**Drug Metabolism in Microorganisms**

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**Abstract**

Several wild type and recombinant microorganisms can transform drugs to the equivalent human metabolites. Fungi, such as *Cunninghamella* spp., and *Streptomyces* bacteria express cytochrome P450 (CYP) enzymes that enable analogous phase I (oxidative) reactions with a wide range of drugs. The gene encoding the bifunctional CYP102A1 in *Bacillus megaterium* can be expressed easily in *E. coli*, and extensive mutagenesis experiments have generated numerous variants that can produce human drug metabolites. Additionally, human cytochrome P450 isoforms have been expressed in various hosts. The application of microbial CYPs to the production of human drug metabolites is reviewed, and additional applications in the field of drug development are considered.

Keywords: *Bacillus megaterium*; *Cunninghamella*; cytochrome P450; metabolism; *Streptomyces*

**Introduction**

Smith and Rosazza (Smith and Rosazza 1974) first explored the possibility of using microorganisms as models of mammalian drug metabolism having recognised the potential similarity between microbial and mammalian cytochromes P450 (CYP). They tested their hypothesis by screening a number of bacteria and fungi for their ability to transform model aromatic compounds, whose mammalian transformation products were well characterised, and found that in most cases the pattern of metabolites formed in the microbial incubations was analogous to that observed in in vitro and in vivo mammalian systems. In the subsequent decades hundreds of studies have examined biotransformations of drugs by microorganisms, demonstrating that certain microorganisms can effectively transform drugs and other xenobiotics to mammalian metabolites. This has led to the application of microorganisms for the production of human drug metabolites as standards and, increasingly, for toxicity testing, typically after a screening step to identify the appropriate strain or mutant. A broader application that takes advantage of microbial CYP activity is functionalization of unreactive centres enabling further modification of biologically active molecules. In this review, the main bacteria and fungi that have been studied for mammalian-like drug metabolism are described alongside the current and potential applications in drug development.

**Microbial models of mammalian drug metabolism**

*Cunninghamella* spp.

In the original Smith and Rosazza study two species of the filamentous fungus *Cunninghamella*, *C. blakesleeana* and *C. bainieri*, were assessed for transformation of the aryl substrates. Ferris et al. (1976) subsequently demonstrated that *C. bainieri* possessed cytochrome P450 activity, and that this was responsible for the hydroxylation reaction. Species of the genus, in particular *C. elegans*, *C. blakesleeana* and *C. echinulata*, have been employed extensively in the transformation of drugs and xenobiotics via phase I (oxidative) and phase II (conjugative) routes (for a comprehensive review on the biotransformations the reader is directed to (Asha and Vidyavathi 2009)). Whilst the oxidative transformation of drugs in *Cunninghamella* spp. is characteristic of CYP activity, few direct data are available on the genes and enzymes responsible. There are 57 CYP isoforms in humans, with myriad functions; 5 isoforms are responsible for the bulk of phase I drug metabolism: 1A2, 2C9, 2C19, 2D6 and 3A4. A gene coding for a cytochrome P450 in *C. elegans* was cloned and expressed in *E. coli* (Wang et al. 2000), and when the amino acid sequence was compared with other fungal CYPs it was found to be closely related to the CYP51 family and was designated CYP509A1. The activity of the gene product was not determined, but the protein was immunostained with antibodies raised against mammalian CYPs, confirming its identity. Lisowska et al. (2006) followed the increased expression of a *cyp* gene by RT-PCR upon incubation of *C. elegans* with the polyaromatic hydrocarbon phenanthrene, and the sequence of the amplicon confirmed that the gene was related to *cyp509A1*. The transformation profile of drugs and xenobiotics in *Cunninghamella* spp. spans a number of reactions catalysed by different mammalian CYP isoforms (Fig 1). For example, PAHs like fluoranthene and phenanthrene are transformed to hydroxylated metabolites by *C. elegans* (Pothuluri et al. 1995; Cerniglia and Yang 1984), and in mammals the corresponding reactions are catalysed by enzymes belonging to the CYP1 family (Omiecinski et al. 1999), albeit with different sites of oxidation. On the other hand, flurbiprofen is transformed to 4’-hydroxyflurbiprofen by *C. elegans*, (Amadio et al. 2010) and in humans this transformation is catalysed exclusively by CYP2C9 (Tracy et al. 1995; Tracy et al. 1996). *Cunninghamella blakesleeana* AS 3.153 was shown to transform dextromethorphan to dextrorphan (Lin et al. 2007), which is a characteristic reaction of CYP2D6. These observations indicate that the *Cunninghamella* spp. either have a CYP with an unusually broad substrate specificity, or a number of CYP isoforms enabling the fungi to catalyse the phase I oxidation of a range of drugs and xenobiotics. The Fungal Genomics Project at Concordia University, Montreal sequenced and partially annotated the *C. elegans* genome; 11 contigs showed sequence similarity to cytochromes P450. However, the homologies are low and cover only small portions of the sequence, furthermore, the translated sequences contain internal stop codons. A reliable, complete annotated genome is very important if the key enzymes involved in drug transformation are to be heterologously expressed, and the full potential of *Cunninghamella* spp. as models of phase I human drug metabolism is understood.

