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Insights into Hydrocarbon Assimilation by Eurotialean and Hypocrealean Fungi: Roles for CYP52 and CYP53 Clans of Cytochrome P450 Genes

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Abstract Several filamentous fungi are able to concomitantly assimilate both aliphatic and polycyclic aromatic hydrocarbons that are the biogenic by-products of some industrial processes. Cytochrome P450 monooxygenases catalyze the first oxidation reaction for both types of substrate. Among the cytochrome P450 (CYP) genes, the family CYP52 is implicated in the first hydroxylation step in alkane-assimilation processes, while genes belonging to the family CYP53 have been linked with oxidation of aromatic hydrocarbons. Here, we perform a

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comparative analysis of CYP genes belonging to clans CYP52 and CYP53 in *Aspergillus niger*, *Beauveria bassiana*, *Metarhizium robertsii* (formerly *M. anisopliae* var. *anisopliae*), and *Penicillium chrysogenum*. These species were able to assimilate *n*-hexadecane, *n*-octacosane, and phenanthrene, exhibiting a species-dependent modification in pH of the nutrient medium during this process. Modeling of the molecular docking of the hydrocarbons to the cytochrome P450 active site revealed that both phenanthrene and *n*-octacosane are energetically favored as substrates for the enzymes codified by genes belonging to both CYP52 and CYP53 clans, and thus appear to be involved in this oxidation step. Analyses of gene expression revealed that CYP53 members were significantly induced by phenanthrene in all species studied, but only CYP52X1 and CYP53A11 from *B. bassiana* were highly induced with *n*-alkanes. These findings suggest that the set of P450 enzymes involved in hydrocarbon assimilation by fungi is dependent on phylogeny and reveal distinct substrate and expression specificities.

Keywords *Aspergillus niger* · *Beauveria bassiana* · Entomopathogenic fungi · Hydrocarbon degradation · *Metarhizium anisopliae* · *Penicillium chrysogenum*

Introduction

Fungi of the order Eurotiales, such as *Aspergillus*, *Penicillium*, and *Talaromyces* species, are dominant inhabitants of aquatic and terrestrial oil-polluted environments [1, 2]. Such fungi can utilize crude oil, and some aliphatic and polyaromatic hydrocarbons, as substrates and are highly tolerant to hydrocarbon-induced and other stresses, so have considerable potential for bioremediation of polluted sites and other environments [3, 4]. Insect-pathogenic fungi, such as *Beauveria* and *Metarhizium* species, are moderately stress-tolerant and able to degrade and penetrate the insect epicuticle, are rich in aliphatic hydrocarbons, and a source of alkanes, to enter the host [5–8]. However, degradation of hydrocarbons, and tolerance to associated stresses, has been little studied in these hypocrealean fungi and is better characterized for the eurotialean fungi [9, 10].

The assimilation of aliphatic and polyaromatic hydrocarbons by fungi has been the focus of considerable research effort during the past 40 years. For instance, some isolates are highly efficient in their use of hydrocarbons as a sole carbon source, and the relevant enzyme systems and metabolic pathways have been well-characterized [11, 12]. The role of cytochrome P450 monooxygenases in the oxidation of polyaromatic hydrocarbons to arene oxides, which is an initial product of their metabolism, is well-established [13]. A P450 system was also shown to be responsible for hydroxylation of both alkanes and fatty acids during alkane assimilation by the yeast *Candida tropicalis* [14] and by insect-associated fungi that act as either symbionts or pathogens [15, 16]. Cytochrome P450 monooxygenases hydroxylate both alkanes and polyaromatic hydrocarbons, but their oxidized derivatives are then degraded via different mechanisms. The oxidized alkanes undergo β-oxidation and are then further oxidized thereby producing CO_2. By contrast, the oxidized polyaromatic hydrocarbons are degraded via conjugation with polar compounds thereby becoming more bioavailable and less toxic, so are then readily broken down (a process known as conjugation or Phase II detoxification reactions) [17].

Cytochrome P450 (CYP) genes constitute one of the largest gene superfamilies thus far identified in nature, and a special nomenclature has become established for them. CYP genes are assigned into families and subfamilies based on amino-acid sequence identity. A higher

order for grouping CYP genes, called the clan, has been applied to studies of cytochrome P450s from the different domains of life [18]. In fungi, CYPs play pivotal roles in facilitating metabolic versatility of fungi and enabling their adaptation to specific ecological niches. While the CYP51 family is involved in sterol biosynthesis - and thus used as antifungal target for the control of fungal diseases in humans, livestock, and crops - other CYP-gene families (e.g., CYP52 and CYP53) play key roles in metabolism and/or detoxification of hydrocarbons, though there is a paucity of information about the molecular mechanisms which underlie these processes [19, 20].

