorph of *Bionectria ochroleuca* (*Bionectriaceae*)**

The styrene biofilter yielding *E. jeanselmei* CBS 814.95 also yielded a *Clonostachys rosea* isolate preserved as CBS 102.94 (Cox *et al.*, 1993,1996). GenBank searches showed that the ITS sequence of this strain are identical to the type strain of *C. rosea*'s corresponding teleomorph, *Bionectria ochroleuca* CBS 710.86 (Table 2). [For historical and practical reasons, the sexual (teleomorph) and asexual (anamorph) forms of the same fungal species may have separate names and type strains.] CBS 103.94, a third strain isolated from the same biofilter and preserved under the name *Gliocladium catenulatum*, a synonym of *C. rosea*, appears to be identical to the previous strain both morphologically and in ITS sequence (H.-J. Schroers, pers. comm.). In a previous study, this species was found to biodegrade 2,4,6-trinitrotoluene partially (Fig. 1) (Weber *et al.*, 2002).

***Sporothrix* sp., related to teleomorphs in *Ophiostoma* (*Ophiostomaceae*)**

Two isolates identified only as '*Sporothrix* sp.' were obtained from an air biofilter loaded with styrene (Cox, 1995). Both could use this substrate as a sole source of carbon and energy. These strains were not preserved but, more recently, a third *Sporothrix*-like fungus capable of growth on toluene was obtained from an air biofilter exposed to toluene, and was deposited in our collection as CBS 110552 (Prenafeta Boldú *et al.*, 2001). GenBank comparisons (Table 2) show that CBS 110552 is highly homologous to a group of strains characterized in a previous molecular study as belonging to the '*Sporothrix schenckii* complex' (*Ophiostomaceae*), a species complex consisting of two major phylogenetic groups (de Beer *et al.*, 2003). The first clade contained only clinical strains associated with the fungal systemic and subcutaneous disease sporotrichosis, whereas the second, with which CBS 110552 has affinity, contained only environmental isolates with the exception of one isolate stated to have caused sporotrichosis. Presently, it is yet not clear whether these two clades within the *S. schenckii* complex represent distinct species. Our own observations, however, show that CBS 110552 lacks the melanized 'secondary conidia' structures found to date in all demonstrated human-pathogenic *S. schenckii* strains (Dixon *et al.*, 1992). In the original publication, it was mentioned that the teleomorphic state of CBS 110552 was observed and identified as *Pseudeurotium zonatum* (*Pseudeurotiaceae*). However, despite using several culture conditions, we were not able to observe

formation of a teleomorph by this strain. Given the similarity of *Pseudeurotium* anamorphs to *Sporothrix*, it is possible that CBS 110552 initially formed incomplete ophiostomataceous perithecial structures that were misinterpreted as the globose ascomata of *Pseudeurotium*. Fully matured ascomata in the *Ophiostoma/Sporothrix* clade have elongated necks and cannot be confused with *Pseudeurotium*.

Anamorphs related to the teleomorph genus *Pseudeurotium* (*Pseudeurotiaceae*)

The toluene enrichment in acidic liquid cultures that yielded the *Exophiala* sp. strain CBS 110555 mentioned previously also yielded an isolate that grew on toluene and that initially was identified as *Leptodontidium* sp. (Prenafeta Boldú *et al.*, 2001). The ITS sequence of this strain, CBS 110554, however, is highly homologous with that of *Teberdinia hygrophila* (Table 2) related to teleomorphic members of the genus *Pseudeurotium* (Sogonov *et al.*, 2005). The strain does not, however, form a teleomorph *in vitro*. Genetically similar strains in the CBS collection have been isolated from acidic peaty soils in alpine environments (CBS 102670 and CBS 102671). The proposed type strain of *T. hygrophila* was obtained from the wastewater of a potato meal factory (CBS 326.81). The type strain of the closely related *Pseudeurotium zonatum* (CBS 329.36) was isolated from soil near a gas leakage.

Metabolic pathways involved in fungal breakdown of monoaromatic hydrocarbons

When the fungi involved in monoaromatic hydrocarbon breakdown and assimilation are correctly identified and placed in a phylogenetic context, it becomes possible to evaluate systematically the occurrence of various metabolic pathways that may be used in biodegradation of such materials. As mentioned in the Introduction, bacteria are able to grow on substituted and unsubstituted monoaromatic hydrocarbons by means of several metabolic pathways. In cases where they degrade alkylbenzenes, the primary substrate oxidation can involve either the aromatic ring or the alkyl side chain, depending on the strain involved (Fig. 3). In zygomycetous fungi, both possibilities have been detected in cometabolic conversions, but only oxidation at the alkyl group has been reported during assimilation (Prenafeta Boldú *et al.*, 2001). Partial oxidation of unsubstituted aromatic hydrocarbons such as benzene, naphthalene, and benzo(*a*)pyrene (Fig. 1) has long been recognized to be widespread among members of at least three of the four recognized fungal phyla, *Zygomycota*, *Ascomycota* and *Basidiomycota* (Smith & Rosazza, 1974; Cerniglia *et al.*, 1978). We have not been able to find convincing evidence

for the utilization of this class of substrates for growth. A recent study asserted that isolate CBS 114326, then identified as *Cladosporium sphaerospermum* but now shown to be a *Cladophialophora* species, could grow at the expense of benzene (Qi *et al.*, 2002). This result, however, was only based on the observation of mycelial development on a ceramic material exposed to benzene vapors, and it contradicts the results of two previous reports on closed liquid cultures of the same strain in which depletion of benzene was not observed (Weber *et al.*, 1995; Prenafeta Boldú *et al.*, 2001). As mentioned above, such growth could be due to scavenging of trace contaminants or inoculum residues alone. In a parallel study, toluene and other alkylbenzenes were oxidized by cellular extracts of this fungus, but no significant biodegradative activity occurred with benzene (Luykx *et al.*, 2003).

