

# Molecular characterization and expression analysis of a suite of cytochrome P450 enzymes implicated in insect hydrocarbon degradation in the entomopathogenic fungus *Beauveria bassiana*

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The insect epicuticle or waxy layer comprises a heterogeneous mixture of lipids that include abundant levels of long-chain alkanes, alkenes, wax esters and fatty acids. This structure represents the first barrier against microbial attack and for broad-host-range insect pathogens, such as *Beauveria bassiana*, it is the initial interface mediating the host–pathogen interaction, since these organisms do not require any specialized mode of entry and infect target hosts via the cuticle. *B. bassiana* is able to grow on straight chain alkanes up to n-C<sub>33</sub> as a sole source of carbon and energy. The cDNA and genomic sequences, including putative regulatory elements, for eight cytochrome P450 enzymes, postulated to be involved in alkane and insect epicuticle degradation, were isolated and characterized. Expression studies using a range of alkanes as well as an insect-derived epicuticular extract from the blood-sucking bug *Triatoma infestans* revealed a differential expression pattern for the P450 genes examined, and suggest that *B. bassiana* contains a series of hydrocarbon-assimilating enzymes with overlapping specificity in order to target the surface lipids of insect hosts. Phylogenetic analysis of the translated ORFs of the sequences revealed that the enzyme which displayed the highest levels of induction on both alkanes and the insect epicuticular extract represents the founding member of a new cytochrome P450 family, with three of the other sequences assigned as the first members of new P450 subfamilies. The remaining four proteins clustered with known P450 families whose members include alkane monooxygenases.

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## INTRODUCTION

Unlike other insect-pathogenic micro-organisms which must be ingested to initiate disease (virus, bacteria, nematodes and protozoa), entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, mostly invade target hosts by penetrating through their cuticle.

**Abbreviations:** AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; CM, complete medium; MM, minimal medium; PDA, potato dextrose agar; STRE, stress response element; *Ti*Lip, lipids extracted from *Triatoma infestans*.

The GenBank/EMBL/DDBJ accession number for the predicted protein products of the *B. bassiana* cytochrome P450 genes are *p450-1*, GU566074; *p450-2*, AM409327; *p450-3*, GU566075; *p450-4*, GU566076; *p450-5*, GU566077; *p450-6*, GU566078; *p450-7*, GU566079; and *p450-8*, GU566080.

A supplementary table of primer sequences and two supplementary figures, showing *B. bassiana* cytochrome P450 gene sequences and amino acid alignments, are available with the online version of this paper.

The outermost insect surface or epicuticle is covered by a lipid-rich layer, usually composed of very long-chain hydrocarbons together with variable amounts of fatty alcohols, fatty acids and wax esters. The epicuticle protects the insect from desiccation, chemical and biological attack, and acts as a platform for host semiochemicals (Blomquist *et al.*, 1987; Figueiras *et al.*, 2009; Juárez, 1994). Entomopathogenic fungi have the ability to degrade insect cuticular lipids with hydrocarbons, the preferred components capable of supporting fungal growth (Napolitano & Juárez, 1997). The first evidence of the complete catabolism of insect-like hydrocarbons by entomopathogenic fungi was obtained from *B. bassiana* and *M. anisopliae* (Crespo *et al.*, 2000). In addition, alkane-grown *B. bassiana* showed enhanced virulence, by both increasing mortality, i.e. a greater proportion of target hosts killed over time (Crespo *et al.*, 2002), and reducing the time to kill its insect hosts (Pedrini *et al.*, 2009). Although several aspects of the biochemistry of the interaction between entomopathogenic

fungi and insect host cuticular hydrocarbons have been examined (Pedrini *et al.*, 2007), there is no information concerning the enzymes involved in the initial steps of alkane hydrocarbon degradation. In yeasts, the first oxidation round of the hydrocarbon substrate is catalysed by a microsomal cytochrome P450 enzyme system (Tanaka & Fukui, 1989). In some instances, besides producing a fatty alcohol as the primary oxidation product, a single cytochrome P450 form has also been shown to catalyse a cascade of monooxidation products (Scheller *et al.*, 1998). Subsequently, the fatty alcohol – or eventually the fatty acid – provides the appropriate fatty acyl-CoA for complete  $\beta$ -oxidation of the hydrocarbon substrate in peroxisomes (Tanaka & Ueda, 1993).

The cytochrome P450 monooxygenases constitute a large superfamily of haem-thiolate proteins widely distributed in different life forms including prokaryotes and lower and higher eukaryotes. Collectively, these enzymes play an important role in the metabolism of both endogenous and xenobiotic compounds, often catalysing conversion of a wide range of lipophilic compounds to more hydrophilic derivatives (Guengerich, 2001). The enzymes specifically involved in terminal hydroxylation of n-alkanes, named P450alk, are expressed from genes belonging to the CYP52 family. These genes have been identified in the yeasts *Candida tropicalis* (Seghezzi *et al.*, 1991, 1992), *C. maltosa* (Ohkuma *et al.*, 1995), *C. apicola* (Lottermoser *et al.*, 1996), *Yarrowia lipolytica* (Iida *et al.*, 1998) and *Debaryomyces hansenii* (Yadav & Loper, 1999).

