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# CYP52X1, Representing New Cytochrome P450 Subfamily, Displays Fatty Acid Hydroxylase Activity and Contributes to Virulence and Growth on Insect Cuticular Substrates in Entomopathogenic Fungus *Beauveria bassiana*[*] [S]

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**Background:** The lipid-rich insect epicuticle mediates the initial interaction with microbial pathogens.
**Results:** A novel cytochrome P450, CYP52X1, implicated in cuticular hydrocarbon assimilation was characterized from *Beauveria bassiana*.
**Conclusion:** CPY52X1 displays fatty acid hydroxylase activity, contributes to cuticle penetration, but is dispensable for virulence once the cuticle has been breached.
**Significance:** These results expand the enzymatic repertoire entomopathogenic fungi express in targeting insects.

**Infection of insects by the entomopathogenic fungus *Beauveria bassiana* proceeds via attachment and penetration of the host cuticle. The outermost epicuticular layer or waxy layer of the insect represents a structure rich in lipids including abundant amounts of hydrocarbons and fatty acids. A member of a novel cytochrome P450 subfamily, CYP52X1, implicated in fatty acid assimilation by *B. bassiana* was characterized. *B. bassiana* targeted gene knockouts lacking *Bbcyp52x1* displayed reduced virulence when topically applied to *Galleria mellonella*, but no reduction in virulence was noted when the insect cuticle was bypassed using an intrahemoceol injection assay. No significant growth defects were noted in the mutant as compared with the wild-type parent on any lipids substrates tested including alkanes and fatty acids. Insect epicuticle germination assays, however, showed reduced germination of Δ*Bbcyp52x1* conidia on grasshopper wings as compared with the wild-type parent. Complementation of the gene-knock with the full-length gene restored virulence and insect epicuticle germination to wild-type levels. Heterologous expression of CYP52X1 in yeast was used to characterize the substrate specificity of the enzyme. CYP52X1 displayed the highest activity against midrange fatty acids (C12:0 and C14:0) and epoxy stearic acid, 4–8-fold lower activity against C16:0, C18:1, and C18:2, and little to no activity against C9:0 and C18:0. Analyses of the products of the C12:0 and C18:1 reactions confirmed NADPH-dependent regioselective addition of a terminal hydroxyl to the substrates (ω-hydroxylase). These data implicate CYP52X1 as contributing to the penetration of the host cuticle via facilitating the assimilation of insect epicuticle lipids.**

Entomopathogenic fungi such as *Beauveria bassiana* play major roles in the natural regulation of insect populations, often responsible for spectacular epizootic infections. As a broad host range pathogen, strains of *B. bassiana* have been exploited for use against agricultural pests and for insects that act as human and animal disease vectors (1–4). Infection of insects begins with attachment of fungal spores to the cuticles of target hosts, and in response to surface cues the fungus germinates; the emerging germ tubes produce a variety of enzymes that combined with mechanical pressure begins the process of cuticle penetration (5–9). The outermost layer of the insect cuticle, also referred to as the waxy layer, serves as the first barrier against microbial attack and consists of a heterogeneous mixture of lipids that include long-chain alkanes, alkenes, wax esters, and fatty acids (10). Despite interaction with the insect waxy layer, representing the initial surface mediating the host-pathogen interaction, little is known concerning the molecular mechanisms, including any enzymes that might facilitate growth and/or penetration of the constituents of the waxy layer. Hydrocarbon assimilation plays a critical role in the ability of *B. bassiana* to infect certain insects, and alkane growth has been linked to increased virulence. Fungal cells grown on alkane-containing media displayed a 2–4-fold increase in mortality against the bean weevil *Acanthoscelides obtectus* when compared with cells grown on glucose (11), and derepression of the alkane pathway increased fungal virulence against insects that had developed resistance to chemical pesticides (12).

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*B. bassiana* is able to grow on a range of mid- to long-chain alkanes and fatty acids, and the genes encoding a suite of cytochrome P450 enzymes implicated in hydrocarbon degradation in *B. bassiana* have been isolated (7). However, the physiological role(s) of any of these proteins and their substrates specificities has yet to be characterized.

In this report we characterize *B. bassiana* CYP52X1, a member of a new cytochrome P450 subfamily. Targeted disruption of *Bbcyp52x1* in *B. bassiana* resulted in no obvious growth defects under normal growth conditions or in media containing any of the tested alkanes or fatty acids as the sole sources of carbon. The gene was linked to cuticle penetration events, with the Δ*Bbcyp52x1* strain displaying reduced virulence in insect bioassays using the Greater Wax moth, *Galleria mellonella*, when applied topically, which represents the natural route of infection but not when injected directly into the hemoceol, the latter a method by which cuticle penetration events can be bypassed. Heterologous expression of CYP52X1 in yeast was used to probe the substrate specificity of the enzyme. Microsomes derived from transformed yeast cells displayed NADPH-dependent ω-hydroxylation of midrange fatty acids (C12:0 and C14:0) and epoxy stearic acid; lower activity was observed against C16:0, C18:1, and C18:2, and little to no activity was observed against C9:0 and C18:0. Taken together our results suggest that CYP52X1 plays a role in epicuticle lipid assimilation but is dispensable for further downstream infection processes. Because the composition of lipid constituents can vary greatly between insects, the role of CYP52X1 in waxy layer assimilation may vary depending upon the insect target; thus, these types of enzymes may act as host range determining factors.

## MATERIALS AND METHODS

*Chemical Reagents and Cultivation of Fungi—B. bassiana* (ATCC 90517) was routinely grown on potato dextrose agar (PDA) or Czapek-Dox plates. Plates were incubated at 26 °C for 10–15 days, and aerial conidia were harvested by flooding the plate with sterile distilled $H_2O$. Conidial suspensions were filtered through a single layer of Miracloth, and final spore concentrations were determined by direct count using a hemocytometer. Blastospores were produced in Sabouraud dextrose supplemented with 0.5–1% yeast extract liquid broth cultures (SDY) using conidia harvested from plates to a final concentration of $0.5–5 \times 10^5$ conidia/ml as the inoculum. Cultures were grown for 3–4 days at 26 °C with aeration. Cultures were filtered (2×) through glass wool to remove mycelia, and the concentration of blastospores was determined by direct count. The *Saccharomyces cerevisiae* WAT11 strain was used for heterologous expression of the CYP52X1 cytochrome P450. Yeast cells were grown at 30 °C in Difco yeast nitrogen base medium without amino acids (6.7 g/liter) containing glucose or galactose at 2% (w/v) with supplements as indicated. Chemical reagents were obtained from either Fischer or Sigma chemicals unless otherwise noted. Phosphinothricin was purchased from Gold Biotech or purified in the laboratory from the herbicide Finale (AgrEvo, Montvale, NJ) as described (13). The silylating reagent *N*,*O*-bistrimethylsilyltrifluoroacetamide containing 1% of trimethylchlorosilane was obtained from Pierce. NADPH and thin layer chromatography (TLC) plates (Silica Gel G60 F254; 0.25 mm) were purchased from Sigma and Merck, respectively. Uniformly radiolabeled $^{14}$C-fatty acid substrates, pelargonic (C9:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and 9,10-epoxystearic acids were obtained from ISOBIO (Fleurus, Belgium).

