Applications of microbial cytochrome P450 enzymes in biotechnology and synthetic biology
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Cytochrome P450 enzymes (P450s) are a superfamily of monoxygenase enzymes with enormous potential for synthetic biology applications. Across Nature, their substrate range is vast and exceeds that of other enzymes. The range of different chemical transformations performed by P450s is also substantial, and continues to expand through interrogation of the properties of novel P450s and by protein engineering studies. The ability of P450s to introduce oxygen atoms at specific positions on complex molecules makes these enzymes particularly valuable for applications in synthetic biology. This review focuses on the enzymatic properties and reaction mechanisms of P450 enzymes, and on recent studies that highlight their broad applications in the production of oxochemicals. For selected soluble bacterial P450s (notably the high-activity P450-cytochrome P450 reductase enzyme P450 BM3), variants with a multitude of diverse substrate selectivities have been generated both rationally and by random mutagenesis/directed evolution approaches. This highlights the robustness and malleability of the P450 fold, and the capacity of these biocatalysts to oxidise a wide range of chemical scaffolds. This article reviews recent research on the application of wild-type and engineered P450s in the production of important chemicals, including pharmaceuticals and drug metabolites, steroids and antibiotics. In addition, the properties of unusual members of the P450 superfamily that do not follow the canonical P450 catalytic pathway are described.

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Introduction
The cytochromes P450 (P450s or CYPs) are an enzyme superfamily, members of which have a vast number of physiological functions in organisms from archaea and bacteria through to man [1]. The majority of P450 enzymes are monoxygenases, catalysing the scission of dioxygen bound to their heme iron, leading to the formation of a highly reactive ferryl iron-oxo intermediate (compound I) that catalyses the insertion of an oxygen atom into the substrate [2,3] (Figure 1). Oxidation of the P450 substrate can lead to several different chemical outcomes — including hydroxylation, epoxidation, demethylation or dealkylation, sulfoxidation, N-oxidation and decarboxylation [4]. Most P450s use NAD(P)H-driven redox partner systems for delivery of the two electrons required for reduction of the ferric heme iron (enabling oxygen binding to ferrous heme) and for the subsequent reduction of the ferrous-oxo (formally ferrie-superoxy) species to form a ferrie-peroxo intermediate in the catalytic cycle (Figure 1). Eukaryotic P450s are bound to membranes with an N-terminal transmembrane helix, whereas prokaryotic P450s are soluble enzymes that lack the N-terminal membrane anchor. The majority of mammalian P450s are attached to the endoplasmic reticulum (microsomal P450s), although a subset involved in steroid biosynthesis are linked to the inner membrane of adrenal gland mitochondria [5]. The microsomal P450s are reduced by NADPH-cytochrome P450 reductase (CPR) — an FAD-binding and FMN-binding enzyme that is also attached to the ER membrane by an N-terminal anchor (a class II P450 redox system) [6]. The mitochondrial P450s interact instead with a soluble iron-sulfur protein (adenodoxin), which sources electrons from the membrane-associated FAD-binding enzyme NADPH-adenodoxin reductase (a class I system) [7]. Most of the characterized prokaryotic P450s also use a class I redox partner system (soluble ferredoxin reductase and ferredoxin; flavodoxins can surrogate in certain systems), though there is considerable further diversity in the redox partner apparatus that drives catalysis in prokaryotic P450 enzymes, as described further below [8**]. The ability of P450s to perform regioselective and often stereoselective oxidation of their substrates makes them attractive catalysts for applications in synthetic biology and in the generation of high value oxochemicals that may not be economical to produce by synthetic chemistry. In the following sections, the diversity of P450 chemistry is described, and descriptions are given of how key P450 enzymes have been engineered for improved catalytic performance and for novel reactivity in the generation of useful chemical products.
The cytochrome P450 catalytic cycle. The canonical P450 catalytic cycle is shown with green arrows. In the resting state (the Cys-Fe$^{3+}$-H$_2$O species in black, common to all of the cycles shown) the heme iron is low-spin, ferric with a loosely coordinated water molecule in the distal position. Binding of substrate (RH) shifts the ferric heme iron equilibrium to the high-spin form, enabling the first electron transfer to reduce the iron to the ferrous state. Binding of dioxygen (O$_2$), transfer of a second electron from a redox partner, two protonation steps (to the ferric-peroxo and ferric-hydroperoxo intermediates) involving active site amino acids, and the release of a water molecule results in the formation of the highly reactive ferryl-oxo porphyrin cation radical species known as compound I. Abstraction of a proton from the substrate forms a protonated ferryl intermediate (compound II) which hydroxylates the substrate radical according to the radical rebound mechanism [49]. Alternative P450 pathways exist for certain members of the P450 enzyme superfamily. Shown with blue arrows is the pathway to nitration of a tryptophan substrate using nitric oxide (NO) and O$_2$, as catalysed by TtxE (CYP1048A1) from S. scabiei and other Streptomyces species in a pathway to form the phytotoxin thaxtomin A [26*]. This pathway deviates from the canonical cycle at the ferric-superoxo species; which reacts with NO rather than being further reduced to the ferric-peroxo form. While the mechanism of tryptophan nitration is not yet resolved, one model would be that the ferric-peroxynitrite species formed by reaction with NO could be protonated and undergo heterolytic scission to form a ferric-hydroxy (Fe$^{3+}$-OH) heme species and a nitronium (NO$_2^+$) ion. The nitronium ion could then react with tryptophan by electrophilic aromatic substitution to form the 1,4-nitrotryptophan product, with further protonation of the Fe$^{3+}$-OH heme leading to restoration of a H$_2$O-coordinated resting state of the P450.
P450 redox partner systems and other diverse routes to driving P450 catalysis