The gene clan CYP52 consists of 10 gene families; these are CYP52, CYP538, CYP539, CYP584, CYP585, CYP655, CYP656, CYP5087, CYP5113, and CYP5203. The CYP52 family was originally identified in alkane-assimilating yeast, but they are absent in *Saccharomyces cerevisiae* and in basidiomycetes [18]. Members of CYP52 family code for proteins able to oxidize *n*-alkyl chains and that constitute the hydroxylase complex responsible for the first (rate-limiting) step of ω-oxidation of *n*-alkanes and fatty acids in *C. tropicalis* [21] and *B. bassiana* [15]. CYP53 is a single-family clan in ascomycete fungi but absent in ascomycete yeasts. CYP53A1, a representative gene of this family, was first described as a component of the beta-ketoadipate pathway in a strain of *A. niger* [22]. This metabolic pathway is present in several microorganisms that are involved in degradation of aromatic compounds such as those released by plants [23].

The present study was carried out to perform a comparative analysis of CYP genes belonging to clans CYP52 and CYP53 in *A. niger*, *B. bassiana*, *M. robertsii* (formerly *M. anisopliae* var. *anisopliae*), and *P. chrysogenum* with emphasis on CYP52 and CYP53 families. Modeling of the molecular docking of alkanes and polyaromatic hydrocarbons to the cytochrome P450 active site, and gene expression analysis of representative members of each clan, will help to shed light on the potential role of these CYP genes in hydrocarbon oxidation.

Materials and Methods

Fungal Isolates and Maintenance

Fungal isolates *A. niger* van Tieghem (LPSC 526) and *P. chrysogenum* Thom (LPSC 495) were obtained from the culture collection of the Instituto Spegazzini, UNLP, Argentina, *B. bassiana* (Balsamo) Vuillemin (strain GHA) was obtained from a commercial wettable powder formulation from Laverlam International, Butte, USA; and *M. anisopliae* (Metchnikoff) Sorokin (CEP 120) was obtained from the culture collection of the Centro de Estudios Parasitológicos y de Vectores, UNLP, Argentina. Fungi were maintained on potato dextrose agar (PDA) (BD Difco, Sparks, USA) at 26 ºC.

Fungal Growth on Hydrocarbons

For culture-based assays, conidia harvested from PDA were inoculated on complete medium agar (CMA; 0.4 g KH_2PO_4, 1.4 g Na_2HPO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 1 g KCl, 0.7 g $NH_4NO_3 \cdot 7H_2O$, 10 g glucose, 5 g yeast extract, and 15 g agar made up to 1 l with distilled water) and cultivated for 14 days at 26 °C. Conidia harvested from CMA plates and filtered through glass wool to remove hyphal fragments and were suspended in and washed twice with sterile distilled water; the suspension was then adjusted to 10^7 conidia ml^{-1}. Twenty microliters

(per well) of the suspensions were placed in 24-well agar plates with 0.05% *w*/*v* Bromocresol Purple (Biopack, Argentina). Each well contained 1 ml of either CMA or minimal medium agar (MMA), the latter overlaid with *n*-hexadecane, *n*-octacosane, or phenanthrene (Sigma Aldrich, USA) as the sole carbon source. MMA is composed of CMA without the glucose and yeast extract. Once the media had solidified, 100 μl of a 10% *w*/*v* hydrocarbon stock solution in either hexane (alkanes) or dichloromethane (phenanthrene) (Carlo Erba, Italy) was added by pipetting onto the center of MMA wells. Solvents were allowed to evaporate before fungal inoculation with 20 μl of a suspension of 10^7 conidia ml^{-1}. Incubation was performed for 7 days at 26 °C. MMA without hydrocarbons was inoculated with fungal suspension as control. A blank control (no fungus) with MMA + hydrocarbons was also performed. The fungal growth was checked daily in order to follow both mycelial development and fungal sporulation. Images of both sides of the plates (front and back) were recorded at each observation. The experiment was repeated three times.

Homology Modeling

The CYP amino acid sequences from *A. niger* were downloaded from Nelson's P450 Database (http://drnelson.uthsc.edu/CytochromeP450.html) [18]. The CYP amino acid sequences from *P. chrysogenum* are not included in this database and have no official CYP names; thus, they were downloaded from the Fungal Cytochrome P450 Database (p450.riceblast.snu.ac.kr) [24]. The hypocrealean CYP amino acid sequences were downloaded from their respective genomes at the National Center for Biotechnology Information (NCBI). As CYP genes obtained from *M. anisopliae* genome sequences are not classified in the usual CYP nomenclature, the related species *M. robertsii* [25] was used for the assays below. The NCBI accession numbers were ADAH00000000 (*B. bassiana*) and ADNJ02000000 (*M. robersii*). CYP sequences were modeled online using Phyre2 V 2.0 software available at http://www.sbg.bio.ic.ac.uk/phyre2 [26]. The server uses PSI-BLAST to find homolog templates and model the 3D structure of provided sequence accordingly. The coordinates for heme were obtained from the template 1TQN and positioned as in the template in homology-modeled proteins using UCSF Chimera v1.10.2 tool. The modeled proteins were energy minimized by Gromacs steepest descent method using server online available at http://lorentz.immstr.pasteur.fr. The energy-minimized modeled protein was accessed with PROCHECK for geometric evaluation.

