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Characterisation of CYP102A25 from *B. marmarensis* and CYP102A26 from *P. halophilus*: P450 homologues of BM3 with preference towards hydroxylation of medium chain fatty acids

Joanne L. Porter, Jack Manning, Selina Sabatini, Michele Tavanti, Nicholas J. Turner and Sabine L. Flitsch*

Abstract: Cytochrome P450 monooxygenases are highly desired biocatalysts due to their ability to catalyse a wide variety of chemically challenging C-H activation reactions. The CYP102A subfamily of enzymes are natural catalytically self-sufficient proteins consisting of a haem and FMN-FAD reductase domain fused in a single component system. They catalyse the oxygenation of saturated and unsaturated fatty acids to produce primarily ω -1, ω -2 and ω -3 hydroxy acids. These monooxygenases have potential applications in biotechnology however, their substrate range is still limited and there is a continued need to add diversity to this class of biocatalysts. Herein we present the characterisation of two new members of this class of enzymes: CYP102A25 (BMar) from *B. marmarensis* and CYP102A26 (PHal) from *P. halophilus*, both of which express readily in a recombinant bacterial host. BMar exhibits the highest activity toward myristic acid and shows moderate activity towards unsaturated fatty acids. PHal exhibits broader activity towards mid chain saturated (C14-18) and unsaturated fatty acids. Furthermore, PHal shows good regioselectivity for hydroxylation of myristic acid targeting the ω -2 position for C-H activation.

Introduction

Cytochrome P450 monooxygenases (P450s or CYPs) are a large family of haem-thiolate enzymes that are found in virtually all organisms (a notable exception being *Escherichia coli*),^[1] where they play critical roles in biosynthetic pathways and the detoxification of exogenous compounds.^[2] P450s are highly attractive biocatalysts due to their ability to catalyse chemically challenging oxidation reactions at unactivated positions, often with a high degree of regio and stereoselectivity.^[3-5] P450s receive electrons from redox partners enabling the activation of molecular oxygen followed by the insertion of one oxygen atom into the substrate and the second reduced to water (Figure 1A).^[6]

P450s are classified based on their respective electron transport systems,^[7] most of which consist of separate redox

Figure 1. (A) General P450 reaction scheme and (B) secondary structural organisation of class VIII CYP102A enzymes

partner proteins (e.g. class I P450 systems are comprised of the requisite haem domain and a separate FAD containing flavodoxin reductase and an iron-sulfur ferredoxin).^[8] Of particular interest are the catalytically self-sufficient P450s (e.g. class VII^[9-10] and class VIII), which contain the haem domain and reductase partners on a single polypeptide strand. The use of these enzymes in biocatalytic applications is preferable to their multi-component counterparts as it removes the need to identify and produce independent redox partners and requires no reconstitution of the electron transport system.^[11]

In particular the class VIII self-sufficient CYP102A1 (P450 BM3) has been heavily studied,^[12] as it was the first of its type identified.^[13] It is a fatty acid hydroxylase with the haem domain C-terminally fused to a eukaryotic class II type redox system: an NADPH cytochrome P450 reductase containing flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) prosthetic groups (Figure 1B).^[14-15] CYP102A1 functions as a dimer with electrons delivered from the reductase domain of one subunit to the haem domain of the neighbouring subunit. Hydroxy fatty acids, such as those produced by CYP102A1, have wide applications in the food industry, in the manufacture of lubricants and industrial fluids, and are also intermediates for the production of polymers.^[16] As such, much research has been aimed at the development of biocatalytic systems utilizing CYP102A1 for the biotransformation of various fatty acid substrates (e.g. Scheme 1). The catalytic activity of CYP102A1 compared to other P450 monooxygenases is vastly superior with k_{cat} values of up to $\sim 285\text{ s}^{-1}$, depending on substrate.^[17] This impressive rate is largely owing to the efficiency of electron transfer through the reductase to the substrate-binding site in the P450 haem domain.^[18] Engineering studies have aimed to generate artificial fusion enzymes with some success,^[19-20] yet

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the coupling efficiency of the wild-type enzymes remains largely unsurpassed. In addition to its native fatty acid substrates, CYP102A1 has been engineered to catalyse the oxyfunctionalisation of a variety of non-physiological substrates ranging from pharmaceutical intermediates^[21-22] and biologically relevant compounds^[23-26] to alkanes,^[27-29] including short chain gaseous alkanes.^[30] This introduced substrate diversity and demonstrated mutational robustness further highlights the importance of CYP102A1.