For the majority of P450 enzymes in vivo, redox partner systems are required for monooxygenase function. However, in recent years several different types of redox partner systems have been identified to expand the class I/class II paradigm [8**,9]. Fulco’s group discovered the first major outlier in *Bacillus megaterium* P450 BM3 (BM3, CYP102A1), a natural fusion of a soluble fatty acid hydroxylase P450 to a soluble CPR [10]. BM3 catalyses arachidonic acid oxidation with a reported *k*<sub>cat</sub> of ~280 s<sup>−1</sup>; substantially faster than any mammalian P450 [11]. BM3 naturally forms a dimer, and inter-monomer (CPR, to P450<sub>2</sub> and vice versa) electron transfer can support catalysis [12]. Attempts to mimic BM3’s structural organization by fusion of its efficient CPR module to heterologous P450s have resulted in functional flavocytochrome enzymes, but these do not yet replicate the catalytic efficiency of BM3, likely due in part to their failure to generate dimers that facilitate domain–domain interactions as efficiently as in BM3, and/or to lower stability of the fusion enzymes [8**,13,14].

More recently, a novel class of bacterial P450s fused to a NAD(P)H-dependent flavin-containing and 2Fe-2S cluster-containing reductase related to phthalate dioxygenase reductase (PDOR) was uncovered [15]. Members of this CYP116B family were shown to catalyse oxidation of, for example, thiocarbamate herbicides (CYP116B1) and alkanes (CYP116B5) [16,17]. Fusions of heterologous P450s to the CYP116B-type PDOR domain has proven successful in various instances, presumably in part due to these fusions being monomeric. Examples of reconstitution of catalytic activity in such fusions include a *Rhodococcus jostii* P450 linked to the CYP116B2 PDOR, producing an enzyme capable of N-demethylation of the drugs imipramine and diltiazem; a fusion of the P450balk enzyme from the marine bacterium *Alcanivorax borkumensis* to the same reductase, generating a catalytically self-sufficient octane hydroxylase (producing 1-octanol) [18,19]. Recent studies also used fusion of a PDOR to an engineered mutant of *Amycolatopsis orientalis* CYP105AS1 to facilitate hydroxylation of the natural product pactamycin and formation of pravastatin for industrial scale production [20*]. Other natural bacterial P450 fusions to flavodoxin and ferredoxin partners are known; involved in catabolism of explosives and in sterol demethylation, respectively [21,22]. Such enzymes still require electron transfer from a separate NAD(P)H-dependent reductase, but do simplify this class I-like apparatus to a 2-component system. Fusions between P450s and native or heterologous ferredoxin/flavodoxin proteins have also been generated with a view to improving electron transfer efficiency to the P450, including a recent study in which the cinnole oxidising P450cin (CYP176A1) from *Citrobacter braakii* was fused to its native flavodoxin (CinA) using peptides of different lengths. Variants were reconstituted with NADPH/Escherichia coli flavodoxin reductase; ultimately identifying a 10 amino acid linker sequence as optimal through studies of the conversion of cinnole into its 2-β-hydroxy-1,8-cineole product [23].

Unusual microbial P450s and reactions

While most P450s adhere to the paradigm involving NADPH-dependent electron transfer from redox partners to the P450 heme iron, there are a number of P450 that have distinctive mechanisms. Indeed, some P450s function without requirement for redox partners. P450nor (CYP55A1) from the fungus *Fusarium oxysporum* (and related P450s in other fungi) catalyses the reduction of two molecules of nitric oxide (NO) to form dinitrogen monoxide (N<sub>2</sub>O) in the final step of a respiratory pathway in which nitrate/nitrite are converted into N<sub>2</sub>O [8**,24]. NADH is used directly, and the crystal structure of a P450nor mutant in complex with a NADH analogue (NAAD — nicotinamide pyridine dinucleotide) revealed the binding mode of the nicotinic acid ring moiety above the heme plane, in a position suitable (with reference to the NO-bound form) for reduction of the complex by hydride transfer [25] (Figure 1). In the case of TtxE (CYP1048A1) from *Streptomyces scabies* (and orthologues from other plant pathogen *Streptomyces* spp.), the normal P450 catalytic cycle progresses as far as the ferric-superoxide intermediate, but thereafter there is binding of NO (produced by a *Streptomyces* NO synthase enzyme from the same gene cluster) and the formation of a reactive ferric peroxynitrite intermediate that catalyses the nitration of the P450 substrate 1-tryptophan to produce 1-4-nitrotryptophan in the first committed step in the production of the phytotoxin thaxtomin A [26*]. More recent studies on TtxE have analysed the enzyme’s ability to nitrate other molecules, and have included exploration of