Molecular Docking

Docking calculations for target enzymes and *n*-octacosane and phenanthrene compounds were performed using AutoDockVina 1.1.2,32 with a working grid that involved the whole protein surface in order to cover all possible binding sites. The parameters used in AutoDockVina were as follows: number of modes = 8; exhaustiveness = 8; and the MGLTools parameters, which consisted of a grid with a spacing of 1.0 Å, a box size which includes the number of points to contain all the protein, and a central point of the macromolecule in the x, y, and z dimensions. The affinity scoring function in the AutoDockVina docking program was used as a measure of binding affinity between protein and the corresponding ligand. In addition to CYP52- and CYP53-gene clans, the CYP504-gene clan was included in this analysis since (together with clan CYP53) it has been linked with degradation of aromatic substrates [24].The candidate genes for this study and for gene expression (see below) were selected based on previous reports involving with hydrocarbon oxidation (when available, such as CYP52X1,

CYP52X2, CYP53A1, CYP53A11, and CYP53A24). For those species in which no information was available, the genes within each clan were selected randomly.

Gene Expression Analysis by Quantitative PCR

For RNA extractions, fungi were grown in complete medium broth (CMB) for 3 days at 26 °C with aeration (180 rpm), and then harvested and grown for additional 7 days in three different conditions: CMB and minimal medium broth (MMB) supplemented either with *n*-hexadecane (1% *v*/*v*) and *n*-octacosane (1% *w*/*v*) or phenanthrene (1% *w*/*v*) as the sole carbon source. CMB and MMB are composed of CMA and MMA without the agar. The cells were harvesting by centrifugation and used for RNA extraction (see below), and the remaining liquid media was filtered and the pH was measured employing a pH meter Orion model 720A (Orion Research Inc., USA).

Total RNA was extracted from fungi grown in CMB and MMB supplemented with alkanes and phenanthrene employing Tri Reagent (MRC, Cincinnati, USA). For further purification, a clean-up of RNA was performed by using RNAeasy Mini Kit (Qiagen, Hilden, Germany) with an on-column DNA digestion step (DNAse I, Qiagen, Hilden, Germany). RNA was quantified by a NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, USA), and its integrity was assessed on a 1% (*w*/*v*) agarose gel. Two-step real-time polymerase chain reaction (RT-PCR) was carried out with iScript cDNA Synthesis kit and iQ SYBR Green Supermix (Bio-Rad, Hercules, USA). PCR was performed in an Mx3000P QPCR System (Stratagene, La Jolla, USA) employing 40 ng reverse-transcribed total RNA for each sample. The primers used are listed in Table S1. The following amplification program was used: denaturation at 95 °C for 10 min, followed by 40 cycles with three-segment amplification (30 s at 95 °C for denaturation, 30 s at 55 °C for annealing, and 30 s at 72 °C for DNA chain elongation). In order to confirm that only single products were amplified, a temperature-melting step was then performed. Negative controls were performed by using templates generated without reverse transcriptase. Reactions containing primer pairs without template were also included as blank controls. The assay was performed in triplicate. The ΔΔCt method was used to perform a relative quantification, and the statistical analysis was done with the REST software (version 2009, Qiagen) [27].

Results

More abundant mycelia were observed in CMA compared with minimal medium MMA supplemented with with *n*-hexadecane, *n*-hexadecane plus *n*-octacosane, or phenanthrene as the sole carbon source(s), and this was expected given that hydrocarbons impose cellular stress [28–30]. No growth was observed in MMA without any hydrocarbon added, confirming that the fungi must be provided with an exogenous carbon source within the medium. *A. niger* and to a lesser extent, *P. chrysogenum*, acidified the CMA by day 1 according to the medium color (yellowish); then, it reverted to violet (pH > 6.8) from day 3 until the end of the trial (day 7) (Fig. 1a, b). In MMA supplemented with either alkanes or phenanthrene, acidification of the culture media was more evident for *A. niger* than for *P. chrysogenum* (Fig. 1a, b). Moreover, *P. chrysogenum* alkalized the culture medium after growing in CMB supplemented with either *n*-hexadecane (alone or combined with *n*-octacosane) or phenanthrene for 7 days. By contrast, *A. niger* cultured in these same nutrient broths, and for the same period, acidified these media

