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# CYP267A1 and CYP267B1 from *Sorangium cellulosum* So ce56 are Highly Versatile Drug Metabolizers[S]

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## ABSTRACT

**The guidelines of the Food and Drug Administration and International Conference on Harmonization have highlighted the importance of drug metabolites in clinical trials. As a result, an authentic source for their production is of great interest, both for their potential application as analytical standards and for required toxicological testing. Since we have previously shown promising biotechnological potential of cytochromes P450 from the soil bacterium *Sorangium cellulosum* So ce56, herein we investigated the CYP267 family and its application for the conversion of commercially available drugs including nonsteroidal anti-inflammatory, antitumor, and antihypotensive drugs. The CYP267 family, especially CYP267B1, revealed the interesting ability to convert a broad range of substrates. We established substrate-dependent extraction protocols and also optimized the reaction conditions for the in vitro experiments and *Escherichia coli*–based whole-cell bioconversions. We were able to detect activity of CYP267A1 toward seven out of 22 drugs and the ability of CYP267B1 to convert 14 out of 22 drugs. Moderate to high conversions (up to 85% yield) were observed in our established whole-cell system using CYP267B1 and expressing the autologous redox partners, ferredoxin 8 and ferredoxin-NADP$^+$ reductase B. With our existing setup, we present a system capable of producing reasonable quantities of the human drug metabolites 4′-hydroxydiclofenac, 2-hydroxyibuprofen, and omeprazole sulfone. Due to the great potential of converting a broad range of substrates, wild-type CYP267B1 offers a wide scope for the screening of further substrates, which will draw further attention to future biotechnological usage of CYP267B1 from *S. cellulosum* So ce56.**

## Introduction

The emergence of new diseases, rising concerns about drug resistance, and the decreasing efficacy of the existing drugs are of great pharmaceutical concern. As a result, drug research during the past century, driven by chemistry, pharmacology, and clinical science, has shown an increasing contribution to the development of new therapeutic agents (Drews, 2000). Despite the success in combating the majority of genetic, infectious, and bacterial diseases, novel drugs and drug derivatives are consistently demanded. However, the efficacy of such drugs and their related metabolites need to be tested and approved. The guidelines published by the Food and Drug Administration and the International Conference on Harmonization highlight the importance of qualifying metabolite exposure in clinical trials, in which a metabolite formed greater than 10% needs to be specifically tested for toxicity (Food and Drug Administration, 2008; International Conference on Harmonization, 2009, 2012). Due to the frequent introduction of novel drugs and drug candidates with new or modified chemical structure, implementation of costly multistep chemical syntheses may not be sufficient enough to overcome the demand of the respective metabolites (Rushmore et al., 2000).

Although the purification of major metabolites from urine is relatively easy and cheap (Gao et al., 2012), a constant and subject-independent large-scale production of drug metabolites, for instance, by using human liver, is hindered by very limited availability. To circumvent such limitations, alternative approaches of using microorganisms have been practiced, in which several microorganisms such as the fungus *Cunninghamella* sp. or bacterial variants of *Streptomyces* strains were shown to transform drugs and xenobiotics to mammalian metabolites (Zhang et al., 1996; Asha and Vidyavathi, 2009; Bright et al., 2011; Murphy and Sandford, 2012; Diao et al., 2013). However, because of the release of side products and the difficulty on handling the microbes during such biotransformation process, there is now a great interest in cytochrome P450 (P450) enzymes for the production of drug metabolites. In general, P450 enzymes are versatile, heme-containing enzymes, which catalyze a variety of reactions highlighting them as essential candidates for biotechnological and pharmaceutical research (Bernhardt and Urlacher, 2014). It has been shown that the utilization of human P450 enzymes enables the sufficient production of human drug metabolites employing baculovirus-infected insect cells expressing CYP3A4 or CYP2D9 (Rushmore et al., 2000), fission yeast expressing CYP2D6 (Peters et al., 2007) or CYP2C9 (Drăgan et al., 2011; Neunzig et al., 2012), and *Escherichia coli* cells expressing CYP3A4, CYP2C9, and CYP1A2 (Vail et al., 2005). Since it is not mandatory to employ associated human P450 enzymes to synthesize human drug metabolites (Schroer et al., 2010; Geier et al., 2015), microbial, especially bacterial, P450 enzymes serve as a good alternative because they are convenient to handle and usually hold higher expression levels and activities, recommending the possibility to employ them as useful

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**ABBREVIATIONS:** AdR, adrenodoxin NADP$^+$ reductase; Adx$_{4\text{-}108}$, truncated adrenodoxin; BM3, cytochrome P450 102A1; CO, carbon monoxide; FdR_B, ferredoxin-NADP$^+$ reductase B; Fdx8, ferredoxin 8; Fpr, ferredoxin NADP$^+$ reductase; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; P450, cytochrome P450.

biocatalysts (Bernhardt, 2006). The genetic manipulation of bacterial P450 enzymes toward a drug metabolizing activity has been successfully demonstrated for several P450 enzymes including the most studied P450 102A1 (BM3), CYP102A1 (Whitehouse et al., 2012; Ren et al., 2015). However, the engineering of enzymes against their native, narrow substrate range, or in general the screening for enzymes to produce certain drug metabolites, is time consuming and complex, which can be overcome by employing versatile wild-type P450 enzymes showing an untypically broad substrate range (Yin et al., 2014).

During our recent investigations on P450 enzymes from the myxobacterium *Sorangium cellulosum* So ce56, several interesting enzymes displaying new properties and substrate specificities have been discovered, which lead us to investigate the potential of these P450 enzymes for an application as drug metabolizing biocatalysts (Khatri et al., 2010, 2013; Schifrin et al., 2015; Litzenburger and Bernhardt, 2016). Therefore, we used bioinformatics analysis to identify myxobacterial P450 enzymes that are closely related to drug metabolizing P450 enzymes. Among others, the two members of the CYP267 family, CYP267A1 and CYP267B1, were found to be potential candidates. Although purified CYP267A1 and CYP267B1 have been previously shown to convert certain drugs (Kern et al., 2015; Litzenburger et al., 2015) and CYP267A1 was found to catalyze the hydroxylation of fatty acids (Khatri et al., 2015), a broader analysis of their substrate spectrum has never been tested. Therefore, in this study, we have tested the in vitro and whole-cell conversion of 22 widely used drugs (Fig. 1) by CYP267A1 and CYP267B1 for the first time. Compounds showing significant in vitro conversion were further chosen for a whole-cell biotransformation to upscale the metabolite production for the structural elucidation of the product(s) via NMR spectroscopy. We demonstrate that seven out of 22 drugs can be converted by CYP267A1 and that CYP267B1 shows activity toward 14 out of 22 drugs.

## Materials and Methods

**Chemicals.** Amitriptyline, amodiaquine, haloperidol, losartan, olanzapine, quinine, repaglinide, ritonavir, tamoxifen, and thioridazine were kindly provided by Dr. Stephan Lütz (Novartis, Basel, Switzerland). Isopropyl-$\beta$-D-1-thiogalactopyranoside and $\delta$-aminolevulinic acid were purchased from Carbolution Chemicals (Saarbruecken, Germany). Bacterial media were purchased from Becton Dickinson (Heidelberg, Germany). All other chemicals were obtained from standard sources at the highest purity available.

