Biosystematics and ecology of hydrocarbon-degrading fungi from air biofilters

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ABSTRACT. The identity of a collection of published isolates obtained from biofilters exposed to volatile aromatic hydrocarbons has been re-evaluated by ribosomal DNA sequence analysis. A number of misidentifications were revealed and hydrocarbon-degrading black yeasts commonly belonged to the closely related genera *Exophiala* and *Cladophialophora*, both anamorphs of *Capronia* in the *Herpotrichiellaceae* family (*Chaetothyriales*). Relatively little is known on the life cycle of these taxa, but based upon a survey on related strains deposited at the CBS collection (Utrecht, The Netherlands) it was evident that they can generally be characterized as micro-extremophiles. They occupy hostile micro-niches, such as the bark of trees, poisonous materials like oil spills or creosoted wood. These ecological features explain the exceptional biodegradation capacity and tolerance towards the growth-limiting conditions present in air biofilters. Some species in the group exhibit a remarkable pathogenic potential, their mycoses in healthy humans sometimes being fatal. Some of the strains examined appear to be closely related to this second group. Hence, additional studies on the potential biohazard of fungal biofiltration of aromatic hydrocarbons are strongly recommended prior to the full-scale implementation of this technique.

1 INTRODUCTION

Biofiltration is considered as a cost-effective technology for the treatment of off-gases containing relatively low concentrations of volatile organic compounds (van Groenestijn and Hesselink, 1993). Initially, biofilters were designed to promote the biodegradative capacity of bacterial populations attached onto the biofilter bed. This configuration faces a number of problems concerning the elimination rate of relatively hydrophobic compounds, such as monoaromatic hydrocarbons, due to their relatively poor diffusion into bacterial biofilms. Moreover, biofilter operational stability is often hampered by acidification and drying out of the filter bed. In order to overcome these inconvenients, biofilters dominated by fungal populations have been proposed. Fungi are generally more resistant to acid and dry conditions than bacteria. Consequently, a less frequent supply of water in combination with a larger contact area between the gas phase and the hydrophobic biomass surface resulting from the filamentous fungal growth improves the substrate mass transfer and the stability of the system (Cox, 1995). The biodegradation of aromatic hydrocarbons by fungi has traditionally been considered to be of a co-metabolic nature, until Cox and co-workers (1993) experimented with the biofiltration styrene-polluted air and identified a number of styrene-utilizing fungi (Cox, 1995). Their findings prompted the interest for the use of fungi in the biofiltration of air polluted with of volatile organic compounds, and have resulted in the isolation of an increasing number of strains growing on volatile aromatic hydrocarbons, mainly on toluene (Weber *et al.*, 1995; Garcia Peña *et al.*, 2001; Prenafeta Boldú *et al.*, 2001; Woertz *et al.*, 2001; Estévez *et al.*, 2005). Several of these isolates belong to the so-called black yeast-like fungi, which are characterized by a heavily melanized thallus and by the ability of dimorphic growth, as filaments or as yeasts, depending on the environmental conditions. The black yeasts are phylogenetically heterogeneous encompassing two different orders of ascomycetes: *Dothideales* and *Chaetothyriales*. These fungi, however, possess few phenetic markers and accurate identification based only on morphology is often troublesome (de Hoog and Guarro, 2000).

In the present study, a number of fungal strains isolated from biofilters and other related projects, that are known to assimilate aromatic hydrocarbons as the sole source of carbon and energy, were collected from authors worldwide and their identity reassessed trough molecular analysis. Based on this information, the phylogenetic relations and ecology of these fungi were discussed.

2 MATERIALS AND METHODS

Fungi with a proven capacity to grow on aromatic hydrocarbons (Table 1) were deposited at the CBS collection (http:/www.cbs.knaw.nl/; Utrecht, The Netherlands). For molecular analysis, fragments of the ribosomal genes encompassing the internal transcriber spacer domains and the 5.8S subunit (ITS1-5.8S-ITS2 rDNA) were amplified and sequenced as previously described (de Hoog *et al.*, 2003). The generated sequences were submitted and blasted into the GenBank database (http://www.ncbi.nlm.nih.gov/; National Center for Biotechnology Information, Maryland), as well as other reference strains in our research databases. For phylogenetic analysis, the sequences were aligned with the ClustalW software (http://www.ebi.ac.uk/; European Bioinformatics Institute, Cambridge, United Kingdom), and a tree was generated with the Treecon program (http://bioinformatics.psb.ugent.be/; Ghent University, Gent, Belgium).