(Fig. 1f). Regarding growth of *B. bassiana* and *M. anisopliae*, this occurred later in both species compared with that observed for eurotialean fungi. Acidification was also observed by the end of day 3 when the insect-pathogens *B. bassiana* and *M. anisopliae* were grown in CMA (Fig. 1c, d). In MMA supplemented with either *n*-hexadecane (alone or combined with *n*-octacosane) or phenanthrene, *M. anisopliae* changed the color of culture media (pH < 6.8) during growth in all hydrocarbons tested, but *B. bassiana* did so only in MMA added with alkanes (Fig. 1c, d). By day 7, *B. bassiana*, but not *M. anisopliae*, had strongly acidified (pH = 4.4) the MMB supplemented with *n*-hexadecane plus *n*-octacosane (Fig. 1f). No color changes were observed in control plates (Fig. 1e).

In order to understand the enzyme-substrate interaction, sequences belonging to clans CYP52, CYP53, and CYP504 were docked with *n*-octacosane and phenanthrene as selected models of alkanes and polyaromatic substrates, respectively. The negative values of free energy binding indicate an energetically favorable bond between substrates and enzyme active sites. Table 1 shows negative values of binding energy for all the enzymes, suggesting favorable conformations for substrate binding. However, phenanthrene exhibited lower values than *n*-octacosane for all enzymes codified by the genes tested; thus, a more accurate docking in the active sites is expected with the former substrate. A representative member of each CYP52 and CYP53 families was chosen for modeling the interactions between enzymes and both substrates and for gene expression analysis. Docking with phenanthrene (Fig. 2) showed the lowest binding energy for *P. chrysogenum* CYP53PCR017 (− 9.0 kcal/mol) and *B. bassiana* CYP53A11 (− 8.0 kcal/mol); on the other hand, docking with *n*-octacosane (Fig. 3) exhibited more favorable conformations for *A. niger* CYP52Q1 (− 6.6 kcal/mol) and *B. bassiana* CYP53A11 (− 6.2 kcal/mol). The candidate residues that the model predicts to be involved in the interactions are HIS 116, PHE 119, VAL 110, ALA 310, ASN 316, LEU 319, ALA 322, ILE 321, ASN 382, THR 387, and GLY 498.

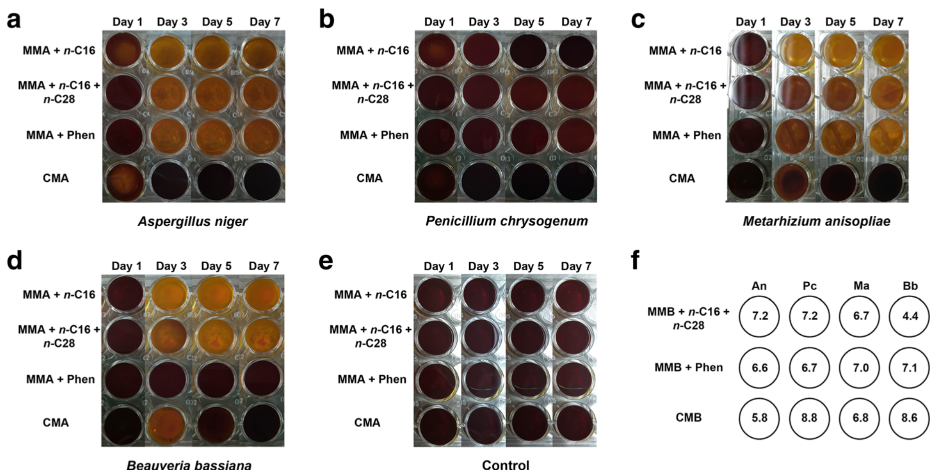

Fig. 1 Underside of 24-well agar microplates containing fungal biomass. **a** *Aspergillus niger*. **b** *Penicillium chrysogenum*. **c** *Metarhizium anisopliae*. **d** *Beauveria bassiana*. **e** Control (which was not inoculated) produced in complete medium agar (CMA) and minimal medium agar (MMA) supplemented with aliphatic and polyaromatic hydrocarbons as the sole carbon source—all media were supplemented with the pH indicator bromocresol purple (0.05% *w*/*v*). **f** pH of liquid cultures at day 7 of fungal growth in complete medium broth (CMB) and minimal medium broth (MMB) supplemented with aliphatic and polyaromatic hydrocarbons as the sole carbon source. *n*-C16: *n*-hexadecane, *n*-C28: *n*-octacosane, Phen: phenanthrene

Table 1 Docking analysis of genes belonging to clans CYP52, CYP53, and CYP504, with selected ligands phenanthrene and *n*-octacosane, for *Aspergillus niger*, *Penicillium chrysogenum*, *Beauveria bassiana*, and *Metarhizium robertsii*