Fig 1

 Phase II metabolism of drugs by *Cunninghamella* spp. has been reported less frequently. Zhang et al. (1996) detected enzyme activities associated with phase II drug metabolism in cytosolic and microsomal fractions of *C. elegans*, including glutathione *S*-transferase, aryl sulfotransferase, UDP-glucosyl and –glucuronosyl transferase. The phase II metabolites formed by *Cunninghamella* spp. are sometimes different to those known in humans, for example, Rydevik et al. (2013b) demonstrated that *C. elegans* metabolised selective androgen receptor modulators (SARMs) to the corresponding glucosides, whereas in humans glucuronides are formed via phase II metabolism. Interestingly, the same group (Rydevik et al. 2013a) described a convenient chemoenzymatic method of generating the SARM-glucuronides by combining transformation of the drug via *C. elegans* metabolism and subsequent oxidation of the glucoside with tetramethylpiperidinyl-1-oxy (TEMPO) generating the glucuronide that was identical to the human metabolite (Fig 2).

Fig 2

*Streptomyces* spp.

Unlike fungal CYPs, which are typically membrane-bound proteins, bacterial CYPs are soluble, thus easier to purify and characterise. Sariaslani and Kunz (1986) demonstrated that *S. griseus* expressed a cytochrome P450 when grown in a soybean-based medium and deduced that the enzyme was responsible for previously observed biotransformations of 7-ethoxycoumarin (Sariaslani and Rosazza 1983). The enzyme was purified and initially designated P450soy (Trower et al. 1989) and latterly CYP105D1, and enables *S. griseus* to transform a range of drugs and xenobiotics in an analogous fashion to mammals (Taylor et al. 1999). Other species of *Streptomyces* have also been examined for their abilities to transform drugs in vivo and demonstrate similar capabilities for phase I oxidation as *S. griseus* and *Cunninghamella* spp. (Bright et al. 2011). As *Streptomyces* spp. are important antibiotic producers, several have had their genomes sequenced revealing multiple *cyp* genes, for example in *S. coelicolor* A3(2) there are 18, which have been heterologously expressed in *E. coli* to confirm their protein identity (Lamb et al. 2002). With easier access to genome data, the prospect of in silico prediction of the drug substrates that the various CYP will transform is raised. However, Lamb et al. (2013) concluded that as a consequence of the demonstrable promiscuity of known CYPs, and structural investigations showing the plasticity of CYP active sites enabling interaction with a variety of substrates, that sequence analysis alone cannot predict if a particular CYP will turnover a given drug.

*Bacillus* spp.