*Nucleic Acid Manipulations and Construction of Δ*Bbcyp52x1 *and Complemented Strains*—All primer sequences for the nucleic acid manipulations, RT-PCR, Southern blot probe generation, and yeast expression vector construction are listed in Supplemental Table 1. To generate the vector for construction of the *Bbcyp52x1* knock-out strain in *B. bassiana* via homologous recombination, a 3.8-kb fragment of *Bbcyp52x1* was amplified from genomic DNA using the primer pair P52X1F and P52X1R. The PCR product was cloned into the pCR2.1-TOPO blunt-end vector (Invitrogen) generating pTOPO-*Bbcyp52x1*. Long-range deletion inverse PCR using the primer pair P52X1KOF and P52X1KOR was then used to produce a 7.7-kb linear fragment lacking 54 bp of internal *Bbcyp52x1* sequence using the pTOPO-*Bbcyp52x1* plasmid as template. The generated linear DNA was then blunt end-ligated to a PCR product corresponding to the herbicide resistance gene (*bar*) cassette amplified from pBAR-GPE (14) using primer pair pBARF and pBARR. The integrity of the resultant gene replacement plasmid designated as pKO-*Bbcyp52x1* was confirmed by PCR and sequencing. Preparation of competent cells, transformation, and screening of recombinant clones was performed as described using a PEG-LiAc mediated protocol (15, 16). The transformation mixture (0.25–0.5 ml) was plated onto Czapek-Dox medium containing 200 μg/ml phosphinothricin, 0.01% bromcresol purple (pH 6.3), in 150-mm diameter Petri dishes overlaid with a sheet of sterilized cellophane. Genomic DNA was isolated as described (17). Putative *B. bassiana* gene knock-out clones were screened and verified by PCR analysis using primers P52X1CF and P52X1CR designed to the *Bbcyp52x1* gene. PCR reactions were performed using the following protocol: 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min. The complementation vector, pSUR-*Bbcyp52x1*, was constructed using the entire open reading frame along with 1 kb of upstream and 0.9 kb of downstream flanking sequences This region was amplified from using *B. bassiana* genomic DNA as the template and primers P52X1F/P52X1R. The product was cloned into pCB1536 that contains the selection marker for sulfonylurea resistance. Transformation of pSUR-*Bbcyp52x1* into *B. bassiana* Δ*Bbcyp52x1* was performed as described previously, and putative transformants were isolated on Czapek-Dox plates supplemented with 10 μg/ml sulfonylurea (15). Transformants were confirmed by PCR, RT-PCR, and Southern blotting. PCR confirmation was performed on genomic DNA extracted from transformants using primers P52X1CF and P52X1CR. Southern blotting was performed using 10 μg of genomic DNA digested with BstxI. The digested DNA was separated in 1.0% agarose gel and subsequently transferred to Biodyne B nylon membrane (Gelman Laboratory, Shelton, WA) using standard protocols. Blots were probed with a 457-bp PCR-amplified product corresponding to genomic DNA, generated using the P52X1SF and P52X1SR primers. Probe preparation, membrane hybridization, and visualization were performed using DIG High

Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science).

*Growth and Grasshopper Wing Germination Assays*—The grasshopper wings sterilized using 37% $H_2O_2$ were immersed in a conidial suspension in water at a concentration of $1 \times 10^6$ spores/ml for 20 s and placed on 0.7% water agar. After incubation for 18 h, the germinated conidia were counted under a light microscope.

*Heterologous Expression of CYP52X1 in Yeast*—The coding region of the *Bbcyp52x1* gene was cloned from a *B. bassiana* cDNA library by PCR using the primer pair P52X1EF/P52X1ER. The resultant PCR product corresponding to a 1605-bp fragment containing 18 bp (6 amino acid) histidine tag and the 1587-bp *Bbcyp52x1* gene was cloned into pYeDP60 under the control of a GAL1 promoter to yield pYe-Bbcyp52x1. The sequence integrity of the plasmid insert was confirmed by sequencing, and the plasmid was then transformed into *S. cerevisiae* WAT11, a yeast strain engineered and optimized for cytochrome P450 expression using a lithium acetate protocol (18). Transformants were selected on nitrogen base medium with and without amino acids, 2% glucose, and auxotrophic supplements, and the expression strain was designated as *Sc-Bbcyp52x1*.

Yeast cultures were grown, and CYP52X1 expression was induced as described in Pompon *et al.* (18) from one isolated transformed colony. Briefly, after growth cells were harvested by centrifugation and manually broken with glass beads (0.45-mm diameter) in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 600 mM sorbitol. The homogenate was centrifuged for 10 min at 10,000 × *g*. The resulting supernatant was centrifuged for 1 h at 100,000 *g*. The pellet consisting of microsomal membranes was resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 30% (v/v) glycerol with a Potter-Elvehjem homogenizer and stored at −30 °C. The volume of resuspension buffer is proportional to the weight of yeast pellet; microsomes extracted from 6 g of yeast are resuspended in 3 ml of buffer. All procedures for microsomal preparation were carried out at 0–4 °C. Western blots were performed using standard protocols. Mouse anti-His monoclonal antibodies were obtained from Invitrogen.

*Enzyme Activities*—Enzymatic activities of CYP52X1 were determined by following the formation rate of metabolites produced in incubations of substrates with microsomes of transformed yeast. The standard assay (0.1 ml) contained 0.6 mg of microsomal protein, 20 mM sodium phosphate (pH 7.4), 1 mM NADPH, and substrate (100 μM). The reaction was initiated by the addition of NADPH and was stopped after 20 min by the addition of 20 μl of acetonitrile (containing 0.2% acetic acid). Incubation media were directly spotted on TLC plates. For separation of metabolites from residual substrate, TLC plates were developed with a mixture of diethyl ether/light petroleum (boiling point, 40–60 °C)/formic acid (50:50:1, v/v/v). The plates were scanned with a radioactivity detector (Raytest Rita Star). The area corresponding to the metabolites were scraped into counting vials and quantified by liquid scintillation, or they were eluted from the silica with 10 ml of diethyl ether, which was removed by evaporation. They were then derivatized and subjected to GC/MS analysis.