In the study of Fedorak & Westlake (1986), utilization of alkylbenzenes of different side-chain length, ranging from toluene to dodecylbenzene (Fig. 1), was surveyed in several poorly documented fungal strains apparently belonging to the ascomycetous orders *Hypocreales* (*Verticillium sensu lato*, *Paecilomyces sensu lato pro parte*, *Beauveria*) and *Eurotiales* (*Penicillium*, *Paecilomyces sensu lato pro parte*). The results showed that growth on dodecylbenzene resulted in the transient accumulation of the side-chain oxidation products benzoic acid and phenylacetic acid. Of the substrates that supported fungal growth, butylbenzene (Fig. 1) had the shortest alkyl group. It was argued that a minimum aliphatic chain length was required for growth of each of the isolates tested, with '*Paecilomyces* sp.' being the only isolate able to use the 4-carbon substituent as well as all longer substituents. However, because substrates were added at water saturation, the lack of fungal growth seen with lighter alkylbenzenes such as toluene or ethylbenzene could have been an artifact of toxicity owing to the higher solubility of these compounds. Whereas styrene and toluene are toxic towards fungi at concentrations well below saturation (Cox *et al.*, 1997; Prenafeta Boldú *et al.*, 2001), several fungal strains have been shown to grow at the expense of these substrates when the substrates are added at relatively low concentrations (Cox *et al.*, 1993; Weber *et al.*, 1995; Woertz *et al.*, 2001; Prenafeta Boldú *et al.*, 2001; Kennes & Veiga, 2004). Styrene pregrown cultures of *Phialophora sessilis* CBS 238.93 (then identified as *Exophiala jeanselmei*), *Clonostachys rosea* CBS 102.94, and six nonpreserved *Penicillium* strains, collectively representing a wide phylogenetic range of ascomycetous fungi, all oxidized styrene at the alkene double bond, resulting in the formation of phenylacetaldehyde through styrene oxide (Fig. 3). This product was subsequently dehydrogenated to phenylacetic acid, and ring oxidation and fission occurred through formation of homogentisic acid (Cox, 1995; Cox *et al.*, 1996). Toluene assimilation in the herpotrichiellaceous *Cladophialophora*

and *Exophiala* strains CBS 114326, CBS 110551, CBS 110553, and CBS 110555, and in the distantly related strains CBS 110552 and CBS 110554, proceeded in all cases via hydroxylation of the methyl group to benzyl alcohol, which was oxidized further to benzoic acid via benzaldehyde; benzoic acid was then hydroxylated to protocatechuic acid (Fig. 3). In fungi, ring cleavage of catecholic compounds most commonly occurs at the *ortho* position, giving rise to muconic acids, which are incorporated into core metabolism via the 3-oxoadipate pathway (Cain *et al.*, 1968). This process has been substantiated in '*Exophiala jeanselmei*' isolate CBS 658.76, reidentified here as *Exophiala oligosperma* (Fig. 2), which grew on phenol by bringing about its oxidation to catechol (Boersma *et al.*, 1998). In tandem with the previous pathway, phenol is also hydroxylated in the *para* position to produce hydroquinone, which is then converted into 1,2,4-trihydroxybenzene (Fig. 1) for ring fission by *ortho*-cleavage. This metabolic variant has been found in *Aspergillus fumigatus* ATCC 28282 (family *Eurotiaceae*) and in the very distantly related ascomycetous yeast *Candida albicans* (an anamorphic member of the family *Saccharomycetaceae*) (Jones *et al.*, 1995; Claußen & Schmidt, 1998). The strains growing on toluene were not able to assimilate dimethylated xylene isomers, but these substrates were partly oxidized by *Cladophialophora* sp. isolate CBS 110553 at the side chains in the same manner as was seen for toluene (Prenafeta Boldú *et al.*, 2002). The distinctive and uniform pattern of toluene and styrene oxidation seen in the fungi studied so far differs from the variety of pathways found in bacteria (Fig. 3), and suggests that an evolutionarily conserved enzymatic capability is uncommonly, but more or less uniformly, preserved as a symplesiomorphy among members of a phylogenetically disparate assemblage of ascomycetous fungi.

With regard to enzymatic involvement, detailed studies on the metabolism of styrene by *P. sessilis* CBS 238.93 and of toluene by *Cladophialophora* sp. CBS 114326 have revealed activity of a cytochrome-P450 monooxygenase NADPH-reductase enzyme complex in the oxidative attack (Cox *et al.*, 1996; Luykx *et al.*, 2003). Fungal P450 monooxygenases are responsible for the oxidation of a wide variety of aromatic and aliphatic hydrocarbons (van den Brink *et al.*, 1998). The side-chain oxidation of alkylated benzenes in fungi very much resembles that seen in alkane degradation, which most commonly starts with the hydroxylation of the terminal methyl group (monoterminal oxidation), and proceeds to the fatty acid via the corresponding alcohol and aldehyde. Fatty acids are then incorporated into the central pathways of cellular catabolism through the β -oxidation pathway (Rehm & Reiff, 1981). Some fungi oxidize alkanes at both terminal methyl groups (diterminal oxidation), a process giving rise to dicarboxylic acids, or at an