**Strains.** The *E. coli* strains Top 10 and NovaBlue Singles Competent Cells for cloning purpose were obtained from Invitrogen (San Diego, CA) and Merck (Duesseldorf, Germany). The *E. coli* strains BL21-Gold(DE3) for the heterologous expression of CYP267A1 and C43(DE3) for the heterologous expression of CYP267B1 were purchased from Agilent Technologies (Santa Clara, CA), whereby C43(DE3) was also used for whole-cell conversions.

**Plasmids.** The genes of CYP267A1 and CYP267B1 were cloned into a pET22b plasmid (Novagen, Darmstadt, Germany) as described elsewhere (Litzenburger et al., 2015). The pKKHC plasmids for the heterologous expression of the autologous redox partners ferredoxin 8 (Fdx8) and ferredoxin-$NADP^+$ reductase B (FdR_B) originate from previous work done in our laboratory (Ewen et al., 2009).

For the expression of the redox partners in the whole-cell system, the pCDF_dFA plasmid from Litzenburger et al. (2015) was used and changed as follows. The gene encoding ferredoxin $NADP^+$ reductase (Fpr) was excised using the restriction enzymes NdeI and KpnI and substituted with the FdR_B gene obtained from pKKHC_FdR_B using the same restriction enzymes. The ligation reactions were performed with the Fast-Link DNA Ligase Kit (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). From the resulting plasmid pCDF_AFB containing the truncated adrenodoxin ($Adx_{4\text{-}108}$) from *Bos taurus* and ferredoxin-$NADP^+$ reductase FdR_B from *S. cellulosum* So ce56, the gene of $Adx_{4\text{-}108}$ was excised with NcoI and HindIII. The resulting open plasmid was ligated with the gene of Fdx8 previously obtained from pKKHC_Fdx8 using the mentioned restriction enzymes. The resulting plasmid pCDF_F8B contains the genes for expressing the autologous Fdx8 and FdR_B from *S. cellulosum* So ce56 (see also Supplemental Fig. 1).

**Expression and Purification.** CYP267A1 and CYP267B1 have been expressed and purified as previously described (Khatri et al., 2015) using the T7 promoter–based expression construct of CYP267A1 and CYP267B1 (pET22b_CYP267A1 and pET22b_CYP267B1). The electron transfer partners Fdx8 and FdR_B were expressed and purified as noted elsewhere (Ewen et al., 2009).

**Media and Buffers.** For the heterologous expression of CYP267A1 and CYP267B1, terrific broth medium (24 g yeast extract, 12 g peptone, 4 ml glycerol, 2.31 g $K_2HPO_4$, and 12.54 g $KH_2PO_4$ per liter $H_2O$) was used. The whole-cell conversions were performed in M9CA medium (6 g $Na_2HPO_4$, 3 g $KH_2PO_4$, 0.5 g NaCl, 1 g $NH_4Cl$, 4 g Bacto Casamino Acids (BD Diagnostics, Sparks, NV), 4 g glucose, 50 $\mu$l 1M $CaCl_2$, 2 ml 1 M $MgSO_4$, 2 ml of trace elements solution per liter $H_2O$; trace elements solution contained 2.5 g EDTA, 250 mg $FeSO_4$, 25 mg $ZnCl_2$, and 5 mg $CuSO_4$ per 50 ml $H_2O$).

**Spectral Characterization of the CYP267 Family.** UV-visible spectra for the purified P450 enzymes were recorded at room temperature on a double-beam spectrophotometer (UV-2101PC, Shimadzu, Kyoto, Japan). The enzyme solution (2 $\mu$M) in 10 mM potassium phosphate buffer, pH 7.4, was dosed with a few grains of sodium dithionite to reduce the heme-iron and the sample was split into two cuvettes. The baseline was recorded between 400 and 700 nm. The sample cuvette was bubbled gently with carbon monoxide (CO) for 1 minute and a spectrum was recorded. The concentration of the P450 enzymes was estimated by CO-difference spectroscopy assuming molar extinction coefficient $\varepsilon$ (450–490 nm) = 91 $mM^{-1}cm^{-1}$ according to the method of Omura and Sato (1964).

**In Vitro Conversions.** A reconstituted in vitro system containing the corresponding P450 (0.5 $\mu$M), FdR_B (1.5 $\mu$M), and Fdx8 (10 $\mu$M), and a cofactor regenerating system with glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (2 U/ml) in potassium phosphate buffer (20 mM, pH 7.4, 1% glycerin) was used. The potential substrates, except olanzapine and omeprazole (both dissolved in dimethylsulfoxide), were dissolved in ethanol (10 mM) and added to an end concentration of 200 $\mu$M. The total volume of the reaction was 250 $\mu$l. The reaction was started by the addition of NADPH (800 $\mu$M) and carried out for 3 hours at 30°C. For substrates 1, 9, 11–15, and 18–21, 1 M glycine buffer (pH 11) or acetate buffer (pH 4) was added after reaction to enable improved recovery of the analytes. Therefore, the reaction was stopped by adding buffer (300 $\mu$l) or organic solvent (500 $\mu$l). The extraction was performed twice with 500 $\mu$l of the appropriate solvent (see (Supplemental Table 0). A negative control without P450 was implemented for each substrate to verify the P450-dependent reaction.

**Whole-Cell Conversions.** The experiments were performed with C43(DE3) cells, which were transformed with two plasmids, one encoding CYP267A1 (pET22b_CYP267A1) or CYP267B1 (pET22b_CYP267B1) and the second one encoding the redox partners Fdx8 and FdR_B (pCDF_F8B). For the main culture, 50 ml M9CA medium containing ampicillin (100 $\mu$g/ml) and streptomycin (50 $\mu$g/ml), inoculated with the corresponding overnight culture in lysogeny broth medium (dilution 1:100), was used. At an optical density of 0.9–1, the induction was initiated by adding 1 mM isopropyl $\beta$-D-1-thiogalactopyranoside and 0.5 mM 5-aminolevulinic acid and the temperature was set to 28°C. After 21 hours of expression, the temperature was set to 30°C and the substrate was added to a final concentration of 200 $\mu$M. To enable higher substrate conversion, EDTA (20 mM) or polymyxin B (32 $\mu$g/l) was added to increase permeability and substrate uptake of the *E. coli* cells (Janocha and Bernhardt, 2013). After 48 hours at 30°C, a 500 $\mu$l sample was removed, quenched by adding buffer or organic solvent, and extracted two times with 500 $\mu$l of organic solvent (see Supplemental Table 0). The organic phases were collected and evaporated to dryness. The extracts were stored at −20°C until analysis. All experiments were done twice, including a negative control (cells only transformed with pCDF_F8B).