3 RESULTS AND DISCUSSION

3.1 Species identity and phylogeny

The molecular analysis showed that fungi growing on aromatic hydrocarbons predominantly belonged to the closely related genera *Cladophialophora* and *Exophiala*, from the family *Herpotrichiellaceae* in the order *Chaetothyriales*, class *Chaetothyriomycetes* (Table 1). The sexually reproducing states of these genera have been identified in the teleomorph genus *Capronia* (de Hoog and Guarro, 2000). No known species were found to match four of the examined strains (CBS 114326, CBS 110551, CBS 110553, and CBS 110555), indicating that these isolates may belong to species that have yet not been described.

Table 1. Fungal strains with a proven capacity to grow on volatile aromatic hydrocarbons examined in this study. Identities were reassessed by molecular comparisons to type strains.

Original name	*Corrected name*	CBS nr.	*Family*	Growth substrate	Geography	Reference
Phialophora sessilis	*Exophiala jeanselmei*	238.93	*Herpotrichiellaceae*	Styrene	The Netherlands	(Cox, 1995)
Gliocladium roseum	*Bionectria ochroleuca*	102.94	*Bionectriaceae*	Styrene	The Netherlands	(Cox, 1995)
Exophiala oligosperma	*Exophiala jeanselmei*	814.95	*Herpotrichiellaceae*	Styrene	The Netherlands	(Cox, 1995)
Cladosporium sphaerospermum	*Cladophialophora sp.*	114326	*Herpotrichiellaceae*	Toluene	The Netherlands	(Weber *et al.*, 1995)
Cladophialophora sp.	*Cladophialophora sp.*	110551	*Herpotrichiellaceae*	Toluene	The Netherlands	(Prenafeta Boldú *et al.*, 2001)
Pseudeurotium zonatum	*Sporothrix sp.*	110552	*Ophiostomaceae*	Toluene	The Netherlands	(Prenafeta Boldú *et al.*, 2001)
Cladophialophora sp.	*Cladophialophora sp.*	110553	*Herpotrichiellaceae*	Toluene	The Netherlands	(Prenafeta Boldú *et al.*, 2001)
Leptodontidium sp.	*Teberdinia hygrophila*	110554	*Pseudeurotiaceae*	Toluene	The Netherlands	(Prenafeta Boldú *et al.*, 2001)
Exophiala sp.	*Exophiala sp.*	110555	*Herpotrichiellaceae*	Toluene	Germany	(Prenafeta Boldú *et al.*, 2001)
Exophiala lecanii-corni	*Exophiala lecanii-corni*	102400	*Herpotrichiellaceae*	Toluene	Texas	(Woertz *et al.*, 2001)
Pseudallescheria boydii	*Paecylomices sinensis*	115145	*Trichocomaceae*	Toluene	Mexico	(Garcia Peña *et al.*, 2001)
Exophiala oligosperma	*Exophiala oligosperma*	113408	*Herpotrichiellaceae*	Toluene	Spain	(Estévez *et al.*, 2005)
Paecylomices variotii	*Paecylomices sinensis*	113409	*Trichocomaceae*	Toluene	Spain	(Estévez *et al.*, 2005)

3.2 Environmental niches

A very specific biodegradation capacity can frequently be explained by a direct selective reinforcement of enzyme systems related to this ability in nature. In case of toluene and other alkylbenzenes, though often thought of as xenobiotics, it has been demonstrated that they can be biosynthesized by very diverse plants (Heiden *et al.*, 1999). Toluene *in vivo* emissions are significant in the bark of trees, particularly under stress conditions like drought or insect attack, and the content of toluene is particularly high in galleries excavated by bark beetles (Vrkocova *et al.*, 2000). This phenomenon could actually originate from the metabolism of the insects involved, as the biosynthesis of toluene has also been demonstrated for bark engraver beetles (Gries *et al.*, 1990). Insect-excavated galleries in trees are populated by various fungi including several herpotrichiellaceous species in the genera *Exophiala*, *Phialophora*, *Rhinocladiella*, and *Ramichloridium* (Kerrigan and Rogers, 2003). In addition, the biological production of toluene has also been detected during the decomposition of leaf litter (Isidorov *et al.*, 2003).