Based on a previous EST dataset (Cho *et al.*, 2006a, b) and newly constructed libraries, we describe the isolation and characterization of eight cytochrome P450 genes of *B. bassiana*. The molecular characterization of these enzymes resulted in their assignment to one novel cytochrome P450 family and four novel subfamilies. Expression analysis of the genes revealed their differential induction by various long chain alkanes and insect lipids, implying overlapping but discrete functions for each enzyme. The potential role of these enzymes in insect hydrocarbon degradation is discussed.

## METHODS

**Cultivation of fungi.** *B. bassiana* (ATCC 90517) was routinely grown on potato dextrose agar (PDA). Plates were incubated at 26 °C for 10–14 days and aerial conidia were harvested by flooding the plate with sterile distilled water. Conidial suspensions were filtered through a single layer of Miracloth and final spore concentrations were determined by direct count using a haemocytometer. For expression studies, fungal cells were grown in complete liquid medium (CM) containing (l<sup>-1</sup> distilled water) 0.4 g KH<sub>2</sub>PO<sub>4</sub>, 1.4 g Na<sub>2</sub>HPO<sub>4</sub>, 0.6 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0 g KCl, 0.7 g NH<sub>4</sub>NO<sub>3</sub>·7H<sub>2</sub>O, 10 g glucose and 5 g yeast extract. Fungi were also cultivated in minimal medium (MM), composed of CM without the glucose and yeast extract, and supplemented with synthetic hydrocarbons (1% w/v) as follows: n-hexadecane (n-C<sub>16</sub>), n-eicosane (n-C<sub>20</sub>), n-tetracosane (n-C<sub>24</sub>) or n-octacosane (n-C<sub>28</sub>). MM was also added with cuticular lipids extracted from the blood-sucking bug *Triatoma infestans* (TiLip).

The extract was prepared by submerging 600 fifth instar exuviae in hexane [6 ml hexane (g exuviae)<sup>-1</sup>] for 48 h. The extract was transferred to another vial, and reduced in volume under nitrogen. Regardless of the carbon source provided, the fungi grew on the surface of the biphasic incubation media. Flasks were incubated at 26 °C for 10 days without aeration.

**Fungal growth on alkanes.** Fungal spores were harvested from PDA plates directly into sterile distilled water and were washed twice with the same solution; the suspension was then adjusted to 10<sup>7</sup>–10<sup>8</sup> conidia ml<sup>-1</sup> after counting using a haemocytometer. Spore suspensions (5–10  $\mu$ l) were then placed into the middle of microtitre agar plates (24- or 48-well). For 24-well plates, each well contained 1 ml MM in Noble agar overlaid with the desired alkane (0.1 ml of a 10% hydrocarbon stock solution in hexane) as a carbon source.

**Confocal microscopy.** For observation of fungal growth on alkanes, 5–10  $\mu$ l of 10% hydrocarbon solution was spotted and allowed to air dry onto glass coverslips, a suspension (10–20  $\mu$ l, 10<sup>8</sup> conidia ml<sup>-1</sup>) of fungal spores was overlaid on the dried spot and incubated at 26 °C, with high humidity (>80% RH), for 24–48 h. Subsequently, a solution containing the fluorescent dye FM4-64 (1 mM stock solution in ethanol; Invitrogen) was added to the coverslip to a final concentration of 2–10  $\mu$ M. Samples were incubated at room temperature for 1 h prior to visualization. Mounted slides were observed using a Zeiss Axiovert PASCAL LSM5 confocal microscope fitted with Nomarski differential interference contrast optics and an LP560 filter (Ex<sub>543</sub>/Em<sub>560</sub>) or LP505 filter (Ex<sub>488</sub>/Em<sub>518</sub>) for FM4-64 and CDCFDA visualization, respectively. Images were recorded with a real-time digital imaging set-up and were arranged by using Adobe Photoshop or similar software.

**Full-length gene sequence isolation.** P450 gene fragments with high homology to P450alk were identified from previously obtained EST libraries (Cho *et al.*, 2006a, b). To obtain full-length cDNA sequences for cytochrome *p450-1* to *-7*, fungal cells were ground in liquid nitrogen and total RNA was extracted using an RNeasy plant mini kit (Qiagen). Several aliquots of total RNA isolated from several samples were pooled in order to construct a representative cDNA library. Full-length sequences were isolated by 5'- and 3'-rapid amplification of cDNA ends (Clontech). Amplified products were cloned into pGEM-T Easy vector system (Promega) and transformed into JM-109 cells. The presence of inserts was detected using the blue/white screening on X-Gal/LB agar plates. The gene-specific primers used are shown in Supplementary Table S1 (available with the online version of this paper). The full-length cDNA sequence for the *B. bassiana* cytochrome *p450-8* and the genomic DNA sequences for all of the P450s were obtained from Drs S. H. Ying and M. G. Feng, Institute of Microbiology, Zhejiang University, Hangzhou, China (*B. bassiana* whole genome sequencing, in progress).

