

Molecular cloning of a family of xenobiotic-inducible drosophilid cytochrome P450s: Evidence for involvement in host-plant allelochemical resistance

(xenobiotic induction/insect–plant interactions/*Drosophila*/cDNA cloning)

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ABSTRACT Cytochrome P450s constitute a superfamily of genes encoding mostly microsomal hemoproteins that play a dominant role in the metabolism of a wide variety of both endogenous and foreign compounds. In insects, xenobiotic metabolism (i.e., metabolism of insecticides and toxic natural plant compounds) is known to involve members of the CYP6 family of cytochrome P450s. Use of a 3' RACE (rapid amplification of cDNA ends) strategy with a degenerate primer based on the conserved cytochrome P450 heme-binding decapeptide loop resulted in the amplification of four cDNA sequences representing another family of cytochrome P450 genes (*CYP28*) from two species of isoquinoline alkaloid-resistant *Drosophila* and the cosmopolitan species *Drosophila hydei*. The *CYP28* family forms a monophyletic clade with strong regional homologies to the vertebrate CYP3 family and the insect CYP6 family (both of which are involved in xenobiotic metabolism) and to the insect CYP9 family (of unknown function). Induction of mRNA levels for three of the *CYP28* cytochrome P450s by toxic host-plant allelochemicals (up to 11.5-fold) and phenobarbital (up to 49-fold) corroborates previous *in vitro* metabolism studies and suggests a potentially important role for the *CYP28* family in determining patterns of insect–host-plant relationships through xenobiotic detoxification.

Cytochrome P450s constitute a superfamily of heme-thiolate proteins characterized by a highly conserved FXXGXXXCXG sequence of amino acids (heme-binding decapeptide) and spectral absorbance peak at 450 nm (1). The diversity of cytochrome P450s is well established, in terms of both the reactions they catalyze and the chemically dissimilar substrates upon which they act. Functions include the oxidative, peroxidative, and reductive metabolism of steroids, fatty acids, pharmaceuticals, agrochemicals, and plant allelochemicals. Excellent reviews of this superfamily may be found in Nelson *et al.* (2) and Feyereisen (3).

Cytochrome P450s are involved in insect growth and development through the processing of such compounds as pheromones and ecdysteroids (4, 5). They have also been implicated in metabolic resistance to insecticides, including carbamates, chlorinated hydrocarbons, organophosphates, and pyrethroids (6, 7). Similarly, cytochrome P450-catalyzed elimination of toxic plant allelochemicals appears to be a key factor in host-plant utilization (8, 9).

The most extensively studied group of insect cytochrome P450s is the CYP6 family, which is related to the major drug-metabolizing CYP3 family in vertebrates. In the house fly

(*Musca domestica*), CYP6A1 metabolizes the insecticides aldrin and heptachlor (10), and CYP6D1 has been linked to deltamethrin metabolism (11). Heterologous expression of *Drosophila melanogaster* CYP6A2 in *Saccharomyces cerevisiae* bioactivates some genotoxins (e.g., aflatoxin B₁) (12). This broad catalytic diversity is thought to have arisen as a result of coevolution between herbivorous animals and toxic allelochemical-producing plants (13). Although this hypothesis is attractive, there have been very few studies that have examined the metabolism of natural substrates by individual cytochrome P450 isoforms. To date, only the *CYP6B* genes of papilionid caterpillars have been shown to metabolize a natural substrate—toxic furanocoumarins present in the organisms' host plants (14).

The well-defined interrelationships between columnar cacti and *Drosophila* in the Sonoran Desert of the southwestern United States and northwestern Mexico provide an ideal model system with which to investigate cytochrome P450-mediated resistance to toxic plant allelochemicals. Four species of *Drosophila* (i.e., *D. mettleri*, *D. nigrospiracula*, *D. mojavensis*, and *D. pachea*) utilize, as a feeding and breeding substrate, the necrotic tissue of five species of columnar cacti—i.e., saguaro (*Carnegiea gigantea*), cardón (*Pachycereus pringlei*), senita (*Lophocereus schottii*), agria (*Stenocereus gummosus*), and organ pipe (*Stenocereus thurberi*). Allowing for geographic differences in host-plant availability, there is essentially a one-to-one relationship between each drosophilid species and the species of host cactus utilized. Furthermore, while the cactophilic drosophilids are endemic to the Sonoran Desert, they are not phylogenetically close and, therefore, are believed to have evolved independently into the desert niche (15).