Many CYP102A homologues have since been identified within sequence databases that are continually expanding and profiting from the advances in DNA sequencing technology. However, the number of enzymes characterised at the protein level is not as substantial as one might expect given the importance of CYP102A1 and potential biotechnological applications. Characterised homologues include the bacterial CYP102A2 and A3 from *Bacillus subtilis*,^[31] CYP102A5 from *Bacillus cereus*^[32] and CYP102A7 from *Bacillus licheniformis*,^[33] the fungal CYP102D1 from *Streptomyces avermitilis*^[34] and CYP505A1 from *Fusarium oxysporum*.^[35] Although CYP102A enzymes have some of the highest catalytic rates among P450 monooxygenases, they typically lack regioselectivity and produce several distinct products with hydroxylation near the ω -terminus (most commonly ω -1, ω -2 and ω -3). The discovery or engineering of enzymes capable of higher levels of regioselectivity is highly desirable and an important factor that would further increase the appeal of these biocatalysts.^[36]

Herein we have characterised two new bacterial CYP102A homologues: CYP102A25 (BMar) from the alkaliphile *Bacillus marmarensis* and CYP102A26 (PHal) from the halophile *Pontibacillus halophilus*. These enzymes are distinct from other class VIII family members (sequence I.D of the full length enzymes $\leq 65\%$) and as such expand the diversity of this class of biotechnologically relevant enzymes.

Results and Discussion

Identification and cloning of two new class VIII self-sufficient CYP102A homologues

A BLAST search was performed using the amino acid sequence of CYP102A1 (P450 BM3) as the query sequence. The aim was to identify CYP102A homologues that shared moderate to low sequence identity (40-60%) not just to A1 but also to other characterised class VIII enzymes, in order to introduce more diversity to this class (Figure S1, Supporting Information). Resultantly, the self-sufficient P450s from the alkaliphile *Bacillus marmarensis* (CYP102A25, BMar) and from the halophilic bacterium *Pontibacillus halophilus* (CYP102A26, PHal) were selected as targets for cloning and characterization (amino acid sequences can be found on the Prokaryotic P450 database p450.co). These previously uncharacterised P450s share 61-38 % sequence identity to other class VIII enzymes including the prototype CYP102A1 and the fungal class VIII CYP505A1 (P450 Foxy) from *F. oxysporum* (Figure 2).

Scheme 1. Substrate panel for class VIII P450s

Alignment of the P450 haem domains with that of CYP102A1, for which there is structural information,^[15] allowed identification of secondary structural elements and comparison of key substrate binding residues (Figure S3, Supporting Information). In CYP102A1, R47 and Y51 are responsible for tethering of the substrate's carboxylate group through hydrogen bonding and electrostatic interactions.^[37] Interestingly CYP102A25 (R48 and F52) and CYP102A26 (K49 and F53) differ in these residues but possess tyrosines in neighbouring positions (Y43 and Y44 respectively), which could serve an equivalent role. This is difficult to substantiate without direct structural evidence but such a modification could result in subtle differences in substrate binding for these new P450s.

Expression, purification and UV-visible absorption properties

The genes encoding CYP102A25 (BMar) and CYP102A26 (PHal) were cloned from genomic DNA into the pET28a expression vector in frame with an N-terminal polyhistidine tag (Figure S3, Supporting Information). Expression of the correct size constructs (BMar = 122.8 and PHal = 121.6 kDa including cleavable linker and polyhistidine tag) was achieved using LB and M9 media with IPTG induction as well as using auto-induction LB. However, SDS-PAGE analysis indicated that at

Figure 2. Phylogenetic analysis of the amino acid sequences of BMar and PHal with known self-sufficient P450 monooxygenases. Evolutionary analysis was performed in MEGA7 with the sequence alignment done using Clustal and evolutionary history inferred using the neighbour-joining method. The evolutionary distances were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site.

Figure 3. Absorption spectra of purified (A) BMar and (B) PHal and their respective ferrous carbon monoxide bound complexes.