*GC/MS Analysis*—Metabolites generated were methylated with diazomethane and trimethylsilylated with *N,O*-bistrimethylsilyltrifluoroacetamide containing 1% (v/v) trimethylchlorosilane (1:1, v/v) then subjected to GC-MS analysis carried out on a gas chromatograph (Agilent 6890 Series) equipped with a 30-m capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 μm (HP-5MS). The gas chromatograph was combined with a quadrupole mass selective detector (Agilent 5973N). Mass spectra were recorded at 70 eV. Temperature increases were from 60 to 310 °C at 4 °C/min.

A mass spectrum of derivatized metabolite generated in incubation with lauric acid showed ions at *m/z* (relative intensity %) 73 (33%) $(CH_3)_3Si^+$, 75 (34%) $((CH_3)_2Si^+ = O)$, 103 (18%) $(CH_2(OSi(CH_3)_3))$, 146 (5%) $(CH_2 = C^+(OSi(CH_3)_3\text{-}OCH_3)$, 159 (8%) $(CH_3\text{-}O^+ = C^+(OSi(CH_3)_3)CH = CH_2)$, 255 (100%) (M-47) (loss of methanol from the (M-15) fragment), 271 (5%) (M-31) (loss of $OCH_3$ from the methyl ester), 287 (44%) (M-15) (loss of $CH_3$ from trimethylsilane group). This fragmentation pattern is characteristic of derivatized 12-hydroxylauric acid (M = 302 g/mol) (19).

Mass spectrum of derivatized metabolite generated in incubation with oleic acid showed ions at *m/z* (relative intensity %) 73 (86%) $(CH_3)_3Si^+$, 75 (100%) $((CH_3)_2Si^+ = O)$, 103 (31%) $(CH_2(OSi(CH_3)_3))$, 146 (15%) $(CH_2 = C^+(OSi(CH_3)_3\text{-}OCH_3)$, 159 (20%) $(CH_3\text{-}O^+ = C^+(OSi(CH_3)_3)CH = CH_2)$, 337 (65%) (M-47) (loss of methanol from the (M-15) fragment), 353 (8%) (M-31) (loss of $OCH_3$ from the methyl ester), 369 (23%) (M-15) (loss of $CH_3$ from trimethylsilane group). This fragmentation pattern is characteristic of derivatized 18-hydroxyoleic acid (M = 384 g/mol) (19).

*Insect Bioassays*—Fungal virulence bioassays were performed using *G. mellonella* larvae (Pet Solutions). Two different assay conditions were tested for each cell type (1). In a topical application, the larvae were dipped for 5–10 s in solutions of $1 \times 10^8$ conidia/ml harvested in sterile distilled $H_2O$, and the excess liquid on the insect bodies was removed by placement on dry paper towel. Controls were treated with sterile distilled $H_2O$ (2). In an intrahemoceol injection, conidia were harvested in Ringer's solution, and the larvae were injected with 5 μl of a solution of $1 \times 10^6$ conidia/ml into the hemocoel cavity. Controls included both untreated and larvae injected with 5 μl buffer. Experimental and control larvae were placed in plastic chambers or large (150 mm) Petri dishes and incubated at 26 °C. For each experimental condition, 20–40 larvae were used, and all experiments were repeated three times. The number of dead insects was recorded daily, and median lethal mortality time ($LT_{50}$) was calculated by Probit analysis.

## RESULTS

*Generation of Bbcyp52x1 Disruption Mutant and Gene Complementation Strains*—Phylogenetic analyses of a number of *B. bassiana* cytochrome P450s implicated in hydrocarbon degradation and the coding sequence corresponding to the *B. bassiana* CYP52X1 protein were previously reported (7). To investigate the role of *Bbcyp52x1*, a gene disruption strategy was used, and a 54-bp region of the *Bbcyp52x1* coding sequence was replaced by the (942 bp) *bar* gene cassette conferring resistance to phosphinothricin via homologous recombination (Fig.

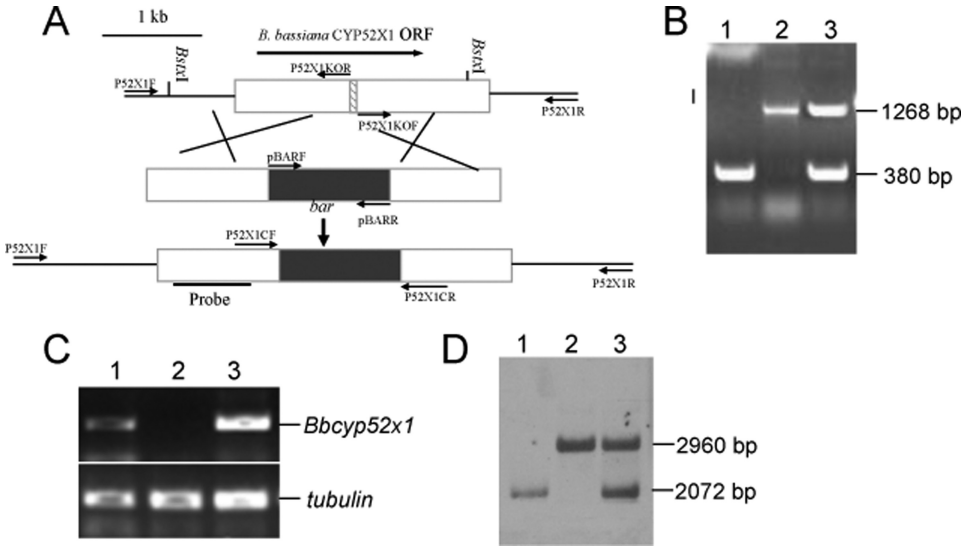


FIGURE 1. **Construction and verification of *B. bassiana Bbcyp52x1* deletion and complementation strains.** *A*, shown is a schematic diagram of vector, *Bbcyp52x1* genomic locus, and homologous recombination event. *B*, shown is PCR verification of the correct integration event. *Lanes 1–3*, shown are PCR products using *Bbcyp52x1* gene amplification primers and genomic DNA from wild type (*lane 1*), Δ*Bbcyp52x1* (*lane 2*), and Δ*Bbcyp52x1:Bbcyp52x1* (complemented strain, *lane 3*). *C*, RT-PCR examination of loss of Bbcyp52x1 transcripts is shown. *Lanes 1–3*, *top panel*, RT-PCR products using Bbcyp52x1 amplification primers and cDNA from wild-type (*lane 1*), Δ*Bbcyp52x1* (*lane 2*), and Δ*Bbcyp52x1:Bbcyp52x1* (*lane 3*). *Lanes 1–3*, *bottom panel*, RT-PCR primers using actin amplification primers for the same samples are as in the *upper panel*. *D*, Southern blot analysis of *Bbcyp52x1* mutants is shown. Genomic DNA was digested with BstxI and probed with a *Bbcyp52x1* ORF gene fragment; *lane 1*, wild type; *lane 2*, Δ*Bbcyp52x1*; *lane 3*, Δ*Bbcyp52x1:Bbcyp52x1*.