![Figure 1 Legend Continued](An alternative mechanism might involve homolytic scission of the ferric-peroxynitrite intermediate to produce NO<sub>2</sub> and a reactive ferryl-oxo species. In this case the ferryl-oxo heme would abstract a hydrogen from the tryptophan substrate and enable a reaction with the NO<sub>2</sub>. However, there is no evidence to date for formation of a transient ferryl-oxo species in TtxE [26*]. Shown with purple arrows is the pathway for conversion of NO to N<sub>2</sub>O catalysed by P450nor from *Fusarium oxysporum* in the final step of a fungal denitrification pathway. The reaction does not require additional redox partners, instead deriving its electrons directly from NADH [50]. The reaction is proposed to proceed via a heme iron-NO complex (presented here as a ferrous-NO<sup>−</sup> form, isoelectronic with the ferric-NO<sup>−</sup> form), which is reduced (by NADH) and then protonated (using Asp393 in P450nor) to form a doubly protonated, singlet Fe<sup>III</sup>−NHOOH<sup>−</sup> species. Further reaction with a second NO radical involves a spin-recoupling reaction in which a N−N bond is formed and a transient Fe<sup>III</sup>−(NO<sub>2</sub>−H)<sup>−</sup> species occurs, which decomposes to release N<sub>2</sub>O, resulting in the restoration of the H<sub>2</sub>O-bound, ferric resting form of the P450 [51,52]. Shown with a yellow arrow is the peroxynitrite reaction catalysed by a number of P450s from the CYP152 family. Hydrogen peroxide is used indirectly to form the reactive ferric-hydroperoxo species compound 0, which then continues on the canonical P450 catalytic cycle [30*,31,53,54].)
the determinants of substrate binding in TxtE [27], as well as producing nitrate derivatives of 4-fluoro-l-tryptophan and 5-fluoro-l-tryptophan using TxtE fused to the BM3 CPR domain or to a CYP116B (PDOR)-type reductase [28].

A particularly interesting group of P450 enzymes (with potential in the biofuels area) are the P450 peroxygenases [29]. These are P450s that have evolved to use hydrogen peroxide (H₂O₂) in place of redox partner systems, with H₂O₂ converting the ferric P450 form directly to the reactive compound 0 species (Figure 1). This ‘peroxide shunt’ mechanism is a well known, but typically inefficient, method for driving P450 catalysis, due to competing oxidative damage to heme and protein by the peroxide [30]. The peroxygenases BS₈ from Bacillus subtilis (CYP152A1) and SP₂ from Sphingomonas paucimobilis (CYP152B1) were the first P450 peroxygenases characterized — and catalyse predominantly β-hydroxylation and exclusively α-hydroxylation of fatty acid substrates, respectively [8,29]. However, more recent studies showed that a novel member of this P450 class (OleT from a Jeotgalicoccus sp., classified as CYP152L1) catalyses mainly oxidative decarboxylation of long chain fatty acids to produce n-1 terminal alkenes, while BS₈ also produced a proportion of terminal alkenes along with its major β-hydroxylated (and minor α-hydroxylated) products. Evidence for peroxide-dependent 1-pentadecene production from palmitic acid was also produced using E. coli cells expressing CYP152 family P450s from other bacteria [30]. The structure of OleT in substrate-free and arachidic acid (C20:0)-bound forms demonstrated how the lipid carboxylate group binds close to the P450 heme and suggested a mechanism for substrate decarboxylation [31]. Other studies demonstrated that use of a heterologous class I redox partner system could also be used to drive OleT to produce a range of different alkenes [32]. OleT and other CYP152 P450s clearly have potential applications in production of alkenes for use as biofuels or in fine chemical applications.

**Engineering of bacterial P450 enzymes for novel oxymetal production**

A large number of bacterial P450s have been expressed and characterized, and these enzymes have an enormous repertoire of substrates including fatty acids, steroids, polyketides and terpenes [33,34]. Recent studies have sought to expand this repertoire using protein engineering of P450s. This has typically been achieved using knowledge from P450 structure to guide rational redesign of the P450 active site, or by random mutagenesis or directed evolution approaches, or by a combination of these approaches. P450 BM3 has proven a popular model system, based on its catalytic self-sufficiency (with P450 and CPR units fused) and its high catalytic rate [1,11]. Recent examples include use of rational mutagenesis to facilitate the binding of the gastric proton pump inhibitor (PPI) drug omeprazole. The combined F87V and A82F mutations resulted in increased active site space and in a conformational change in the P450 to enable oxidation of omeprazole at the 5-methyl position (Figure 4a), mimicking the activity of the major human omeprazole-metabolising P450 CYP2C19 [35]. Subsequent work showed that other PPI drugs (e.g. lansoprazole) were also transformed to human P450-type metabolites by the same mutants [36]. The efficient production of human drug metabolites (for drug safety testing) is widely researched, and other examples include BM3 mutants that can make the human-type 4′-hydroxylated and 5-hydroxylated metabolites of the non-steroidal anti-inflammatory drug diclofenac [37]. BM3 mutants (generated using both site-directed and random mutagenesis) have also been used to produce metabolites of 17β-estradiol; including 2-hydroxy-β-estradiol, a product formed by human CYP1 family P450s [38]. Further examples of the exploitation of BM3 mutants in oxymetal production are given in Figure 2.