**Sequence analyses.** The sequences were analysed using programs implemented at the Biology WorkBench website (<http://workbench.sdsc.edu/>); splice site analysis was performed using the ESLICE program at the SoftBerry server (<http://www.softberry.com/berry.phtml>). Introns were detected by visual comparison to both cDNA and DNA sequences, in zones where the former were available, by either EST or full-length cDNA. Coding sequences were translated to amino acid sequences and were aligned using the ExpASy tools (ExpASy Proteomics Server). Phylogenetic analysis was performed with the Phylogeny.fr platform (Dereeper *et al.*, 2008), which uses the programs MUSCLE for multiple alignment, Gblocks for automatic alignment curation, PhyML for tree building and TreeDyn for tree drawing.

The sequences obtained were submitted both to GenBank for gene annotation and to David Nelson (University of Tennessee, Memphis,

USA) for assignment of the official cytochrome P450 names, based on criteria established by the P450 Nomenclature Committee (<http://drnelson.uthsc.edu/CytochromeP450.html>).

**Gene expression analysis.** Total RNA was obtained as described above, which included an on-column DNA digestion step. RNA was quantified by using the RiboGreen RNA quantification kit (Molecular Probes), and the integrity was assessed on a 1% (w/v) agarose gel. Two-step RT real-time PCR was carried out with the iScript cDNA synthesis kit and iQ SYBR Green supermix (Bio-Rad). The primers used are shown in Supplementary Table S1. Amplification was performed in an iCycler Thermal Cycler (Bio-Rad), employing 20 ng reverse-transcribed total RNA for each sample (CM, n-C<sub>16</sub>, n-C<sub>20</sub>, n-C<sub>24</sub>, n-C<sub>28</sub> or *TiLip*). The following amplification programme 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 64 °C for annealing and 30 s at 72 °C for polymerase elongation). In order to confirm that only single products were amplified, a temperature melting step was then performed. The calibration curve method was used for the analysis of data obtained from the RT-PCR system, with ribosomal 5.8S RNA as the housekeeping gene. Negative controls were performed by using 'cDNA' generated without reverse transcriptase as templates. Reactions containing primer pairs without template were also included as blank controls. The assay was done in duplicate for each of the three independent biological replicates performed.

## RESULTS

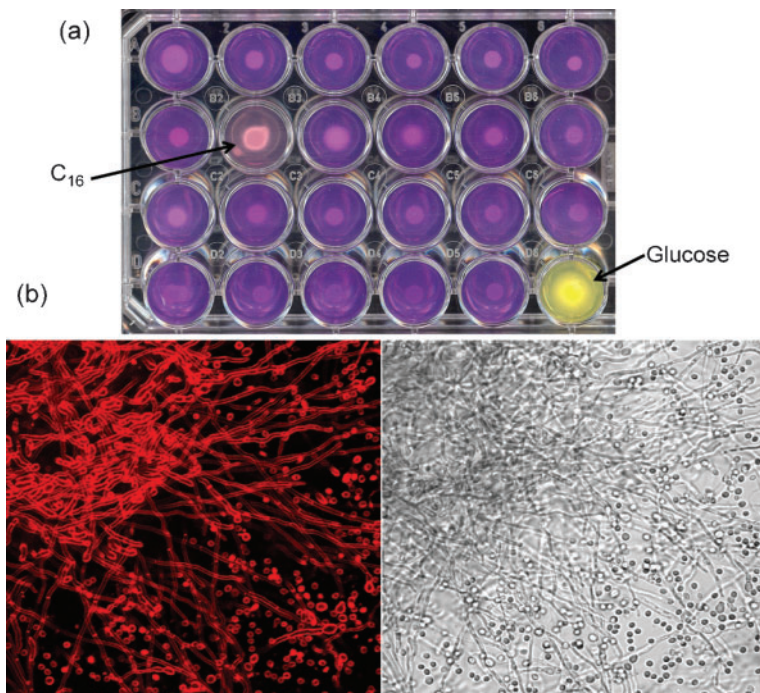
### Fungal growth on alkanes

Although *B. bassiana* is capable of growing on insect cuticular hydrocarbons, the chain length range that could support growth of the fungus as the sole source of carbon and energy is not known. Therefore, the growth of *B. bassiana* on a range of aliphatic alkanes, including n-C<sub>9-24</sub>,