1*A*). Approximately 82 transformants were initially screened for the correct integration event by PCR, of which 4 appeared to represent targeted disruptions of *Bbcyp52x1*, with the rest representing ectopic integration events (Fig. 1*B*). RT-PCR experiments using primers designed to the *Bbcyp52x1* cDNA sequence showed complete loss of the transcript in the knock-out strain (Fig. 1*C*). Targeted gene disruption clones were also verified by Southern blotting (Fig. 1*D*). Complementation vectors were constructed using 3.8 kb of genomic sequence (1.9 kb of ORF plus 1.0 and 0.9 kb of upstream and downstream flanking sequences, respectively). At least two independent strains were examined for the gene knock-out strains and complemented strains. The two clones of each respective strain were identical in all phenotypic aspects examined (only data for one knock-out and one complemented strain are shown). Furthermore, the complemented strains using the entire *Bbcyp52x1* ORF were indistinguishable from the wild-type strain in all phenotypic aspects examined below.

*Mutation of Bbcyp52x1 Results in Impaired Germination on Insect Epicuticle*—No significant differences were seen between the Δ*Bbcyp52x1* strain and its wild-type parent in growth and conidiation in standard mycological media including rich media (Sabouraud-dextrose) or minimal media containing sucrose as the carbon source (Czapek-Dox). To test whether loss of *Bbcyp52x1* had any effect on growth on various lipids, the wild-type and mutant strains were inoculated onto minimal agar plates (IDEM) containing alkanes or fatty acids as the sole source of carbon. No differences between the mutant and parent strain were seen on plates containing the alkanes (0.1%) $C_9$, $C_{10}$, $C_{12}$, $C_{14}$, $C_{16}$, $C_{18}$, $C_{24}$, or $C_{28}$, substrates considered to be metabolized by the wild-type strain. Because fatty acid utilization has not been examined in *B. bassiana* before, a brief description of the growth phenotypes of the wild-type strain is given; however, no difference was noted between the wild-type strain and the Δ*Bbcyp52x1* strain for any of the substrates listed below (tested at 0.1%). *B. bassiana* displayed good growth on olive oil, conidiating on the substrate within 14 days. Both oleic (C18:1) and linoleic (C18:2) acids supported good growth of *B. bassiana*; however, noticeably less conidiation occurred within the same time frame (14 days) as compared with growth on olive oil. Growth on stearic (C18:0), palmitic (C16:0), and myristic (C14:0) acids was poor, and only small colonies were visible after 14 days of incubation, with some conidiation apparent. In contrast, better mycelia growth was apparent on lauric acid (C12:0) as compared with the latter set of compounds tested, and a zone of clearing could be seen on plates, likely because of the more even distribution of the substrate within the media. No conidiation, however, was seen on plates containing lauric acid within the 14-day period. No growth and in fact inhibition of growth was seen in plates containing pelargonic acid (9:0).

To examine whether disruption of *Bbcyp52x1* might affect growth on insect cuticular substrates directly, a grasshopper wing assay was used where the fungal conidia were deposited onto dissected wings, and the percent germination was monitored over time (Fig. 2). These data showed impaired germination for the Δ*Bbcyp52x1* strain as compared with the wild-type control ($p < 0.05$). In this and all other phenotypes examined, the complemented (Δ*Bbcyp52x1:Bbcyp52x1*) strain was identical to the wild type.

*Disruption of Bbcyp52x1 Affects Pathogenicity via Topical Application but Not When Directly Injected into Host Hemoceol*—Two types of insect bioassays using the Greater Wax Moth *G. mellonella* as the target host were used to determine the effect of the *Bbcyp52x1* knock-out on virulence. In the first, conidia of *B. bassiana* wild-type and the Δ*Bbcyp52x1* strain were adjusted to $1 \times 10^8$ spores/ml and applied topically, which represents the natural route of infection, and the mortality was determined over a 5-day

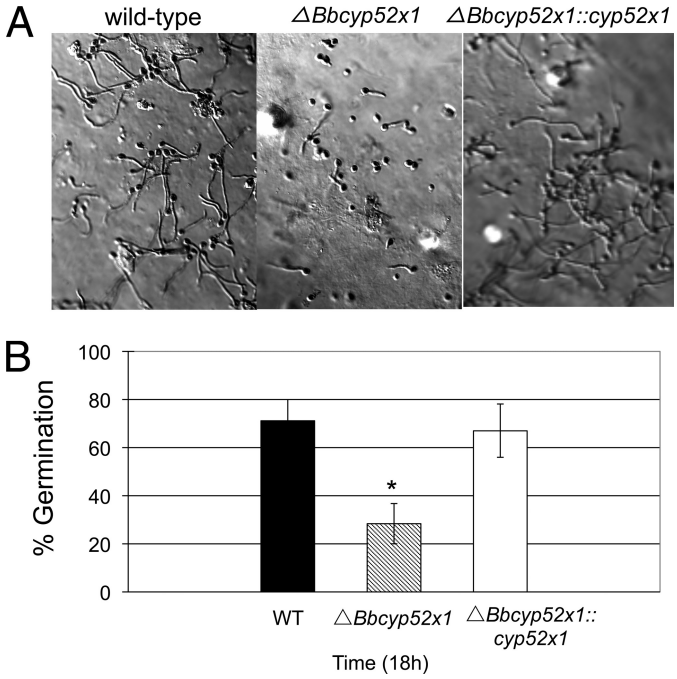


FIGURE 2. **Grasshopper wing germination assay.** *A*, shown are differential interference contrast images of *B. bassiana* conidia incubated on top of dissected grasshopper wings for 18 h as described under "Materials and Methods." *B*, shown is quantification of *B. bassiana* wild type, Δ*cyp52x1*, and Δ*cyp52x1:cyp52x1* conidial germination on grasshopper wings. The *asterisk* indicates significant difference ($p < 0.05$).