Other recent examples of bacterial P450s engineered for synthetic biology applications include the fusion of several P450s from Rhodococcus josii RHA1 with a PDOR module, leading to identification of catalytically self-sufficient enzymes generating drug metabolites, including CYP257A1 that produced N-demethylated products of the vasodilator diltiazem and the antidepressant imipramine [39]. In studies towards production of 1-butanol from n-butane, Nebel et al. used the Polaromonas sp. JS666 CYP153A4 for butane hydroxylation, and improved the 1-butanol product yield by introducing a G254A mutation in the P450. Further enhancement of product yield was achieved by fusing the mutant P450 to the P450 BM3 reductase to create a catalytically self-sufficient enzyme. Subsequent improvements were achieved by performing reactions at high pressure (15 bar) in efforts to overcome the limited aqueous solubility of n-butane [40]. P450sca-2 from Streptomyces carophilus is an industrially important P450 that stereo-selectively hydroxylates compactin to form pravastatin. A functional triple fusion enzyme (P450sca-2/Pdx/Pdr) was created using putidaredoxin/putidaredoxin reductase partners. This construct was used to produce more active mutants by site-directed saturation mutagenesis focused on the substrate access site and substrate binding pocket regions, as well as on the proposed P450-Pdx interaction site, leading to substantial increases in product formation in whole cell transformations [41].

**Synthetic biology applications of P450 enzymes**

The P450s have diverged into a vast number of biological catalysts that bind and oxidise substrates of widely varying size and chemical composition. As described above, P450 substrate selectivity can be further broadened by
Diverse catalytic activities of mutant and wild-type forms of P450 BM3. P450 BM3 is a natural, soluble fusion of a fatty acid hydroxylase P450 to a cytochrome P450 reductase, and has the highest reported monooxygenase activity among the P450s [11]. Variants of P450 BM3 with novel substrate selectivity have been produced using both rational (structure-guided) and random/directed evolution mutagenesis procedures. Figure 2 shows several examples of P450 BM3 mutants with novel substrate selectivity and applications in production of novel oxychemicals. (1) Conversion of 17β-estradiol to 2-hydroxy-17β-estradiol by BM3 mutants made using both site-directed and random mutagenesis methods. The 2-hydroxy-β-estradiol product is also a metabolite made by human CYP1A1, CYP1A2 and CYP1B1 [38]. (2) Conversion of diclofenac to 4’ and 5-hydroxy diclofenac products from members of a library of BM3 variants carrying multiple mutations. These are the same metabolites produced by human CYP2C9 and CYP3A4 [37]. (3) Conversion of testosterone to 15-β-hydroxytestosterone using other mutants from the same library as in (2) [37]. (4) Conversion of the terpene (−)-limonene to (−)-perillyl alcohol, a potential anti-tumour drug, using the A264V/A238V/L437F BM3 triple mutant [55]. (5) Production of 9-hydroxy-β-cembradienol or 10-hydroxy-β-cembradienol from β-cembradienol using F78A/I263L and L75A/V78A/F87G mutants, respectively. The tobacco diterpene β-cembradienol has antibacterial and antifungal properties [56]. (6) Conversion of the non-steroidal anti-inflammatory drug mafenamic acid to 3-OH, 4-OH and 5-OH human-type metabolites using isolates from a BM3 mutant library [57]. (7) Hydroxylation of 1-tetralone derivatives using BM3 mutants from a directed evolution programme [58]. (8) Conversion of the gastric PPI drugs omeprazole and esomeprazole to their 5-hydroxylated, human CYP2C19-type metabolites by the BM3 F87V/A82F double mutant [35, 36]. (9) Transformation of 1-hexene to 1,2-epoxyhexane by BM3 mutants produced by saturation mutagenesis of the active site and by recombination of beneficial mutations [59]. (10) Conversion of the sesquiterpene (+)-valencene to (+)-nootkatol and on to (+)-nootkatone using mutants of the camphor hydroxylase P450cam (CYP101A1), for oxidation of (+)-trans-nootkatol and wild-type P450 BM3 for production of cis-(+)-nootkatol and trans-(+)-nootkatol, and (+)-nootkatone [60].

protein engineering. Several P450s and engineered variants have now been used in synthetic biology approaches for valuable chemical production by fermentation in microbial cells. Figure 3 shows recent important examples.