37 °C expression was mostly insoluble with negligible amounts observed in the soluble fraction, expression of the highest level of soluble protein was obtained using LB or M9 with typical IPTG induction at lower temperatures (< 25 °C) (Figure S4, Supporting Information). Conditions were further optimised at 20 °C to give consistently high expression of $\sim 200 \text{ nmol}_{\text{P450}} \text{ g}_{\text{cdw}}^{-1}$ for CYP102A25 and CYP102A26 (Table S4, Supporting Information). The enzymes were purified by IMAC and the purified samples were analysed by SDS-PAGE, which showed purification of the full-length enzymes (Figure S5, Supporting Information).

The UV-visible absorption profile of the purified class VIII P450s CYP102A25 and CYP102A26 are typical of P450 haemoproteins (Figure 3). The absorption maximum for the major Soret band of the oxidised enzymes is at 420 nm, while the smaller α and β bands lie at 569 and 537 nm for CYP102A25 and 569 and 535 nm for CYP102A26. Upon reduction with sodium dithionite the main Soret band shifted to 416 and 419 nm respectively and decreased in intensity. The expected shift in absorbance of the Soret peak to 450 nm was induced by the addition of CO and subsequent formation of the thiolate-ligated

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Fe(II)-CO complex. Smaller absorbance bands could be observed at ~ 420 nm representing the inactive P420 form.

Buffer optimisation

Assay buffer, pH and salt concentration were varied across several experiments in order to determine the influence of buffer composition on enzyme performance. Myristic acid **4** was selected as substrate since both enzymes display good activity toward this medium chain fatty acid. 0.1 M potassium phosphate (pH 7, 7.5 and 8), TRIS (pH 7.5, 8, 8.5, 9) and borate buffer (pH 8, 8.5, 9, 9.5 and 10) were used across their respective buffering ranges to cover combined pH values between 7 and 10 (Figure 4). CYP102A25 showed moderate relative activity (30 – 40 %) at pH 8.5 – 9.5 in borate buffer, however CYP102A26 displayed negligible activity (less than 10 %) relative to the NADPH oxidation rates by either enzyme in 0.1 M TRIS at pH 7.5. Both P450s exhibited considerably higher rates of NADPH oxidation in TRIS buffer at pH 7.5 and 8 than in KPi buffer over the same pH range. In 0.1 M TRIS at pH 7.5 CYP102A25 displayed a 3.9 ± 0.6 fold higher, and CYP102A26 a 3.2 ± 0.8 fold higher NADPH oxidation rate than at the same pH in KPi buffer. Consequently, this buffer was used for all subsequent experiments unless otherwise stated.

Figure 4. (A) General P450 reaction scheme and (B) secondary structural organisation of class VIII CYP102A enzymes

Since both enzymes originate from halophilic bacteria, the effect of salt on enzyme activity was assessed based on NADPH oxidation as above. Surprisingly reduction in activity with increasing sodium chloride concentration was significant with over 50 % decrease in NADPH oxidation observed with the addition of 25 mM NaCl (Figure S6, Supporting Information).

Nucleotide cofactor preference

The ability of CYP102A25 (BMar) and CYP102A26 (PHal) to reduce cytochrome *c* using either NADPH or NADH was investigated in steady-state reactions to determine cofactor preference (Table 1 and Figure S7, Supporting Information). Both of these class VIII self-sufficient P450s showed preference toward NADPH with K_M values of 37 and 102 μM for CYP102A25 and A26 respectively compared to values greater than 1 mM with NADH. This is consistent with previously characterised CYP102A enzymes all of which exhibit preference to NADPH as electron donor.

Table 1. Kinetic properties for electron transfer by BMar and PHal using either NADPH or NADH as reductant and cytochrome *c* as electron acceptor.

P450	Cofactor	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{M}^{-1} \text{s}^{-1}$)
CYP102A25 (BMar)	NADPH	37 ± 5	16 ± 1	$4.3 \pm 0.9 \times 10^5$
	NADH	> 1000	Not determined	
CYP102A26 (PHal)	NADPH	102 ± 9	33 ± 2	$3.2 \pm 1 \times 10^5$
	NADH	> 1000	Not determined	

Substrate screening

The respective activities and substrate preference of CYP102A25 (BMar) and CYP102A26 (PHal) were initially assessed based on NADPH oxidation rate in the presence of a panel (Chart 1) of saturated and unsaturated fatty acids (C8 to C18). Figure 5 shows initial NADPH oxidation rates displayed as a relative value compared to the respective rates by CYP102A25 and A26 with myristic acid **4**. Both P450s showed negligible activity towards the shorter C8 (**1**) and C10 (**2**) fatty acids and low but detectable activity toward the C12 lauric acid (**3**). CYP102A25 exhibited the most activity in the presence of C14 myristic acid (**4**), with NADPH oxidation activity also seen in the presence of C16 **5** and C18 **6** saturated acids and the three unsaturated fatty acids (**7**, **8** and **9**). However, the oxidation rate in the presence of **4** was more than double that observed for any other compound from the substrate panel.