n-C<sub>26-28</sub>, n-C<sub>30,31,33,35,39,41</sub>, in both liquid and solid media conditions was tested (Fig. 1a). *B. bassiana* was able to grow on all alkanes tested as the sole carbon source except for the three longest chain alkanes, n-C<sub>35</sub>, 39, 41, with the mid-range alkanes (around n-C<sub>16</sub>) appearing to be the preferred alkane substrate for growth as a sole carbon source. Some growth on the three longest chain alkane substrates was noted when spores harvested from n-C<sub>20</sub>-grown cells were used as the inocula, indicating that higher chain alkanes may be degraded under specific conditions, or growth on these substrates may be an adaptive response. Directed and higher density hyphal growth towards the alkane nutrient source could also be visualized in experiments in which fungal conidia were overlaid and/or placed near the alkane substrate source (Fig. 1b). Attempts at visualizing potential hydrocarbon transport by incubation of the fungal cells growing on alkanes (n-C<sub>16</sub>) with the lipophilic dye FM4-64 resulted in bright membrane staining; however, no internal lipid bodies could be detected under the conditions used. Similar directed and higher density growth was seen in experiments using C<sub>20</sub>, C<sub>22</sub> and C<sub>28</sub>, as well as with *TiLip*.

### Full-length sequences and nomenclature

A suite of cytochrome P450 gene fragments (*p450-1* to *p450-8*) was identified in a set of EST libraries, with sequence lengths varying from 207 to 736 bp. Full-length cDNA genomic sequences were obtained as described in Methods. The gene *p450-2* was coincident with one of the genes belonging to a previously characterized contig of 21 932 bp (GenBank accession no. AM409327), which is



**Fig. 1.** (a) Growth of *B. bassiana* inoculated in the centre of microtitre wells containing MM, bromocresol purple (0.05%) and the indicated n-alkane (0.1% w/w) as the sole carbon source. Top row, C<sub>9</sub>–C<sub>14</sub>; row 2, C<sub>15</sub>–C<sub>20</sub>; row 3, C<sub>21</sub>–C<sub>24</sub>, C<sub>26</sub>, C<sub>27</sub>; row 4, C<sub>28</sub>–C<sub>31</sub>, C<sub>33</sub>, glucose. (b) Differential interference contrast and fluorescence images of FM4-64 stained fungal cells growing on C<sub>16</sub>. The droplet containing the alkane is in the upper left-hand corner of the image.

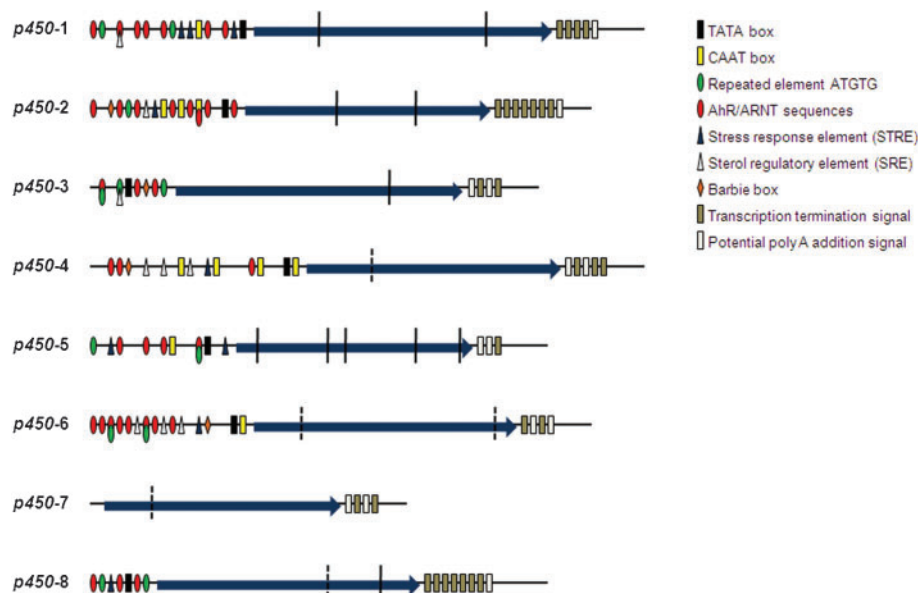
involved in the biosynthesis of the 2-pyridone tenellin in *B. bassiana* (Eley *et al.*, 2007).

Based on the analysis of the predicted protein products of the *B. bassiana* cytochrome P450 gene, according to the established criteria of the P450 Nomenclature Committee, *p450-3* was found to represent the first member of a novel cytochrome P450 family, whereas *P450-1*, *p450-6* and *p450-8* were assigned as the first genes belonging to three novel subfamilies. The official names assigned were CYP52X1 (*p450-1*, GenBank accession no. GU566074), CYP655C1 (*p450-2*, AM409327), CYP5337A1 (*p450-3*, GU566075), CYP52G11 (*p450-4*, GU566076), CYP539B5 (*p450-5*, GU566077), CYP617N1 (*p450-6*, GU566078), CYP53A26 (*p450-7*, GU566079) and CYP584Q1 (*p450-8*, GU566080).