Artemisinin is a sesquiterpene lactone that is a standard antimalarial treatment worldwide. CYP71AV1 performs three successive oxidation reactions to convert amorpha- diene to artemisinic acid as a precursor to artemisinin in a
Biotechnological applications of cytochrome P450 enzymes. There are numerous examples of the exploitation of P450 enzymes in engineered pathways for the production of important biomolecules in microorganisms. (a) The antimalarial artemisinin, a sesquiterpene lactone, is generated chemically from artemisinic acid, which in turn is synthesised from acetyl CoA in an engineered pathway in Saccharomyces cerevisiae. The P450 CYP71AV1 from Artemisia annua is a multifunctional oxidase enzyme that catalyses key steps in hydroxylating amorphadiene to artemisinic acid, and then further to the aldehyde and on to artemisinic acid [42**]. (b) Morphine has been synthesised successfully from sugar feedstock in an engineered pathway in S. cerevisiae [44**]. The pathway exploits the reticuline epimerase from opium poppy, which is a multi-domain protein consisting of the P450 CYP82Y2 fused to an aldo-keto reductase (AKR). CYP82Y2 (1,2-dehydroreticuline synthase, DRS) catalyses the conversion of (S)-reticuline to 1,2-dehydroreticuline; and the AKR module (1,2-dehydroreticuline reductase, DRR) then converts the product to (R)-reticuline [43**]. A second P450, CYP719B1, then converts (R)-reticuline to salutaridine [44**,45]. This reaction involves reorientation and twisting of the (R)-reticuline, and an oxidative C–C bond coupling step catalysed by CYP719B1. Relevant carbon atoms are numbered according to Gesell et al. [45]. (c) The cholesterol-lowering drug pravastatin was synthesised in Penicillium chrysogenum by introduction of the genes encoding the production of the natural product compactin. The conversion of compactin to pravastatin was achieved by also introducing an engineered form of the Amycolatopsis orientalis P450 CYP105A51. This form of the enzyme (P450Prava, containing five mutations) catalyses the stereo-selective hydroxylation of compactin to form the active pravastatin rather than its inactive epimer epi-pravastatin [20*]. (d) A novel biosynthetic pathway for production of the plant flavonoid fisetin was engineered into E. coli. Fisetin has potential anti-cancer, anti-viral and other health benefits. Fisetin was synthesised from tyrosine in a nine-step process, the last step involving an unassigned P450 from Petunia hybrida that catalyses the conversion by hydroxylation of the flavonoid resокаempferol to fisetin [5].

Saccharomyces cerevisiae fermentation. Paddon et al. then used a singlet oxygen source to produce artemisinin from artemisinic acid (Figure 3a) [42**]. S. cerevisiae fermentation has also been used to produce the analgesic morphine. The opium poppy reticuline epimerase is a fusion of a P450 (CYP82Y2) to an aldo-keto reductase (AKR). The CYP82Y2 transforms (S)-reticuline to 1,2-dehydroreticuline, and the AKR converts this intermediate to (R)-reticuline. Another P450 (CYP719B1) then converts (R)-reticuline to salutaridine in a C–C phenol coupling reaction (Figure 3b) [43,44**,45]. In a fermentation process using Penicillium chrysogenum, genes encoding compactin production were introduced into the strain and an esterase gene deleted to prevent product degradation. The introduction of an engineered Amycolatopsis orientalis P450 (P450Prava) facilitated stereoselective hydroxylation of compactin to form the cholesterol-lowering pravastatin (Figure 3c).
X-ray crystallographic data demonstrated clearly how engineering enabled the productive binding of compactin to P450Prava (Figure 4c) [20]. A P450-dependent step was also important for the final hydroxylation step in production of the flavonol fisetin, which has reported anti-cancer properties. The unassigned P450 hydroxylates resokaempferol to form fisetin in an E. coli fermentation process (Figure 3d) [46].

Another successful example of the use of a P450 enzyme in the production of valuable compounds is the 2-step fermentation of tyrosine to caffeic acid using a tyrosine ammonia lyase to form p-coumaric acid. The *Rhodopseudomonas palustris* CYP199A2 proved more efficient for the subsequent conversion of p-coumaric acid to caffeic acid than did a bacterial flavoprotein 4-coumarate 3-hydroxylase. Caffeic acid exhibits anti-cancer and antioxidant activities [47]. In a novel approach, Wlodarczyk et al. showed that two membrane-bound eukaryotic P450s (CYP79A1 and CYP71E1 from the synthetic pathway for the cyanogenic glucoside dhurrin in the grass species *Sorghum bicolor*) can be integrated into the thylakoid membrane of *Synechocystis* sp. PCC 6803 and that light-driven photosystem I and ferredoxin can replace a typical P450 redox partner system to produce substantial amounts of dhurrin in the cyanobacterium [48].

**Summary**

Cytochrome P450 enzymes across Nature have an extraordinarily diverse substrate range. Mutagenesis strategies continue to expand this repertoire of substrates, particularly in the case of soluble microbial P450s for which structural data help to guide protein engineering.
In an era where synthetic biology is striving to replace synthetic chemistry for production of high-value chemicals, P450s have a crucial role. P450s can catalyze the specific addition of oxygen atoms at positions on chemical scaffolds, while this can be very challenging by traditional chemical methods. Many examples in this review highlight the crucial roles played by P450s in producing important molecules through microbial fermentation processes, and point to further applications for P450s in the synthesis of valuable chemicals such as antibiotics, drug metabolites, steroids and terpenes.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- **of outstanding interest

1. Munro AW, Girvan HM, Mason AE, Dunford AJ, McLean KJ: What makes a P450 tick? Trends Biochem Sci 2013, 38:140-150.

2. A concise description of the general properties of cytochrome P450 enzymes.

3. Groves JT: High-valent iron in chemical and biological oxidations. J Inorg Biochem 2006, 100:434-437.

4. Guengerich FP, Munro AW: Unusual cytochrome P450 enzymes and reactions. J Biol Chem 2013, 288:17065-17073.

5. Guengerich FP: Human cytochrome P450 enzymes. In Cytochrome P450: structure, mechanism and biochemistry, 4th ed., Edited by Ortiz de Montellano PR. New York: Springer; 2015:523-785.

6. Waskell L, Kim J-J: Electron transfer partners of cytochrome P450. In Cytochrome P450: structure, mechanism and biochemistry, 4th ed., Edited by Ortiz de Montellano PR. New York: Springer; 2015:33-68.