Each species of cactus is characterized by a suite of allelochemicals that are toxic to all but normal resident species. In agria and organ pipe cacti (which are utilized only by *D. mojavensis*), triterpene glycosides, C₈–C₁₂ fatty acids, and sterol diols are the major toxins. For *D. nigrospiracula* (which lives only on saguaro and cardón), *D. mettleri* (which lives in rot exudate-soaked soils usually found at the base of saguaro and cardón), and *D. pachea* (which lives exclusively on senita), utilization involves resistance to isoquinoline alkaloids. In saguaro, the alkaloids gigantine and carnegine constitute 1–2% of the plant's dry weight, whereas in senita, lophocereine and its trimer, pilocereine, constitute up to 20% of dry tissue weight (16). In contrast to the Sonoran Desert species, *Dro-*

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: RACE, rapid amplification of cDNA ends; rp49, ribosomal protein 49.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. U89746 (*CYP28A1*), U89747 (*CYP28A2*), U91565 (*CYP28A3*), and U91566 (*CYP28A4*)].

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sophila hydei is a cosmopolitan species known to utilize *Opuntia* cactus tissue, which lacks toxic allelochemicals.

The successful utilization of cactus tissue requires the ability to tolerate these toxins. Several studies have established the involvement of cytochrome P450s in cactophilic drosophilid resistance to isoquinoline alkaloids. In particular, *in vitro* alkaloid metabolism and total cytochrome P450 content were significantly induced by exposure of desert drosophilids to cactus alkaloids or phenobarbital (an inducer of many toxin-metabolizing cytochrome P450s). Conversely, the induced *in vitro* metabolism and *in vivo* larval viability were reduced by the cytochrome P450 inhibitor piperonyl butoxide (8, 17).

Among the cactophilic *Drosophila*, only *D. mettleri* displays a behavioral preference for oviposition in rot exudate-soaked soils. As a result of inevitable evaporative water loss from these soils, *D. mettleri* larvae regularly encounter concentrations of toxic alkaloids that may be more than an order of magnitude higher than in necrotic tissue (18). No other cactophilic drosophilid is routinely exposed to such high levels of toxic plant allelochemicals. This is especially true for larvae in soils soaked by rot exudate from senita cactus. In the current study, therefore, the induction of individual *D. mettleri* cytochrome P450s by senita alkaloids was investigated. We have identified a previously undescribed family of insect P450s as being potentially involved in the metabolism of these host-plant compounds.

MATERIALS AND METHODS

***Drosophila* Species and Larval Induction.** Multifemale lines of *D. mettleri* and *D. nigrospiracula* originally collected from mainland regions of the Sonoran Desert and *D. hydei* (strain A820) originally isolated from Jalisco, Mexico, were used in the current study. *Drosophila* stocks were maintained on yeast-supplemented instant *Drosophila* medium (Ward's, Rochester, NY). For the initial cytochrome P450 cDNA cloning work, early third-instar larval *Drosophila* were induced for 48 hr prior to RNA isolation with 20 mg of phenobarbital dissolved in 1 ml of water (2% wt/vol, pH 9.0) and distributed evenly across the surface of 50 g of rehydrated instant *Drosophila* medium. As an inducer of several cytochrome P450s implicated in xenobiotic metabolism, treatment with phenobarbital was used to maximize the message levels of genes potentially involved in metabolism of toxic isoquinoline alkaloids. To obtain RNA for use in Northern analyses, early third-instar larvae were induced for 48 hr with 1 g (dry weight) of one of the following: (i) senita cactus tissue containing lophocereine and pilocereine at concentrations present in fresh tissue (i.e., 15% dry weight), (ii) saguaro tissue supplemented with purified saguaro alkaloids to a concentration of 3 or 10 times that found in fresh tissue (i.e., 4.5% or 15% dry weight), (iii) purified agria triterpene glycosides (≈ 3 times the concentration present in fresh tissue), or (iv) 20 mg of phenobarbital, as described above. All inducers were distributed evenly across the surface of 50 g of instant *Drosophila* medium and rehydrated before the introduction of 1 g of larvae. Uninduced larvae maintained on instant *Drosophila* medium served as controls. Saguaro alkaloids and agria triterpene glycosides were extracted from dried cactus tissue by using standard methods (18, 19).

