A phthalate family oxygenase reductase supports terpene alcohol oxidation by CYP238A1 from *Pseudomonas putida* KT2440

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Abstract

CYP238A1, one of the two P450 enzymes in the genome of *Pseudomonas putida* KT2440, has been produced heterologously in *Escherichia coli*, purified, and found to bind acyclic and cyclic terpene alcohols such as farnesol, nerolidol, linalool, and terpineol. The other P450 enzyme in this organism (gene locus: *PP1950*) was also produced in *E. coli* but no substrate has been identified from a limited screen. A phthalate family oxygenase reductase (PFOR) encoded by the *PP1957* gene, just downstream of the *PP1955* gene for CYP238A1, accepts electrons from the reduced form of both nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate and is able to support monooxygenase activity of CYP238A1, both *in vitro* and in *E. coli*, in which both enzymes are produced. CYP238A1 oxidizes *cis*- and *trans*-nerolidol to the 9-hydroxy product, with no evidence of attack at the olefinic double bonds. The NADH turnover rate of 170 nmol(nmol-P450)$^{-1}$ Min^{-1} for CYP238A1 with *cis*-nerolidol as substrate at a PP1957:CYP238A1 concentration ratio of 8:1 suggests that this PFOR could function as the physiological redox partner for CYP238A1. The physiological role of CYP238A1 may be related to the *PP1955* gene being part of an island/cluster of inducible genes associated with energy metabolism and response to xenobiotics. Volume 60, Number 1, Pages 9–17, 2013

Keywords: *CYP, electron transfer, P450, PFOR, phthalate dioxygenase reductase, terpenes*

1. Introduction

Cytochrome P450 (CYP) enzymes are found in microorganisms, plants, and mammals. P450 enzymes primarily catalyze the chemically challenging reaction of C–H bond oxidation but other activities are known, including dealkylation, desaturation, C–C bond formation, dehydration, decarboxylation, and skeletal rearrangements [1–3]. By virtue of these varied activities, P450 enzymes are involved in biological processes as diverse as carbon source assimilation, biosynthesis, and biodegradation of endogenous compounds, antibiotic synthesis, xenobiotic and drug metabolism, bioprotection, and plant–insect interactions. In addition to these physiological roles, the oxyfunctionalization of organic molecules by P450 enzymes has numerous potential applications in benign synthesis of intermediates and end products [4]. Whole-cell reactions are most commonly carried out in *Escherichia coli*, which can suffer from issues such as substrate and product toxicity and entry of hydrophobic compounds through the outer membrane. Other Gram-negative bacteria, in particular robust Pseudomonads [5–7], have been studied as suitable hosts, and recently the use of Gram-positive organisms such as *Bacillus* was reported [8].

Abbreviations: *CYP, cytochrome P450; PFOR, phthalate family oxygenase reductase; NADH, nicotinamide adenine dinucleotide reduced form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; DMSO, dimethyl sulfoxide; GC, gas chromatography.*

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Pseudomonads are known to be tolerant of organic compounds, a trait associated with their highly adaptable nature [9], notable examples being strains that tolerate high concentrations of toluene [10] or grow on terpenoid compounds as the sole carbon source [11, 12]. The archetypal P450cam (CYP101A1) system was isolated from such a strain [13]. *Pseudomonas putida* GPo12 expressing the CYP153A6 enzyme system was used for the oxidation of *S*-limonene to *S*-perillyl alcohol [5]. When the system was transferred to *P. putida* KT2440, the yield of *S*-perillyl alcohol reached 6.5 g/L of aqueous culture (0.43 g/g-cell-dry-weight) [6]. In screening Pseudomonads for their tolerance to organic compounds, we found that *P. putida* KT2440 was able to grow on *R*-limonene as the sole carbon source (to be published). Although analysis of the organics did not reveal the presence of intermediates of oxidative metabolism such as carveol and perillyl alcohol, we set out to examine the properties of the two P450 enzymes in the genome of this organism. The results of these studies are reported here. PP1950 and CY238A1 in the *P. putida* KT2440 genome were heterologously produced in *E. coli*, as was the protein encoded by the *PP1957* gene—a member of the group of phthalate family oxygenase reductase (PFOR) enzymes. CYP238A1 was found to bind cyclic and acyclic terpene alcohols and PP1957 to transfer electrons to CYP238A1 to support substrate oxidation. (We adopt the convention that the names of genes are italicized, *e.g.*, *PP1957*, whereas the name of the encoded protein is not, *i.e.*, PP1957.) An *in vivo* system of the CYP238A1/PP1957 pair was constructed and CYP238A1 shown to oxidize *cis*- and *trans*-nerolidol to 9-hydroxy-*cis*-nerolidol and 9-hydroxy-*trans*-nerolidol, respectively.

2. Materials and Methods

2.1. General

General DNA and microbiological experiments were carried out using standard methods [14]. The KOD polymerase, used for the PCR steps, and the pET26a and pETDuet expression vectors were from Merck (Beeston, Nottingham, UK). T4 DNA ligase and restriction enzymes for molecular biology were from New England Biolabs (Hitchin, Hertfordshire, UK). General reagents and organic compounds were from Sigma–Aldrich (Gillingham Dorset, UK) or Merck. Isopropyl-β-D-thiogalactopyranoside (IPTG), growth media, and buffer components were from Melford Laboratories (Ipswich, Suffolk, UK). Nicotinamide adenine dinucleotide reduced form (NADH) and nicotinamide adenine dinucleotide phosphate reduced form (NADPH) were from Roche Diagnostics (Burgess Hill, West Sussex, UK).

2.2. Cloning, expression, and purification

Pseudomonas putida KT2240 was stored in 20% (vol%/vol%) glycerol at −80 °C. It was streaked onto Luria-Bertani (LB) agar plates and incubated at 28 °C for 48 H. A broth culture of 5 mL LB in a sterile 30-mL tube was inoculated with a single bacterial colony and shaken at 200 rpm at 28 °C overnight. The genomic DNA was purified using 1 mL of bacterial culture using the Wizard® Genomic DNA Purification Kit (Promega, Southampton, Hampshire, UK) following the manufacturer's instructions for genomic DNA isolation from Gram-negative bacteria.