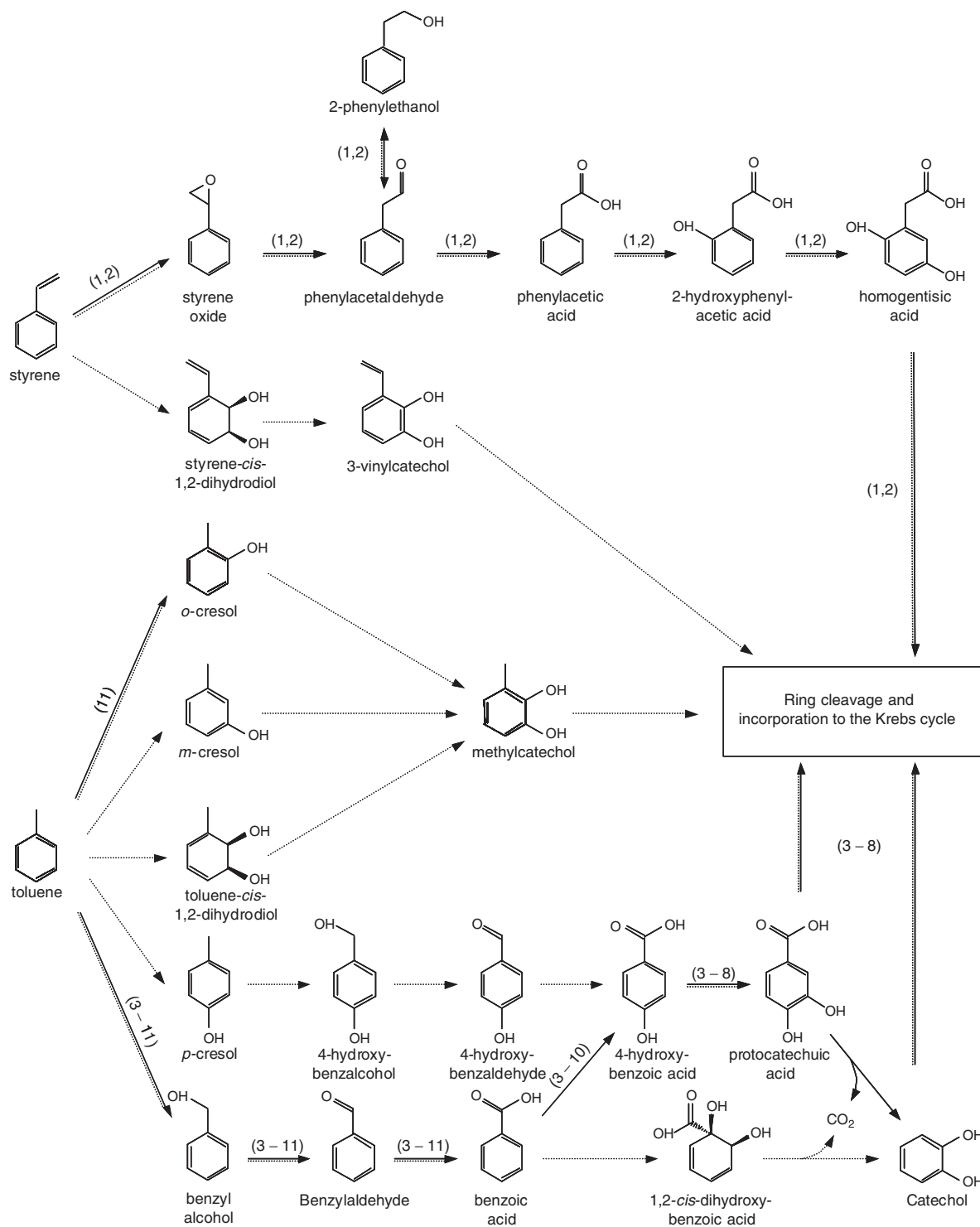


Fig. 3. Summary of the major metabolic pathways for the aerobic assimilation of styrene and toluene known in fungi (bold lines) compared to those in bacteria (dotted lines) composed after Holland *et al.* (1993); Cox (1995); Weber *et al.* (1995); Prenafeta Boldú *et al.* (2001) and O'Leary *et al.* (2002). Numbers associated with each pathway identify the fungi, named according to the updated identifications from Table 1, that have been shown to perform the particular transformation: (1) *Phialophora sessilis* CBS 238.93; (2) *Clonostachys rosea* CBS 102.94; (3-5) *Cladophialophora* spp., including strains CBS 114326 (3), CBS 110553 (4), and CBS 110551 (5); (6) *Exophiala* sp. strain CBS 110555; (7) *Sporothrix* sp. strain CBS 110552; and (8) *Pseudeurotium* sp. CBS 110554. Partial conversions of toluene are also numbered: (9) *Aspergillus niger* CBS 12648; (10) *Cunninghamella echinulata* CBS 596.68; and (11) *Umbelopsis isabellinus* ATCC 42613.

intermediate methylene group (subterminal oxidation), giving rise to secondary alcohols and ketones. Both terminal and subterminal oxidation processes have been reported in fungal cometabolic conversions of long-chained alkylbenzenes. The former process has been documented in *Cunninghamella echinulata* ATCC 26269 (Holland *et al.*, 1987; Uzura *et al.*, 2001) and the latter in a fungus identified by the ambiguous name *Fusarium moniliforme* (now recognized as subtending over 20 different *Fusarium* species) (Holland *et al.*, 1987; Uzura *et al.*, 2001).

Ecology of the alkylbenzene-utilizing fungi

Environmental niches

The appearance in fungi of a metabolic capacity as specific as alkylbenzene assimilation suggests that this type of substrate is present in the natural niche(s) of these organisms. The main known source of alkylated aromatic hydrocarbons in the biosphere is fossil fuels such as petroleum and coal (Boylan & Tripp, 1971; Sinninghe Damste *et al.*, 1992). It is, thus, not surprising that strains of the *Paecilomyces variotii* species complex have commonly been found as fuel biodegradation agents in storage tanks (Domsch *et al.*, 1980; Hettige & Sheridan, 1984; Bento & Gaylarde, 2001). Some *Exophiala*, *Cladophialophora*, and other related species of the family *Herpotrichiellaceae* have also been isolated from environments polluted with petroleum products (Table 1) but, in general, members of this clade appear to be ecologically more specialized than are generalist biodegraders such as *P. variotii*.