RNA Isolation and cDNA Synthesis. Total RNA for the initial 3' rapid amplification of cDNA ends (RACE) (20) reaction was isolated from phenobarbital-induced larvae by following the guanidine-hydrochloride-based method of Skuse and Sullivan (21). Poly(A)⁺ RNA was isolated for 5' RACE reactions (20) and Northern analyses by direct capture of mRNA onto oligo(dT)₂₅-coated paramagnetic beads (Novagen), following the procedure of Jakobsen *et al.* (22).

Because full-length cDNAs are not essential for cloning of the 3' ends of cytochrome P450 cDNAs, total RNA and

standard Moloney murine leukemia virus reverse transcriptase were used for first-strand synthesis primed off the poly(A) tail. Where a high percentage of full-length cDNAs was required (e.g., cloning of cytochrome P450 5' sequences), Superscript II (GIBCO/BRL), which lacks RNase H activity, was employed for first-strand cDNA synthesis.

Cytochrome P450 cDNA Cloning. For the 3' RACE reaction, a fully degenerate (2,048-fold) gene-specific primer [5'-GGICCI(A/C)GIAA(C/T)TG(C/T)ATIGC-3', where I represents deoxyinosine and the parentheses contain mixed bases] was designed on the basis of the highly conserved GPRNCIG heme-binding decapeptide motif common to many insect cytochrome P450s. Deoxyinosine was used at sites of fourfold degeneracy to minimize mismatch instability. Using sequence information obtained from the 3' end of PCR-amplified cytochrome P450 cDNAs, gene-specific reverse primers (CYP28A1, 5'-CCGAGC-GAGTCTTTGGATTGA-3'; CYP28A2, 5'-CCTGGCATTGACACTGACATC-3') were constructed and full-length cDNAs were obtained by 5' RACE. Consensus cDNA sequences were constructed with data from multiple overlapping clones to minimize errors due to nucleotide misincorporation by reverse transcriptase and *Taq* DNA polymerase.

rp49 cDNA Cloning. Partial cDNA sequence for ribosomal protein 49 (rp49) (homologous to human ribosomal protein L32) was cloned by 3' RACE from each of the *Drosophila* species for use as a homologous probe to standardize mRNA loading in the Northern analyses. The sequence of the *rp49* gene-specific primer was 5'-AA(A/G)TTC(C/T)TGGTGCA(C/T)AA(C/T)GT-3'. Ribosomal proteins are highly conserved genes that display stable high-level expression (23). The small size of the *rp49* transcript (approximately 650 bp) made its use as a loading standard ideal because its signal appeared well below that of the cytochrome P450 transcripts.

DNA Sequencing and Analysis. Amplification products were ligated into the pGem-T vector (Promega) and used to transform *Escherichia coli* strain DH5- α . Inserts from transformants were sequenced by the dideoxynucleotide chain-termination method (24) and analyzed for similarity to known genes with the BLAST algorithm at the National Center for Biotechnology Information (25). Using the BLAST-generated regional sequence alignments as a guide, we manually aligned the deduced full-length amino acid sequences for CYP28A1 and CYP28A2 with the sequences for *D. melanogaster* CYP6A2 (GenBank M88009) and CYP4D1 (GenBank X67645), house fly, *Musca domestica*, CYP6A1 (GenBank L27241) and CYP6D1 (GenBank U22362), black swallowtail butterfly, *Papilio polyxenes*, CYP6B1 (GenBank M80828) and CYP6B3 (GenBank U25819), Australian cotton bollworm, *Helicoverpa armigera*, CYP6B2 (GenBank U18085), tobacco budworm, *Heliothis virescens*, CYP9A1 (GenBank U23506), cockroach, *Blaberus discoidalis*, CYP4C1 (GenBank M63798), rat, *Rattus norvegicus*, CYP3A1 (GenBank D29967), and the human CYP3A4 (GenBank J04449) and CYP11A1 (GenBank D00169). Distance matrices and phylograms were generated from aligned sequences by using PAUP software (Phylogenetic Analysis Using Parsimony, by D. Swofford, Smithsonian Institution). Human CYP11A1 served as the outgroup for the phylogenetic analyses.