### Sequence analysis

Other than in *p450-7*, for which not enough 5' flanking sequence was isolated, a variety of genetic elements and repeated sequences were found in the upstream region of the genes (Fig. 2 and Supplementary Fig. S1, available with the online version of this paper). Among these, a pentanucleotide repeat element ATGTG (or its complement CACAT) appeared in *p450-2* at position -939, twice in *p450-1* (beginning at positions -613 and -1033), *p450-5* (-413 and -991), *p450-6* (-561 and -864) and *p450-8* (-29 and -266), and three times in *p450-3* (-19, -322 and -434). These sequences have been previously reported in promoters of P450alk (alkane-specific) genes of the yeast *C. maltosa* (Ohkuma *et al.*,

1995) and *D. hansenii* (Yadav & Loper, 1999). A TATA box was present in the putative promoters beginning at -152 (*p450-1*), -216 (*p450-2*), -277 (*p450-3*), -131 (*p450-4*), -374 (*p450-5*), -57 (*p450-6*) and -131 (*p450-8*). Also, CCAAT box sequences were detected at positions -438 (*p450-1*), -353, -381 and -509 (*p450-2*), -88, -377, -745 and -874 (*p450-4*), -562 (*p450-5*) and -49 (*p450-6*). Several sequences belonging to the core binding motif of the xenobiotic responsive element, which participates in the induction of CYP genes via interactions with the aryl hydrocarbon receptor (AhR) and the AhR nuclear translocator (ARNT) were identified (Swanson, 2002; Tsuchiya *et al.*, 2003). The sequences CACGC or CANNTG, which are potentially recognized by the AhR/ARNT heterodimer and/or the corresponding homodimers, were found at positions -352, -432, -687, -794, -935, -982 and -1035 (*p450-1*), -105, -299, -351, -360, -427, -862, -1005 and -1173 (*p450-2*), -200, -322 and -437 (*p450-3*), -428, -1416 and -1460 (*p450-4*), -412, -633, -773 and -881 (*p450-5*), -444, -517, -561, -657, -846, -865, -1061 and -1107 (*p450-6*), and -78, -150 and -408 (*p450-8*). Also, the pentanucleotide CCCCT (or its complement), corresponding to the stress response element (STRE), was found in all the genes. This sequence was repeated three times in *p450-1* (at positions -235, -505 and -540), once in *p450-2* (-554), *p450-4* (-799), *p450-6* (-222) and *p450-8* (-206), and twice in *p450-5* (-61 and -893). The STRE has been described in several promoters of *Saccharomyces cerevisiae*, and it is related to gene induction by xenobiotic agents and environmental and physiological conditions (Treger *et al.*, 1998). This element was also found in promoters for the P450alk gene of the yeast *D. hansenii*



**Fig. 2.** Scheme of *B. bassiana* P450 genes. The ORFs are represented by a blue arrow. Vertical lines mark positions of predicted (dashed line) and confirmed (solid line) introns. Genetic elements and repeated sequences in both 5' and 3' non-coding regions are shown in different colours.

(Yadav & Loper, 1999), but as far as we know, it was not previously described in P450 promoters of filamentous fungi. Other putative regulatory sequences (Barbie box, sterol regulatory elements) detected in the 5' upstream region of some genes are shown in Fig. 2 and Supplementary Fig. S1. The region surrounding the start codon contains a purine (G or A) at the -3 position, as originally reported for 18 highly expressed genes in *S. cerevisiae* (Hamilton *et al.*, 1987), and later in fungal CYP genes (Iida *et al.*, 1998; Ohkuma *et al.*, 1995; Seghezzi *et al.*, 1991; Yadav & Loper, 1999; Yadav *et al.*, 2003). The 3' non-coding region of all the genes was characterized by the presence of a polyadenylation formation signal (Fig. 2). Only *p450-7* exhibited the common conserved AATAAA-3' sequence, whereas the other P450 genes showed different hexameric variants (Graber *et al.*, 1999; Shen *et al.*, 2008) (Supplementary Fig. S1), confirming that this signal is less conserved in fungi (Shen *et al.*, 2008). Variants of the tripartite signal [5'-TAG...TA(T/A)GT...TTT], which indicate potential transcription termination, were also found in the 3' flanking region (Supplementary Fig. S1). A local alignment between the 5' sequences available for the examined P450 genes found no obvious novel common sequence element.

All the genes analysed contain at least one intron (Fig. 2, Supplementary Fig. S1). *p450-1*, *p450-2*, *p450-6* and *p450-8* have two introns each; sizes were 109 and 167 bp (*p450-1*), 62 and 64 bp (*p450-2*), 65 and 56 bp (*p450-6*), and 68 and 69 bp (*p450-8*). *p450-3*, *p450-4* and *p450-7* contain one intron each with lengths of 77, 61 and 66 bp, respectively. *p450-5* has five small introns of 47, 49, 54, 57 and 78 bp. All the exon/intron boundaries conformed to the canonical GT/AG donor/acceptor rule.