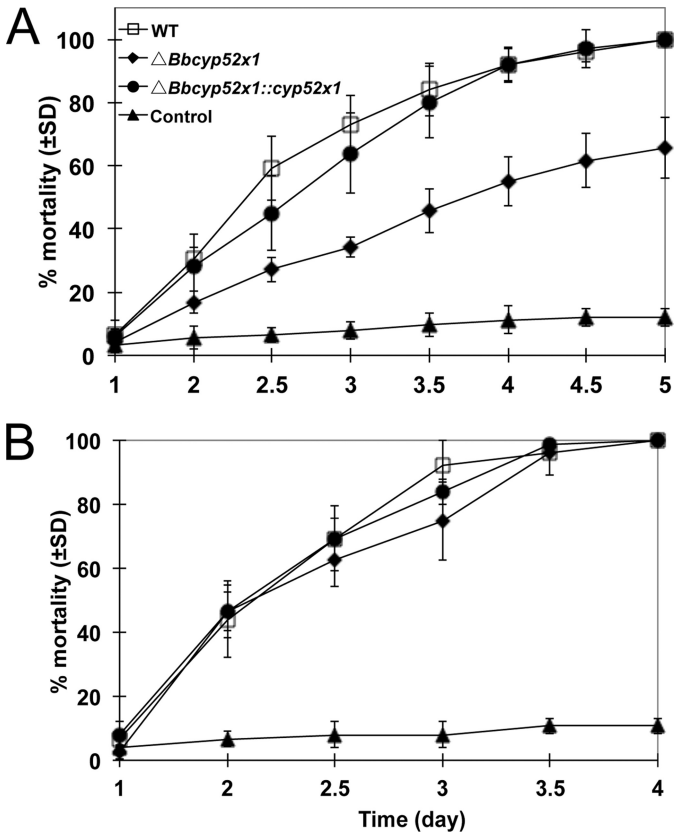


FIGURE 3. ***G. mellonella* larvae were treated topically (*A*) or via intrahemocoel injection (*B*) with conidia from wild-type *B. bassiana* (■), Δ*Bbcyp52x1* (◆), Δ*Bbcyp52x1:Bbcyp52x1* (complemented strain (●)), or mock-treated controls (▲) as described under "Materials and Methods".** The percentage mortality over the indicated time course is presented. The *error bars* represent ± S.D. of at least three independent experiments.

time course (Fig. 3*A*). These data showed a decreased lethal time ($LT_{50}$) to kill for the Δ*Bbcyp52x1* strain as compared with the wild type, where at day 3 the Δ*Bbcyp52x1* strain displayed a 50% reduction in insect mortality as compared with the wild-type parent (~70% total mortality *versus* 35%). The calculated $LT_{50}$ values were as follows: wild type = 2.3 ± 0.2 days, Δ*Bbcyp52x1* = 3.7 ± 0.4 days, and the complemented strain Δ*Bbcyp52x1*::*Bbcyp52x1* = 2.4 ± 0.2 days. In the second assay, the requirement for cuticle penetration was bypassed via direct injection of the spores into the insect hemocoel. Unlike what was observed during topical application, the Δ*Bbcyp52x1* was not significantly different in virulence as compared with the wild-type parent (Fig. 3*B*). The calculated $LT_{50}$ values for the intrahemocoel injection bioassays were as follows: wild type = 1.9 ± 0.3 days, Δ*Bbcyp52x1* = 2.1 ± 0.2 days, and the complemented strain Δ*Bbcyp52x1*::*Bbcyp52x1* = 1.9 ± 0.2 days. Aside from the reduced virulence via topical application, no other obvious defects in disease progression were noted, and the mutant strain was able to sporulate on the host cadaver like the wild-type parent.

*Heterologous Expression of CYP52X1 and Characterization of Its Substrate Specificity*—The *Bbcyp52x1* open reading frame was cloned into the yeast expression vector, pYeDP60, using *B. bassiana* cDNA as the template (*i.e.* no introns) as described under "Materials and Methods," yielding expression construct pYe-*Bbcyp52x1*, which included a C-terminal His tag on the protein. Heterologous expression was performed in the *S. cerevisiae* WAT11 strain, which contains only a minimal complement of the endogenous cytochrome P450s that are expressed at low levels under the growth conditions tested and which do not use the substrates examined. Furthermore, this strain overexpresses a cytochrome P450 reductase that funnels electrons from NADPH to the P450 enzyme (18). Endoplasmic reticulum-derived microsomes were prepared from control (empty vector) and pYe-*Bbcyp52x1*-containing strains as described under "Materials and Methods." Expression of CYP52X1 was verified by Western blotting (Fig. 4*A*). The isolated microsomes showed a low spin iron heme spectrum ($\lambda_{max}$ = 418), but no CO difference spectral shift was observed, indicating the absence of CO binding when dithionite was added (data not shown).

Yeast microsomes were incubated with radiolabeled substrate (lauric acid) in the presence and absence of NADPH followed by analysis of the products and reactants by thin layer (radio)chromatography (TLC). The radioactivity contained in fractions from the developed TLC plates was quantified in a liquid scintillation counter (Fig. 4*B*). These radiochromatograms revealed the formation of a new peak (*peak 1*, Fig. 4*B*) that migrated slower than the substrate and that was not found in incubations performed in the absence of NADPH or in reactions containing microsomes derived from yeast transformed with an empty vector ± NADPH. The product of the reaction using lauric acid (C12:0) as the substrate was purified and identified as 12-hydroxylauric acid by GC/MS analysis (Fig. 5) as described under "Materials and Methods." Similarly, to test the effect of fatty acid chain length on enzyme regioselectivity, the product of reaction mixtures containing oleic acid (C18:1) was