The following oligonucleotide primers (with the restriction sites used for cloning highlighted in bold and underlined) were used to clone the two P450 enzymes and the phthalate dioxygenase reductase from the genomic DNA of *P. putida* KT2240 (Eurofins MWG Operon, Ebersberg, Germany): PP1950_5′, 5′-ttaatt**catatg**tccgaaaccattcgtgttaatgc-3′, PP1950_3′, 5′-ttaatt**aagcttggtacc**ctattagccaaccaggcgtaatggcagag-3′; PP1955_5′, 5′- ttaatt**catatg**gaaatacttgatcgtcctcaag-3′, PP1955_3′, 5′-ttaatt**aagcttggtacc**ctattaccagattacttgcaaag agagg-3′; PP1957_5′, 5′-ttaatt**ccatgg**ccaattggttagaactagaaata tcc-3′, PP1957_3′, 5′-ttaatt**ggatccgaattc**ctattacagctcaagcac caggcgagg-3′. The genes encoding the CYP enzymes PP1950 and PP1955 (henceforth referred to as CYP238A1) were cloned into the expression vector pET26a(+) between the *Nde*I (**catatg**) and *Hin*dIII (**aagctt**) restriction sites. The *PP1957* gene encoding the phthalate dioxygenase reductase-like protein was cloned into the pETDuet vector using the *Nco*I (**ccatgg**) and *Eco*RI (**gaattc**) restriction sites. The sequences of all the genes were confirmed by DNA sequencing (Geneservice, Cambridge, Cambridgeshire, UK).

The whole-cell oxidation system was constructed by excising the *PP1955* gene from the pET26 vector using the *Nde*I and *Kpn*I restriction enzymes and ligating it into the pETDuetPP1957 vector digested with the same two restriction enzymes to generate the pETDuetPP1957/PP1955 recombinant plasmid [15, 16].

All three enzymes were produced using *E. coli* strain BL21(DE3). The cells were grown in 2YT media containing 30 μg L^{-1} kanamycin (pET26a) or 100 μg L^{-1} carbenicillin (pETDuet) at 37 °C for 5 H, and recombinant protein was produced by induction with 0.1 mM IPTG for a further 10 H at 25 °C. The cells were harvested by centrifugation and lysed by sonication at 4 °C. The lysates were centrifuged at 20,000*g* for 1 H to remove the cell debris.

Both P450 enzymes were purified by loading the soluble fraction onto a DEAE FastFlow Sepharose column (200 mm × 50 mm; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and eluting with a linear salt gradient of 50–250 mM KCl in 50 mM Tris, pH 7.4, 1 mM dithiothreitol (buffer T) developed over eight column bed volumes at a flow rate of 8 mL Min^{-1}. The red colored fractions were collected, concentrated by ultrafiltration, and desalted using a Sephadex G-25 column (200 mm × 40 mm), eluting with buffer T. The final purification step was anion-exchange chromatography using a Source-Q column (120 mm × 26 mm; GE Healthcare), and the protein was eluted using a linear gradient of 0–130 mM KCl in buffer T developed over 20 column bed volumes at a flow rate of 8 mL Min^{-1}. The fractions with the greatest ratios of A_{418}/A_{280} were collected and the purity of the CYP enzyme was estimated to be greater than 95% using an A_{418}/A_{280} ratio of ∼1.5. P450 enzyme concentrations were estimated

using $\varepsilon_{450–490} = 91$ mM^{-1} cm^{-1} for the ferrous-CO species [17]. The PP1957 phthalate dioxygenase reductase was produced and purified as described for the analogous enzyme from *Pseudomonas cepacia* [18]. Its concentration was estimated using $\varepsilon_{455} = 24.5$ mM^{-1} cm^{-1} [19]. All enzymes were stored at −20 °C in 50 mM Tris, pH 7.4 containing 50% (vol%/vol%) glycerol.

2.3. Substrate binding

Glycerol was removed immediately before use by gel filtration on a 5-mL PD-10 column (GE Healthcare) by eluting with 50 mM Tris, pH 7.4. UV/vis spectra were recorded at 30 ± 0.5 °C on a Varian CARY-50 or CARY-1E spectrophotometer (Agilent, Yarnton, Oxfordshire, UK). For spin-state shift assays the PP1950 and CYP238A1 (PP1955) enzymes were diluted to about 2 μM in 50 mM Tris, pH 7.4 (1 mL). Aliquots of substrate (0.5–2 μL) were added using a Hamilton syringe from a 100 mM stock solution in dimethyl sulfoxide (DMSO). The high-spin heme content was estimated (to approximately ±5%) by comparison to a set of spectra generated from the sum of the appropriate percentages of the spectra of the substrate-free (>95% low-spin, Soret peak at 418 nm) and camphor-bound (>95% high-spin, Soret peak at 392 nm) forms of WT CYP101A1.

To determine the nerolidol binding constant, the CYP238A1 enzyme was diluted to about 2 μM in 50 mM Tris, pH 7.4 (2.5 mL). Aliquots of substrate (0.5–2 μL) were added using a Hamilton syringe from 1, 10, and 100 mM stock solutions and the sample was mixed and the difference spectrum was obtained between 700 and 250 nm. The peak-to-trough difference in absorbance was recorded. Further aliquots of substrate were added until the peak-to-trough difference did not change further. The apparent binding constant, K_d, of farnesol to CYP238A1 was sufficiently high ($K_d > 5[E]$) for its determination by fitting the peak-to-trough difference versus substrate concentration to a rectangular hyperbola:

$$\frac{\Delta A}{\Delta A_{max}} = \frac{[S]}{K_d + [S]}$$

where ΔA is the peak-to-trough absorbance difference, ΔA_{max} is the maximum absorbance difference, and $[S]$ is the substrate concentration. In contrast, the much lower binding constant, K_d, of *cis*-nerolidol ($K_d < 5[E]$) required fitting of the peak-to-trough difference versus substrate concentration to the quadratic equation for tight binding:

$$\frac{\Delta A}{\Delta A_{max}} = \frac{[E] + [S] + K_d - \sqrt{([E] + [S] + K_d)^2 - 4[E][S]}}{2[E]}$$

where ΔA is the peak-to-trough absorbance difference, ΔA_{max} is the maximum absorbance difference, $[S]$ is the substrate concentration, and $[E]$ is the enzyme concentration.