Fungus	Gene	Binding affinity with phenanthrene (kcal/mol)	Binding affinity with *n*-octacosane (kcal/mol)
Aspergillus niger	CYP52Q1	− 8.2	− 6.6
	CYP52P1	− 5.8	− 0.9
	CYP52H4	− 7.6	− 5.7
	CYP52G6	− 7.7	− 5.7
	CYP53A1	− 8.8	− 5.6
	CYP504E6	− 8.9	− 4.9
	CYP539D3	− 7.4	− 5.8
	CYP584E6	− 8.9	− 5.2
	CYP584E7	− 8.0	− 6.0
	CYP584E8	− 8.3	− 5.8
	CYP584H1	− 7.6	− 5.7
	CYP5087B2	− 7.7	− 6.1
Penicillium chrysogenum	CYP52PCR066	− 8.7	− 6.5
	CYP52PCR096	− 7.6	− 5.2
	CYP52PCR051	− 8.4	− 5.9
	CYP52PCR017	− 9.0	− 6.2
	CYP52PCR039	− 8.7	− 6.0
	CYP504PCR024	− 8.5	− 6.4
	CYP539PCR092	− 8.5	− 6.5
	CYP504PCR080	− 8.5	− 6.6
	CYP504PCR067	− 8.1	− 6.7
	CYP53PCR002	− 6.8	− 5.0
	CYP53PCR084	− 7.2	− 4.8
	CYP584PCR055	− 9.2	− 5.2
Beauveria bassiana	CYP52X1	− 7.7	− 5.5
	CYP52G11	− 7.6	− 6.1
	CYP52T1a	− 7.8	− 6.0
	CYP52T1b	− 9.0	− 6.2
	CYP52T1c	− 7.4	− 5.4
	CYP52T1d	− 7.8	− 5.7
	CYP53A11	− 8.0	− 6.2
	CYP504A6	− 7.8	− 5.4
	CYP504B10	− 7.2	− 6.4
	CYP504E1	− 7.2	− 4.8
	CYP504E5	− 7.6	− 4.5
	CYP539B1	− 7.9	− 6.2
	CYP584D4	− 7.3	− 5.6
	CYP584E2	− 7.5	− 4.8
	CYP584E7	− 8.0	− 5.3
	CYP584G1	− 8.2	− 5.8
	CYP584Q1	− 7.0	− 5.1
	CYP655C1	− 8.3	− 5.8
Metarhizium robertsii	CYP52X2	− 7.7	− 5.8
	CYP53A24	− 7.5	− 5.5
	CYP53A25	− 7.7	− 5.7
	CYP504A15	− 8.7	− 5.6
	CYP504A21	− 7.9	− 6.0
	CYP504B7	− 8.0	− 6.4
	CYP504E5	− 7.5	− 4.8
	CYP504H1	− 7.9	− 5.1
	CYP504J1	− 8.7	− 6.4
	CYP539A10	− 8.3	− 6.5
	CYP539B4	− 7.9	− 5.7

Table 1 (continued)

Fungus	Gene	Binding affinity with phenanthrene (kcal/mol)	Binding affinity with *n*-octacosane (kcal/mol)
	CYP539K1	− 8.0	− 6.5
	CYP584E14	− 8.9	− 7.4
	CYP584P1	− 7.5	− 6.2

In all the species studied, the genes from the family CYP53 were marked induced in fungi grown in MMB supplemented with phenanthrene when compared with fungi grown in CMB (Fig. 4), with values of relative expression of 66.7-fold induction (CYP53PCR002, *P. chrysogenum*), 21.6-fold induction (CYP53A1, *A. niger*), and 9.4-fold induction for both CYP53A11 (*B. bassiana*) and CYP53A24 (*M. anisopliae*). In contrast, no induction was observed for some members of CYP52 family, i.e., *B. bassiana* CYP52X1 and *P. chrysogenum*