*Bacillus megaterium* ATCC14581 produces CYP BM3 (or 102A1), which in the wild type strain hydroxylates fatty acids (Narhi and Fulco 1986). Unusually, the enzyme has a reductase domain to transfer electrons to the haem domain, rather than employing a separate reductase which is commonly the case with other CYPs. The wild type enzyme also transforms some drugs that are substrates for CYP3A4, CYP2E1 and CYP1A2 (Di Nardo et al. 2007), albeit with elevated Km values compared with the human enzymes. The enzyme is expressed efficiently in *E. coli*, which has enabled in-depth structural and mutational analysis. The availability of a crystal structure of BM3 bound with palmitoleic acid (Li and Poulos 1997), coupled with mutagenesis investigations (Noble et al. 1999) revealed the key active site residues for substrate recognition. Combinations of site-directed and random mutagenesis have generated BM3 variants with altered substrate specificity enabling the efficient biotransformation of drugs to metabolites analogous to those generated in mammals. Notably, mutations at residues remote to the active site can significantly affect the oxidation of non-natural substrates. For example, through error-prone PCR Tsotsou et al. (2012), generated the double mutant Asp251Gly/Gln307His, which can hydroxylate the CYP2C substrates diclofenac, ibuprofen and tolbutamide, despite the amino acids being located on the surface of the enzyme and not directly involved in substrate binding and turnover. X-ray crystallographic analysis revealed that the mutations were likely to yield a protein with increased flexibility, in particular the Asp251Gly mutation, which eliminates the salt bridge between the Asp and Lys224. Most recently, Kolev et al. (2014) introduced unnatural amino acids into the BM3 CYP employing the orthogonal aminoacyl-tRNA synthetase/tRNA pairs approach, to improve the selectivity of hydroxylation of *S*-ibuprofen methyl ester, which is hydroxylated at the 1’ and 2’ positions by wild type BM3 in a ratio of 62:38 %. Incorporation of unnatural *p*-acetyl phenylalanine at residue 78 resulted in enhanced regioselectivity of the oxidation, yielding 87% 1’-hydroxy-*S*-ibuprofen methyl ester.

 Efforts have been made to identify a minimal set of BM3 mutants that are capable of transforming a broad range of drugs, thus providing a convenient method for metabolite screening. Sawayama et al. (2009) identified 120 BM3 variants that could produce 12 out of 13 known mammalian metabolites of the drugs verapamil and astemizole. Reinen et al. (2011) constructed a panel of four BM3 mutants that produced phase I metabolites for 41 out of 43 drugs tested. Variants of the enzyme are commercially employed in 96-well metabolite screening kits manufactured by Codexis (Microcyp™). Most investigations with this enzyme have concentrated on tuning the substrate specificity to transform different drugs; however, other factors have been acknowledged to play an important role in the application of these enzymes as biocatalysts, in particular, the relatively low turnover rate, poor coupling efficiency and enzyme stability.

Mammalian CYP expression in microbial hosts

The most intuitive way to study mammalian drug metabolism in microorganisms is to express the key CYP in a suitable host, such as *E. coli* or yeast. This has been a fertile ground for research for over two decades (Gonzalez and Korzekwa 1995), and has led to the heterologous expression of the key xenobiotic-metabolising CYPs. In *E. coli*, it is necessary to modify the amino terminal of the protein to encourage a high level of expression, and to co-express the CYP reductase, since there is no endogenous activity. In contrast, expression of human CYP in yeast, such as *Saccharomyces cerevisiae*, does not require the same degree of manipulation. *Pichia pastoris*, which is a popular host for the expression of eukaryotic genes, has also been used for the expression of CYP2D6 and 3A4 (Geier et al. 2012; Martina et al. 2013). Cornelissen et al. (2012) compared the expression of mammalian (human and rat) CYP1A1 in *E. coli*, *S. cerevisiae* and *Pseudomonas putida*, via the ability of the recombinant strains to deethylate 7-ethoxyresorufin. Higher activities of rat CYP1A1 were measured in *E. coli* strains compared with the human enzyme, indicating that the species from which the gene originates is a factor in the workflow, and overall, the *E. coli* strains displayed higher enzyme activities than the other hosts. In contrast, Geier et al. (2012) found that *P. pastoris* was more efficient at expressing CYP2D6 compared with *E. coli* as a host organism; however, the *cyp* gene was unmodified in this case, thus *E. coli* expression would have been expected to be compromised.