**Analysis of the In Vitro and Whole-Cell Conversions via High-Performance Liquid Chromatography (HPLC).** The HPLC analysis was performed on a Jasco (Gross-Umstadt, Germany) HPLC 2000 system consisting of a PU-2080 Plus Pump, a AS-2050 Plus Sampler and a UV-2075 Plus UV/Vis-Detector. For the HPLC analysis, samples of substrates 3, 10, 13, and 22 were dissolved in 75 $\mu$l acetonitrile and 75 $\mu$l Milli-Q water. The remaining substrates were dissolved in the same volumes of solvents containing 0.1% trifluoroacetic acid. Analyses were performed on a reversed-phase column (125/4 Nucleodur

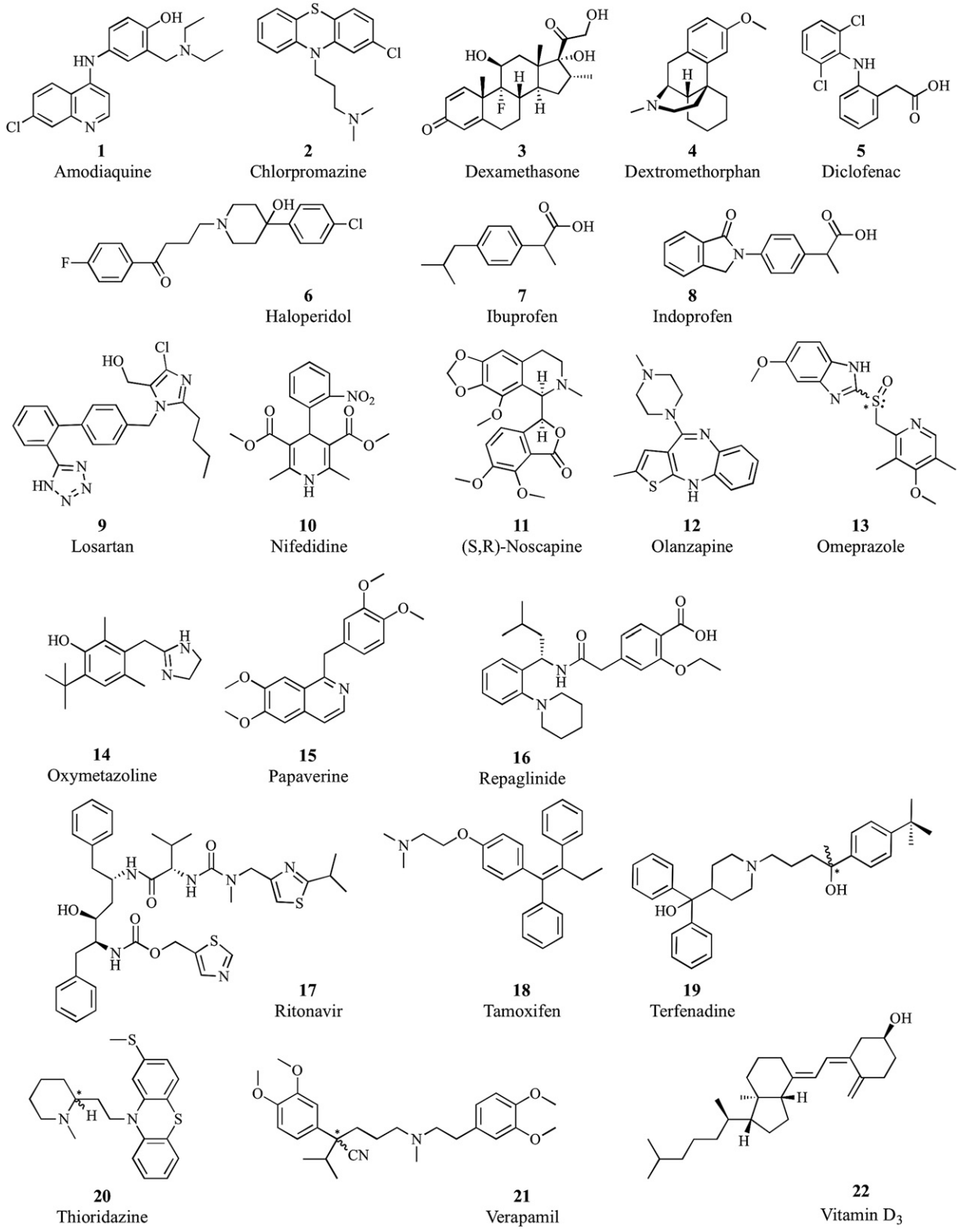


**Fig. 1.** Structural illustration of the tested drugs. In the case of drugs 13, 19, 20, and 21, racemic mixtures were used in all experiments.

100-5 C18ec; Macherey Nagel, Düren, Germany) at a flow rate of 1 ml/min and a temperature of 40°C. The injection volume was set to 30 $\mu$l. Due to the amount of different substrates the HPLC methods (including detection wavelengths) were substrate dependent and are presented in the Supplemental Material (Supplemental Table 1).

**Upscaling of the Whole-Cell Biotransformation System and Purification of the Products.** To obtain sufficient amounts of products for structure elucidation via NMR analysis, the previously described whole-cell conversions were up-scaled to a total volume of 2.5 l. After 48 hours, the reaction was stopped with the same volume of the appropriate solvent (see Supplemental Table 0). Before extraction of

the product of substrate 13, the culture was set to a pH of 11 using 2 M KOH and subsequently purified as described previously (Litzenburger et al., 2015). The purification of product 13a was done in the absence of trifluoroacetic acid.

**NMR Analysis.** The structures of the products were analyzed by NMR spectroscopy (Institute for Pharmaceutical Biology, Saarland University). The $^{1}$H- and $^{13}$C-NMR were recorded on a Bruker (Rheinstetten, Germany) 500 MHz NMR spectrometer. Two-dimensional NMR spectra were recorded as gs-H, H-COSY, gs-HSQC, and gs-HMBC. All chemical shifts are relative to $CDCl_3$ ($\delta$ = 77.00 for $^{13}$C-NMR; $\delta$ = 7.24 for $^{1}$H-NMR) or $CD_3OD$ ($\delta$ = 49.00 for $^{13}$C-NMR; $\delta$ = 3.31 for $^{1}$H-NMR) using the standard $\delta$ notion in parts per million.

**Tandem Mass Spectrometry (MS/MS) Analysis.** Product 13a was additionally characterized by MS/MS analysis (Institute of Bioanalytical Chemistry, Saarland University) with a API 2000 Qtrap (ABSciex, Darmstadt, Germany). Detailed settings of the MS/MS experiments can be found in section 8 (MS/MS settings) of the Supplemental Material.