2.4. Product formation

Steady state NADH consumption assays were performed with mixtures (1.2 mL) containing 50 mM Tris, pH 7.4, 0.5 μM CYP238A1, 1 μM PP1957 (and 4 μM), and 100 μg/mL bovine liver catalase. The mixtures were equilibrated at 30 °C for 2 Min. NADH or NADPH was added to about 320 μM (final $A_{340} = 2.00$) and the absorbance at 340 nm was monitored. Substrates were added as a 100 mM stock solution in DMSO to a final concentration of 1 mM. The rate of NAD(P)H turnover was calculated using $\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}.

Products were extracted from the enzyme mixture using ethylacetate (EtOAc). Gas chromatography (GC) analyses were performed on a ThermoFinnigan Trace GC instrument (ThermoFisher Scientific, Lutterworth, Leicestershire, UK) equipped with an autosampler and a CP-Sil 8 fused silica column (15 m × 0.25 mm; Varian) using helium as the carrier gas (a flow rate of 2.0 mL Min^{-1}) and flame ionization detection. The injector and flame ionization detector were held at 200 and 250 °C, respectively. The retention times for the different organic compounds were 4.7 Min for *cis*-nerolidol, 4.9 Min for *trans*-nerolidol, 5.9 Min for 9-hydroxy-*cis*-nerolidol, 6.2 Min for 9-hydroxyl-*trans*-nerolidol, and 6.3 Min for 9-hydroxyfluorene (internal standard, which was not added to the whole-cell oxidation reaction of *cis*- or *trans*-nerolidol).

2.5. Growth and expression of the *in vivo* system

The plasmid pETDuetPP1957/PP1955 was transformed into competent BL21(DE3) cells and grown on LB plates containing 100 μg/mL carbenicillin (LB_{carb}). A single colony was inoculated into 500 mL $2YT_{carb}$ broth in a 2-L flask and grown at 37 °C overnight. Protein expression was induced by the addition of 60 μM IPTG (from a 0.4 M stock in H_2O); the temperature was reduced to 20 °C. The growths were allowed to continue for another 24 H before the cell pellet was harvested by centrifugation and washed in *E. coli* minimal media (EMM) [20].

The cell pellet (wet cell weight 5–6 g/L) was resuspended in an equal volume of antibiotic-containing media (EMM_{carb}). The nerolidol substrate (2 mM from a 1 M stock in EtOH was added to the resuspended cells and a 1-mL aliquot of the media was taken. The reactions were then shaken at 220 rpm and 20 °C and 1-mL samples were taken and analyzed to monitor the course of the reaction. On completion, the whole culture was extracted with dichloromethane, the organic extracts pooled, and the solvent was removed by vacuum distillation and then under a stream of nitrogen. The products were purified using silica gel chromatography using a hexane/ethyl acetate stepwise gradient ranging from 0% to 100%. The residue was dissolved in $CDCl_3$ and the organics characterized by nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were acquired on a Varian Unity-plus and Bruker AvII spectrometer, both operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C. The structures were determined using a combination of ^{1}H, ^{13}C, COSY, HSQC, and HMBC experiments.

9-Hydroxy-*cis*-nerolidol: δ_H (500 MHz, Chloroform-d) 1.27 (3 H, s, Me15), 1.56 (4 H, dt, *J* 9.9, 6.2, H4), 1.66 (3 H, d, *J* 1.0, Me13), 1.71 (3 H, s, Me12), 1.72 (3 H, s, Me14), 1.96–2.10 (1 H, m, H8), 2.05–2.14 (2 H, m, H5), 2.41 (1 H, dt, *J* 13.4, 7.9, H8), 4.48 (1 H, td, *J* 8.5, 5.4, H9), 5.05 (1

H, dd, *J* 10.8, 1.2, H1), 5.16–5.20 (1 H, m, H10), 5.21 (1 H, dd, *J* 17.3, 1.2, H1), 5.28 (1 H, t, *J* 7.2, H6), 5.89 (1 H, dd, *J* 17.3, 10.8, H2). δ_C (126 MHz, Chloroform-d) 18.15(13), 22.70(5), 23.83(14), 25.73(12), 27.97(15), 39.95(8), 42.02(4), 66.79(9), 73.44(3), 111.83(1), 127.72(10), 128.50(6), 131.43(7), 134.81(11), 144.80(2).

9-Hydroxy-*trans*-nerolidol: δ_H (500 MHz, Chloroform-d) 1.27 (3 H, s, Me15), 1.53–1.63 (2 H, m, H4), 1.65 (3 H, d, *J* 1.3, Me14), 1.68 (3 H, d, *J* 1.4, Me13), 1.71 (3 H, d, *J* 1.4, Me12), 2.05–2.09 (2 H, m, H5), 2.12 (1 H, t, *J* 5.6, H8), 4.43 (1 H, tdd, *J* 7.8, 5.5, 1.7, H9), 5.05 (1 H, dd, *J* 10.8, 1.3, H1), 5.15 (1 H, dt, *J* 8.4, 1.4, H10), 5.21 (1 H, dd, *J* 17.3, 1.3, H1), 5.24–5.30 (1 H, m, H6), 5.90 (1 H, ddd, *J* 17.4, 10.8, 4.2, H2). δ_C (126 MHz, Chloroform-d) 16.36(14), 18.33(13), 23.05(5), 25.89(12), 28.09(15), 41.84(4), 48.20(8), 66.08(9), 73.61(3), 111.85(1), 127.62(10), 128.64(6), 131.93(7), 135.04(11), 145.12(2).

3. Results

3.1. P450 enzyme production and substrate screening

Two potential CYP genes (*PP1950* and *PP1955*) were detected in the genome of *P. putida* KT2240. They are both found in a region of the genome having an atypical G+C composition (insertion size 64.8 kb, genes *PP1919* to *PP1965*), which has been reported to be a mobile element linked to energy metabolism [9].

CYP family and subfamily assignments are made according to the P450 nomenclature [21]. The assignments are based on the amino-acid sequences of the P450 enzymes: 40% identity places a P450 in the same family (unless homologues with lower identity are demonstrated to be functionally equivalent), and 55% places it in the same subfamily. The *PP1955* gene has been assigned as CYP238A1 by David Nelson and the other has some sequence similarity to the CYP199 family of enzymes found in *Rhodopseudomonas palustris* species and *Bradyrhizobium japonicum* [22, 23]. Both the *PP1950* and *PP1955* genes have been reported to be upregulated in a study scanning the genome-wide gene expression on RNA extracted from sterilized soil inoculated with *P. putida* KT2440/pSL1, which contains a chloroaromatic degrading plasmid (pSL1) using microarray techniques [24].