The herpotrichiellaceous species mentioned above as growing on alkylbenzenes, as well as closely related species, tend to be isolated in a restricted range of habitats that frequently are not known to contain aromatic hydrocarbons. Based on numbers of isolates and diversity of alkylbenzene-degrading species obtained so far, the *Herpotrichiellaceae* may be the most important alkylbenzene-degrading fungal group worldwide – in many habitats, at least. Only a small number of isolates in the *Herpotrichiellaceae* have been tested for alkylbenzene degradation, but the wide dispersal of known positive strains in the dendrogram (Fig. 2) suggests that such abilities may be widespread in this group and that further testing of these abilities in additional strains and species is warranted. There is therefore sufficient reason to review briefly the known habitats of these fungi in general. For many species such as *Exophiala dermatitidis*, *Fonsecaea pedrosoi* and *Cladophialophora bantiana*, most isolates are obtained as agents of human disease (De Hoog & Guared, 2000). *Exophiala oligosperma*, one of the most frequently isolated alkylbenzene-degrading species, has re-

peatedly been found in warm human-associated environments poor in nutrients, such as inert materials lining saunas and swimming pools (de Hoog *et al.*, 2003). This species has also been isolated from humans, in whom it has caused infections that in several cases might have had waterborne contamination as a source. A very specialized infection cycle that does not involve free water or known alkylbenzene sources has been observed in the related species *Cladophialophora carrionii* and *Fonsecaea pedrosoi*, which grow as endophytes in cacti. They colonize the young spines, and after the spines have dried out and become stiff, these fungi are transmitted to humans via skin perforation and cause the distinctive subcutaneous disease chromoblastomycosis (Zeppenfeldt *et al.*, 1994; Salgado *et al.*, 2004). Human pathogenicity is particularly conspicuous in *Cladophialophora bantiana*, because nearly all available strains originate from infected humans (van den Ende & de Hoog, 1999). This fungus has only rarely been isolated from the environment, usually after soil, wood, or bark collected from endemic geographic regions has been inoculated into laboratory animals. To our knowledge, only two environmental isolates of *C. bantiana* with identities confirmable by sequence analysis have been preserved (van den Ende & de Hoog, 1999). They are CBS 647.96 from rotten lumber (sometimes wrongly reported as sawdust), and CBS 982.96 from soil (Klite *et al.*, 1965; Dixon *et al.*, 1980). The highly related alkylbenzene-degrading strain CBS 110553 was isolated from polluted soil (Prenafeta Boldú *et al.*, 2001), and has successfully been cultivated in a soil batch (Prenafeta Boldú *et al.*, 2004). *Cladophialophora arxii* and *C. devriesii* species, both closely related to the alkylbenzene-degrading *Cladophialophora* strain CBS 110551, have not as yet been recorded from natural sources and are only known from human infections. Whether the very rare incidence of infections caused by these species signals a degree of specialization for occasional growth in animal hosts, or merely represents a repeatedly occurring ecological fortuity and an evolutionary 'dead end', is yet not clear. The fact that such infections are always acquired from the environment rather than from other infected humans or animals suggests an as yet undiscovered primary niche, the nature of which may be hinted at by the unusual hydrocarbon degrading abilities of the fungi involved.

The relatively low number of strains of pathogenic herpotrichiellaceous fungi that have been obtained directly from the environment have mostly been isolated from rotten wood, leaf litter, bark and rhizosphere soil (Klite *et al.*, 1965; Conti-Diaz, 1977; Dixon *et al.*, 1980; Vicente *et al.*, 2001). Most isolations and collections of the *Capronia* teleomorphic states formed by the sexually reproducing members of the *Herpotrichiellaceae* have also originated from plant material, particularly from bark, wood, and resins of coniferous trees (de Hoog *et al.*, 1999; Untereiner &

Malloch, 1999). Collections are also made from the tough, relatively durable senescent fruiting bodies of lignin-degrading fungi. Despite these trends, *Capronia* and its related anamorphic species are generally unable to grow on the main plant and wood polymers starch, cellulose, and lignin, and they are also unable to utilize the fungal wall component chitin. Most species, however, exhibit a strong lipase activity (Untereiner & Malloch, 1999). This activity is consistent with the occurrence of these species as lignicolous and fungicolous organisms, because decaying woody and poly-poroid/steroid substrates are known to accumulate long chain fatty acids like oleic and linoleic acids (Fig. 1) (Hafizoglu & Reunanen, 1994; Gao *et al.*, 1995; Gutierrez *et al.*, 2002; Hafizoglu *et al.*, 2002). Association with white-rot fungi might also be reinforced by the fact that *Capronia* anamorphs grow very efficiently on aromatic acids, such as protocatechuic (Fig. 3), syringic, vanillic, and ferulic acids (Fig. 1), which are released during lignin decomposition. A study on the fungal assimilation of various oxidized aromatic compounds revealed that *Exophiala oligosperma* CBS 658.76 (then identified as *E. jeanselmei*) was able to degrade a very broad range of these substrates (Middelhoven, 1993).

Lignin is also broken down into its basic monomeric aromatic constituents artificially during the manufacture of paper. Early inventories on fungi inhabiting slimes from wood pulp and paper mills reported the regular isolation of *Rhinoctadiella atrovirens*, a close relative of the known alkylbenzene-degrading *Exophiala* species; *Pseudeurotium zonatum* was also regularly isolated (Brewer, 1958). When fungi from pulp and paper mills were surveyed for the capacity to grow on syringic acid, it was found that '*E. jeanselmei*' and *Paecilomyces variotii*, along with a member of the *Fusarium solani* complex identified under the ambiguous name *F. eumartii*, were the most efficient degraders of this compound. Significant biodegradation was also measured with an isolate identified as *Rhinoctadiella* sp. (Bergbauer, 1991). In fact, CBS 232.33, the type strain of *Exophiala heteromorpha* (a species previously named *Rhinoctadiella mansonii*) was isolated from wood pulp, as was the type strain of *Ophiostoma stenoceras*, a teleomorphic species closely related to the *Sporothrix schenckii* complex (de Beer *et al.*, 2003). A survey of about 1000 filamentous fungi for growth on different aromatic acids showed that a *P. variotii* strain had a particularly remarkable capacity for metabolizing a wide range of these compounds (Rahouti *et al.*, 1999).

Besides lignin by-products, large quantities of volatile terpenoid hydrocarbons such as *p*-cymene and α -pinene (Fig. 1) are released during bleaching of wood and paper pulp (Stomvall & Petersson, 1992). These volatiles cause odor nuisance, leading to experimentation designed to bring about their safe and rapid degradation. Experimental bio-filters used for treating α -pinene vapors were shown to become enriched with unspecified dematiaceous fungi (van Groenestijn & Liu, 2002).