## Results

### Bioinformatics Studies and Comparison of CYP267A1 with CYP267B1

To identify homologs of potentially drug metabolizing P450 enzymes from *S. cellulosum* So ce56, all of the 21 P450 enzymes (Khatri et al., 2010) from this bacterium were aligned with the known bacterial drug metabolizing P450 enzymes (Supplemental Table 2). We observed that the CYP265A1, CYP266A1, and the CYP267 family of this bacterium clustered within the same clan of the drug metabolizing P450 enzymes such as CYP107E4 from *Actinoplanes* sp. ATCC 53771 and CYP105 and CYP256 from *Rhodococcus jostii* RHA1, showing amino acid sequence identities of 38.9%, 34.2%, and 33.4%, respectively (Fig. 2; Supplemental Table 2). These bacterial P450 enzymes are considered to be active drug metabolizers for several drugs including amitriptyline, chlorpromazine (2), and diclofenac (5) (Prior et al., 2010; Kulig et al., 2015). In addition, we have previously shown that CYP264A1 was able to convert tricyclic drug molecules (Litzenburger et al., 2015), and CYP265A1 and CYP266A1 were able to hydroxylate the antitumor drug epothilone D (Kern et al., 2015). As a result, our homology study with the drug metabolizing bacterial P450 enzymes suggested that the two members of the CYP267 family (in addition to CYP265A1 and CYP266A1) are potential candidates for metabolizing certain drugs. The amino acid sequence alignment of CYP267A1 and CYP267B1 showed an identity of 38%. CYP267B1 possesses a conserved heme-binding domain ($_{347}$**F**GG**G**IHF**C**L**G**$_{356}$), the conserved threonine in the I-helix ($_{243}$AGHE**T**T$_{248}$), and glutamic acid and arginine in the K-helix ($_{281}$E**E**AL**R**$_{285}$), whereas in CYP267A1 the conserved phenylalanine in the heme-domain is replaced by leucine (L366) (Khatri et al., 2015). Amino acid sequence alignment of the CYP267 family with human P450 enzymes demonstrated that CYP267B1 showed the highest identities of 20.7%, 20.2%, 19.6%, 19.3%, and 19.0% with CYP2W1, CYP2C8, CYP2A6, CYP2D6, and CYP3A4, respectively (Supplemental Table 2), which are considered to be efficient drug metabolizers (Wrighton and Stevens, 1992; Guengerich, 1999).

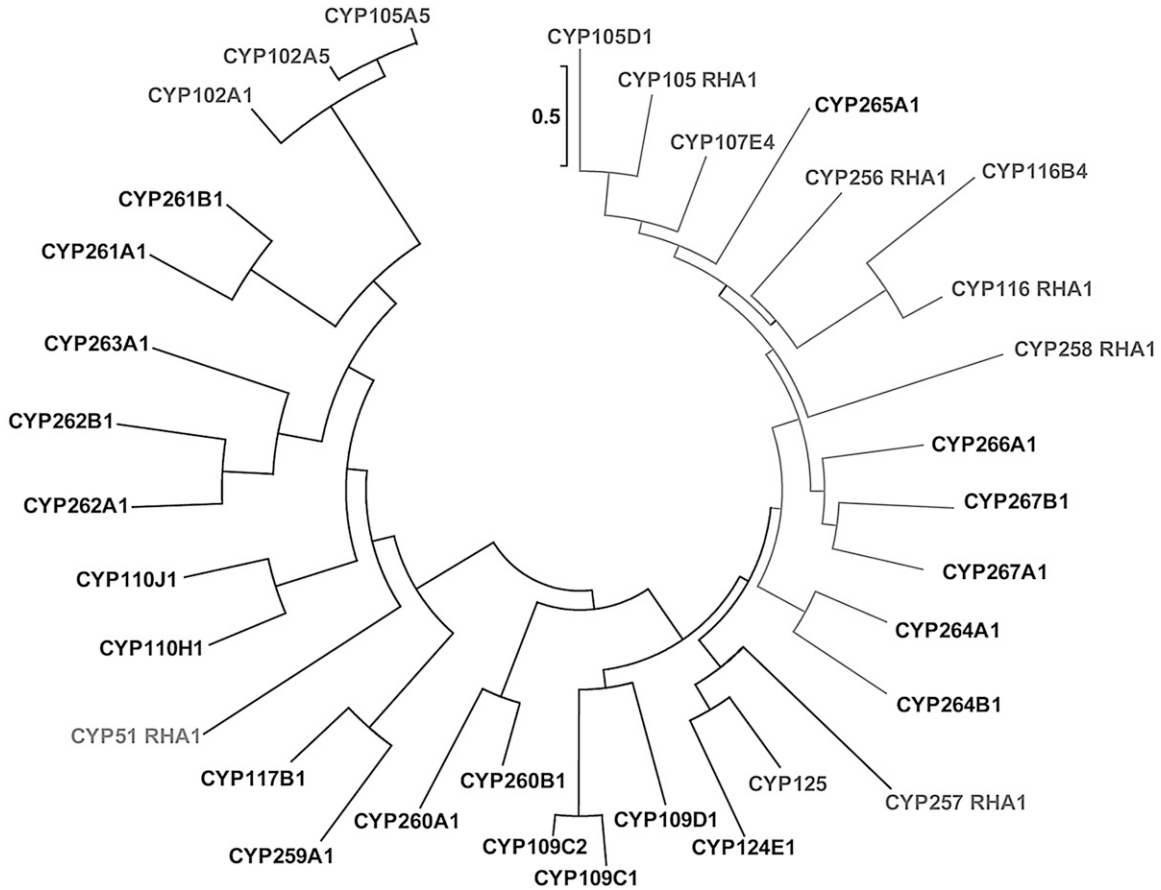


**Fig. 2.** The radial view of an unrooted phylogenetic tree obtained by MEGA4 (version 4.0, (Tamura 2007)) analysis for the determination of relatedness of the 21 P450 enzymes from *S. cellulosum* So ce56 (in black) with respect to drug metabolizing bacterial P450 enzymes CYP105D1 (P26911.1) from *Streptomyces griseus*; CYP51_RHA1 (Q0S7M9), CYP105_RHA1 (Q0SDH7), CYP116_RHA1 (Q0RUR9), CYP125_RHA1 (Q0S7N3), CYP256_RHA1 (Q0RXF8), and CYP257_RHA1 (Q0RVH0) from *Rhodococcus jostii* RHA1; CYP107E4 (ACN71221.1) from *Actinoplanes* sp. ATCC 53771; and CYP116B4 (EAV41564.1) from *Labrenzia aggregate* (in gray). The cluster of drug metabolizing P450 enzymes is shown in the gray clan. The bar in the tree indicates 0.5 amino acid substitutions per amino acid for the branch length.

### Expression, Purification, and Characterization of the CYP267 Family Members

First expression studies for CYP267B1 were realized using the vector pCWori$^+$. However, the yield of CYP267B1 using the pCWori$^+$-based expression construct was very low (<20 nmol/l *E. coli* culture after purification). Therefore, the protein has been expressed and purified using a T7-based expression construct (pET22b_CYP267B1) (Litzenburger et al., 2015), in which the protein yield was increased 5-fold (100 nmol/l).

As shown in Fig. 3, the UV-visible absorption spectrum of the oxidized CYP267B1 showed the presence of the Soret band ($\gamma$) at 416 nm, and the *Q*-bands at 567 nm ($\beta$) and 533 nm ($\alpha$). The reduction of CYP267B1 with sodium dithionite showed a slightly diminished absorption peak for the Soret band at 410 nm and a single peak in the *Q*-region (538 nm). The reduced CO-complex of CYP267B1 showed a typical peak maximum at 449 nm. The purified CYP267A1 showed the same spectroscopic features as previously described (Khatri et al., 2015), with the characteristic peak maximum at 448 nm in the CO-difference spectroscopy experiment and a peak maximum at 418 nm in the oxidized form of CYP267A1.