CYP102A26 displayed a wide substrate scope with comparable high levels of activity in the presence of the C14 **4** and C16 **5** saturated fatty acids as well as palmitoleic (**7**) and oleic (**8**) unsaturated fatty acids. In addition, significant NADPH oxidation rates (60 – 40 % compared to **4**) were also observed with stearic **6** and linolenic acid **9**.

Separate reactions were conducted using 0.5 or 0.05 μM purified enzyme with a cofactor recycling system and were analysed by GC-FID to directly monitor fatty acid substrate

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Figure 5. Activity of CYP102A25 (BMar) and CYP102A26 (PHal) towards various saturated and unsaturated fatty acids based on NADPH oxidation. Reactions were performed in 0.1 M TRIS pH 7.5 and the initial rate of oxidation is expressed as a relative value compared to that for myristic acid **4**.

depletion (Figure S8, Supporting Information). Conversions were observed for substrates **3–9** (C12–C18) with almost full conversion (< 90 %) observed for substrates **3, 4, 5, 7, 8** and **9** by both CYP102A25 and A26 used at 0.5 μ M concentration. Stearic acid was converted to a lesser extent by both P450s, this is likely in part attributed to the lower water solubility of this long chain fatty acid.

Product identification and distribution

Having determined the substrate preference and scope using the substrates outlined in Chart 1, the regioselectivity of CYP102A25 and A26 catalysed fatty acid oxidation was assessed (Table 2). The hydroxylation position was determined by analysis of the specific MS fragmentation of the trimethylsilyl derivatised hydroxy fatty acid products. Similarly to other characterised members of the CYP102A family,^[31, 33] no terminal hydroxylation was detected with instead the ω -2 and ω -1 positions preferred over ω -3. CYP102A25 showed a slight preference to hydroxylation at the ω -2 position (41–45 %) over ω -1 (35–41 %) and ω -3 (14–20 %) positions for the entire range of tested saturated fatty acids **3–6** (C12–C18). The product distribution for CYP102A26 was substrate dependent with the hydroxylation of lauric acid **3** (C12) occurring primarily at the ω -1 position (54.1 %). However for longer chain saturated fatty acids from C14 to C18 **4–6**, the major products were the respective ω -2 hydroxy fatty acids. Of particular interest is the biotransformation of myristic acid (C14) (**4**) by CYP102A26 to give predominantly the ω -2 hydroxyacid (73 %) with the ω -1 (21 %) and ω -3 (6 %) hydroxyacids as minor products.

The prototype CYP102A1 (P450 BM3) is known to hydroxylate fatty acids near the ω terminus, however the exact position is substrate dependent. In the case of myristic acid (C14) **4**, preference is shown for hydroxylation at the

subterminal ω -1 position. Although several P450s of this family have been shown to preferentially hydroxylate at the ω -2 position (CYP102A2, **3** and **7**), none possess any high degree of regioselectivity. Table 3 provides a comparison of the product distribution for the CYP102A25 and A26 catalysed hydroxylation of myristic acid (C14) **4** with previously reported values for other CYP102A enzymes. CYP102A26 displays the highest level of regioselectivity producing the ω -2 hydroxyacid (12-hydroxymyristic acid) as the major product at 73 % with the ω -1 and ω -3 as minor products. This is greater than CYP102A2 which gives 62 % 12-hydroxymyristic acid, followed by CYP102A7, A25 and A3 all of which have only a slight preference (≤ 50 %) to the ω -2 hydroxyacid.