Northern Analyses. Induction of individual cytochrome P450s by alkaloid-containing cactus tissue, agria triterpene glycosides, or phenobarbital was investigated by high-stringency Northern hybridization using poly(A)⁺ RNA blotted onto Hybond N⁺ nylon membrane (Amersham) and digoxigenin-UTP-labeled probes. Detection of bound probe employed anti-digoxigenin Fab conjugated to alkaline phosphatase, which catalyzed the degradation of the chemiluminescent substrate CDP-Star (Tropix, Bedford, MA), following the protocol of Engler-Blum *et al.* (26). Photographic images (X-Omat AR film, Kodak) of the chemiluminescent signal for each cytochrome P450 and a subsequent probing of the same

blot for *rp49* transcripts were superimposed, scanned at 300 dots per inch, and analyzed using Photoshop software. Signal strength was quantified on the basis of the above-background pixel counts of the scanned image and standardized to signal from a second probing of message for *rp49* based on the linear (for the blots analyzed) relationship between the amount of RNA loaded and the resulting signal intensity.

RESULTS

Sequencing and BLAST analysis of 123 subcloned cDNAs amplified by 3' RACE using a moderately degenerate oligonucleotide primer targeted to sequence encoding the heme-binding region of insect cytochrome P450s resulted in the identification of 101 partial cytochrome P450 cDNAs. These sequences represented 15 distinct forms of cytochrome P450, which displayed strong regional similarity to members of the CYP6 (5 forms), CYP4 (6 forms), and CYP9 (2 forms) cytochrome P450 families.

Each of the 15 partial cDNA sequences from *D. metleri* was used as a probe to screen the corresponding cytochrome P450 transcripts (by Northern hybridization) for xenobiotic responsiveness to senita cactus alkaloids. Only three cytochrome P450 genes (initially designated *DU43*, *DU108*, and *DU369*) demonstrated a greater than 3-fold increase in message following exposure to senita alkaloids. In accordance with established cytochrome P450 nomenclature, *DU369* and *DU43* have been designated *CYP28A1* (GenBank U89746) and *CYP28A2* (GenBank U89747), respectively (*DU108* has not been assigned a CYP designation).

Northern blots for the CYP28 transcripts are shown in Fig. 1. While *CYP28A2* (Fig. 1B) and *DU108* were induced to similar degrees following exposure to senita cactus alkaloids (7.7-fold and 7.1-fold, respectively), *CYP28A1* displayed greater than 11-fold induction (Fig. 1A). Interestingly, xenobiotic induction of *CYP28A1* was specific for senita alkaloids, as neither chemically similar saguaro alkaloids (at an equivalent concentration) nor toxic agria cactus triterpene glycosides were able to induce a similar response. By contrast, *CYP28A2* and *DU108* were crossinducible by saguaro cactus alkaloids (8.4-fold and 9.3-fold, respectively) but not by agria triterpene glycosides. Finally, all three genes showed a greater than 10-fold increase in message with exposure to phenobarbital (*CYP28A2*, 48.9-fold; *DU108*, 26-fold; and *CYP28A1*, 16.5-fold).