The molecular examination from Figure 1 confirms that species in the *Herpotrichiellaceae* encompass environmental strains isolated from polluted sites containing petroleum and coal derivatives, plant litter, as well as from arboreal beetle galleries. Members in this group, however, have predominantly been isolated from wooden objects treated with the preservative creosote, such as railway ties and power poles. Creosotes are commonly obtained by high-temperature treatment of coal tar, and are variable, complex mixtures of aromatic hydrocarbons, including toluene and phenols. Phenolic compounds are released during the decomposition of lignin, naturally by the white-rot fungi, or artificially during the pulp bleaching processes for the manufacture of paper, both environments from which herpotrichiellaceous fungi have also been found (Figure 1).

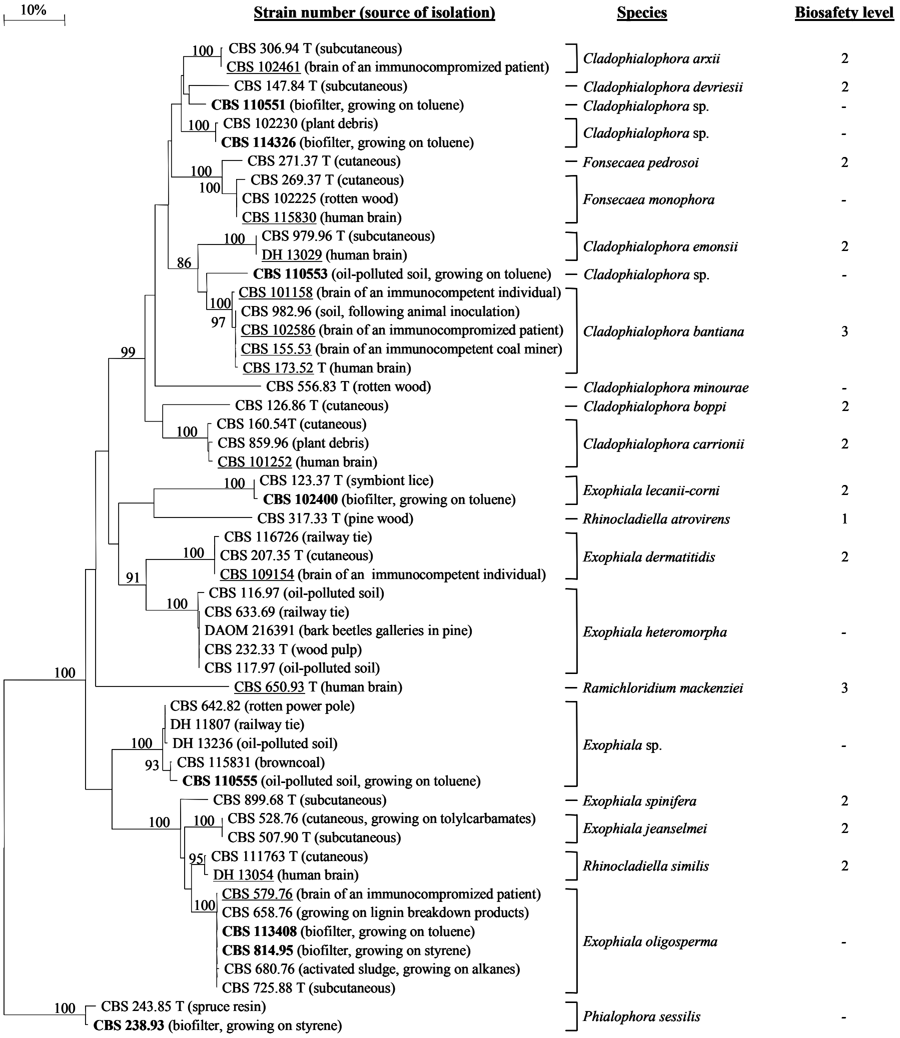

Figure 1. Phylogenetic tree based on confidently aligned ITS1-5.8S-ITS2 rDNA sequences from selected herpotrichiellaceous fungi. The tree was generated with the Neighbor-joining algorithm and the Kimura correction and was bootstrapped 100 times; values above 85 % are indicated near the branches. The *Phialophora sessilis* clade was selected as an outgroup. Reference type strains of described species are indicated as "T"; strains able to grow on aromatic hydrocarbons are indicated in bold and neurotropic isolates are underlined. The biosafety level is rated according to (de Hoog and Guarro, 2000): pathogens potentially able to cause severe deep mycoses in otherwise healthy individuals (3); agents of cutaneous and subcutaneous mycoses, they may cause deep mycoses in immune-compromised patients (2); infections are coincidental, superficial and non-invasive, or mild (1); not defined yet (−). Culture collections: CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands), DAOM (National Mycological Herbarium, Ottawa, Canada), DH: Personal collection of G.S. de Hoog (CBS).