A phthalate dioxygenase reductase-like gene (*PP1957*, phthalate family oxygenase reductase—PFOR) was found close to these two *CYP* genes. PP1957 is similar to the P450 redox partner domain of the CYP116 family of fusion proteins first described in *Rhodococcus* sp. (P450Rhf) [25] and subsequently found in *Cupriavidus metallidurans* [26] and *Rhodococcus ruber* [27].

Both *CYP* genes and the *PFOR* gene from *P. putida* KT2240 were readily amplified by PCR and cloned into standard expression vectors. The determined nucleotide sequences of the cloned genes were in complete agreement with those in the genome database. The two P450 enzymes were produced in soluble form in *E. coli* and the heterologous expression of these P450s supports the correct identification of the initiation sites. Both enzymes were purified by two anion-exchange chromatography steps and found to be in their low-spin ferric forms with Soret maxima at about 418 nm. In addition, both showed the characteristic 448–450 nm absorption for the $Fe^{II}(CO)$ complex, with minimal amounts of the inactive P420 form (P420 formation was induced by CO bubbling) (Figs. 1a and 1b). The P450 enzymes were stable and retained their activity on storage at −20 °C for prolonged periods (>12 months).

The *PP1957* gene was also cloned and the protein produced in soluble form in *E. coli*. The purified PFOR protein was yellow/orange in color and showed very similar spectral properties to those reported for phthalate dioxygenase reductase [18] with maxima at 277, 348, 416, 460, and 485 nm (Fig. 1c). The initial data suggested that this enzyme is similar to PFOR from *P. cepacia* in containing an FMN domain and a 2Fe–2S ferredoxin domain [28].

The substrate specificity of the two P450 enzymes was investigated by screening classes of compounds that were known to be metabolized by *P. putida* or oxidized by other close P450 enzyme homologues. These included cyclic and acyclic mono- and sesquiterpenoids, short and medium-chain (C_6—C_{12}) linear alkanes, benzene and polycyclic aromatic hydrocarbons, substituted phenols, substituted benzoic acids, polychlorinated benzenes, and steroidal compounds. Substrate binding was assayed by the classical type I shift of the Soret band from 418 nm in the substrate-free form to 390 nm for the high-spin form when a substrate was bound in close proximity to the heme to displace the water bound to the iron.

Although 3-chlorobenzoic acid was reported to induce the expression of the cluster of genes that included those for the two P450 enzymes [24], neither it nor 2- and 4-chlorobenzoic acid induced a significant spin-state shift (<10%) with PP1950 or CYP238A1. None of the other tested compounds induced a spin-state shift with PP1950. However, CYP238A1 showed type I shifts with a variety of terpenoid compounds (Table 1). *R*-limonene induced a shift of about 10%, whereas terpineol gave a slightly greater shift of 20% (Fig. 2a), as did β-ionone, whereas both α-ionone and bisabolol gave 55% shifts. Linalool and nerolidol (Fig. 2b) induced 40% and 50% spin-state shifts, respectively, but lower shifts were observed with farnesol (25%) and geraniol (10%; Figs. 2c and 3).

3.2. P450 activity reconstitution and product characterization

To reconstitute the monooxygenase activity of CYP238A1, we used the PFOR to mediate electron transfer from NAD(P)H to the P450. Under steady-state turnover conditions (0.5 μM P450 and 1 μM PFOR) with *cis*-nerolidol as a substrate, the NADH oxidation rate was 45 ± 2 nmol(nmol-P450)$^{-1}$ Min^{-1} versus an NADPH oxidation rate of 37 ± 3 nmol(nmol-P450)$^{-1}$ Min^{-1}. When the PFOR concentration was increased to 4 μM, the rate with NADH and NADPH increased to 170 and 160 nmol(nmol-P450)$^{-1}$ Min^{-1}, respectively.