### Deduced proteins

Based upon the isolated cDNA sequences as well as conceptual translation of the genomic DNA sequences using predicted splicing of identified introns, ORFs with typical lengths and domain structures of cytochrome P450s were reconstructed (Supplementary Fig. S2). The length, estimated molecular mass and isoelectric point for each enzyme are shown in Table 1. All the deduced proteins had at least one hydrophobic transmembrane segment (ranging from 17 to 24 aa) as predicted by TMpred analysis, indicating the membrane-bound nature of the P450s. The alignment showed a lower identity in the N-terminal region than in the distal extreme. The three conserved regions helix-I, the site for alkane-binding (Sanglard & Loper, 1989; Yadav & Loper, 1999), helix-K and HR-2 (haem-binding) are boxed in Supplementary Fig. S2.

### Phylogenetic analysis

A phylogenetic analysis of the eight P450 deduced amino acid sequences isolated from *B. bassiana* was performed using the Phylogeny.fr platform (Dereeper *et al.*, 2008). The tree was constructed to include one member of each

**Table 1.** Properties of deduced *B. bassiana* cytochrome P450 monooxygenase proteins

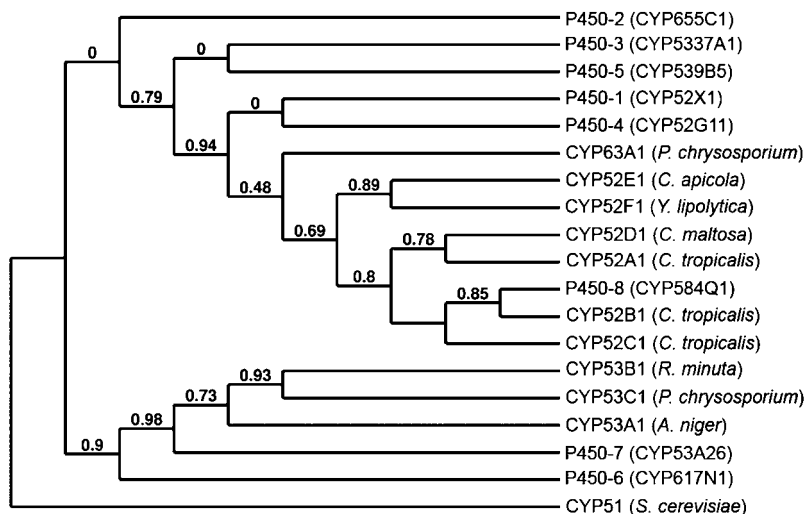
Protein	Length (aa)	Molecular mass (kDa)	pI	Transmembrane segment position*
P450-1	528	59.5	7.72	2–26
P450-2	508	58.4	8.45	8–29
P450-3	534	58.3	8.82	1–20
P450-4	528	59.3	8.24	2–18
P450-5	515	58.7	6.48	10–32
P450-6	552	61.0	6.45	11–29, 36–62
P450-7	513	57.6	7.66	6–26
P450-8	539	61.3	8.32	20–39

\*Position in no. of amino acids from N-terminus, starting Met.

CYP52 subfamily (P450alk) from yeast, and members of the CYP53 subfamily (benzoate hydroxylase), and the CYP63 subfamily from filamentous fungi. The CYP51 from *S. cerevisiae* (lanosterol 14-demethylase) was used as reference, since it is the most conserved P450 gene common to all fungi (Aoyama *et al.*, 1996). The *B. bassiana* sequences separate into two distinct clusters (Fig. 3). The first, consisting of the CYP53 family, included *B. bassiana* P450-6 and P450-7. The second major cluster included the CYP52 and 63 families; however, only P450-8 clustered within these two families, whereas the rest of the *B. bassiana* P450 proteins, (P450-1 to -5) subclustered together but appeared distantly related to P450-8.

### Cytochrome P450 induction by alkanes and insect lipids

The expression patterns of the eight *B. bassiana* cytochrome P450 genes isolated were examined by quantitative PCR. In order to examine whether the P450 genes were specifically induced by alkanes, fungal cultures were grown on n-hexadecane (n-C<sub>16</sub>), n-eicosane (n-C<sub>20</sub>), n-tetracosane (n-C<sub>24</sub>), or n-octacosane (n-C<sub>28</sub>) as the sole carbon source and the expression patterns of the P450 genes were compared with cells grown on CM (glucose) (Fig. 4). In all instances, the expression pattern of ribosomal 5.8S RNA was used as an internal control. The expression of *B. bassiana p450-1* was slightly induced by growth on the shorter chain hydrocarbons (n-C<sub>16</sub> and n-C<sub>20</sub>), displayed ~10-fold induction by the intermediate chain length alkane (n-C<sub>24</sub>), but was induced almost 100-fold by the very long chain alkane (n-C<sub>28</sub>). In contrast, *p450-2*, *p450-3*, *p450-6* and *p450-7* expression was greatly induced by all alkanes tested (at least more than 50-fold), with *p450-3* showing the largest fold induction (300–600-fold) of the genes examined when *B. bassiana* was grown on all of the alkane substrates tested. Induction of *p450-4* expression was nominal under the conditions tested but appeared somewhat enhanced during growth on the highest alkane chain lengths tested (three- to fourfold after growth on n-C<sub>24</sub> and n-C<sub>28</sub>). In contrast with the other P450 genes