Terpene hydrocarbons formed from the polymerization of isoprene units (Fig. 1) and are naturally emitted by a wide variety of higher plants (Buckingham *et al.*, 1995). Along with *p*-cymene, other nonterpenoid aromatic hydrocarbons such as toluene and styrene, as well as trace amounts of benzene, have been detected in coniferous litter, compost facilities, and damp wooden houses, all environments related to the decomposition of lignocellulosic materials by fungi (Ström *et al.*, 1994; Pöhle & Kliche, 1996; Isidorov *et al.*, 2003). Such emissions might result from the secondary metabolism of fungi, because styrene, benzene, and cyclohexane (Fig. 1) are produced by axenic fungal cultures (Shimada *et al.*, 1992; Ezeonu *et al.*, 1994; Larsen, 1998).

Although often thought of as artificial, industrial molecules, toluene and styrene were originally obtained by pyrolytic distillation of resins of tolu (*Myroxylon balsamum*, *Fabales*) and storax (*Styrax benzoin*, *Ericales*), plants to which they owe their names (Buckingham *et al.*, 1995). Thus, under certain circumstances, fungal enzyme systems could be subject to direct selective reinforcement related to the ability to degrade these compounds in nature. Toluene, ethylbenzene, xylenes, trimethylbenzenes and ethylmethylbenzenes, as well as branched decanobenzenes, have been found in taxonomically very diverse plants (Holzinger *et al.*, 2000; Vrkokova *et al.*, 2000). The complex long-chain alkylbenzene 1-phenyl-5-vinyl-5,9-dimethyl decane (Fig. 1) has been found in wax coatings of leaves of the jaborandi tree (*Pilocarpus jaborandi*, *Sapindales*) (Negri *et al.*, 1998). Toluene biosynthesis has been demonstrated in sunflower (*Helianthus annuus*, *Asterales*) by isotopic labeling experiments (Heiden *et al.*, 1999). Field measurements indicated that toluene *in vivo* emissions are significant in Scots pine (*Pinus sylvestris*, *Coniferales*) and helm oak (*Quercus ilex*, *Fagales*), particularly under stress conditions such as drought or insect attack (Heiden *et al.*, 1999; Holzinger *et al.*, 2000). The content of toluene, along with several other volatile aliphatic and aromatic hydrocarbons, is particularly high in galleries excavated by the oak bark beetle (*Scolytus intricatus*, *Curculionidae*) (Vrkokova *et al.*, 2000). This phenomenon could actually originate from the metabolism of the insects involved, as the biosynthesis of toluene from isotopically labeled phenylalanine (Fig. 1) was demonstrated for the pine engraver beetle (*Ips pini*, *Curculionidae*), a taxonomic relative of the oak bark beetle (Gries *et al.*, 1990). The oak bark beetle is reported as a vector of plant pathogenic *Ophiostoma* fungi, which cause withering and death of oak trees (Doganlar *et al.*, 1984). Fungi in this genus are often tree parasites, such as the famous agents of Dutch elm disease in the *Ophiostoma ulmi* complex, and depend on wood-boring insects for dispersal and for penetration into the vascular tissues of new trees (Klepzig & Six, 2004). Insect-excavated tree galleries are populated by various other fungi, including, interestingly, several

herpotrichiellaceous species in the genera *Exophiala*, *Phialophora*, *Rhinocladiella*, and *Ramichloridium* (de Hoog, 1977; Kerrigan & Rogers, 2003).

Our molecular examination shows that species in the *Herpotrichiellaceae* growing on alkylbenzenes encompass environmental strains isolated from rotten litter/wood, wood pulp and arboreal beetle galleries, as well as from environments containing petroleum and coal derivatives (Fig. 2). Wooden objects treated with the wood preservative creosote, such as railway ties and power poles, represent another common source of isolation for these fungi. A culture independent molecular study showed that fungi from the genera *Cladophialophora* and *Exophiala* were present in a creosote-polluted soil (Stach & Burns, 2002). Creosotes are commonly obtained by high-temperature treatment of coal tar, and are variable, complex mixtures of aromatic hydrocarbons, including toluene and phenols (Buckingham *et al.*, 1995; Kiilerich & Arvin, 1996). The predominance of herpotrichiellaceous species in biofilters used for the biodegradation of toluene and related compounds might very well derive from a natural adaptation of these fungi to assimilate volatile alkylbenzenes and related compounds released from wood and other plant materials. The similarity of the biofilters to the natural habitats where such compounds are encountered probably lies in part in the fluctuating physico-chemical conditions that characterize both environments. Air biofilters and exposed wood or bark both vary over time in temperature and humidity. Both are also low in pH and poor in nutrients.

Human pathogenicity

Some alkylbenzene-degraders such as members of the *Paecilomyces variotii* complex are generally regarded as relatively weak opportunistic human pathogens of immunocompromised patients (Sterflinger *et al.*, 1999), whereas the *Sporothrix* strain is closely related to virulent human pathogens but is not known to be pathogenic itself. By contrast, known alkylbenzene-degrading members of the *Herpotrichiellaceae* and their close relatives are causal agents in a wide diversity of clinical infection types. Individual herpotrichiellaceous fungal species often show highly distinctive patterns of mammalian invasive disease, generally affecting immunocompetent as well as immunocompromised individuals. In the genus *Cladophialophora*, for example, excepting a very few species such as *Cladophialophora minourae* that are only known to be saprobic, there is a strong tendency towards human pathogenicity, and nearly all species that have repeatedly been shown to cause characteristic types of disorders (de Hoog & Guarro, 2000). *C. bantiana* typically causes cerebral infection in otherwise healthy patients. Although the fungus is believed to be acquired by inhalation (Dixon *et al.*, 1987), primary infection is neurological,