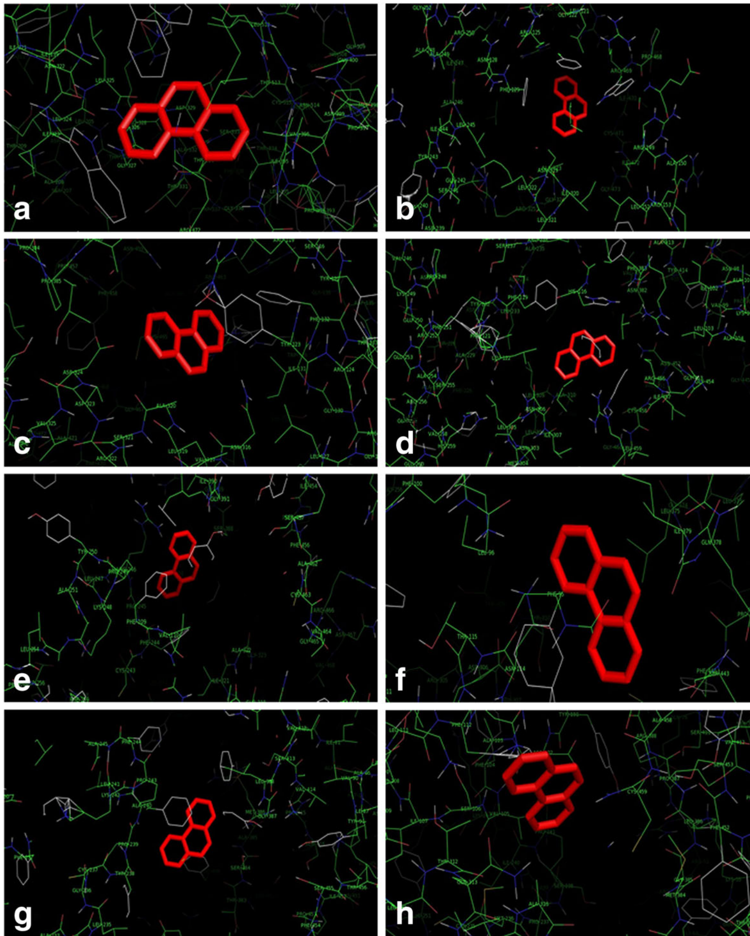

Fig. 2 Molecular docking of phenanthrene with **a** *B. bassiana* CYP52X1, **b** *M. robertsii* CYP52X2, **c** *A. niger* CYP52Q1, **d** *P. chrysogenum* CYP52PCR017, **e** *B. bassiana* CYP53A11, **f** *M. robertsii* CYP53A24, **g** *A. niger* CYP53A1, and **h** *P. chrysogenum* CYP53PCR002, using AutoDock Vina

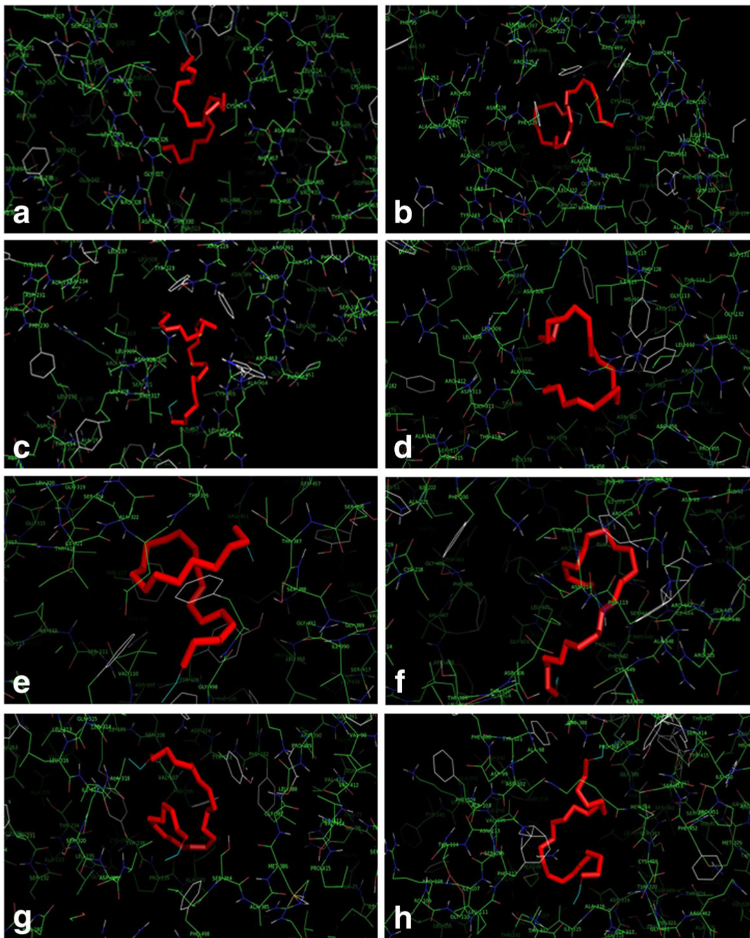

Fig. 3 Molecular docking of *n*-octacosane substrate with **a** *B. bassiana* CYP52X1, **b** *M. robertsii* CYP52X2, **c** *A. niger* CYP52Q1, **d** *P. chrysogenum* CYP52PCR017, **e** *B. bassiana* CYP53A11, **f** *M. robertsii* CYP53A24, **g** *A. niger* CYP53A1 and H *P. chrysogenum* CYP53PCR002, using AutoDock Vina