The electron coupling efficiency for the CYP102A25 and A26 catalysed conversion of myristic acid (**4**) was also assessed. Inefficient coupling of electrons from NADPH to substrate oxygenation can result in the formation of reactive oxygen

Table 2. Distribution of fatty acid hydroxylation products (in %) by CYP102A25 and CYP102A26

Substrate	CYP102A25 (BMar)			CYP102A26 (PHal)		
	ω -1	ω -2	ω -3	ω -1	ω -2	ω -3
Lauric acid (C12) 3	35	45	20	54	26	20
Myristic acid (C14) 4	36	47	17	21	73	6
Palmitic acid (C16) 5	40	42	18	29	56	15
Stearic acid (C18) 6	41	45	14	41	45	14

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Table 3. Comparison of product distribution for the hydroxylation of myristic acid (C14) **4** catalysed by CYP102A homologues

	ω -1 (%)	ω -2 (%)	ω -3 (%)
CYP102A1 (BM3)	55	25	20
CYP102A2	21	62	18
CYP102A3	11	47	43
CYP102A7	20	50	30
CYP102A25 (BMar)	36	47	17
CYP102A26 (PHal)	21	73	6

species (ROS). This uncoupling can influence the reaction rate, furthermore the presence of ROS can affect the stability and performance of the system. Hydroxylation of fatty acids by CYP102A enzymes occurs almost fully coupled to NADPH utilisation (>90 %),^[17, 33] however this is most often not the case for non-physiological substrates. Here the coupling efficiency of the CYP102A25 (BMar) and CYP102A26 (PHal) catalysed

hydroxylation of **4** is comparably high with values of 92 ± 5 and 98 ± 2 % respectively.

Biotransformation of fatty acids by CYP102A25 and A26

To demonstrate the utility of these enzymes, biotransformations were conducted using an increased substrate loading. Cells expressing the appropriate P450 were lysed and clarified cell lysates were used in the biotransformations with substrate loading from 1 to 50 mM myristic acid (C14) **4** (Figure 6). Full substrate conversion (>99 %) was observed with both CYP102A25 and A26 at initial substrate loading concentrations of 1, 2 and 5 mM. Although the percentage conversion decreased at higher substrate concentrations, the total amount of substrate converted increased (i.e. 10 mM, ~60 %, 6.5 mM; 20 mM, 40 %, 8 mM; 50 mM, 40 %, 20 mM). This overall reduction in substrate conversion is likely partially attributed to decreased substrate solubility at these higher concentrations.

It was postulated that implementation of a two-phase system and/or substrate feeding and product removal strategy could aid future preparative scale production of these hydroxy fatty acids. As a preliminary test to assess this feasibility, biotransformations

Figure 6. Biotransformation of myristic acid (C14) **4** catalysed by BMar or PHal with (A) various substrate loadings and (B) 10 mM substrate in the presence of additives or water immiscible solvents. Reactions were performed in 0.1 M TRIS pH 7.5 with 10 % (v/v) DMSO using cell lysate containing 4 μ M P450.

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were conducted as above using 10 mM **4** but in the presence of additives (e.g. methyl- β -cyclodextrin, Tween® 20 and Triton™ X-100) to aid substrate solubility or with a solvent overlay (e.g. heptane, dodecane, methyl tert-butyl ether, ethyl acetate or toluene) in a bi-phasic system (Figure 6B). In the original aqueous reaction buffer (0.1 M TRIS pH 7.5), CYP102A25 and A26 catalyse the oxidation of 10 mM myristic acid (**4**) giving conversions of 50–60 %. Addition of a solvent overlay (10 % v/v) typically lowered conversions, with the exception of dodecane where conversions were within error to those obtained under aqueous conditions. The presence of the nonionic surfactants Tween® 20 or Triton™ X-100 did not improve conversions however, addition of methyl- β -cyclodextrin gave increased conversions by both P450s. Cyclodextrins have been used previously to aid solubility of hydrophobic substrates in aqueous solutions, including steroids,^[38] terpenes^[25] and fatty acids.^[39] Here the addition of 4 % (w/v) methyl- β -cyclodextrin significantly improved the conversion of myristic acid (**4**) (at 10 mM substrate loading) from ~ 60 % in aqueous solution to 80 %. The product distribution by CYP102A26 under these improved conditions was not dissimilar to that reported above, but showed slightly greater selectivity toward the ω -2 position (CYP102A26 product distribution 10 mM **4** with 4 % Me- β -CD: ω -2 = 79, ω -1 = 16 and ω -3 = 5 %).