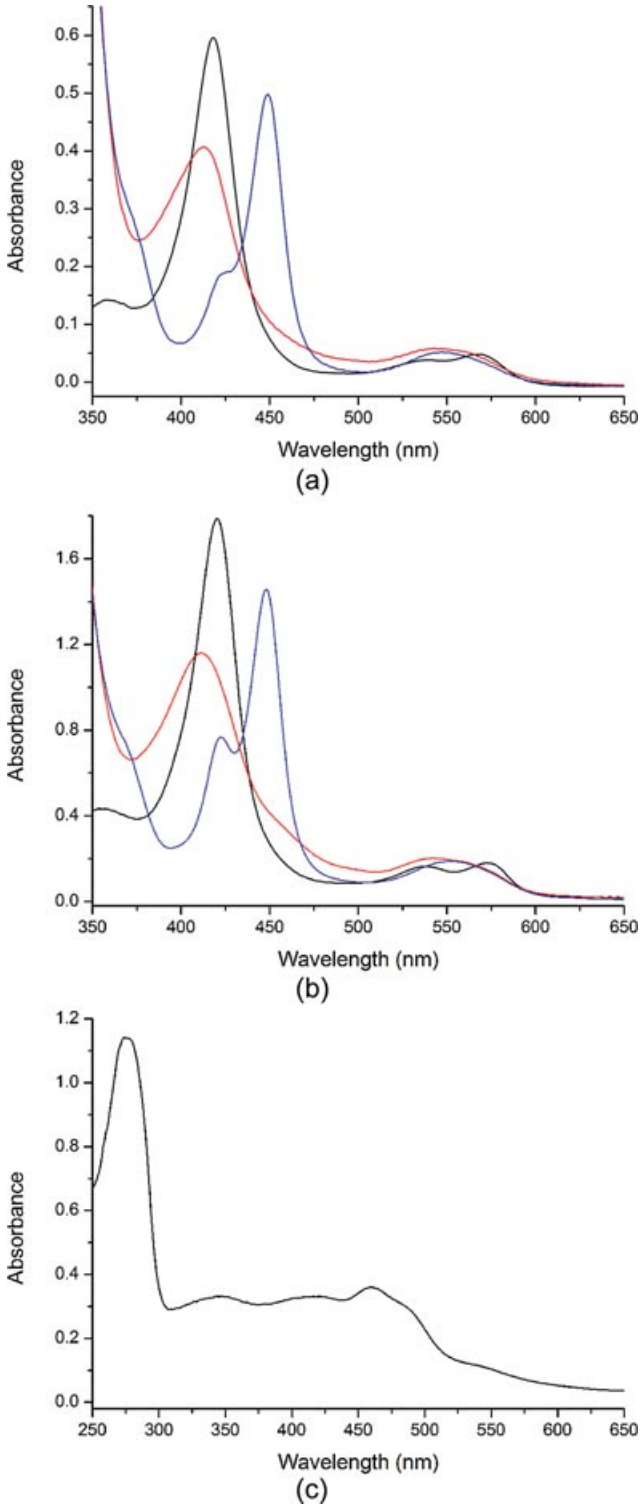

FIG. 1 *The electronic spectra of the P450 enzymes (a) PP1950 and (b) CYP238A1, and of (c) the phthalate dioxygenase reductase PFOR (PP1957). For the P450 enzymes, the spectra of the ferric resting state are in black, the ferrous form in red, and the $Fe^{II}(CO)$ species in blue (c).*

TABLE 1 ***The induced heme spin-state shift (estimated to within 5%) and NADH consumption rates of terpenoid compounds with CYP238A1***

Substrate	*High-spin heme content*	*NADH consumption rate*
R-limonene	10%	BR
Terpineol	20%	BR
Geraniol	10%	BR
Linalool	40%	BR
Farnesol	25% (9.0 ± 1.6 μM)	58 ± 2.1
α-Bisabolol	55%	BR
α-Ionone	55%	BR
β-Ionone	20%	BR
cis-Nerolodiol	55% (1.96 ± 0.08 μM)	45 ± 2.0
trans-Nerolidol[a]	55%	49 ± 2.7

Rates are in nmol(nmol-P450)$^{-1}$ Min^{-1} for incubations with 0.5 μM CYP238A1 and 1.0 μM PFOR and given as mean ± SD for at least three experiments. BR: the rates are not above the background rate [~10 nmol(nmol-P450)$^{-1}$ Min^{-1}] in the absence of an added substrate. The dissociation constant (K_d) of substrate binding for *cis*-nerolidol and farnesol are given in brackets.

[a]The *trans*-nerolidol sample contained ~40% *cis*-nerolidol.

Although α-bisabolol, linalool, and α-ionone induced spin-state shifts similar to nerolidol, the NADH turnover rates with these molecules were barely above the background rate arising from air oxidation of reduced PFOR [~10 nmol(nmol-PFOR)$^{-1}$ Min^{-1}]. On the other hand, farnesol induced just 25% high-spin heme content but gave a NADH turnover rate of 58.0 ± 2.1 nmol(nmol-P450)$^{-1}$ Min^{-1} for reactions with 0.5 μM P450 and 1 μM PFOR. The lack of correlation between the extent of the spin-state shift and NADH turnover rate is not uncommon in P450 catalysis.

Gas chromatography analysis of organic extracts from the incubations showed products from *cis*- and *trans*-nerolidol and farnesol oxidation. The highest product concentration was observed for *cis*- and *trans*-nerolidol, and both gave just one product (Figs. 4 and 5). A similar amount of product was formed with either NADH or NADPH. Farnesol gave much smaller quantities of four products. However, no products were detected in the reactions with the other tested substrates, including α-(−)-bisabolol, linalool, and α-ionone that gave comparable spin-state shifts to the nerolidols. These low product yields compared to the amount of NADH consumed indicated low coupling efficiencies for the reactions. The highest coupling was estimated to be ~20% for *cis*-nerolidol, the most productive substrate in this study, by comparison of the substrate and product peak areas in the GC analysis. The coupling did not vary