### Optimization of the In Vitro Conversion

Since P450 enzymes are monooxygenases, they require electrons from NADPH, which are transferred by autologous or heterologous redox partners (Hannemann et al., 2007). The substrate turnover also depends on the coupling and the efficiency of the redox partner proteins. In the case of the CYP267 family we observed limited in vitro conversions (Kern et al., 2015; Litzenburger et al., 2015) when using the bovine redox partners $Adx_{4\text{-}108}$/adrenodoxin NADP$^+$ reductase (AdR). Therefore, we substituted the heterologous $Adx_{4\text{-}108}$ and AdR with the autologous redox partners Fdx8 and FdR_B from *S. cellulosum* So ce56, which were previously shown to increase the CYP267B1-dependent epothilone D conversion (Kern et al., 2015). In our previous studies on the conversion of tricyclic antipsychotics and antidepressants (Litzenburger et al., 2015), thioridazine (20) could be identified as a substrate for CYP267A1 and amitriptyline as well as chlorpromazine (2) for CYP267B1, respectively; however, with low in vitro and whole-cell conversion. The substitution of $Adx_{4\text{-}108}$/AdR with the autologous redox partners Fdx8/FdR_B showed increased thioridazine-5-sulfoxide (20a) formation (from 43% to 50%) during the in vitro conversion of substrate 20. Likewise, the in vitro conversion of amitriptyline by CYP267B1 showed a significant enhancement of 10-hydroxyamitriptyline formation (from 15% to 60%), whereas the in vitro conversion of chlorpromazine (2) showed no difference.

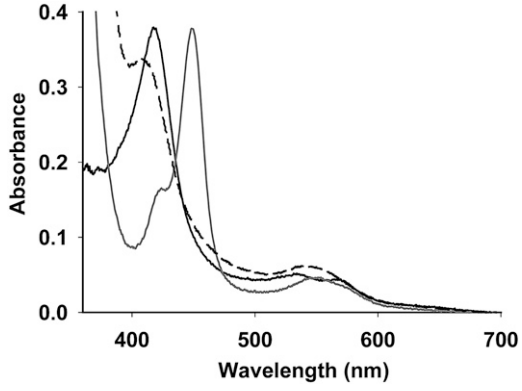


**Fig. 3.** Spectroscopic characterization of CYP267B1. The UV-visible spectra of oxidized (black line), dithionite reduced (dashed line), and CO-bound (gray line) CYP267B1 were recorded in 10 mM potassium phosphate buffer, pH 7.5.

### Optimization of the *E. coli*–Based Whole-Cell Bioconversion System

Since we observed a significant increase in the product formation when using the autologous redox partners, we investigated the coexpression of the autologous redox partners Fdx8 and FdR_B for our whole-cell bioconversion experiments. For comparison, a whole-cell system coexpressing $Adx_{4\text{-}108}$ and Fpr was used, since it has been shown previously that the whole-cell conversion of 4-methyl-3-phenyl-coumarin by CYP264A1 from *S. cellulosum* So ce56 increased when Fpr instead of AdR was used (Ringle et al., 2013). However, when substituting $Adx_{4\text{-}108}$/Fpr with the autologous redox partners Fdx8/FdR_B, the yield of the product was even further increased. In the case of CYP267A1, a nine times higher conversion of substrate 20 was observed (from 5% to 45% thioridazine-5-sulfoxide 20a) (Table 1). Likewise, the formation of chlorpromazine sulfoxide (2a) was nearly doubled by the CYP267B1-Fdx8-FdR_B whole-cell bioconversion system compared with CYP267B1-$Adx_{4\text{-}108}$-Fpr (from 18% to 30% 2a) (Table 1). In addition, we also observed a higher yield (from 7.5% to 26%) of the product 10-hydroxyamitriptyline from amitriptyline (Table 1). On the basis of these results, all further drug conversions were performed using Fdx8/FdR_B as redox partners in all in vitro and *E. coli*–based whole-cell bioconversion experiments.

When establishing a whole-cell system for P450 enzymes, indole-dependent inhibition should also be considered. The metabolism of tryptophan by the tryptophanase TnaA of *E. coli* results in the formation of indole (Li and Young, 2013). Since tryptophan is present in terrific broth medium, a concentration of over 600 $\mu$M indole was detected after 72 hours (Ringle et al., 2013). It was observed that indole acts as an inhibitor of CYP264A1 from *S. cellulosum* So ce56 (Ringle et al., 2013) and CYP109B1 from *Bacillus subtilis* (Girhard et al., 2010). In addition, some P450 enzymes are known to convert indole, and in this way competing with the normal substrate (Gillam et al., 2000). In case of the two members of the CYP267 family, the presence of 600 $\mu$M indole decreases the product formation in vitro by up to 40% for CYP267B1 and by up to 85% in the case of CYP267A1 (Supplemental Fig. 3). Therefore, we performed the whole-cell bioconversions in a defined M9CA minimal medium, which was previously shown to exhibit a very low amount of indole when using *E. coli* as a host (<5 $\mu$M) (Ringle et al., 2013).

Furthermore, the effect of the additives EDTA and polymyxin B was also investigated during the whole-cell conversions, since it has been shown previously that the presence of EDTA or polymyxin B enhances substrate uptake of *E. coli* cells for resin acid diterpenoid conversion by a CYP105A1-based whole-cell biocatalyst (Janocha and Bernhardt, 2013). We observed that the highest product formation was obtained when 20 mM EDTA for substrates 1, 2, 5, 7, 9, 15, and 16, and 32 $\mu$g ml$^{-1}$ polymyxin B for substrates 11, 13, 18, 19, and 20 were applied in the whole-cell system. Higher concentrations of both additives did not alter the product pattern for the tested drugs.

### Optimization of Product Extraction and HPLC Conditions for the Investigation of Drug Conversions

Due to the diverse chemical structures and functional groups of the tested drug molecules, we established a substrate-dependent extraction protocol to improve our experimental conditions for efficient analyses and product purification (Supplemental Table 0). We also optimized the HPLC conditions as listed in the Supplemental Table 1.

### CYP267-Dependent Substrate Conversion

**In Vitro and Whole-Cell Conversions by CYP267A1.** The in vitro conversions revealed that seven out of 22 drugs were converted by CYP267A1. In comparison with CYP267B1, the product pattern of

TABLE 1

Comparison of the whole-cell conversions with CYP267A1 and CYP267B1 using different redox partners

| Model Substrate | CYP267 | Conversion with heterologous $Adx_{4\text{-}108}$/Fpr[a] | Conversion with autologous Fdx8/FdR_B | Product |
|---|---|---|---|---|
| | | % | % | |
| Amitriptyline | B1 | 7.5 | 26 | 10-Hydroxyamitriptyline[a] |
| Chlorpromazine (2) | B1 | 18 | 30 | Chlorpromazine sulfoxide[a] (2a) |
| Thioridazine (20) | A1 | 5 | 45 | Thioridazine-5-sulfoxide[a] (20a) |

[a]Taken from Litzenburger et al. (2015).