7. Ewen KM, Kleser M, Bernhardt R: Adrenodoxin: the archetype of vertebrate [2Fe-2S] cluster ferredoxins. Biochim Biophys Acta 2011, 1814:111-125.

8. McLean KJ, Leys D, Munro AW: Microbial cytochromes P450. ** In: Cytochrome P450: structure, mechanism and biochemistry, 4th ed., Edited by Ortiz de Montellano PR. New York: Springer; 2015:261-408.

A thorough and up to date review of the properties of the major microbial P450 enzymes and their properties.

9. McLean KJ, Luciakova D, Belcher J, Tee KL, Munro AW: Biological diversity of cytochrome P450 reduct partner systems. Adv Exp Med Biol 2015, 851:299-317.

10. Narhi LO, Fulco AJ: Characterization of a catalytically self-sufficient 119,000-Dalton cytochrome P450 monoxygenase induced by barbiturates in Bacillus megaterium. J Biol Chem 1986, 261:7160-7169.

11. Noble MA, Miles CS, Chapman SK, Lysek DA, MacKay AC, Reid GA, Hannzik RP, Munro AW: Biochem J 1999, 339:371-379.

12. Neeli R, Girvan HM, Lawrence A, Warren MJ, Leys D, Scrutton NS, Munro AW: The dimeric form of flavocytochrome P450 BM3 is catalytically functional as a fatty acid hydroxylase. FEBS Lett 2005, 579:5582-5588.

13. Lundemo MT, Notonier S, Striedner G, Hauer B, Woodley JM: Process limitations of a whole-cell P450 catalyzed reaction using a CYP153A-CPR fusion construct expressed in Escherichia coli. Appl Microbiol Biotechnol 2016, 100:1197-1208.

14. Helvig C, Capdevila JH: Biochemical characterization of rat P450 2C11 fused to rat or bacterial NAPDH-P450 reductase domains. Biochemistry 2000, 39:5196-5205.

15. De Mot R, Parret AH: A novel class of self-constituted cytochrome P450 monoxygenases in prokaroytes. Trends Microbiol 2002, 10:502-508.

16. Warman AJ, Robinson JW, Luciakova D, Lawrence AD, Marshall KR, Warren MJ, Cheesman MR, Rigby SE, Munro AW, McLean KJ: Characterization of Cupriavidus metallidurans CYP116B1 – a thio-carbamate herbicide oxygenating P450: pthaldehyde dioxygenase reductase fusion protein. FEBS J 2012, 279:1675-1693.

17. Minieri D, Sadeghi SJ, Di Nardo G, Rua F, Castignano P, Gilardi G: CYP116B5: a new class VII catalytically self-sufficient cytochrome P4504F10 resistant to chloroform that enables growth on alkanes. Mol Microbiol 2015, 95:539-554.

18. Kulig JK, Spandolf C, Hyde R, Ruzzini AC, Eltsa LD, Gröninger G, Hayes MA, Grogan G: A P450 fusion library of home domains from Rhodococcus jostii RHA1 and its evaluation for the biotechnological production of drug molecules. Biorg Med Chem 2015, 23:5603-5609.

19. Nodate M, Kubota M, Misawa N: Functional expression system for cytochrome P450 genes using the reductase domain of self-sufficient P450R rbh from Rhodococcus sp. NCIMB 9784. Appl Microbiol Biotechnol 2006, 71:455-462.

20. McLean KJ, Hans M, Mejirmk B, van Schepening WB.

21. A novel route to industrial scale production of pravastatin using an engineered bacterial P450.

22. Jackson CJ, Lamb DC, Marczylo TH, Warriow AG, Manning NJ, Lowe DJ, Kelly DE, Kelly SL: A novel sterol 14alpha-demethylase/ferredoxin fusion protein (MCCYP51F1X) from Methylcoccus capsulatus represents a new class of the cytochrome P450 superfamily. J Biol Chem 2002, 277: 46959-46965.

23. Chong CS, Sabir DK, Lorenz A, Bontemps C, Andeer P, Stahl DA, Strand SE, Ryllott EL, Bruce NC: Analysis of the xplAB-containing gene cluster involved in the bacterial degradation of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine. Appl Environ Microbiol 2014, 80:6601-6610.

24. Belisa KD, Ruff AJ, Martinez R, Shivange AV, Munhada H, Holtmann D, Schrader J, Schwanenberg U: P-Link: a method for generating multicomponent cytochrome P450 fusions with variable linker length. Biotechniques 2014, 57: 13-20.

25. Shoun H, Fushinobu S, Jiang L, Kim S-W, Wakagi T: Fungal denitrification and nitric oxide reductase: cytochrome P450Nor. Philos Trans R Soc B 2012, 367:1186-1194.

26. Oshima R, Fushinobu S, Su F, Zhang L, Takaya N, Shoun H: Structural evidence for direct hydride transfer from NADH to cytochrome P450Nor. J Mol Biol 2004, 342:207-217.

27. Barry SM, Kers JA, Johnson EG, Song L, Aston PR, Patel B, Krassoff SB, Crane BR, Gibson DM, Loria R, Challis GL: Cytochrome P450-catalyzed L-tryptophan nitrification in thaxtomin phytoxin biosynthesis. Nat Chem Biol 2012, 8: 814-816.