Because of the magnitude and specificity of alkaloid responsiveness displayed by *CYP28A1*, the partial cDNA sequence was used to design a gene-specific reverse primer and the remaining cDNA was obtained by 5' RACE. Full-length

cDNA sequence was also obtained for *CYP28A2*, the cytochrome P450 most closely related to *CYP28A1*. The consensus nucleotide sequences and conceptual amino acid translations of the full-length cDNAs obtained are shown in Fig. 2 A and B. The 1,831-bp cDNA for clone *CYP28A1* and the 1,764-bp cDNA for clone *CYP28A2* contain open reading frames encoding proteins of 506 and 505 residues, respectively. The 3' untranslated regions of *CYP28A1* and *CYP28A2* both contain a putative polyadenylation signal [AATAAT and AATAAA, respectively (27)] within 15 bases of the poly(A) tail.

Alignment of the deduced amino acid sequence for both *CYP28A1* and *CYP28A2* by BLAST revealed strong regional similarity (up to 64%) to several CYP3A and CYP6A sequences for the region encompassing the C terminus of helix I through the middle of helix L (*CYP28A1* residues G³⁰⁸–F⁴⁷¹ and *CYP28A2* residues G³⁰⁷–F⁴⁷⁰). The heme-binding decapeptide is located just N-terminal to helix L. Total positional identity for full-length CYP28 sequences aligned with previously described cytochrome P450s, however, did not exceed 25%. By contrast, an alignment between *CYP28A1* and *CYP28A2* revealed 55.6% sequence identity. The relatedness of the CYP28 sequences to each other and their phylogenetic uniqueness among insect cytochrome P450s is also evident in the single, most parsimonious, phylogenetic tree generated by heuristic analysis of full-length protein sequences (Fig. 3). The CYP28 sequences form a monophyletic clade that is separate from the clades formed by members of the CYP3, CYP6, and CYP9 families. Both CYP3 and CYP6 have been implicated in xenobiotic metabolism. The function of CYP9 has not been determined. While a phylogenetic tree based on maximum parsimony suggests that the CYP6 and CYP28 families may have been derived from a common ancestor, bootstrap analysis produced a polytomy, with a bootstrap value of 69, among the monophyletic clades for the CYP3, CYP6, CYP9, and CYP28 sequences.

Sequence similarity was used to identify additional members of the CYP28 family from among 3' RACE-generated partial cytochrome P450 cDNAs for two other drosophilid species, *D. nigrospiracula* and *D. hydei*. An alignment of the deduced amino acid sequences encoded by these genes with the corresponding region (helix L through the translational stop codon) of *CYP28A1* and *CYP28A2* is presented in Fig. 4. In pairwise comparisons of clone *DU33ng* (the most closely related sequence from *D. nigrospiracula*) and clone *DU4hy* (the most closely related sequence from *D. hydei*) with *CYP28A1*, amino acid positional identity was 72% and 81%, respectively. Compared with *CYP28A2*, identity dropped to 56% in both cases. This was the same level of identity seen between *CYP28A1* and *CYP28A2*. The sequences for *DU33ng* and *DU4hy* have been