occurring only after the fungus has been vascularly translocated to the brain. This fungus, which is associated with wood dust and with particular endemic areas such as the US states of North and South Carolina and parts of India, South Africa, Japan and southern Europe, is classified as being one of the most dangerous pathogenic fungi known. Without combined brain surgery and antifungal drug therapy, the infection is nearly always fatal within months (de Hoog *et al.*, 2003). Primary neurotropism has also been observed in the related species *Exophiala dermatitidis* and *Ramichloridium mackenziei*. These fungi also probably enter the human body via inhalation. *Exophiala dermatitidis* is frequently isolated from saunas, hot tubs, and similar habitats worldwide, although for reasons that remain unclear it mainly causes cerebral infections in east Asians (Matos *et al.*, 2002). *R. mackenziei* is a rare fungus for which the habitat is completely unknown, and all isolates obtained so far have been from brains of patients who have resided on the Arabian peninsula (Horre & De Hoog, 1999). Sporadic cases of brain infection have also been caused by *Cladophialophora modesta* (of which only one isolate is known so far), *C. arxii*, *C. emmonsii*, and *Exophiala oligosperma* (de Hoog & Guarro, 2000; de Hoog *et al.*, 2003; Saberi *et al.*, 2003). Two additional herpotrichiellaceous species, *Fonsecaea pedrosoi* and *Rhinocladiella atrovirens*, have also been reported to cause brain infection, but molecular studies have recently shown that the neurotropic isolates involved belong to the newly described species *Fonsecaea monophora* and *R. similis*, respectively (de Hoog *et al.*, 2003, 2004). *Cladophialophora devriesii* and *C. arxii*, in the rare cases from which they are known, acted as agents of invasive systemic mycosis, often fatal. In the much more common infections caused by *C. carrionii* and *F. pedrosoi*, the fungus remains localized in the dermal skin layers, causing a chronic disease called chromoblastomycosis (Usuki *et al.*, 1996). In some areas, such as parts of Venezuela, this distinctive tropical disease is relatively common. Other causative agents of chromoblastomycosis include *Cladophialophora boppii*, *Exophiala jeanselmei*, *Phialophora verrucosa* and, rarely, *E. spinifera* (de Hoog & Guarro, 2000). Although these fungi grow as ordinary moulds in pure culture, they also, under certain cultural conditions, can be induced to form strongly melanized, isodiametrically expanding 'meristematic' cells that divide by fission. This type of cell, traditionally known as a sclerotic fission cell, is characteristically found in infected human tissue but can also be observed when these fungi grow in natural niches that can be roughly characterized as 'extreme', as in *F. pedrosoi* growth within desiccating cactus thorns (Sterflinger *et al.*, 1999). Various herpotrichiellaceous species such as *Exophiala lecanii-corni*, *E. spinifera* and *E. dermatitidis* may cause human subcutaneous infections of the 'phaeohyphomycosis' type that are associated with melanized hyphae and yeast cells in tissue rather than meristematic cells (de Hoog & Guarro, 2000).

Extremophily and virulence

Fungi that have an evolutionary niche as virulent, contagious or commensal agents of human and animal disease may have very sophisticated virulence factors reflecting coevolution with mammalian hosts (de Hoog & Guarro, 2000). Enumeration of these factors is beyond the scope of the current review. A different situation is found in respect to environmental fungi that only fortuitously cause disease in mammals. In these cases, factors conferring virulence, or at least, invasive capability, are likely to be pre-existing attributes that may be relatively simple in nature, starting with the mere ability to grow at human body temperature. The coincidental assembly of a critical number of such factors may allow certain environmental fungi to 'break through' as regular agents of disease in immunocompetent persons, even though there are no known mechanisms of pathogenicity-related evolutionary reinforcement that would tend to maintain them in that role. One of the best-studied fungal groups in this regard is the *Herpotrichiellaceae*.

Many *Herpotrichiellaceae* have been cited as classic examples of fungal extremotolerance (Sterflinger *et al.*, 1999). This generalized suite of adaptations for dealing with extreme environments, particularly high temperatures, is expected to contribute to pathogenic potential. Many of the natural and artificial habitats that are associated with growth of *Herpotrichiellaceae*, such as decaying tree bark and creosoted poles and ties, are likely to favor fungi that, in addition to being able to break down the aromatics occurring in these substrata, have a generalized set of adaptations to conditions that at least occasionally become highly stressful (very hot, dry, very cold, very low in micronutrients and growth factors, very high or low in pH, etc.). Many of these adaptations may fortuitously predispose fungi towards human opportunistic pathogenesis. Tolerance of human body temperature is, of course, critical for pathogenesis, but can be fortuitously acquired via adaptation to warm environmental habitats. The melanin pigment formed by herpotrichiellaceous fungi as well as many others may also reflect extremotolerance: it is thought to function primarily as an ultraviolet light shield, and is thus clearly connected to survival in hot, sunlit environments. It is also, however, known to serve as a critically important virulence factor in various pathogenic fungi (Butler & Day, 1998; Langfelder *et al.*, 2003), most of them probably fortuitous opportunists not specifically adapted as mammalian pathogens. The conversion of some *Herpotrichiellaceae* to meristematic growth forms both in human hosts and in extreme environmental conditions (as detailed in the previous section) is another factor connecting extremophily and attributes associated with at least some forms of pathogenicity (Sterflinger *et al.*, 1999).

It must be noted, though, that some completely non-pathogenic members of the *Herpotrichiellaceae*, such as

Coniosporium species inhabiting rock surfaces in Mediterranean areas (Sterflinger *et al.*, 1997), also show melanogenesis, tolerance of extreme (including very warm) conditions, and muriform cell formation. This indicates that these attributes, although known to be connected to mammalian pathogenic potential, are in and of themselves insufficient to confer pathogenic capability. Pathogenic species must also possess other, as yet undiscovered attributes fortuitously serving as virulence factors. The metabolism of alkylbenzenes and related compounds, for reasons discussed in the next section, may be an additional member of the list of contributory factors cumulating towards a sufficient explanation of environmental fungal pathogenicity. The various virulence-related factors operating in different environmentally occurring fungi, besides contributing to the fundamental ability to survive and grow in mammalian tissue, should also help to explain the site-specificity and pattern of infection shown by the various pathogens within the human body. We will therefore consider how the metabolism of alkylbenzenes and related hydrocarbons may be related to both the potential of certain environmental fungi for causing human disease, and to the specific disease manifestations seen. In particular, the distinctive neurotropism of many herpotrichiellaceous pathogens must be evaluated for a possible connection to specialized hydrocarbon-degrading capabilities.