**Fig. 3.** Phylogenetic tree of the *B. bassiana* P450 genes. The analysis was based on deduced amino acid sequences of P450 genes with known function from different species of yeast and filamentous fungi. Phylogenetic analysis was performed with the Phylogeny.fr platform as described in Methods. Numbers at nodes indicate bootstrap values. CYP51 (*S. cerevisiae*) was used as reference since it has the most conserved P450 gene that is common to all fungi.

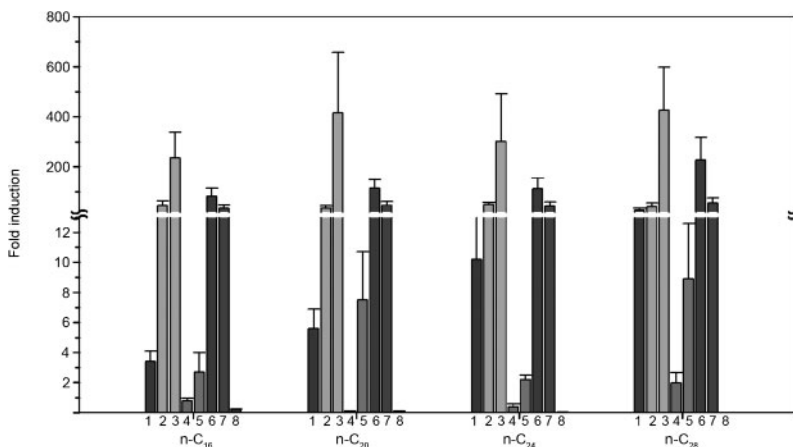
examined, induction of *p450-5* did not seem to follow a trend; cells grown on  $n\text{-C}_{20}$  and  $n\text{-C}_{28}$  were induced ~10-fold and slightly lower induction was observed when the cells were grown on  $n\text{-C}_{16}$  and  $n\text{-C}_{24}$  (two- to fourfold), although overall induction remained low in the presence of the alkanes tested compared with some of the other P450 genes. Little to no induction of *p450-8* was observed in any of the alkane growth conditions examined.

In order to examine the expression of the P450 genes induced by insect-derived lipids, *B. bassiana* was grown on a cuticular lipid extract obtained from the blood-sucking bug *T. infestans*, as described in Methods. Three distinct induction profiles were noted: highly induced *B. bassiana* P450 genes (>200 fold), including *p450-2* and *p450-6*; genes moderately induced (>30-fold by the insect extract), including *p450-1*, *p450-3* and *p450-7*; and genes that displayed low to no induction, *p450-4* (fourfold), *p450-5* (12-fold) and *p450-8* (no induction) (Fig. 5). These values were similar to those obtained with the synthetic alkanes, although *p450-3* induction was noticeably higher when

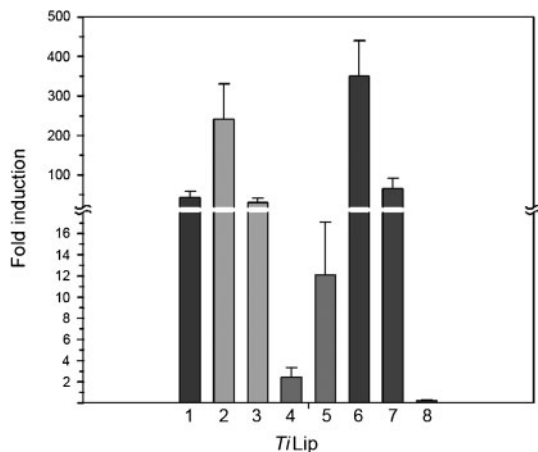
grown on the alkanes than on the insect-derived lipid, whereas the insect lipid was the best inducer for *p450-5*.