CYP267A1 differs only for chlorpromazine (2; 2.4%) for which one single product was observed. For ibuprofen (7), tamoxifen (18), and terfenadine (19) the yields of the product formation were significantly lower compared with the respective CYP267B1-dependent conversions (7: 1.5%, 18: 3.7%, and 19: 2.1%). Despite having lower conversions with CYP267A1, five new substrates for CYP267A1 were identified (4, 6, 7, 18, and 19) (Fig. 4A). However, during in vitro conversion of substrates 18 and 20, one side product has been observed for substrate 18 (1.9% ± 1.1%) and in the case of substrate 20 two minor side products (3.7% ± 0.3% and 2.6% ± 0.1%) were found.

Only dextromethorphan (4), haloperidol (6), and thioridazine (20) were further employed for the investigation in the whole-cell system with CYP267A1-Fdx8-FdR_B, in which compounds 4 and 6 showed no conversion. Due to the higher in vitro conversion of substrates 2, 18, and 19 with CYP267B1, these substrates were only tested in the CYP267B1-Fdx8-FdR_B whole-cell system. However, using CYP267A1, substrate 20 was successfully converted to product 20a yielding a 44.7% product formation in our *E. coli*–based whole-cell bioconversion (Fig. 4A).

**In Vitro and Whole-Cell Conversions by CYP267B1.** It is very interesting to note that the in vitro conversions of the 22 tested drugs showed that CYP267B1 was able to convert 14 out of 22 compounds (Fig. 4B), seven more than CYP267A1. The highest in vitro yield was observed for oxymetazoline (14; 77.7%) and moderate in vitro conversions were detected for chlorpromazine (2; 37%), diclofenac (5; 37.5%), ibuprofen (7; 31.1%), and repaglinide (16; 41.4%). In addition, amodiaquine (1, 10.2%), losartan (9; 8.9%), noscapine (11; 12.1%), olanzapine (12; 16.5%), omeprazole (13; 13.7%), papaverine (15; 12.6%), and tamoxifen (18; 15%) were converted by CYP267B1. Very low conversion was observed for ritonavir (17; 1.4%) and terfenadine (19; 4.1%). However, during the in vitro experiments, also one minor side product was observed for substrates 7 (2.2% ± 0.1%), 9 (1.9% ± 0.4%), 11 (1.85% ± 0.45%), 12 (2% ± 0.1%), 13 (7% ± 0.4%), 15 (2.2% ± 0.1%), 16 (7.8% ± 1.4%), and 18 (2.55% ± 0.45%). In the case of substrate 14, two minor side products were found (5.1% ± 1.0% and 3% ± 0.5%).

The 14 drugs identified as substrates for CYP267B1 during the in vitro experiments were further investigated in the corresponding whole-cell experiments, where the highest yield was observed for omeprazole (13; 78.1%). The compounds 2 (30.3%), 5 (38%), and 7 (44.1%) showed similar yields compared with the corresponding in vitro experiments. We also observed a high conversion of losartan (9) to one product; however, without expressing CYP267B1 in the whole-cell experiment. This observation leads to the assumption that substrate 9 might have been oxidized by *E. coli* C43(DE3) to losartan carboxy acid. Due to the limited availability of a reference standard, we were not able to further investigate this assumption. The CYP267B1-Fdx8-FdR_B whole-cell system was also able to convert substrates 1, 11, 15, 16, 18, and 19, but in lower yields (≤10%). In the CYP267B1-dependent whole-cell experiments, minor side products were only formed in the case of substrates 11 (1.25% ± 0.15%), 13 (5.3% ± 0.2%), 15 (5.4% ± 0.5%), and 16 (5.2% ± 0.4%), showing identical retention time to those

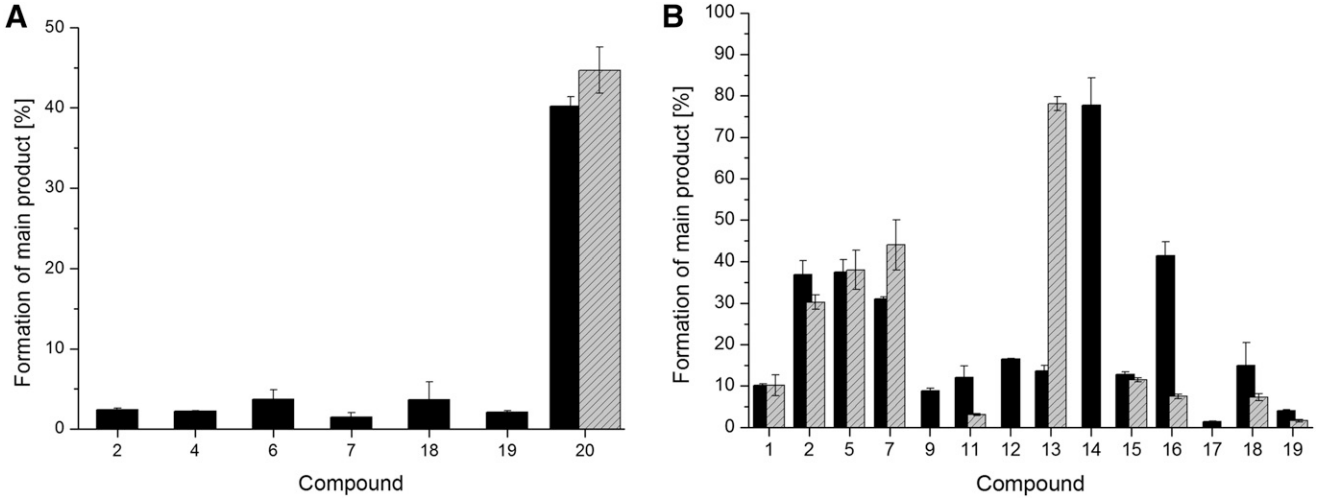


**Fig. 4.** The main metabolite formation in vitro (black bar) and in the whole-cell (gray bar) system by CYP267A1-Fdx8-FdR_B (A) and CYP267B1-Fdx8-FdR_B (B). (A) The compounds 1, 3, 5, 8–17, 21, and 22 were not converted by CYP267A1 and are therefore not shown. Only the substrates 4, 6, and 20 were further tested in the whole-cell system, whereby compounds 4 and 6 showed no conversion. The highest whole-cell conversion for substrate 20 was achieved with the supplement of polymyxin B (32 μg/ml). (B) The highest yields for the whole-cell conversions of compounds 1, 2, 5, 7, 9, 15, and 16 by CYP267B1 were achieved in the presence of 20 mM EDTA. In the case of compounds 11, 13, 18, and 19, the highest conversions were observed with the addition of polymyxin B (32 μg/ml). Despite the in vitro conversions of compounds 12 and 14 by CYP267B1, no conversion was observed in the corresponding whole-cell experiments. Due to the absence of conversion in the in vitro experiments, compounds 3, 4, 6, 8, 10, and 20–22 are not presented in this diagram.

observed in the corresponding in vitro experiments. For compounds 12 and 14, all attempts to utilize them in the whole-cell conversion system were unsuccessful, despite having high in vitro conversions (16.5% for compound 12 and 83% for compound 14). For the substrates that were not converted (3, 4, 6, 8, 10, 20, 21, and 22) or showed poor conversion (17) during the in vitro assay, attempts of investigating these drugs within the whole-cell system were discarded. Due to the high conversion in the whole-cell experiments, compounds 2, 5, 7, and 13 were further chosen for upscale and product characterization.