**Applications of microorganisms for the production of drug metabolites**

In addition to acting as models of mammalian drug metabolism, microorganisms are easily scalable, thus can be used for the production of drug metabolites that may be required as authentic standards or in toxicity screening programmes during drug development. The microbiological approach is more environmentally friendly than the synthetic alternative, more efficient and cost effective than using microsomal preparations from hepatocytes and raises no ethical issues such as those encountered with the dosing of animals. Preparative-scale biosynthesis of drug metabolites has been demonstrated in recombinant *E. coli* expressing CYP 3A4, 2C9 and 1A2. In 1 L reactions, it was possible to acquire 59 mg 6β-hydroxytestosterone, 110 mg 4’-hydroxydiclofenac and 88 mg acetaminophen from the parent drugs (Vail et al. 2005). The fission yeast *Schizosaccharomyces pombe* has proven to be a good host for the expression of human CYP and the application of recombinant *S. pombe* expressing reductase and CYP for preparative scale synthesis has been demonstrated for several hydroxylated metabolites of designer drugs (Peters et al. 2007; Peters et al. 2009a; Peters et al. 2009b), and most notably for 4’-hydroxydiclofenac (Dragan et al. 2011). For the latter metabolite, 2.8 g of it was isolated from 6 l of culture. Interestingly, *S. pombe* can also be used to produce phase II metabolites when engineered to express human UDP-glycosyltransferase. Buchheit et al. (2011) demonstrated that upon expression of UGT2B7 in *S. pombe* it was possible to generate ibuprofen acyl glucuronides, and that the productivity could be improved if an endogenous UDP-glucose pyrophosphorylase was also overexpressed to increase the supply of UDP-glucose.

Fig 3

 The preparative production of drug metabolites in *E. coli* and *S. pombe* described above was conducted in shake flasks, using suspended cultures, thus at the end of the incubation period the cells were removed and discarded. Some researchers have examined alternative culture methods to the shake flask to enable the re-use of the microbial biocatalyst. Osorio-Lozada et al. (2008) employed a hollow fibre reactor composed of a bundle of semi-permeable membrane fibres, into which cells of *Actinoplanes* sp. ATCC53771, which express CYP107E4 (Prior et al. 2010), were loaded into the extracapillary space (Fig 3). The semi-permeable membrane enabled transfer of nutrients, oxygen, substrate and metabolites between the cells and bulk liquid. With this configuration, 4’-hydroxy- and 5’-hydroxy-diclofenac were generated from the parent drug (50 and 500 µM), and the reactor could be used several times over. Amadio et al. (2013) observed that the fungus *C. elegans* could grow as a biofilm under specific incubation conditions, and transform flurbiprofen to 4’-hydroxyflurbiprofen. Biofilms are typically stable and can remain active for long periods of time, which are attractive characteristics for biocatalytic applications. In the case of *C. elegans*, the supernatant containing the metabolite was easily decanted allowing the cells to be reused several times in a semi-continuous fashion.

**Application of microbial drug metabolism in guiding drug development**

Although it has been clearly demonstrated that some microorganisms metabolise drugs in an analogous fashion to mammals, particularly in phase I, unique metabolites are generated on occasion, and these may have medicinal value. For example, Paludo et al. (2013) discovered that *C. elegans* forms two glucosides of the potential anti-cancer compound β-lapachone, whereas in humans the drug is conjugated with glucuronic acid. Glucosidation at position 6 of ring B (Fig 4) led to a derivative that was less toxic towards normal cells compared with the parent drug, albeit with a reduced activity towards breast cancer cells.

Fig 4

 Cytochromes P450 enable oxyfunctionalisation at chemically inaccessible sites on important molecules, thereby allowing further modification of drug structure and potency. Parthenolide (Fig 5) is a plant-derived sesquiterpene lactone, which is of medicinal relevance owing to it anti-tumour properties. Whilst some chemical modification of the structure has been possible at C-13, the derivatives have reduced potency compared with the parent molecule (Han et al. 2009); furthermore, modification of other positions, notably sp3-hybridized C-9 and C-14, is not possible using the currently available synthetic methods. Kolev et al. (2014) enhanced the ability of CYP BM3 to oxidise these positions using a combination of high throughput active-site mapping and reactivity prediction coupled with mutagenesis. The hydroxylation of these positions enabled further chemical modifications to generate a series of aryl esters that were assessed for antitumour activity; most showed improvement compared with the parent compound and the enzymatically generated hydroxylated compounds.