CYP52PCR017, whereas lower expression levels were observed for *A. niger* CYP52Q1 and *M. anisopliae* CYP52X2 (0.39- and 0.01-fold expression, respectively) grown in MMB supplemented with phenanthrene. In fungi grown in MMB supplemented with a mix of the alkanes *n*-hexadecane and *n*-octacosane, we found a significant induction in *B. bassiana* CYP52X1 (14.5-fold) but not in the other CYP52 family members studied. Concerning the CYP53 family, we observed high levels of transcripts only in *B. bassiana* and *P. chrysogenum*, with relative expression values of 12.5-fold induction for CYP53A11 and 2.9-fold induction for CYP53PCR002, respectively (Fig. 5).

Discussion

Microbial systems are subject to a complex interplay between metabolic responses to stress and upregulation of systems to catabolize xenobiotics and assimilate hydrocarbons. Although

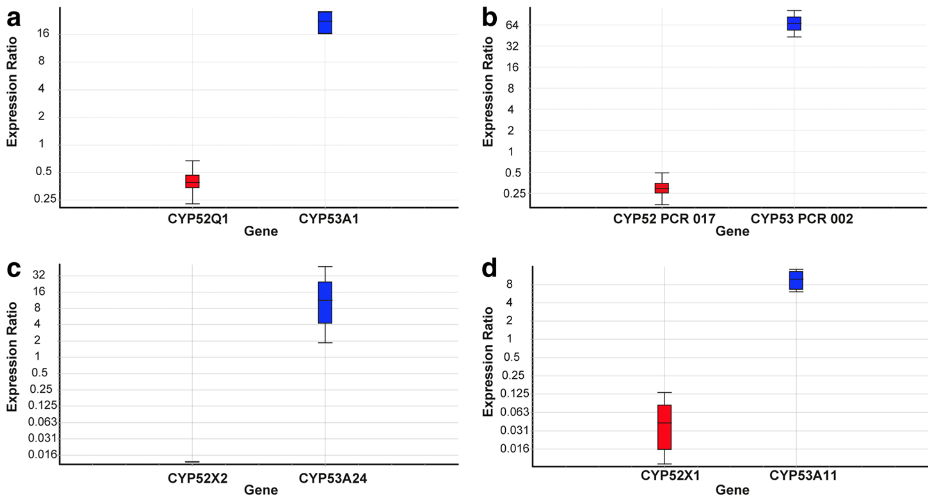

Fig. 4 Boxplots showing gene expression analysis for selected members from families CYP52 and CYP53 in fungi grown on phenanthrene-supplemented media as described in the text. **a** *A. niger*, **b** *P. chrysogenum*, **c** *M. anisopliae*, **d** *B. bassiana*; determined using REST 2009. The box area encompasses 50% of all observations; the dotted line represents the sample median, and the vertical bars represent the outer 50% of observations

fungi assayed in the current study were able to assimilate both types of hydrocarbon, more abundant mycelia were observed in minimal medium added with alkanes rather than with phenanthrene. Only *B. bassiana* was associated with significant acidification of the media after growth in alkanes (pH = 4.4 at day 7). This species is known to secrete virulence factors as part of the pathogenic cycle within insects [31, 32]; for instance, oxalic acid has a pH-dependent activity as a virulence factor against ticks [33]. Oxalic acid secretion was dependent on the

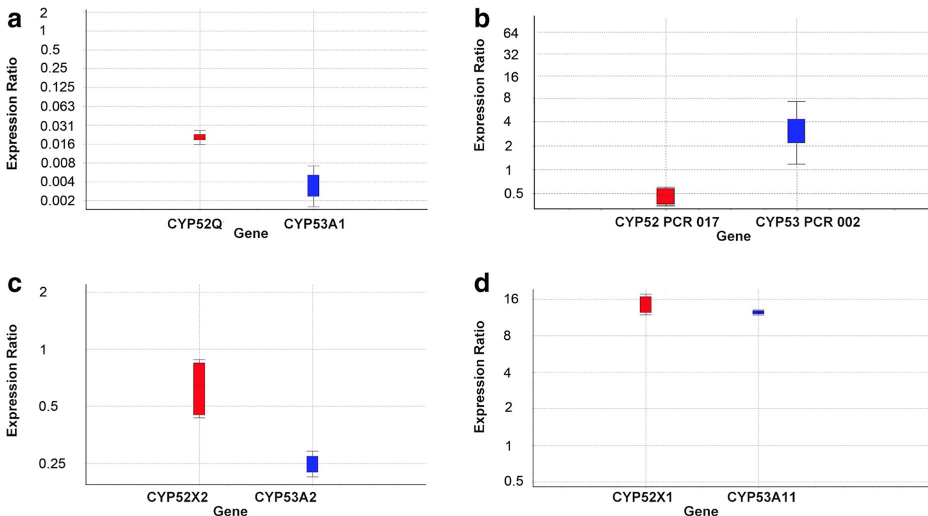

Fig. 5 Boxplots showing gene expression analysis for selected members from families CYP52 and CYP53 in fungi grown on alkane-supplemented media as described in the text. **a** *A. niger*, **b** *P. chrysogenum*, **c** *M. anisopliae*, **d** *B. bassiana*; determined using REST 2009. The box area encompasses 50% of all observations; the dotted line represents the sample median, and the vertical bars represent the outer 50% of observations