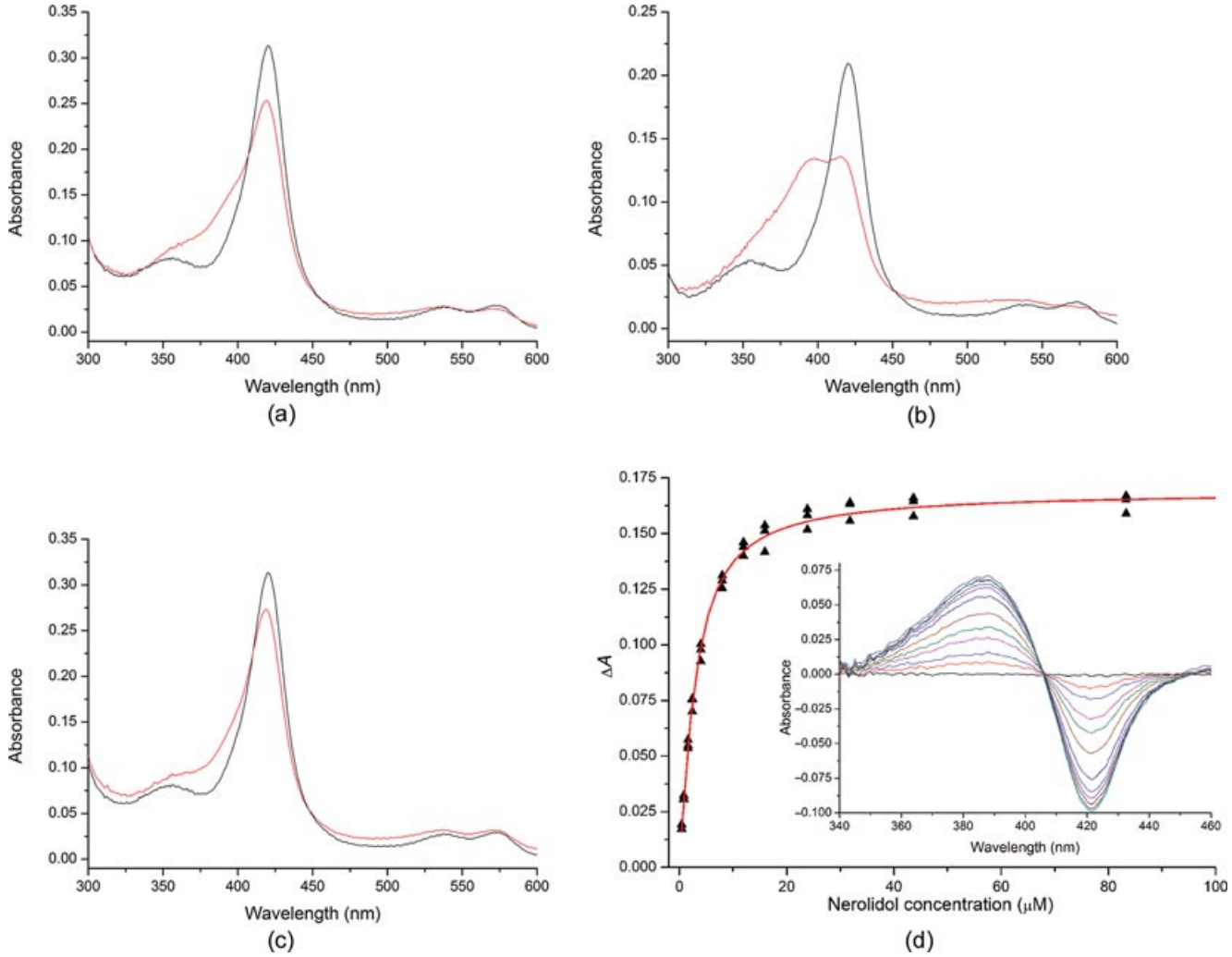

FIG. 2

The heme spin-state shift of CYP238A1 upon binding of (a) terpineol, (b) cis-nerolidol, and (c) geraniol. The substrate-free form is shown in black and the substrate-bound form in red. (d) Spectroscopic titration for cis-nerolidol binding to CYP238A1 and the hyperbolic fit of the peak-to-trough absorbance difference against nerolidol concentration.

significantly with the CYP238A1 to PFOR ratio. Spectroscopic binding titrations with *cis*-nerolidol gave a binding constant of 1.96 ± 0.08 μM (Fig. 2d) and 9.0 ± 1.6 μM for farnesol (data not shown).

To characterize the product of nerolidol turnovers, a whole-cell substrate oxidation system expressing the PFOR electron-transfer protein and CYP238A1 was constructed. This enabled *in vivo* preparative scale reactions for product isolation and characterization. The pETDuetPP1957/PP1955 plasmid was utilized to express the *PP1955* and *PP1957* genes with each gene having its own promoter. The plasmid was transformed into *E. coli* hosts and the cells were grown and proteins overproduced as described in *Materials and Methods*. The growths produced 5–6 g L^{-1} of a dark red-brown cell pellet, indicating successful expression of the P450 components of the system. The whole-cell system was found to be capable of oxidizing *cis*-nerolidol to a single product with the same retention time as that found *in vitro* (Fig. 6). The oxidation of a mixture of *cis*- and *trans*-nerolidol led to two products, one of which had the same retention time as the *cis*-nerolidol product. There was an excess of the *trans*-nerolidol product, suggesting that *trans*-nerolidol may be oxidized preferentially to the *cis* isomer. In both cases not all of the substrate was converted to product; the reaction stopped after several hours but there were no issues with further oxidation of the products. Both products were extracted from the growth medium and isolated via silica chromatography. NMR analysis identified these as 9-hydroxy-*cis*-nerolidol and 9-hydroxy-*trans*-nerolidol (Figs. 3 and 4). Because the stereochemistry at C3 of both nerolidols is not defined, it is not possible to assign the stereochemistry at C9 of the oxidation product. In addition, a minority product was observed in the ^{1}H NMR spectrum of both 9-hydroxynerolidol products, for example, the resonances at 4.95 and 5.35 ppm in Fig. 4a. The 4.49 ppm resonance is consistent with *cis/trans* diastereomeric C9-alcohols but full assignment was not possible because of the spectral overlap. Further work is required to

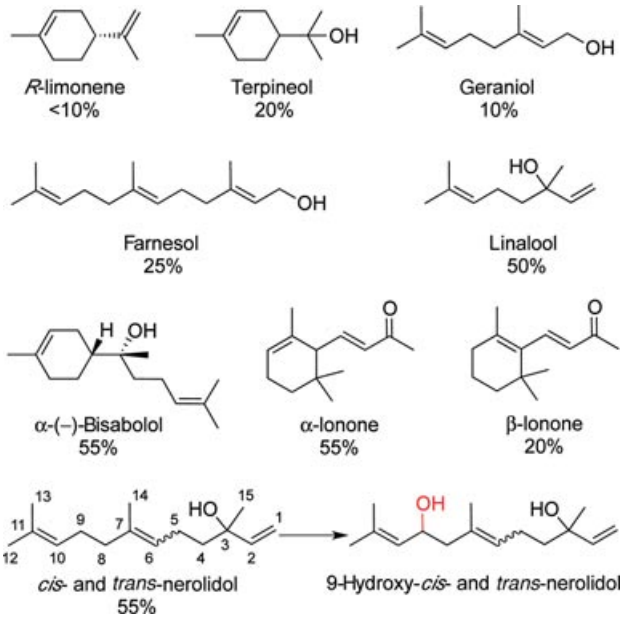

FIG. 3 *Terpenoid compounds shown to bind to CYP238A1 (PP1955) from Pseudomonas putida KT2440. The percentage high-spin heme content induced by binding of the substrates is also given, as is the product of the oxidation of cis- and trans-nerolidol.*

establish the substrate specificity of CYP238A1 for the binding of geometric and stereochemical isomers of nerolidol and the diastereoselectivity of C9 oxidation.

4. Discussion

The *Pseudomonas* genus of Y-proteobacteria are soil-dwelling bacteria that come into contact with numerous toxic compounds such as heavy metals, detergents, biocides, and organic solvents. Many Pseudomonads are found in association with plants where they are exposed to defense agents and organic compounds, especially the terpenoid group of molecules. Their ability to thrive in these hostile environments reflects the metabolic diversity and genetic adaptability of these organisms that readily acquire metabolic pathways through gene transfer. *P. putida* are also suitable hosts for heterologous expression of genes for biotransformation applications [7]. *P. putida* KT2440 is a safe strain and particularly attractive for such purposes. One of the highest yields of a P450-catalyzed biotransformation is reported in this organism [6]. The inducibility and properties of the endogenous P450 enzymes in this organism are thus of interest.