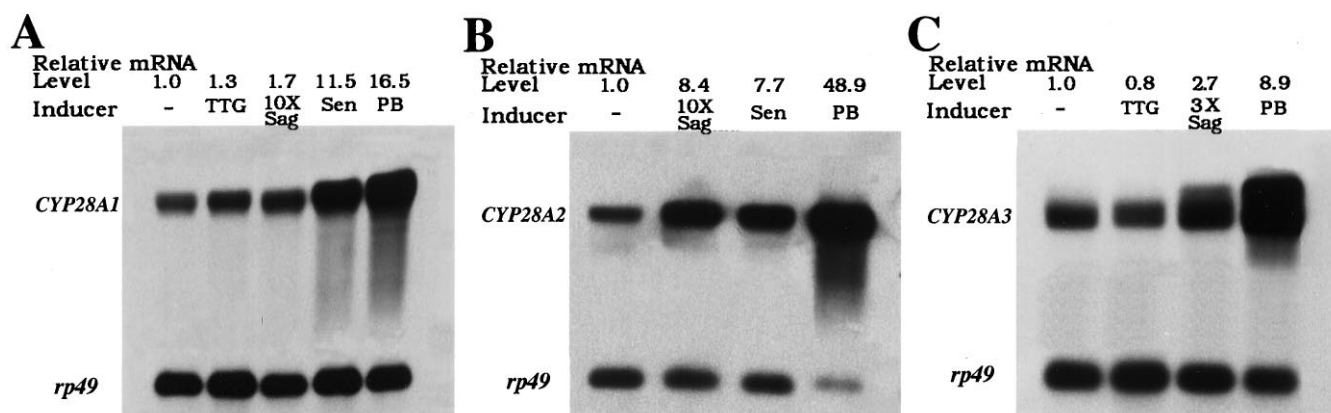


FIG. 1. Analysis by Northern hybridization of the responsiveness of *CYP28A1* (A), *CYP28A2* (B), and *CYP28A3* (C) to agria cactus triterpene glycosides (TTG), saguaro cactus alkaloids (Sag), senita cactus alkaloids (Sen), and phenobarbital (PB). Induction relative to untreated controls (–) was standardized to signal from a homologous *rp49* probe. Images of the sequential cytochrome P450 and *rp49* probings of the same blot have been superimposed. (Note: Interference from residual rRNA produces the “doublet-like” bands in the 3× Sag and PB lanes of C.)

Northern hybridization of mRNA from third-instar *D. mettleri* larvae that had been previously exposed to senita alkaloids (lophocereine and pilocereine) demonstrated significantly in-

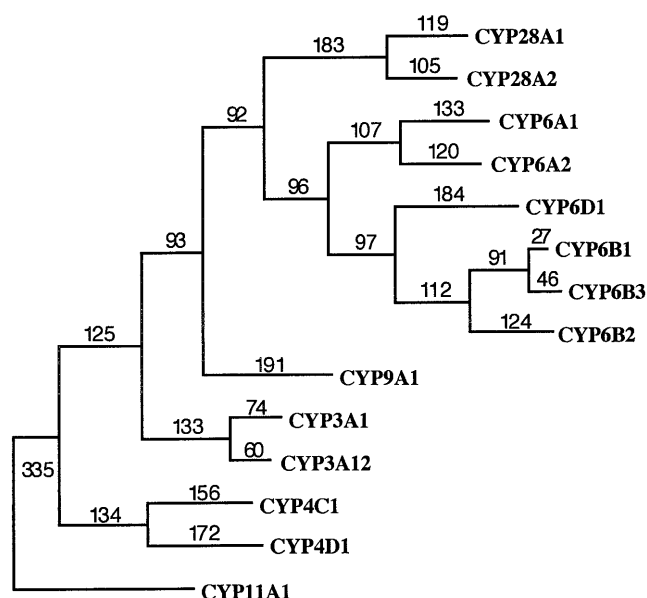


FIG. 3. Phylogram based on the single most parsimonious tree generated from the aligned full-length amino acid sequences of CYP28A1, CYP28A2, and representatives of the CYP3, CYP4, CYP6, and CYP9 families. The human mitochondrial CYP11A1 served as the outgroup. Branch lengths are indicated in terms of the predicted number of amino acid changes.

creased message levels for only 3 of 15 putative cytochrome P450s. Only one of the 3 senita alkaloid-inducible sequences, however, appeared to be a member of the CYP6A family, based on its strong sequence identity of 69% and 64% when aligned with *D. melanogaster* CYP6A9 and *Ceratitis capitata* CYP6A10, respectively. The corresponding region (helix L through the translational stop codon) of the remaining two senita alkaloid-responsive sequences displayed only weak sequence identity when aligned with known cytochrome P450 genes (e.g., 33% identity with CYP6B4v1).

Full-length cDNA sequences for these genes, CYP28A1 and CYP28A2, were subsequently obtained, and their conceptual amino acid translations reveal proteins clearly having the three salient characteristics of members of the cytochrome P450 superfamily. These are as follows: the conserved FXXGXXX-CXG sequence of the heme-binding decapeptide near the C terminus, the DGXXT motif associated with formation of the oxygen-binding pocket of helix I (30), and the conserved EXXR pair within helix K that is thought to hydrogen bond with the "meander," a stretch of residues N-terminal to the heme-binding decapeptide (31).