Neurotropic pathogenicity and metabolism of aromatic compounds

Primary neurotropism in fungi is rare outside the family *Herpotrichiellaceae*. It is interesting, therefore, that the few nonherpotrichiellaceous fungi potentially occurring as neurotropic human pathogens are often associated with the decomposition of wood, petroleum and coal products, just as the *Herpotrichiellaceae* are. These neurotropic fungi are also strongly thermophilic (a *sine qua non* of growth in the brain) and many are melanogenic. A good example is the ascomycetous anamorph *Ochroconis gallopava* (*Leotiaceae*), which is a relatively common agent of epidemic encephalitis in poultry. In human, it causes brain infection and disseminated disease predominantly in immunocompromised patients (de Hoog & Guarro, 2000); there is also one case report documenting an occupationally acquired lung infection in an immunocompetent wood pulp worker (Odell *et al.*, 2000). Environmental isolates of *O. gallopava* have been obtained in acidic warm environments such as hot springs, heated streams, composting broiler-house litter, stored lumber, and coal waste piles (Mekin & Nannfeldt, 1934; Evans, 1971; Tansey & Brock, 1973; Waldrip *et al.*, 1974; Rippon *et al.*, 1980).

Other pathogenic ascomycetes with a marked predilection towards brain infections, such as *Pseudallescheria*

boydii, *Candida albicans*, and *Aspergillus fumigatus* (de Hoog & Guarro, 2000), have also been isolated from environments containing hydrocarbons or phenols, and are known to assimilate these substrates (Sorkhoh *et al.*, 1990; Jones *et al.*, 1995; Gaylarde *et al.*, 1999). The common fuel biodeteriogen *Paecilomyces variotii* also occurs as an opportunistic pathogen, occasionally causing central nervous system infections in immunocompromised patients (de Hoog & Guarro, 2000; Kantarcioglu *et al.*, 2003). Infections with *Sporothrix schenckii* (a species mentioned above as closely related to an alkylbenzene-degrading isolate surveyed here) are usually localized in the subcutaneous and lymphatic tissues, but disseminated infections with neurotropic involvement have occasionally been observed in individuals with impaired immunity (Rocha *et al.*, 2001). One unexpected case of brain infection was caused by a dematiaceous fungus identified as a *Nodulisporium* sp. (*Xylariaceae*) (Umabala *et al.*, 2001). Members of this taxon are lignin-degrading organisms typically found in decaying wood, but thermophilic relatives have also been isolated from coal tips (Evans, 1971; de Hoog, 1973).

Neurotropism is also found in *Blastomyces dermatitidis* (*Onygenaceae*; teleomorph name *Ajellomyces dermatitidis*) and in the basidiomycetous yeast species *Cryptococcus neoformans* and its close relative *C. gattii* (*Filobasidiaceae*; teleomorph names *Filobasidiella neoformans* and *F. bacillisporus*) (de Hoog & Guarro, 2000). These three species may cause disease outbreaks associated with occupational or recreational activities in endemic natural areas (Klein *et al.*, 1987; Kidd *et al.*, 2004). *Blastomyces dermatitidis* infection generally manifests itself as pulmonary disease with dissemination to the skin and internal organs, and brain involvement is relatively uncommon, although it is regularly seen in case surveys. *C. neoformans* and *C. gattii*, although generally initiating a subclinical or frank pulmonary infection, frequently translocate to the meninges, where they cause cryptococcal meningitis. Blastomycosis is often contracted around streams or rivers with high content of moist soil enriched with organic matter and rotting wood, and environmental isolates of *B. dermatitidis* have also been obtained from woodpiles and from the soil of a shed used for drying tobacco leaves (Denton *et al.*, 1961; Baumgardner & Paretsky, 1999). One case of blastomycosis was reported in a petroleum technician, and *B. dermatitidis* was isolated from the earthen floor of a petroleum filtering shed where the patient worked (de Hoog & Guarro, 2000). Infections with *C. neoformans* and *C. gattii* have been related to the presence of these fungi in decaying wood in trunk hollows of a wide variety of tree species (Randhawa *et al.*, 2001; Kidd *et al.*, 2004).

The fungal ecological and physiological patterns reviewed here, although showing the variability that can only be expected in surveys of diverse biological species, clearly

suggest that there is a pattern of association between the ability to metabolize plant-derived or artificially produced phenols and hydrocarbons, and the ability to cause serious human disease with a tendency towards neurotropism. Phenolic and hydrocarbon assimilation in fungi may represent an additional important virulence factor to add to the list of those already known that may come into play particularly in infections of the central nervous system. The brain contains monoaromatic catecholamine neurotransmitters such as dopamine (Fig. 1) that accumulate and polymerize to form the characteristic dark pigmented neuromelanin in the so-called *substantia nigra* (Zecca *et al.*, 2003). Dopamine is also catabolized in the brain by a two-step process involving the enzymes monoamine oxidase and catechol-*ortho*-methyltransferase, leading to the formation of 3,4-dihydroxyphenylacetic acid and homovanillic acid (Fig. 1), compounds which are also found as products of lignin degradation (Takada *et al.*, 2004). Similarly, vanillic acid has also been detected in the human brain and in cerebrospinal fluid, and as intermediate of lignin decomposition (Ebinger & Verheyden, 1976; Takada *et al.*, 2004). In addition to this, the lipid content of the human body is known to be particularly high in the brain, where up to 50% of the tissue dry weight is lipid-like in nature, mainly consisting of aliphatic aminoalcohols such as sphingosine (Fig. 1).