### Production of Drug Metabolites Using an *E. coli*–Based Whole-Cell Bioconversion System and Purification of Products via Preparative HPLC

The whole-cell system of CYP267B1-Fdx8-FdR_B was up-scaled to 2.5 l of M9CA medium for substrates 2, 5, and 7. For compound 13, an upscaling to 500 ml was sufficient enough to produce 5 mg of product 13a. In the case of whole-cell system CYP267A1-Fdx8-FdR_B and substrate 20, the conversion was also up-scaled to 2.5 l M9CA medium. For the compounds converted in a larger scale, comparable yields as previously described (Fig. 4, A and B) were observed, revealing the great potential of the established bioconversion system for future biotechnological upscaling. The products were purified via preparative HPLC and the purity of the isolated products was further verified by an additional HPLC measurement. The chromatograms of the purified products (5a, 7a, and 13a) and the pure substrates (5, 7, and 13) are shown in the Supplemental Material (Supplemental Fig. 2), confirming the high purity of the corresponding products. For products 2a and 20a, the chromatograms coincide with previous data and can be found in the Supplemental Material (Litzenburger et al., 2015).

### Drug Metabolites Formed by the CYP267 Family

As previously presented, CYP267A1 is able to convert seven out of 22 drugs and CYP267B1 catalyzes the conversion of 14 out of 22 drugs in vitro. However, only drug 20 for CYP267A1 and 10 drugs (1, 2, 5, 7, 11, 12, 15, 16, 18, and 19) for CYP267B1 were successfully converted in our whole-cell system. For drugs 2, 5, 7, 13, and 20 showing high yields after whole-cell biotransformation, the respective metabolites were additionally elucidated with an up-scaled production and via NMR measurements. A comprehensive overview of the analyzed drugs, and the human metabolites produced by the two members of the CYP267 family, is presented in (Supplemental Fig. 6). CYP267B1 is able to catalyze an aromatic hydroxylation of drug 5 to the human metabolite 4′-hydroxydiclofenac (5a) and an aliphatic hydroxylation of drug 7 to 2-hydroxyibuprofen (2a). Furthermore, the sulfoxidation of drugs 2 and 13 is catalyzed by CYP267B1 and the sulfoxidation of drug 20 is catalyzed by CYP267A1. All products were obtained with high purity and sufficient amounts (5–10 mg) for the structure elucidation via NMR spectroscopy. The NMR ($^{1}$H and $^{13}$C) data for products 5a, 7a, and 13a are shown in the (Supplemental Material). In the case of product 13a, an additional MS/MS measurement provided an unambiguous assignment to omeprazole sulfone (Supplemental Fig. 5). The NMR data of products 2a and 20a were identical to those previously described (Litzenburger et al., 2015) and match the corresponding reference standards (Zhang et al., 1996; Morrow et al., 2005). However, we achieved significantly increased yields in this study by our new whole-cell constructs (see Supplemental Fig. 1), which also gave better access to high product amounts for characterization of the respective products.

## Discussion

In recent years, the number of publications about the potential applications of P450 enzymes for the production of drug or drug-related compounds of pharmaceutical interest has continuously grown (Julsing et al., 2008). This progress can directly be of use for efficient and time-saving production of human drug metabolites. High yields and conversion rates can already be achieved by using corresponding human (Rushmore et al., 2000; Vail et al., 2005; Schroer et al., 2010; Geier et al., 2012; Schiffer et al., 2015) or suitable nonhuman (Taylor et al., 1999; Otey et al., 2006; Sawayama et al., 2009; Reinen et al., 2011; Di Nardo and Gilardi, 2012; Kiss et al., 2015; Ren et al., 2015) P450 enzymes in a whole-cell system to produce respective metabolites. The majority of published bacterial P450 enzymes used for the conversion of drugs are mutants of CYP102A1 (BM3) from *Bacillus megaterium*. We investigated the native myxobacterial P450 enzymes from *S. cellulosum* So ce56 for their application as drug metabolizers since soil bacteria should be able to convert and metabolize different xenobiotics present in their environment. Therefore, special attention was given to the CYP267 family.

It is interesting to note that CYP267B1 revealed the remarkable ability to accept substrates with completely different chemical structures and functions. In addition to the capability of converting tricyclic compounds (Litzenburger et al., 2015), the large 16-membered macrolide epothilone D (Kern et al., 2015), and small structures such as apocarotenoids (Litzenburger and Bernhardt, 2016), CYP267B1 also showed activity toward the conversion of 14 out of 22 different drugs. In contrast, CYP267A1 was only able to convert seven out of 22 drugs. The drugs converted by CYP267A1 and CYP267B1 feature a variety of chemical structures such as heterocyclic aromatics, morphinan class compounds, and alkaloids, thus increasing both the known substrate spectrum for this P450 family and the conceivable fields of their application. The metabolism of a drug molecule by a human P450 usually results in the formation of several side products (Table 2) since the main aspect of drug metabolism is excretion out of the body. Hence, the application of myxobacterial P450 enzymes is favorable in enabling the production of a single human drug metabolite, highlighting their ability for biotechnological processes to produce a metabolite in large quantity.

However, an important bottleneck in the application of P450 enzymes in biotechnological processes is often the efficiency of the redox system (Bernhardt and Urlacher, 2014). Therefore, we first identified efficient autologous redox partners to transfer electrons to the CYP267 family. Although the autologous redox system Fdx8/FdR_B from *S. cellulosum* So ce56 has already been shown to transfer electrons to myxobacterial CYP109D1, CYP260A1, and CYP264A1 (Khatri et al., 2010; Ringle et al., 2013), the heterologous bovine $Adx_{4\text{-}108}$ with AdR or the *E. coli* Fpr has been shown to be more efficient (Khatri et al., 2013; Ringle et al., 2013). However, in this study, the substitution of $Adx_{4\text{-}108}$/Fpr by Fdx8/FdR_B showed a significant increase of product yields for drug molecules (Table 1) when using the members of the CYP267 family. As a result, an *E. coli*–based whole-cell bioconversion system has been established containing the autologous redox partners of the CYP267 family.