PP1950 has been confirmed to be a P450 enzyme with its signature 450 nm Soret band for the $Fe^{II}(CO)$ species. We were not able to identify a potential substrate for this enzyme by screening a representative collection of common substrates for Pseudomonads with the type I heme spin-state shift assay. The *PP1950* gene was upregulated when *P. putida* KT2440 was exposed to 3-chlorobenzoic acid [24],

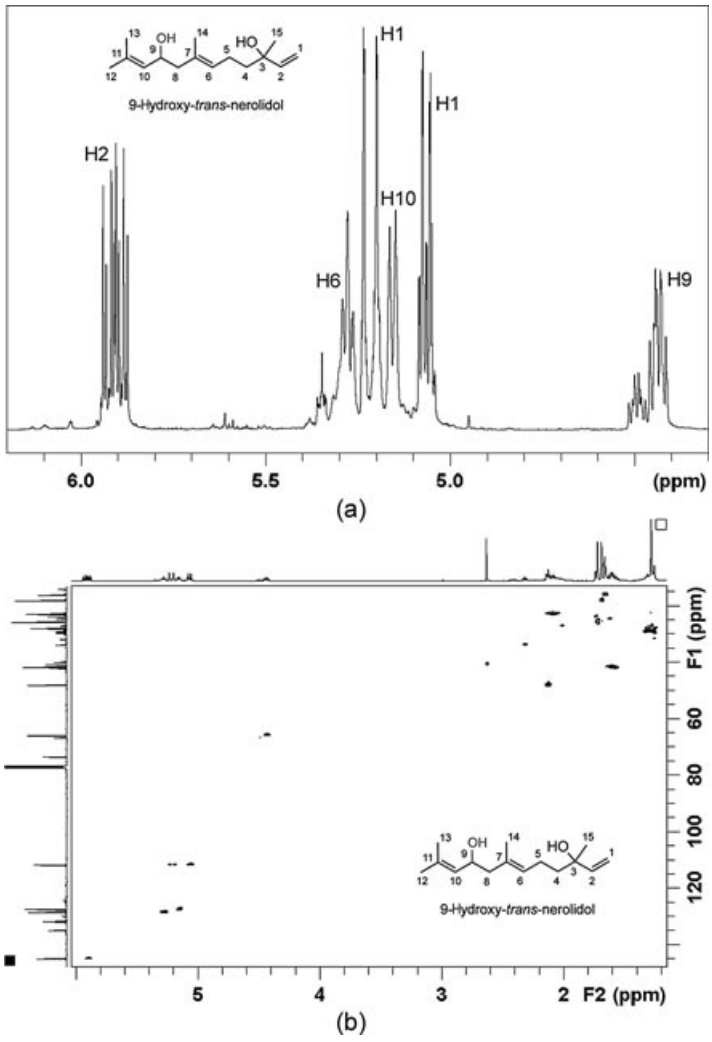

FIG. 4 *(a) The olefinic region, with assignments, of the ^{1}H NMR spectrum of 9-hydroxy-trans-nerolidol with the allylic alcohol proton H9 assigned to the resonance at 4.43 ppm also shown. The resonances at 4.49 and 5.35 ppm are likely to arise from a diastereoisomic C9 alcohol but definitive assignment is not possible because of the extensive spectral overlap. (b) The ^{1}H–^{13}C HSQC spectrum enabling the full assignment of both the ^{1}H and ^{13}C NMR spectra of the majority diastereoisomer of 9-hydroxy-trans-nerolidol (see Materials and Methods).*

but this molecule did not elicit a spin-state shift. PP1950 shares 37% identity with CYP199A2 from *R. palustris* CGA009, which oxidizes *para*-substituted benzoic acids at the 4-position [23]. However, comparison of critical residues involved in 4-methoxybenzoic acid binding to CYP199A2, as revealed by the crystal structure of the enzyme–substrate complex [29], with their counterparts in PP1950 suggested that these residues, for example, Arg94, Glu101, and Ser247 in CYP199A2, are not conserved. It is possible that the PP1950 enzyme does not

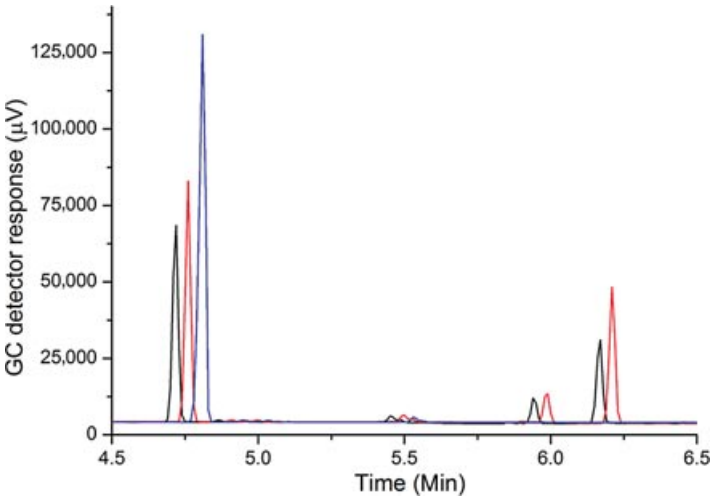

FIG. 5 *Gas chromatography analysis of CYP238A1 in vitro turnovers with cis-nerolidol and NADH with 1 μM PFOR (black) and 4 μM PFOR (red). The cis-nerolidol substrate eluted at RT 4.7 Min (a cis-nerolidol control is shown in blue) with the product eluting at RT 5.9 Min. The 9-fluorenol internal standard eluted at RT 6.3 Min. For clarity the chromatograms have been offset along the x axis. There is an impurity present in commercial cis-nerolidol (blue), which eluted at RT 5.45 Min.*