media used; a high concentration was produced by *B. bassiana* when cultured in Sabouraud dextrose broth, but none was detected in potato dextrose broth [33].

Although a higher mycelial mass was obtained in alkane-supplemented media compared with phenanthrene-supplemented media, modeling of the molecular docking in all sequences assayed (clans CYP52, CYP53, and CYP504) showed more favorable conformations in their active sites binding phenanthrene than *n*-octacosane. Moreover, a significant increase in the transcription level of CYP53 genes was observed for all fungi when grown in phenanthrene-supplemented media, but only for *B. bassiana* and *P. chrysogenum* grown in alkane-supplemented media. Collectively, the data on fungal growth, molecular docking, and gene expression confirm that other mechanisms, including other enzymes than P450s (or at least other than CYP52 and CYP53 clans), are implicated in hydrocarbon assimilation. Furthermore, both CYP52X1 and CYP53A11 genes were highly induced in *B. bassiana* after 10 days of growing on various *n*-alkanes (ranging from 16- to 28-carbon in length) without aeration [34]. In this study, the fungi were first grown on complete medium for 3 days (until abundant biomass was present), and then on minimal medium supplemented with alkanes for additional 7 days. Thus, the different culture conditions strongly regulate the gene expression. Both CYP53 and CYP504 gene- family members from the entomopathogens *M. anisopliae* and *M. acridum* were upregulated during insect-cuticle infection [35], which suggest a potential participation of the enzymes encoded by these genes in detoxification of the phenylacetate released by both plants and insects (which has antimicrobial properties; [36]) . However, the sole CYP53 gene in *B. bassiana* is also induced in fungi grown either in insect cuticles or synthetic aliphatic hydrocarbons [34].

B. bassiana has six genes belonging to the CYP52 family, whereas *M. anisopliae* and *M. robertsii* have only two (Table S2). Using targeted gene-knockout approaches, members of this family in both *B. bassiana* (CYP52X1) and *M. robertsii* (CYP52X2) have been found to be involved in assimilation of insect epicuticular hydrocarbons [15, 37]. However, only CYP52X1 (not CYP52X2 and CYP52G11 in *B. bassiana*) are involved in virulence against insect hosts [10, 15, 37]. These results, together with the differential expression patterns of P450s observed after *B. bassiana* growth on either insect-derived lipids or synthetic alkanes [34], suggest that cytochrome P450s play multiple roles in cellular properties/processes, such as germination, penetration through the cuticle, and virulence. Another gene belonging to the CYP52 clan that was upregulated in *B. bassiana* grown in both alkanes of different chain length and insect lipids was CYP655C1 [37]. This gene is involved in biosynthesis of the toxic secondary metabolite tenellin, which is produced from aromatic intermediates, acting specifically in an oxidative ring expansion step [38].

Further insight can be gained by considering the CYP63 family that is present in *Phanerochaete chrysosporium* (Basidiomycota) but absent in Ascomycotina representatives. It contains seven members, should be classified under the CYP52 clan [39] according to sequence homology. All seven genes have shown a transcriptional induction with alkanes, mono aromatic and polycyclic aromatic hydrocarbons, and also alkyl-substituted aromatic compounds [40, 41]. One gene of this family, namely CYP63A2, contains one of the largest active-site cavities among P450s, probably responsible for the extraordinary oxidation activity toward larger and diverse substrates, e.g., polyaromatic hydrocarbons of up to six rings, alkyl phenols, and several alkanes [42].

The current study shows that eurotialean fungi can assimilate phenanthrene better than hypocrealean fungi are able to, as revealed by fungal growth, molecular docking, and gene expression analysis of CYP53 family. Further work is needed to confirm the role of these genes in the overall process. Although all species exhibited more abundant mycelial

development after growth on alkanes rather than phenanthrene, the CYP52 family appears to be involved just in *B. bassiana* and *P. chrysogenum*, at least in the conditions tested here. Indeed, there are other questions which have yet to be resolved to better understand this process, including the potential implications for microbes in the context of their natural ecologies where they are exposed to hydrocarbons of plant, microbial, or abiotic origin [43–48].

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