A detailed study of a biotechnologically important P450 capable of substrate nitrification.

28. Dodani SC, Cahn JK, Heinisch T, Brinkmann-Chen S, McIntosh JA, Arnold FH: Structural, functional, and spectroscopic characterization of the substrate scope of the novel nitratoriy cytochrome P450 Txe. ChemBioChem 2014, 15:2259-2267.
28. Zuo R, Zhang Y, Huquet-Tapia JC, Mehta M, Dedic E, Bruner SD, Loria R, Ding Y; An artificial self-sufficient cytochrome P450 directly nitrates fluorinated tryptophan analogs with a different regio-selectivity. Biotechnol J 2016. (in press).

29. Hrycay EG, Bandiera SM: Monoxygenase, peroxidase and peroxide properties and reaction mechanisms of cytochrome P450 enzymes. Adv Exp Med Biol 2015, 851:1-61.

30. Rude MA, Baron TS, Brubaker S, Alibhai M, Del Cardayre SB, • Schirmer A: Terminal olefin (1-alkene) biosynthesis by a novel P450 acid decarboxylase from Jeotgalicoccus species (2011). Appl Environ Microbiol 2011, 77:1718-1727.

First description of a novel type of P450 decarboxylase enzyme able to produce terminal alkenes.

31. Belcher J, McLean KJ, Matthews S, Woodward LS, Fisher K, Rigby SE, Nelson DR, Potts D, Baynham MT, Parker DA, Leys D, Munro AW: Structure and biochemical properties of the alken producing cytochrome P450 OleT in the Jeotgalicoccus sp. 8456 bacterium. J Biol Chem 2014, 289:6535-6550.

32. Dening A, Kuhn M, Tassotti S, Thiessenhusen A, Gilch S, Büttler T, Haas T, Hall M, Faber K: Oxidative decarboxylation of short-chain fatty acids to 1-alkenes. Angew Chem Int Ed Engl 2015, 54:8819-8822.

33. Janocha S, Schmitz D, Bernhardt R: Terpene hydroxylation with microbial cytochrome P450 monoxygenases. Adv Biochem Eng Biotechnol 2015, 148:215-250.

34. Lee GY, Kim DH, Kim D, Ahn T, Yun CH: Functional characterization of steroid hydroxylase CYP106A1 derived from Bacillus megaterium. Acc Pharm Res 2015, 38:98-107.

35. Butler CF, Peet C, Mason AE, Voice MW, Leys D, Munro AW: Key mutations alter the cytochrome P450 BM3 conformational landscape and remove inherent substrate bias. J Biol Chem 2013, 288:25387-25399.

Demonstration that conformational perturbation can radically alter the substrate selectivity profile in a biotechnologically important P450 enzyme.

36. Butler CF, Peet C, McLean KJ, Baynham MT, Blankley RT, Fisher K, Rigby SE, Leys D, Voice MW, Munro AW: Human P450-like oxidation of diverse proton pump inhibitor drugs by “gatekeeper” mutants of flavocytochrome P450 BM3. Biochim Biophys Acta 2013, 1824:235-245.

37. Ren X, Yorke JA, Taylor E, Zhang T, Zhou WH, Wong LL: Drug oxidation by cytochrome P450 BM3: metabolite synthesis and discovering new P450 reaction types. Chem Eur J 2015, 21:15039-15047.

38. Cha GS, Ryu SH, Ahn T, Yun CH: Regioselective hydroxylation of 17 beta-estradiol by mutants of CYP102A1 from Bacillus megaterium. Biotechnol Lett 2014, 36:2501-2506.

39. Kulig JK, Spandolf C, Hyde R, Ruzzini AC, Ellis LD, Grönborg G, Hayes MA, Grogan G: A P450 fusion library of heme domains from Rhodococcus jostii RHA1 and its evaluation for the biotransformation of drug molecules. Bioorg Med Chem 2015, 23:5603-5609.

40. Nebel BA, Scheps D, Honda Malca S, Nesti BM, Breuer M, • Wagner HG, Breitscheid B, Kratz D, Hauer B: Biooxidation of n-butyne to 1-butanol by engineered P450 monoxygenase under increased pressure. J Biotechnol 2014, 191: 86-92.

Interesting combination of P450 protein engineering, redox partner fusion and use of high pressure to enhance oxidation of a gaseous substrate.

41. Ba L, Li P, Zhang H, Duan Y, Lin Z: Semi-rational engineering of cytochrome P450oxa-2 for enhanced catalytic activity: insights into the important role of electron transfer. Biotechnol Bioeng 2013, 110:2815-2825.

42. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K et al.; • High level semi-synthetic production of the potent antimalarial artemisinin. Nature 2013, 496:528-532.

Development of a P450-dependent process for production of an important antimalarial therapeutic.

43. Farrow SC, Hagel JM, Beaudoin GA, Burns DC, Facchini PJ: • Stereochemoical inversion of (S)-reticuline by a cytochrome P450 fusion in opium poppy. Nat Chem Biol 2015, 11: 728-732.

Characterization of an unusual P450/aldo-keto reductase and its application in synthesis of morphine.

44. Galanie S, Thodey K, Trenchard IJ, Filsinger Interrante M, • Smolke CD: Complete biosynthesis of opioids in yeast. Science 2015, 349:1095-1100.