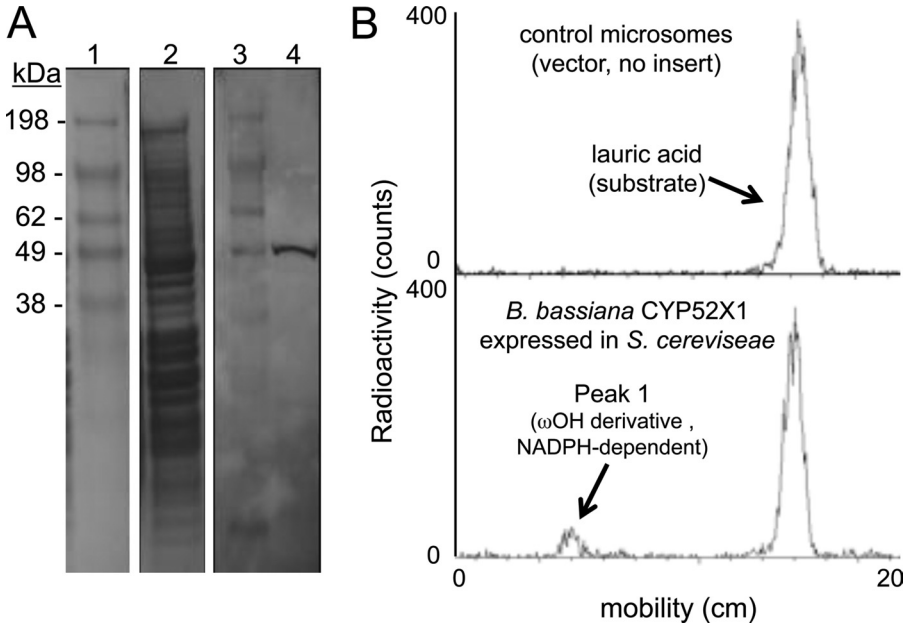


FIGURE 4. **Heterologous expression and thin layer chromatography of enzymatic activity of *B. bassiana* CYP52X1 in yeast.** *A*, SDS-PAGE and Western blot of *S. cerevisiae* WAT11 cells expressing *B. bassiana cyp52x1* are shown. Construction of the *B. bassiana* cytochrome P450 expression vector containing an C-terminal His-tag as described under "Materials and Methods." Shown are SDS-PAGE of molecular weight standards (*lane 1*) and Coomassie-stained gel of yeast crude extract (*lane 2*), Western blot standards (*lane 3*), and crude extract (*lane 4*) probed with mouse anti-His monoclonal antibody. No bands were observed in crude extracts derived from WAT11 cells transformed with the empty vector control. *B*, *B. bassiana* P450 activity is expressed in a yeast heterologous system. Data are radiochromatograms obtained after scanning of thin layer chromatography plates loaded with reaction mixtures and developed in ether/petroleum ether/formic acid (50/50/1). Microsomes were prepared as described under "Materials and Methods." *Top panel*, activity of control (yeast transformed with empty vector) microsomes with lauric acid as the test substrate. *Bottom panel*, CYP52X1 activity (microsomes, 0.6 mg of protein in 20 mM phosphate buffer (pH 7.4), 100 $\mu$M lauric acid, 100 $\mu$M NADPH, 30 min at 27 °C) shows emergence of new peak (*arrow*). No activity was seen in reaction mixtures lacking NADPH.

purified and confirmed as being the $\omega$-OH metabolite by GC/MS analysis (Fig. 6).

The substrate specificity of CYP52X1 was further probed via use of a series of radiolabeled substrates including saturated and unsaturated fatty acids; pelargonic (C9:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), and linoleic (C18:2) acids as well as epoxide 9,10-epoxystearic acid. The enzyme displayed optimal activity against the midrange fatty acids, *e.g.* lauric and myristic acids, as well as against the 9,10-epoxystearic acid and 4–8-fold lower activity against oleic acid followed by 20–40-fold lower activity (as compared with lauric acid) *versus* pelargonic, palmitic, and linoleic acids, with little to no activity against steric acid (Fig. 7). No epoxides were detected in reaction mixtures containing either oleic or linoleic acids.

## DISCUSSION

*B. bassiana* is a facultative pathogen of insects that can grow as a saprophyte and does not require an insect host yet maintains a broad host range and is virulent toward a diverse range of insect and other arthropod species. Intriguingly, although *B. bassiana* clusters phylogenetically with plant pathogenic fungi, it has not been shown to act as a plant pathogen. Instead, it can form intimate relationships with plants and is able to endophytically colonize a variety of plant species (20). Hydrocarbons constitute the outer layer of both plant and insect substrata. Cross-linked $\omega$-hydroxy fatty acids are important constituents of cutin, the outermost layer of the plant cuticle, and plant pathogenic fungi contain cutinases that facilitate penetration of the this layer (21). For plant pathogenic fungi, the resultant release of cutin monomers via the action of cutinases acts as a signal that leads to the formation of fungal penetration structures (appressoria) as well as stimulating increased cutinase production. Regarding the insect epicuticle, fatty acids and alkanes are prevalent, but it is unclear whether any significant amounts of cross-linked lipids are present.

Adhesion of fungal conidia, which represents the main infectious particle, to the insect cuticle is mediated in part by cell surface proteins known as hydrophobins (22, 23). Once attached, the conidia germinate and produce a battery of enzymes targeting cuticular substrates. Although the production and regulation of proteases and other hydrolases by *B. bassiana* in response to insect cuticles has received some attention, little is known concerning the degradation and/or penetration of the initial barrier that must be overcome for successful infection to occur, in particular the hydrocarbons that constitute the insect epicuticle or waxy layer. Alternations in hydrocarbon content during fungal infection of various insects have been noted (9, 24). Differences in the hydrocarbon content of the waxy layer can have profound effects on fungal pathogenesis. Some hydrocarbons inhibit spore germination, whereas others stimulate germination and growth (25, 26). Cuticular hydrocarbons can also promote (27, 28) or inhibit (29) fungal attachment to cuticle, and specific components may act as chemical inducers for the production of penetrant germ tubes on hosts (30, 31). Pentane extracts of two closely related tick species, one highly susceptible to *B. bassiana* (*Amblyomma*