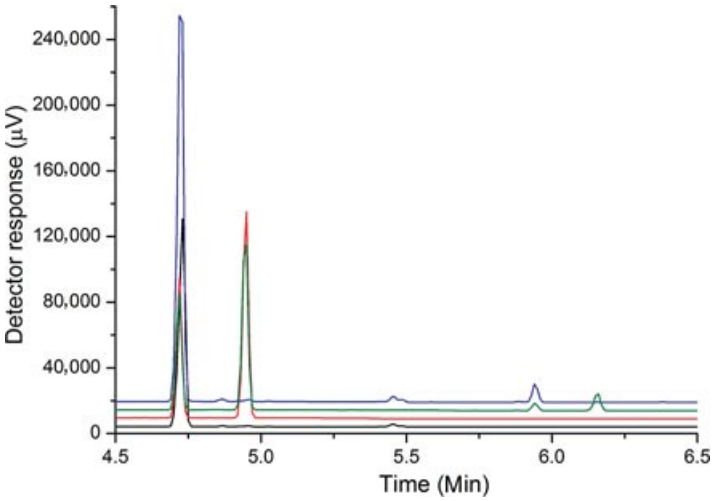

FIG. 6 *Gas chromatography analysis of the CYP238A1 in vivo turnovers of cis-nerolidol (blue) and a mixture of cis- and trans-nerolidol (green) using the pETDuetPP1957/PP1955 plasmid whole-cell expression system in E. coli BL21(DE3). The cis-nerolidol and trans-nerolidol substrates (controls in black and red, respectively) eluted at RT 4.7 and 5.0 Min, respectively. The 9-hydroxy-cis-nerolidol eluted at RT 5.9 Min and 9-hydroxy-trans-nerolidol at RT 6.2 Min. For clarity, the chromatograms have been offset along the y axis.*

oxidize 3-chlorobenzoic acid but its metabolite(s) after initial attack by other enzymes.

The *PP1955* gene was also upregulated by 3-chlorobenzoic acid but the encoded CYP238A1 enzyme did not show a spin-state shift with this compound. On the other hand, mono- and sesquiterpenoid compounds induced spin-state shifts, and the CYP238A1 enzyme showed a distinct preference for compounds with longer chain lengths and tertiary alcohols, for example, farnesol (25% high spin) versus geraniol (10%), linalool (40%) versus geraniol (10%), and nerolidol (55%) versus farnesol (25%).

We found that the PFOR (PP1957) was able to support substrate oxidation by CYP238A1 with NAD(P)H turnover rates increasing from ~50 to ~200 Min^{-1} when the PFOR:CYP238A1 ratio was increased from 2 to 8 with *cis*-nerolidol as the substrate. Interestingly, only *cis*- and *trans*-nerolidol and farnesol gave NADH turnover rates above background even though other molecules, such as linalool and bisabolol, induced similar or greater heme spin-state shifts. Because such a shift is generally indicative of a lower barrier to the first electron-transfer step that initiates the P450 catalytic cycle, the fact that slow NADH turnover is nevertheless observed indicates that subsequent steps in the catalytic cycle have become rate limiting. We note that similar, facile first electron transfer but slow pyridine nucleotide turnover has been reported for a group of mutants of CYP102A1 ($P450_{BM3}$) [30, 31]. It was proposed that the second electron transfer (or some subsequent step such as O–O bond activation) might require substrate-induced conformational changes, and such a mechanism may also operate for CYP238A1.

It is interesting that only the longer nerolidol and farnesol molecules were able to elicit product formation. Furthermore, CYP238A1-catalyzed oxidation of *cis*- and *trans*-nerolidol was highly specific, both forming the 9-hydroxy-derivative as the sole product while the much more reactive internal but especially the terminal olefinic double bonds were not attacked. This indicates that the nerolidol isomers are bound in specific conformations such that only C_9 is close to the heme. It seems reasonable to suggest that specific binding interactions involving the substrate alcohol group and CYP238A1 active-site residue side chains hold the C_3 end of nerolidol (and the terminal alcohol group in farnesol) away from the heme iron such that only compounds with a sufficiently long chain are readily oxidized. The crystal structure of the enzyme–substrate complex will be of interest.

Nerolidol degradation by *Alcaligenes eutrophus* has been reported to proceed via initial epoxidation of the terminal olefinic double bond followed by epoxide ring opening to form the 1,2,3-triol. [32]. The plant pathogen *Glomerella cingulata* [33] and *Streptomyces cinnamonensis*, a Monensin A producing strain [34], both metabolize *cis*-nerolidol by epoxidation at the C_{10}–C_{11} double bond followed by ring opening to form the 3,10,11-triol, whereas the latter also oxidizes *trans*-nerolidol at either the C_{10}–C_{11} or C_6–C_7 double bonds, eventually forming

the 3,10,11- and 3,6,7-triols. 9-Hydroxynerolidol was not observed in these reactions. Although the monooxygenases involved in nerolidol oxidation by these organisms are not known, the involvement of P450 enzymes related to CYP238A1 cannot be ruled out. Wang et al. [24] proposed that *P. putida* KT2440 acquired the *PP1955* gene via horizontal gene transfer from *Actinobacteria*. Some of these organisms are known to be associated with plants, which may account for the terpenoid oxidation activity of CYP238A1 if this hypothesis was correct. In this regard, farnesol oxidation by CYP238A1 may be significant as it is a signaling molecule in *planta*.

The physiological function of the PFOR encoded by the *PP1957* gene is not known. It shares up to 50% sequence identity with vanillate demethylases (vanB) and other PFOR enzymes, including 42% identity with the PFOR from *P. cepacia* and 35% with the reductase domain of CYP116B2 (P450Rhf). Although not fused to the P450 enzyme, unlike the PFOR domains in CYP116B2 and B3, PP1957 supports CYP238A1 monooxygenase activity with a reasonable turnover activity of 170 Min^{-1} with nerolidol as the substrate at a 8:1 PFOR:CYP238A1 ratio. Although this activity is low as compared to about 1,000 Min^{-1} turnover rates of highly active systems such as CYP101A1/Pdx, CYP199A2/Pux [35], and CYP101D2/Arx [36], nerolidol only induces a 50% spin-state shift with CYP238A1, that is, the intrinsic activity at 100% high-spin heme may be much higher. The simple construction and good activity of the *E. coli* whole-cell substrate oxidation system will enable rapid screening of this PFOR for supporting the activity of other P450 enzymes [37–40].

In summary, CYP238A1 (PP1955) of *P. putida* KT2440 shows terpene alcohol monooxygenase activity, forming the novel metabolite 9-hydroxynerolidol with nerolidol. The PFOR (PP1957) supports CYP238A1 activity in what appears to be a potentially new class of P450 electron-transfer chain organization.

5. References

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