Above and beyond the presence of the same type of susbstrates, the coincidental isolation of related species in biofilters and woody habitats may also be reinforced by the fluctuating physicochemical conditions that characterize both environments. Air biofilters and exposed wood or bark both vary over time in temperature and humidity, and are also low in pH and poor in nutrients. Hence, it is not surprising that fungi isolated from these environments, particularly the in the *Herpotrichiellaceae* clade, tend to be oligotrophic, highly melanized, and thermotolerant. The last two characters, melanization and thermotolerance, are well known virulence factors towards infection of the mammal tissue (de Hoog and Guarro, 2000).

3.3 Human pathogenicity

In addition to the environmental trends described previously, the alkylbenzene-degrading members of the *Herpotrichiellaceae* are close relatives of agents of human invasive disease, affecting immunocompetent as well as immunocompromised individuals (Fig 1). Pathogenicity is particularly remarkable in the genus *Cladophialophora* and nearly all known species cause characteristic types of human disease (de Hoog and Guarro, 2000). One of the *Cladophialophora* isolates assimilating toluene here studied (CBS 110553) is very closely related to *Cladophialophora bantiana*, a species often know from cerebral infections in otherwise healthy patients. Though *C. bantiana* is believed to be acquired by inhalation, primary infection is neurological, occurring only after the fungus has been vascularly translocated to the brain. Therefore, this fungus has been classified as being one of the most dangerous pathogenic fungi known and without combined brain surgery and antifungal drug therapy, the infection is nearly always fatal within months. A comparable tendency towards primary brain infection and virulence has been observed in the species *Exophiala dermatitidis* and *Ramichloridium mackenziei*, but sporadic cases of brain infection have also been caused by *Cladophialophora arxii*, *Cladophialophora carrionii*, *Cladophialophora emmonsii*, *Exophiala oligosperma*, *Fonsecaea monophora*, and *Rhinocladiella similis*. In addition, these and other related species have recurrently been isolated from cutaneous and subcutaneous infections (Figure 1).

Just as growth on alkylbenzenes, the capacity to invade the brain tissue appears to be scattered among the *Herpotrichiellaceae* family and both properties coincides at least in one species, *E. oligosperma* (Figure 1). A possible explanation for the unexpected pattern of association between these two abilities might arise from the high content of monoaromatic catecholamine neurotransmitters like dopamine that accumulate in the mammal brain. Interestingly, aromatic intermediates common in the mammal brain, such as 3,4-dihydroxyphenylacetic, vanillic, and homovanillic acids, are also found as products of lignin degradation (Ebinger and Verheyden, 1976; Tornkvist *et al*., 2004). Although it is, as yet, speculative to say so, a link between brain infection and assimilation of aromatic substrates may exist, and may be one of the factors conferring pathogenic competence on fungi fortuitously seeded to the human brain. 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.

The thermotolerant members of the family *Herpotrichiellaceae* may provide a fungal parallel to the bacterial groups (including *Mycobacteriaceae* such as *Mycobacterium*, *Nocardia* and *Rhodococcus*, as well as *Staphylococcus*, *Pseudomonas*) that are recognized as degraders of aromatic hydrocarbons associated with polluted sites (Burback Brian and Perry Jerome, 1993; Foght *et al*., 1996), and are also well known as important human pathogens more-or-less regularly connected with dissemination of infection to brain tissue (Kim *et al*., 1991; Luby, 1992). *Nocardia*, the only truly filamentous bacterium in this category, also shares the propensity of the *Herpotrichiellaceae* for causing chronic subcutaneous infection (Kwon-Chung and Bennett, 1992). Also, though it is perhaps less relevant, the fast-growing mycobacteria, staphylococci and pseudomonads include species well known for association with abscesses and other deep skin lesions. These parallels suggest a possibility that diverse groups of hydrocarbon-degrading organisms may share some common factors predisposing them to particular patterns of human pathogenicity. Though tentative, the connection appears to be worth exploring as more genomic information becomes available about the groups in question.

3.4 General conclusions

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. 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 resulting in a significant inhalation hazard.

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. 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 developing 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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