Historically, cytochrome P450 nomenclature has been based on a seemingly arbitrary set of criteria where proteins exhibiting <40% amino acid sequence identity have been assigned to separate gene families. In recent years, however, the cloning of cytochrome P450s from more divergent species, including arthropods, has necessitated a shift to a nomenclature system that focuses more on regional sequence identity and that takes into account the results of phylogenetic analyses. The cock-

roach CYP4C1, therefore, was included in the CYP4 family despite having only 32–36% positional identity with its vertebrate homologs (32). Similarly, conservation around the heme-binding decapeptide shows the evolutionary relatedness of CYP6A1 and CYP6B2 despite their <40% global sequence identity (2). The members of the CYP28 family, therefore, represent a distinct branch of xenobiotic-inducible cytochrome P450s. Evidence for the uniqueness of this gene family is found in the single most parsimonious tree of the aligned full-length sequences for 11 insect and 3 vertebrate cytochrome P450s, most of which have been linked to xenobiotic metabolism (Fig. 3). The clustering of the CYP28 sequence in the same clade as the xenobiotic-metabolizing vertebrate CYP3 and invertebrate CYP6 cytochrome P450s, rather than with the invertebrate CYP4 P450s [which are thought to play a role in energy mobilization rather than toxin metabolism (32)], suggest both evolutionary relatedness and the possibility of similar physiological functions. At the same time, bootstrap analyses point to the distinctiveness of the CYP28 sequences among xenobiotic-metabolizing families. When CYP4C1 was used as an outgroup, the CYP6 and CYP28 sequences in Fig. 3 formed separate monophyletic clades with bootstrap values of 80 and 100, respectively. Inclusion of the CYP4, CYP9, and CYP3 clades (each being monophyletic) with CYP11A1 as the outgroup produced a polytomy among all but the CYP4 sequences. This is consistent with the view that the major families of xenobiotic-metabolizing cytochrome P450s arose after the CYP4 family and suggests that the CYP28 family may be as distant from the invertebrate toxin-metabolizing enzymes as it is from vertebrate CYP3 cytochrome P450s.

The induction of the *D. mettleri* DU108 and CYP28 cytochrome P450s by the toxic alkaloids present in the tissue of one of the species' host plants (i.e., senita cactus) and phenobarbital corroborate the results of earlier *in vitro* alkaloid metabolism studies (8, 17). These studies demonstrated a significant increase in alkaloid metabolism by isolated microsomes from *D. mettleri* larvae exposed to senita tissue and an even greater increase following phenobarbital exposure. Of greater significance with respect to possible involvement of these cytochrome P450s in xenobiotic metabolism, though, was the strong and highly specific induction of CYP28A1 by alkaloids that are present in senita cactus tissue at concentrations well in excess of those for any alkaloid in any other species of columnar cacti (i.e., 15–23% of the plant's dry weight). Interestingly, exposure to an equivalent concentration of saguaro alkaloids did not produce a significant increase in CYP28A1 message. This is in sharp contrast to CYP28A2 and DU108, which were both crossinducible by senita as well as the chemically similar saguaro cactus alkaloids. This pattern suggests that resistance to senita alkaloids in *D. mettleri* may involve the cooperative action of multiple cytochrome P450s, which include at least one "specialist" that responds only to senita alkaloids and multiple "generalists" capable of responding to a broader range of chemically similar compounds. The action of such alkaloid-inducible "generalist" cytochrome P450s may also be reflected in the greater *in vivo* resistance of *D. mettleri* to another plant alkaloid, nicotine, relative to a non-alkaloid-resistant strain of *D. melanogaster* (33).