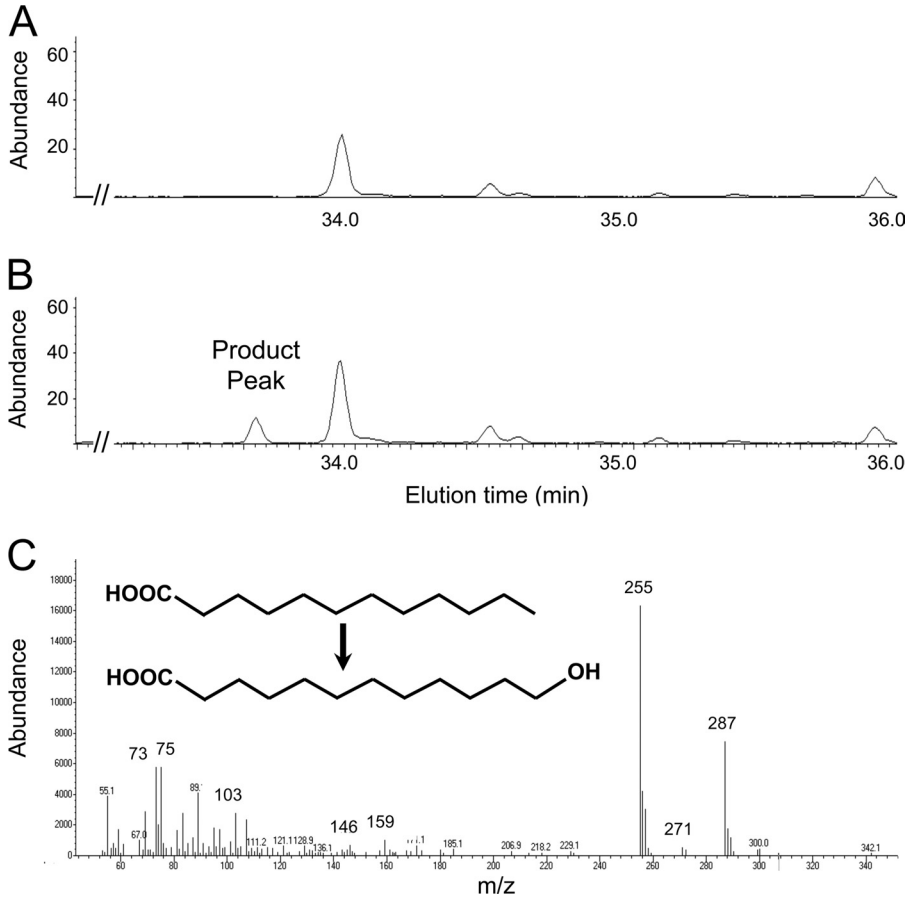


FIGURE 5. **Gas chromatographic resolution of metabolites generated in incubations of lauric acid with microsomes from yeast expressing *CYP52X1*.** Microsomes were incubated with 100 $\mu$M lauric acid in the absence (*A*) or in the presence (*B*) of 100 $\mu$M NADPH. Incubations were performed with 600 $\mu$g of microsomal proteins at 27 °C. Reaction products were extracted with diethyl ether, derivatized, and subjected to GC/MS analysis. *C*, the MS fragmentation pattern of metabolite from the product peak is characteristic of derivative of 12-hydroxylauric acid.

*maculatum*) and the other somewhat resistant to fungal infection (*Amblyomma americanum*), revealed inhibition of fungal germination in the case of *A. americanum* but good growth on the *A. maculatum* extracts (32). *B. bassiana* has been shown to utilize several insect hydrocarbons including aliphatic and methyl branched alkanes (33). $C_{28}$ and $C_{24}$ alkanes were degraded by *B. bassiana* mainly into free fatty acids, phospholipids, and acylglycerols, with alkane grown cells producing *n*-decane as a volatile organic compound as a byproduct of the $\beta$-oxidation reactions (34, 35). Similarly, the major components of the larvae of the blood-sucking bug *Triatoma infestans* (an important vector of human disease causing microbes) epicuticle includes $C_{29}$, $C_{31}$, and $C_{33}$, which are able to promote *B. bassiana* growth (33). Radiolabeled hydrocarbons have been used to investigate the catabolic pathways of alkane degradation in *B. bassiana*, and these data support a degradative pathway involving $\beta$-oxidation by a cytochrome P450 enzyme system followed by peroxisome-mediated successive transformations to yield the appropriate fatty acyl-CoA (36, 37).

Many lipid utilizing fungi express a battery of cytochrome P450s involved in alkane and fatty acid metabolism. The degradative pathways in alkane-assimilating yeasts such as *Candida* sp. (*maltosa* and *tropicalis*) and *Yarrowia lipolytica* represent the best characterized systems in which metabolic genes including cytochrome P450s, alcohol, and aldehyde dehydrogenases, acyl-CoA oxidases, and various catalases have been isolated, and the subcellular distribution of these enzymes has been described (38–40). At least eight cytochrome P450s, including CYP52X1, implicated in hydrocarbon assimilation have been reported in *B. bassiana* (7). However, none of these proteins has been biochemically characterized, and their role(s) in fungal growth and other phenotypes, *e.g.* virulence, remains obscure. Expression of *cyp52x1* was only marginally induced after growth on $n-C_{16}$ and $n-C_{20}$ (2–6-fold) but was elevated greater than 10-fold when the fungus was grown on $n-C_{24}$ and $n-C_{28}$. Intriguingly, in the same report, *cyp52x1* expression was increased greater than almost 50-fold during growth on a cuticular lipid extract derived from *T. infestans*. Although CYP52X1 represents a member of a novel subfamily, the CYP52 family encompasses several enzymes with known activity toward alkanes and/or fatty acids as well as members that have yet to be characterized biochemically. *B. bassiana cyp52x1* was heterologously expressed in the WAT11 yeast strain. This yeast strain constitutively expresses a reductase needed for cytochrome P450 activity. Microsomes isolated from the *cyp52x1*-expressing strain failed to show the typical P450 CO spectrum. Although unusual, such an observation is not without precedent. Poor or atypical CO spectra have been reported for some plant P450s that have weak affinity for CO (41), and P450 19A1

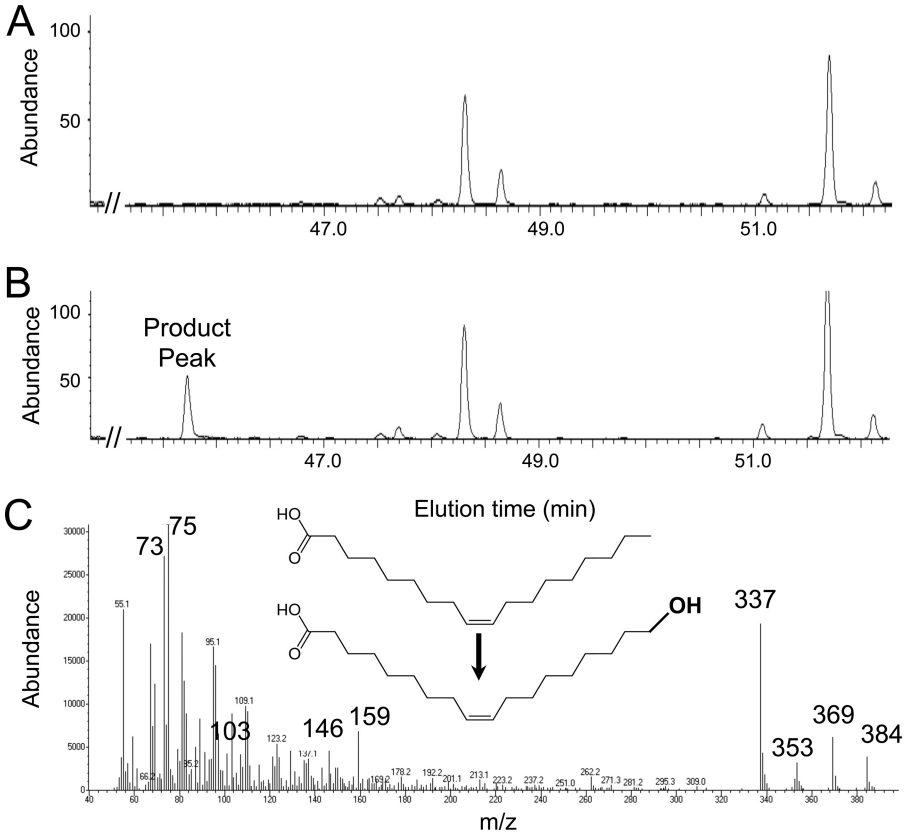


FIGURE 6. **Gas chromatographic resolution of metabolites generated in incubations of oleic acid with microsomes from yeast expressing *CYP52X1*.** Microsomes were incubated with 100 $\mu$M oleic acid in the absence (*A*) or in the presence (*B*) of 100 $\mu$M NADPH. Incubations were performed with 600 $\mu$g of microsomal proteins at 27 °C. Reactions products were extracted with diethyl ether, derivatized, and subjected to GC/MS analysis. *C*, the MS fragmentation pattern of metabolite from product peak is characteristic of derivative of 18-hydroxyoleic acid.