## DISCUSSION

The entomopathogenic fungus *B. bassiana* belongs to a group of broad-host-range insect pathogens that do not require any specialized route of infection. Fungal infection of hosts begins with cuticular attachment and degradation, followed by penetration and proliferation within the insect body (Holder & Keyhani, 2005; Holder *et al.*, 2007; Wanchoo *et al.*, 2009). Although interaction with the outer insect waxy layer (epicuticle) is the first step in pathogenesis, the fungal genes that underlie insect waxy layer degradation remain almost completely unexplored. Our results show that *B. bassiana* is capable of growing on straight chain alkanes up to  $n\text{-C}_{33}$ , as the sole source of carbon and energy. The lipophilic dye FM4-64 has been previously used to examine nutrient uptake in *B. bassiana* (Lewis *et al.*, 2009), and here we have used it to show



**Fig. 4.** *B. bassiana* cytochrome P450 gene expression analysis as determined by qRT-PCR during growth on various alkanes normalized to expression during growth on glucose-containing media as described in the text. Columns indicate analysis of *p450-1* to *-8*. Values are means  $\pm$  SEM.



**Fig. 5.** Expression analysis of *B. bassiana* cytochrome P450 during growth on *T/Lip*. Columns indicate analysis of *p450-1* to *-8*. Values are means  $\pm$  SEM.

directed growth of *B. bassiana* hyphae towards the alkane source.

Cytochrome P450s constitute a superfamily of haem-thiolate monooxygenases that act on a wide variety of endogenous and xenobiotic molecules, of which alkanes, a major hydrocarbon component of many insect cuticles, represent a subset of the substrates for these enzymes (Montellano, 2005). The pathway for alkane oxidation in eukaryotic micro-organisms begins with a cytochrome P450 enzyme system. A P450 monooxygenase (P450alk or alkane hydroxylase), acting together with an NADPH cytochrome P450 reductase, was initially described in a number of yeasts, with their distribution in filamentous fungi being scarce (Tanaka & Fukui, 1989; van Beilen *et al.*, 2003). Our results, the first regarding P450alk genes in entomopathogenic fungi, indicate that *B. bassiana* contains at least seven or eight P450alk genes, corresponding to seven different families. The expression pattern of the enzymes was examined using long chain alkanes as well as epicuticular lipids extracted from *T. infestans*. This latter growth substrate was composed of 37% hydrocarbons, 29% triacylglycerols, 15% fatty alcohols, 8% free fatty acids and 7% waxes, and our strain of *B. bassiana* displays similar levels of virulence to this host, as has been reported for other *B. bassiana* strains (Juarez & Fernandez, 2007; Pedrini *et al.*, 2009). Additional cytochrome P450 sequences were also detected in the available EST dataset; however, they were not included in this study because of their low homology with known P450alk sequences.

Other than *p450-1*, all of the genes contained small introns, in agreement with what has been reported for the CYP63 family of the fungus *Phanerochaete chrysosporium* (ranging from 35 to 82 bp) (Yadav *et al.*, 2003). The introns found in *p450-1* were similar to those observed for CYP53B1 of *Rhodomonas minuta* (Fujii *et al.*, 1997) and CYP53C2 in *P.*

*chrysosporium* (Matsuzaki & Wariishi, 2005), which ranged from ~50 to ~250 bp. Within a P450 family, the intron–exon organization has been proposed to be conserved (Gotoh, 1993); however, this was not the case for all of the *B. bassiana* genes examined. For example, *p450-7* is a member of the CYP53 family, which normally contain multiple introns (CYP53B1 of *R. minuta* has seven), but the *B. bassiana* gene has only one intron. Furthermore, *p450-1* has introns similar in length to those observed in the CYP53 family; however, phylogenetically this gene clusters remotely from this family.

*B. bassiana p450-3* represents the first member of a new cytochrome P450 family, CYP5337, and it also was the gene whose expression was most highly induced in response to the hydrocarbons tested. In contrast, *B. bassiana p450-4*, classified within the alkane-substrate-utilizing CYP52 family, was not highly induced under any of the conditions tested, unlike in alkane-utilizing yeasts (van Beilen *et al.*, 2003). However, *p450-1*, also a CYP52 family member, was induced by the longer chain alkanes *n*-C<sub>24</sub> and *n*-C<sub>28</sub>, as well as by the insect epicuticular extract, but was not induced significantly by the shorter chain alkanes tested.

*B. bassiana p450-2*, *p450-6* and *p450-7* were induced by all the substrates tested, including the insect-derived extract, reaching ~250–350-fold induction levels, suggesting an important role for these genes in the first step of insect lipid oxidation. Intriguingly, *p450-2* has been previously demonstrated to be involved in tenellin synthesis (with aromatic intermediates) (Halo *et al.*, 2008). Although further experiments are needed, our results might suggest multiple roles (i.e. diverse substrates) for some P450s in different cell processes.

In conclusion, our data indicate that *B. bassiana* probably contains multiple hydrocarbon-assimilating cytochrome P450 enzymes that may display overlapping substrate specificities. Molecular approaches, including targeted gene knock-outs of specific P450 genes, in order to examine their substrate specificities and confirm their role in insect hydrocarbon degradation, are warranted. Furthermore, these genes would be appropriate potential candidates for overexpression in *B. bassiana* in order to increase its targeting and virulence towards insect pests.

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