Another bottleneck in whole-cell conversions that we faced during our studies was substrate uptake and indole inhibition. As a result, we established a substrate-dependent protocol, where EDTA and polymyxin B, which are shown to enhance substrate uptake into *E. coli* cells (Janocha and Bernhardt, 2013), significantly increased the limited whole-cell conversions (Fig. 4, A and B). To overcome inhibition of CYP267A1 and CYP267B1 by indole (Supplemental Fig. 3), we performed the whole-cell experiments in defined M9CA medium. However, for substrates 2, 11, 14, 16, 18, and 19 the CYP267B1-dependent whole-cell conversion showed lower yields compared with the in vitro assay, which might be a limitation caused by the low permeability of the *E. coli* cells for these substrates during the whole-cell

TABLE 2

Comprehensive overview of human drug metabolites formed by the members of the CYP267 family from *S. cellulosum* So ce56 and the corresponding human P450 enzymes

Metabolites marked with # and * are selectively formed by CYP267A1 and CYP267B1, respectively. The order of the listed metabolites is not representative for the product distribution.

| Drug | Human P450 | Desired Metabolite | Expected Side Products of Human P450 Enzymes | Reference |
|---|---|---|---|---|
| Chlorpromazine (2) | CYP2D6<br>CYP3A4 | Chlorpromazine sulfoxide (2a) * | 7-Hydroxychlorpromazine<br>Chlorpromazine-*N*-oxide | Cashman et al. (1993) |
| Diclofenac (5) | CYP1A2 | 4′-Hydroxydiclofenac (5a) * | — | Bort et al. (1999) |
| | CYP2C8<br>CYP2C18<br>CYP2C19 | | 5-Hydroxydiclofenac<br>4,5-Dihydroxydiclofenac | |
| | CYP2C9 | | 3-Hydroxydiclofenac<br>5-Hydroxydiclofenac | |
| Ibuprofen (7) | CYP2C8<br>CYP2C9 | 2-Hydroxyibuprofen (7a) * | 3-Hydroxyibuprofen<br>Carboxyibuprofen | Hamman et al. (1997); Neunzig et al. (2012) |
| Omeprazole (13) | CYP3A4 | Omeprazole sulfone (13a) * | 5-Hydroxyomeprazole | Yamazaki et al. (1997); Abelö et al. (2000) |
| Thioridazine (20) | CYP2D6 | Thioridazine-5-sulfoxide (20a) # | *N*-desmethylthioridazine<br>7-Hydroxythioridazine<br>Mesoridazine | Daniel et al. (2000) |

conversion. In contrast, substrates 7 and 13 showed higher yields in the whole-cell system compared with the in vitro conversion, suggesting that the conditions established for these substrates support an efficient metabolite production with our whole-cell system.

It has been shown recently that several members of a P450 fusion library, constructed by P450 enzymes and their autologous redox system RhfRED from *R. jostii* RHA1, are able to convert five out of 48 selected drugs (Kulig et al., 2015). Compared with our system consisting of the wild-type CYP267B1 and its autologous redox partners Fdx8 and FdR_B, we observed a larger substrate range (14 out of 22 drugs) and significantly higher activity toward the conversion of drugs. This leads to the suggestion that CYP267B1 features a great potential for the biotechnological production of various drug metabolites when using bacterial P450 enzymes. Our thus far not optimized whole-cell system was able to convert 38% of 160 mg of diclofenac (5) within 48 hours, which is a good starting point for the production of 4′-hydroxydiclofenac (5a). In this regard, the production of product 5a has previously been shown using an optimized fermentation process. The recombinant expression of CYP2C9 in fission yeast strain CAD68 resulted in an efficient formation of product 5a (468 mg/l) after the optimization of the pH value, the glucose concentration, and the establishment of a favorable host organism for the hydroxylation of substrate 5 (Drăgan et al., 2011). The engineering of BM3 toward the metabolism of drugs resulted in the BM3 mutant Asp251Gly/Gln307His, capable of the metabolism of drug 5 to product 5a in vitro (Tsotsou et al., 2012). This BM3 mutant was also shown to produce 2-hydroxyibuprofen (7a) from ibuprofen (7) (Tsotsou et al., 2012); however, experiments were only done in vitro or in a microtiter plate. Likewise, the production of product 7a in a preparative in vitro scale was described yielding 74.3 mg of product 7a (96% conversion) (Rentmeister et al., 2011). However, in this study, we were able to produce product 7a in a more relevant, biotechnological way using an *E. coli*–based whole-cell bioconversion system for CYP267B1 consisting of autologous redox partners and necessary cofactors within the cells. In this regard, the conversion of 115 mg of drug 7 to product 7a giving 44% product yield by the wild-type CYP267B1 demonstrated a promising scope for further optimization since we have not yet focused on the optimization or the engineering of the respective P450 toward higher space-time yields. In addition, our CYP267B1-Fdx8-FdR_B whole-cell system also presents the first method to produce omeprazole sulfone (13a) using a biotechnological approach with a high conversion yield (nearly 80%, 68 mg/l of drug 13) and high selectivity (<5% formation of unknown side product). Although CYP3A4 is responsible for the formation of omeprazole sulfone (13a) in the human body (Yamazaki et al., 1997), to the best of our knowledge, the biotechnological production of product 13a with CYP3A4 (Table 2) or another P450 has thus far not been described.

The established CYP267-Fdx8-FdR_B whole-cell systems are an excellent starting point for further optimizations in view of biotechnological upscaling and optimization (Bernhardt and Urlacher, 2014). Optimizations such as changing expression and reaction conditions or engineering P450 enzymes could be potential topics of interest. Several approaches for increasing the performance of the whole-cell system have been published, describing that an increased number of ferredoxin gene copies (Schiffer et al., 2015) or coexpressing a NADPH regenerating system (Zehentgruber et al., 2010) could enhance product formation. In a recent review, numerous approaches and examples were presented to enhance the catalytic activity of P450 enzymes toward potential practical purposes (Gillam, 2008). It is remarkable that the wild-type CYP267B1 is already able to catalyze three different reaction types (hydroxylation, sulfoxidation, and epoxidation) without any directed or evolutionary modification. In fact, the catalyzed hydroxylation reactions can be diversified to aliphatic (in the case of drug 7), allylic (as described for sesquiterpenes) (Litzenburger and Bernhardt, 2016), and aromatic (as shown for drug 5) hydroxylations, whereby the aromatic hydroxylation has not yet been described for a myxobacterial P450 enzyme.

Although in previous studies the bioconversion of drugs and xenobiotics was performed using different strains of *Streptomyces* or *Cunninghamella* sp. (Zhang et al., 1996; Asha and Vidyavathi, 2009; Bright et al., 2011; Murphy and Sandford, 2012; Diao et al., 2013), the systems were not selective and side products were usually observed. In addition, the nonoptimized media conditions used in these approaches could also interfere with the product identification. In contrast, in this study, we established a whole-cell biocatalyst for the conversion of widely used therapeutically important drugs and xenobiotics using two bacterial P450 enzymes (CYP267A1 and CYP267B1) and we also optimized this whole-cell system to allow convenient and effective product isolation for identification by NMR and MS/MS. In this way, the substrate spectrum of the CYP267 family—and especially for CYP267B1—was extended to commercially used drugs and their associated diverse chemical structures, demonstrating the potential of

myxobacterial P450 enzymes as drug metabolizers. Due to the great potential to convert a broad range of substrates, it can be concluded that CYP267B1 is an efficient and promising candidate for further substrate screening and protein engineering attempts, particularly with regard to its biotechnological applicability.

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**Authorship Contributions**

*Participated in research design:* Kern, Bernhardt.

*Conducted experiments:* Kern, Litzenburger, Khatri.

*Performed data analysis:* Kern, Litzenburger, Khatri.

*Wrote or contributed to the writing of the manuscript:* Kern, Khatri, Litzenburger, Bernhardt.

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