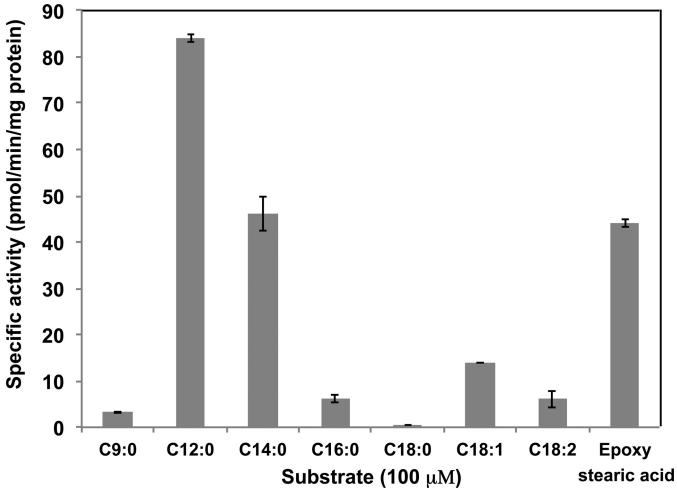


FIGURE 7. **Substrate specificity of heterologous expressed *B. bassiana* CYP52X1.** The specific activity of *B. bassiana* CYP52X1 expressed in *S. cerevisiae* WAT11 cells was determined using the indicated substrates via radiochromatography using labeled substrates. In all cases no activity was observed in reactions mixtures lacking NADPH, and no activity was observed in control WAT11 derived microsomes.

(aromatase) does not bind CO (42, 43). These results remain unexplained and may also be rather under-reported in the field.

Subsequent biochemical assays using radiolabeled fatty acids varying in their chain length and in their degree of unsaturation were used to confirm that the enzyme displayed $\omega$-hydroxylase activity able to add terminal hydroxyl groups to various fatty acids and epoxides. For each substrate tested only a single product peak was detected by TLC, and subsequent GC/MS analysis was used to demonstrate that this enzyme hydroxylates lauric acid (C12) and oleic acid (C18:1) with strict regioselectivity on the terminal methyl. No other metabolites were detected in reaction mixtures; in particular no epoxide formation was seen in incubation with the unsaturated fatty acids (oleic and linoleic acids). CYP52X1 displayed robust activity (>40 pmol/min/mg of protein) against lauric (C12:0), myristic, and 10-epoxysteric acids, 2–10 pmol/min/mg of protein of activity against C(9:0), C(16:0), C(18:1), and C(18:2), and ~ 0.5 pmol/min/mg protein of activity against C18:0. These data indicate that the enzyme prefers the mid-range (possibly $C_{10-11}$, $C_{12-14}$, and possibly $C_{15}$) saturated fatty acids. Intriguingly, the enzyme displayed better activity toward unsaturated and epoxy-$C_{18}$ fatty acids than toward the saturated $C_{18}$ fatty acid. An important future direction would be to test branched chain fatty acids, compounds that are enriched in insect wax; however, at this time these substrates are not readily available.

Ablation of *cyp52x1* in *B. bassiana* did not result in any noticeable growth defects under any of the conditions tested that included the substrates for the enzyme as described above. Furthermore, the substrate specificity of the enzyme appeared in several instances to conflict with the data regarding the growth of the fungal organism on different fatty acids. Thus, myristic acid was a good substrate for the enzyme but a poor growth substrate, and stearic acid was a poor substrate for the

enzyme but could support growth (albeit not very vigorous). It is unclear what can account for the former observation. One possibility is that transport of the substrate into the cell is the limiting step. This would imply, however, that larger (precursor) substrates could enter the cell. Regarding the latter findings, these data support a model in which multiple cytochrome P450 alkane/fatty acid substrate utilizing enzymes, some with overlapping substrate specificities, are produced by *B. bassiana*. This redundancy could, therefore, account for the lack of any obvious discernable growth phenotype in the Δ*Bbcyp52x1* mutant and also highlights the importance of alkane/fatty acid metabolism in this fungus. This model would also predict that the other P450 enzymes would act on shorter and longer chain fatty acids (than the ones used by CYP52X1). Similarly, in *Y. lipolytica*, which contains 12 P450alk genes in its genomes, single knockouts of most of these genes do not result in loss of that ability to metabolize alkanes. The exception was Δ*alk1* mutants, which were unable to grow on decane (44). Grasshopper wing germination assays, however, did show a difference in the germination rate between the wild-type and Δ*Bbcyp52x1* conidia. Furthermore, topical application, which represents the natural route of infection, of mutant Δ*Bbcyp52x1* conidia onto larvae of the Greater Wax moth, *G. mellonella*, resulted in reduced virulence as compared with the wild-type parent. In contrast, no difference in virulence was noted when conidia were directly injected into the hemoceol of the insect, a procedure that bypasses any fungal-epicuticular (hydrocarbon and other) interactions and cuticle penetration. Such a result supports the idea that *B. bassiana cyp52x1* is important in epicuticle interactions but is dispensable once the cuticle is breached. There are at least two possible explanations for these results. First, additional natural substrates for the enzyme might exist on or near the host surface whose degradation contributes to the pathogenic process. Alternatively, endogenous fungal substrate(s) for the CYP52X1 enzyme might need to be mobilized sometime during the cuticle penetration event.

Although, as mentioned, it is likely that the array of hydrocarbon substrate utilizing cytochrome P450s found in *B. bassiana* have overlapping substrate specificities, they may also utilize unique substrates found on insect cuticles. Because insect epicuticular hydrocarbon profiles vary not only among different insect species but also between the different instars during insect development, our data lead to the hypothesis that specific cytochrome P450s may contribute at least in part to the host range of the fungus by acting on unique substrates. Further biochemical and genetic characterization of the other *B. bassiana* hydrocarbon substrates utilizing cytochrome P450s is likely to shed light on this issue.

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