 Fluorine is an important atom in pharmaceuticals, with approx. 20 % of drugs currently available containing at least one fluorine atom. The presence of fluorine can alter the properties of the drug by increasing metabolic stability, improving bioavailability or increasing bioactivity. A novel method of introducing fluorine into unactivated carbon centres of drugs and drug-like molecules was developed by Rentmeister et al. (2009), who used cytochrome P450 BM3 variants to hydroxylate the substrates in different positions; the hydroxyl was then substituted for fluorine using the nucleophilic fluorinating reagent diethylaminosulfur trifluoride (DAST). This approach enabled the rapid synthesis of a series of fluorinated compounds with a cyclopentenone moiety that is found in many bioactive natural products. These researchers also used this strategy to generate mono and di-fluorinated derivative of ibuprofen methyl ester (Fig 6), and when assessed for membrane permeability these derivatives showed improvement compared to the parent (non-fluorinated) compound, which is relevant for the development of drugs to more easily cross the blood brain barrier, for example. Bright et al. (2013) employed *Cunninghamella elegans* and *Streptomyces griseus* to identify the metabolically-labile site of drug-like substrates (biphenyl), which allowed for the synthesis of a fluorinated derivative so that the fluorine atom was positioned appropriately. The fluorinated biphenyl was then tested for metabolic stability by incubating with the same microorganisms, and it was observed that by blocking the metabolically labile site, no CYP-catalysed oxidation occurred. The same approach was taken with the non-steroidal anti-inflammatory drug flurbiprofen, leading to the targeted fluorination of the 4’-position and yielding a much more metabolically stable derivative (Fig 7).

Fig 6

Fig 5

Fig 7

**Outlook**

Since the initial discovery by Smith and Rosazza that microorganisms could transform drugs in a similar fashion to mammals, there has been an extensive research effort to examine drug biotransformation in a range of microorganisms. The work has deepened our understanding of microbial CYP and led to genuine applications for drug metabolite production and drug development. Jezequel (1998) critically discussed the predictive value of microbial drug metabolism as it related to humans and concluded that whilst microorganisms could be biocatalytically useful in the generation of known metabolites, the products of microbial metabolism were often different in both relative amounts and structure compared with those in humans. Despite the detailed understanding gained from mutational and structural studies, in particular with CYP102A1 (BM3), the predictive value of microbial drug metabolism is low, and prior knowledge of the human/mammalian metabolism is still required. However, with the introduction of the US Food and Drug Administration’s MIST (Metabolites In Safety Testing) guidelines, which recommend that drug metabolites that are identified only in humans or are present at disproportionately higher levels in humans compared with animals should undergo nonclinical in vitro toxicity testing, has underscored the need for effective methods of metabolite production. Whilst conventional synthetic chemical methods can often deliver required metabolites, there are challenges in achieving functionalization at non-activated carbon centres, and there is a general acceptance that where possible biological methods of production are more desirable from environmental and waste-management perspectives. It has been convincingly demonstrated through decades of research that wild type and recombinant microorganisms can produce human drug metabolites. Although there are some reports of up-scaling microbial cultures for drug metabolite production, there has been less effort to optimise productivity so that the industry has a viable alternative to the current chemical methods of metabolite generation. Consequently, the focus of research in this area in the short term should be on optimising the bioprocess, quantifying productivity and developing conditions that are cost-effective.

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**Figure Legends**

Figure 1. The biotransformation of phenanthrene, flurbiprofen and dextrorphan to human phase I metabolites by *Cunninghamella* spp.

Figure 2. Chemoenzymatic production of Selective Androgen Receptor Modulator (SARM) S1 glucuronide conjugate.

Figure 3. Cross-section view of hollow fibre cartridge used to immobilise *Actinoplanes* sp. ATCC 53771 (reproduced with permission from Osorio-Lozada et al. 2008).

Figure 4. Structure of β-lapachone glucosidated at position 6 by *C. elegans*

Figure 5. Structure of parthenolide

Figure 6. Regiospecific chemoenzymatic fluorination of ibuprofen methyl ester via sequential hydroxylation and fluorination with a cytochrome P450 BM3 mutant and diethylaminosulfur trifluoride (DAST).

Figure 7. Strategy for designing metabolically stable fluorinated drugs. Step 1, flurbiprofen incubated with *C. elegans* revealing metabolically labile site. Step 2, synthesis of regiospecifically fluorinated flurbiprofen via Suzuki-Miyaura coupling. Step 3, incubation of modified drug with *C. elegans* to assess stability.

Fig 1.



Fig 2



Fig 3



Fig 4



Fig 5



Fig 6



Fig 7