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CYP28A3 (PCR Primer) MRFALIQIKAAVVEVITKFNVRVNPKTRKDNEYEPTAFITSLKGGIWLD FESRP *
CYP28A1 (FGDGPRVCIG) .....G.L..LL...II.....S...D..T..GTC.....L.Q *
CYP28A2 (FGDGPRICIG) ...G.A.A...L..ILVN.D.S..AR.....L.D.KNLLST.E.....AA.S *

CYP28A4 (PCR Primer) MRFAMTQIKGALVEVLTKFNVRVNPKTRTDNEYEPTRFITTLLKGGIWLD FEP RQ *
CYP28A1 (FGDGPRVCIG) ....LI.....L...II.....S...D..T..G.C.....L.. *
CYP28A2 (FGDGPRICIG) ...GLA.A.A...I.VN.D.S..AR...K..L.D.KNLLS..E.....AA.S *

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FIG. 4. Alignment of the deduced amino acid sequences (helix L through translational stop) of CYP28A3 and CYP28A4 with CYP28A1 and CYP28A2. Identical amino acids are indicated by periods and differences, by the appropriate amino acid single-letter symbol.

Finally, none of the 15 *D. mettleri* cytochrome P450s were induced by toxic triterpene glycosides isolated from the agria cactus. Insofar as induction has been indicative of enzymatic activity, the absence of induction suggests the lack of involvement of these genes in triterpene glycoside metabolism. With respect to the senita alkaloid-responsive cytochrome P450s, in particular, this was expected, given the chemical dissimilarity between isoquinoline alkaloids and triterpene glycosides. The general lack of triterpene glycoside inducibility was also not surprising, as *D. mettleri* larvae are unable to utilize agria rot exudate-soaked soils.

CYP28A3 and *CYP28A4* (isolated from *D. nigrospiracula* and *D. hydei*, respectively) represent two additional members of the CYP28 family. It appears more likely that both are orthologs of *CYP28A1* rather than *CYP28A2*, in view of the greater positional identity of these genes *vis-à-vis* *CYP28A1*. Because *D. nigrospiracula* utilizes alkaloid-containing saguaro cactus tissue as a feeding and breeding substrate, it was possible to begin to determine whether the xenobiotic responsiveness of the CYP28 genes is a feature unique to *D. mettleri* or a more generalized characteristic of the gene family as a whole. A total of eight different *D. nigrospiracula* cytochrome P450 genes were screened by Northern hybridization for induction by saguaro alkaloids, triterpene glycosides, and phenobarbital. The general pattern of induction was remarkably similar to that of the CYP28 cytochrome P450s in *D. mettleri*. Phenobarbital induction of *CYP28A3* was greater than for any other *D. nigrospiracula* cytochrome P450 tested. Furthermore, it was the only one of eight cytochrome P450s cloned from *D. nigrospiracula* to show induction by saguaro alkaloids. No induction by triterpene glycosides was observed. As before, these findings are consistent with previous *in vitro* alkaloid metabolism studies and the inability of *D. nigrospiracula* to utilize the triterpene glycoside-containing agria cactus as a host plant.

The demonstrated xenobiotic-inducibility of at least three members of the CYP28 family certainly points to a potentially significant role for the CYP28 family in host-plant utilization. At the same time, however, *D. nigrospiracula* clearly expresses an inducible *CYP28* gene but is unable to utilize senita as a host plant, and *D. hydei* is incapable of utilizing any alkaloid-containing cactus species. Thus, while the CYP28 family, as a whole, may be readily induced by and possibly capable of conferring resistance to some plant allelochemicals, coevolutionary processes unique to the natural history of each species may account for the obvious differences in the observed patterns and magnitude of induction.

In conclusion, the use of a PCR-based cloning strategy has resulted in the isolation of four members of a previously unknown family of insect cytochrome P450s, designated CYP28, from three species of *Drosophila*. The CYP28 family is one of only two families of insect P450s (the other being CYP6) that have been found to display xenobiotic responsiveness at the mRNA level and may, therefore, play a role in the detoxification of natural and/or anthropogenic compounds. The ability of toxic isoquinoline alkaloids and phenobarbital to induce CYP28 message significantly in *D. mettleri* larvae suggests that (along with at least one other CYP6 cytochrome P450) these genes may be involved in utilization of soils soaked by senita cactus rot exudate. Similarly, at least one CYP28 gene may be a significant factor in saguaro cactus utilization by *D. nigrospiracula*.

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