Although it is, as yet, speculative to say so, a link between neurotropism and assimilation of aromatic substrates may exist, and may be one of the factors that confers pathogenic competence on fungi fortuitously seeded to the human brain, with its unique chemical properties. Likewise, the adaptations for extremophilic conditions that are found in many fungi degrading aromatics may connect with competence in brain infections as well as with the ability to grow in environments artificially polluted with alkylbenzenes. In terms of chemical structure, several brain components resemble alkylbenzene and lignin biodegradation intermediates that are present in air biofilters, and in woody materials of many plants (Fig. 1). Such a correlation has previously been pointed in the case of *C. neoformans*, a fungus that produces melanin from 3,4-dihydroxyphenylalanine (Fig. 1) but lacks the tyrosinase enzyme required for endogenous production of catecholic precursors (Polacheck & Kwonchung, 1988). Thus, an environmental source of dehydroxyphenols is required and the brain might represent a favorite target for this purpose. In case of the *Herpotrichiellaceae*, the biosynthesis of melanin is constitutive and is based on the polymerization of 1,8-dihydroxynaphthalene (Fig. 1) (Butler & Day, 1998). Neurotropism within this family could be explained, at least partly, by the ability to efficiently metabolize catecholic substrates as a carbon source. Species causing clinical infections, such as *Cladophialophora bantiana*, *C. carrionii*, *Exophiala dermatitidis*, *Fonsecaea monophora* and *E. oligosperma*, have often been isolated from

environmental sources that may contain phenolic compounds or hydrocarbons (Fig. 2). The ability to assimilate volatile alkylbenzenes appears to be scattered throughout the *Herpotrichiellaceae*, as is the tendency to cause infections of the brain.

As mentioned above, the species that is most strongly and consistently associated with brain infections is *C. bantiana*. It is interesting that one of our toluene-assimilating strains, CBS 110553, is very closely related to this species, and is either conspecific or represents a closely related sister taxon. Although this strain can readily be shown to be thermo-tolerant, to be melanized and to assimilate aromatics, we do not yet know to what degree it may be pathogenic, although animal testing of this question based specifically on a developed *Cladophialophora* infection model is in progress. CBS 110553 was obtained from the Netherlands, an area where just a single case of brain infection suspected to be caused by an unidentified *Cladophialophora* sp. is known (Meis *et al.*, 1999). The most geographically proximal case of brain phaeohyphomycosis confirmed as being caused by *C. bantiana* (isolate preserved as CBS 155.53) was described in a coal miner from Belgium (Dereymaeker & de Somer, 1955), the nation immediately to the south of the Netherlands. Human-induced proliferation of habitats rich in aromatic hydrocarbons may be in the process of extending the geographic range of *C. bantiana* and other such fungi. Such a range extension process, of course, would affect not just the Netherlands, but other geographic areas, and thus would also potentially have an impact on open biofilters working in these areas.

Biohazard potential of air biofilters

The delineation of the natural habitats of microorganisms involved in human pathogenicity has always been of high scientific interest (Restrepo *et al.*, 2000). This knowledge is important in public health management, as it allows the design of control measures aimed at minimizing the risk of human infection. Similar biohazard management must be done in connection with engineered biological systems, especially those, such as wastewater treatment plants or composting facilities, which are known to enrich for human pathogenic microorganisms (Schaub, 2004; Westrell *et al.*, 2004). The biofiltration of air streams polluted with volatile organic compounds is still an emerging technology, and risk assessment studies are therefore comparatively scarce. In such assessments, consideration must be given both to the pathogenic potential of the organisms encountered, and to the inoculum levels that workers may be exposed to – for example, if containment fails. The enriched growth of potentially pathogenic fungi, coupled with the forced aeration taking place in air biofilters, might result in aerosolization of large quantities of conidia, producing a significant

inhalation hazard. Unpublished data on fungal counts measured in the effluent gas of experimental biofilters loaded with styrene ranged from 2×10^3 to 1×10^4 CFU m⁻³, depending on the runtime since the startup, values that were significantly higher than those measured in the influent air (5×10^1 to 3×10^2 CFU m⁻³). About 1% of the colonies grown from the effluent were identified as *Exophiala jeanselmei*. The same fungus (which, as mentioned above, would probably be identified as *E. oligosperma* in current taxonomy) has also been reported in the emissions derived from a commercial biofiltration unit treating mixtures of volatile organic compounds, including toluene (Florance & Cooke, 2003). Members of the *Herpotrichiellaceae* are rarely reported as airborne fungi (Samson *et al.*, 2000) and, owing to the inherent difficulties of identifying members of this family based only on morphological characters, the biohazard levels that may be associated with spore counts done to date are difficult to evaluate. Also, as mentioned above, ambiguity about the biosystematic relationships, and therefore the degree of shared pathogenicity factors, of some isolates such as *Cladophialophora* sp. strain CBS 110553 impedes evaluation of the biohazard levels associated with aerosols derived from biofilters.

A very poignant question is ‘how can we ensure that biofilters treating volatile aromatic hydrocarbons do not represent a biohazard to the operators and to the population in general?’ Inoculation of biofilters with well-known non-pathogenic hydrocarbon-degrading strains in the *Pseudeurotiaceae* or *Bionectriaceae* clades is one tactic that might be used. Because biofilters function as an open enrichment culture, though, overgrowth of this starting inoculum by other fungi from the environment may pose a problem. Displacement of a nonpathogenic pseudoeurotiaceous strain by an unknown black yeast-like fungus has indeed been observed in biofilters after long-term runs (J. A. van Groenestijn, pers. comm.). Therefore, research on conditions favoring nonpathogen growth in efficiently operating biofilters may also be of value. As previously mentioned, melanization is a very important virulence factor and black yeasts with bioremediation potential could be rendered less pathogenic than wild-type strains by mutagenic suppression of melanin biosynthesis (Cheng *et al.*, 2004).

For evaluation of the biosafety of biofilters treating monoaromatic hydrocarbons, precise taxonomic identification of hydrocarbon-degrading strains introduced or encountered in full-scale installation might be needed, at least for strains able to grow at or near human body temperature. Molecular tools have allowed the detection of herpotrichiellaceous fungi in hydrocarbon-rich environments (Stach & Burns, 2002; Prenafeta Boldú *et al.*, 2004). Improved molecular species concepts, facilitating the distinction of conspecific isolates from members of potentially quite differently behaving sibling species, would be useful. A clear alternative is

producing biofilters that may indeed enrich naturally occurring opportunistic pathogens, but that have containment properties and associated handling protocols ensuring that they do not pose a workplace or disposal hazard.

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