Development of a yeast fermentation process for the synthesis of opioids.

45. Gesell A, Rolf M, Ziegler J, Chávez MLD, Huang F-C, Kutchan TM: CYP719B1 is salutaridine synthase, the C–C phenol-coupling enzyme of morphine biosynthesis in opium poppy. J Biol Chem 2009, 284:24432-24442.

46. Stalhut SG, Siedler S, Malla S, Harrison SJ, Maury J, Neves AR, Forster J: Assembly of a novel biosynthetic pathway for production of the plant flavonoid fisetin in Escherichia coli. Metab Eng 2015, 31:84-93.

47. Rodrigues JL, Araújo RG, Prather KL, Kluskens LD, Rodrigues LR: Heterologous production of caffeic acid from tyrosine in Escherichia coli. Enzyme Microbial Technol 2015, 71:36-44.

48. Wlodarczyk A, Gnansasekaran T, Nielsen AZ, Zulu NN, Mellor SB, • Luckner M, Thafner JF, Olsen CE, Mottawie MS, Burow M, Pilbl M, Feusner I, Moller BL, Jensen PE: Metabolic engineering of the light-driven cytochrome P450 dependent pathways into Synechocystis sp. PCC 6803. Metab Eng 2016, 33:1-11.

Engineering of a cyanobacterium to express eukaryotic P450s and driving their catalysis in a light-dependent manner.

49. Rittle J, Green MT: Cytochrome P450 compound I: capture, characterization and C–H bond activation kinetics. Science 2010, 330:933-937.

50. Shiró Y, Fuji M, Iizuka T, Adachi S, Tusakumoto K, Nakahara K, Shoun H: Spectroscopic and kinetic studies on reaction of cytochrome P450nor with nitric oxide – implication for its nitric oxide reduction mechanism. J Biol Chem 1995, 270:1617-1623.

51. McQuarters AB, Wirgau NE, Lehner N: Model complexes of key intermediates in fungal nitric oxide reductase (P450nor). Curr Opin Chem Biol 2014, 19:82-89.

52. Riplier B, Bill E, Daiber A, Ullrich V, Shoun H, Reese F: New insights into the nature of observable reaction intermediates in cytochrome P450 NO reductase by using a combination of spectroscopy and quantum mechanics/molecular mechanics calculations. Chem Eur J 2014, 20:1602-1614.

53. Lee DS, Yamada A, Sugimoto H, Matsunaga I, Ogura H, Ichihara K, Adachi S, Park SY, Shiró Y: Substrate recognition and molecular mechanism of fatty acid hydroxylation by cytochrome P450 from Bacillus subtilis – crystallographic, spectroscopic, and mutational studies. J Biol Chem 2003, 278:9761-9767.

54. Fujishiro T, Shoji O, Nagano S, Sugimoto H, Shiró Y, Watanabe Y: Crystal structure of H2O2-dependent cytochrome P450 (SPo) with its bound fatty acid substrate, insight into the regioselective hydroxylation of fatty acids at the alpha position. J Biol Chem 2011, 286:29941-29950.

55. Seifert A, Antonovici M, Hauer B, Pleiss J: An efficient route to selective bio-oxidation catalysts: an iterative approach comprising modeling, diversification, and screening, based on CYP102A1. ChemBioChem 2011, 12:1346-1351.

56. Le-Huu P, Heidt T, Claesen B, Laschat S, Urlacher VB: Chemo-, regio-, and stereoselective oxidation of the monocyclic diterpenoid beta-cembranediol by P450 BM3. ACS Catal 2015, 5:1772-1780.

57. Venkataraman H, Verkade-Vreeker MC, Caporlini L, Geerke DP, Vermeulen NPE, Commandeur JNM: Application of engineered cytochrome P450 mutants as biocatalysts for the synthesis of benzyl and aromatic metabolites of fenamic acid NSAIDs. Bioorg Med Chem 2014, 22:5613-5620.

58. Roiban GD, Agudo R, Ile A, Lonsdale R, Reetz MT: CH-activating oxidative hydroxylation of 1-tetralones and related
compounds with high regio- and stereoselectivity. Chem Commun 2014, 50:14310-14313.

59. Kubo T, Peters MW, Meinhold P, Arnold FH: Enantioselective epoxidation of terminal alkenes to (R)- and (S)-epoxides by engineered cytochromes P450BM-3. Chem Eur J 2006, 12:1216-1220.

60. Sowden RJ, Yasmin S, Rees NH, Bell SG, Wong LL: Biotransformation of the sesquiterpene (+)-valencene by cytochrome P450cam and P450 BM-3. Org Biomol Chem 2005, 3:57-64.

61. Xu LH, Ikeda H, Liu L, Arakawa T, Wakagi T, Shoun H, Fushinobu S: Structural basis for the 4’-hydroxylation of diclofenac by a microbial cytochrome P450 monooxygenase. Appl Microbiol Biotechnol 2015, 99:3081-3091.

62. Yasutake Y, Kitagawa W, Hata M, Nishioka T, Ozaki T, Nishiyama M, Kuzuyama T, Tamura T: Structure of the quinoline N-hydroxylating cytochrome P450 RauA, an essential enzyme that confers antibiotic activity on aurachin alkaloids. FEBS Lett 2014, 588:105-110.