Conclusions

The biotechnological importance of catalytically self-sufficient P450s, in particular the fatty acid hydroxylase BM3 has already been well established.^[12] Here we expand the diversity of this subfamily of P450s with the characterisation of CYP102A25 (BMar) from *B. marmarensis* and CYP102A26 (PHal) from *P. halophilus*. These P450s show good levels of expression in a recombinant *E. coli* system and like their predecessors preferentially obtain electrons from NADPH through a fused electron transport system. CYP102A25 shows the highest NADPH oxidation rate and the highest conversions with myristic acid (C14) **4** as substrate. CYP102A26 displays a wider substrate scope with significant activity observed for mid chain saturated (C14–18) **4–6** and unsaturated (including palmitoleic **7**, oleic **8** and linolenic **9** acid) fatty acids. Furthermore, CYP102A26 shows high regioselectivity for the production of the ω -2 12-hydroxymyristic acid (> 70 %), where other members of this class generate a more balanced ratio of ω -1, ω -2 and ω -3 hydroxy acids. These new CYP102A members add diversity to this family of industrially relevant enzymes and can present alternatives to the typical CYP102A1 for future applications.

Experimental Section

Chemicals: All chemicals were purchased from Sigma-Aldrich unless otherwise stated and gases were purchased from BOC. Chemically competent *E. coli* NEB5 α and BL21(DE3) cells as well as restriction enzymes and PCR components were purchased from New England Biolabs.

Identification and cloning: A BLAST search was conducted using CYP102A1 (P450 BM3) as the query sequence on the UniProt server to identify BMar (ID: U6SQQ2-1) and PHal (ID: A0A0A5I4I0). Genomic DNA was purchased from DSMZ (*Bacillus marmarensis* DSMZ strain 21297 and *Pontibacillus halophilus* DSMZ strain 19796). Complementary primers for PCR were designed with suitable 5' overhangs for subsequent In-fusion cloning (Clontech) into the pET28a vector between *Bam*HI and *Sal*I restriction sites (Table S3).

Expression and purification: *E. coli* BL21(DE3) cells were transformed with the pET28a plasmid carrying either the BMar or PHal genes and a single colony was inoculated into the expression media (800 mL LB in 2 L flask) from a starter culture. Cultures were grown at 37 °C to an OD₆₀₀ of ~ 0.8 then induced with IPTG (0.4 mM) and expressed at 20 °C for 20 h at 200 rpm. Purification of BMar and PHal was carried out by immobilised metal affinity chromatography (IMAC). Resuspended cell pellets (in 0.1 M KPi pH 8) were lysed by sonication and clarified by centrifugation then incubated with Ni-agarose (Qiagen) for 1 h at 4 °C and 200 rpm. The resin bound protein was washed with 20 mM imidazole then eluted with 250 mM imidazole and analysed by SDS-PAGE. The spectral absorption properties and concentration of BMar and PHal were determined on a CARY 50 UV/Visible spectrophotometer (Agilent Technologies). CO difference spectroscopy was performed using an extinction coefficient of 91 mM⁻¹ cm⁻¹ and following an established protocol.^[40]

NADPH oxidation assays: Depletion of NADPH was monitored at 340 nm on a microtitre platereader (Tecan Infinite M200). Reactions were carried out using 0.2 mM NADPH (Prozomix Ltd.), 0.2 mM substrate (Scheme 1) in DMSO (used at a final concentration of 5 % (v/v)) and 0.04 – 0.15 μ M enzyme in a total reaction volume of 200 μ L. Initial reaction rates were calculated using Magellan™ data analysis software and are expressed as relative values, data was obtained in triplicate and the error is the standard deviation.

Buffer optimisation: Reactions were performed monitoring NADPH consumption as specified above with myristic acid **4** as substrate. Potassium phosphate buffer (KPi), TRIS and borate buffer were assessed at various pH values between 7 and 10 and salt concentration (0 – 250 mM NaCl) was also varied in a subsequent experiment.

Cytochrome c assay for nucleotide cofactor preference: Reduction of cytochrome c was monitored by change in absorbance on a microtitre platereader (Tecan Infinite 200) at 550 nm with temperature control at 25 °C. Reactions were carried out in 0.1 M TRIS, pH 7.5 with 10 nM enzyme and 50 μ M cytochrome c ($\Delta\epsilon_{550} = 26.35 \text{ mM}^{-1} \text{ cm}^{-1}$) in a total reaction volume of 100 μ L. NADPH and NADH were tested as cofactors over concentration ranges of 2 – 500 μ M for NADPH and 0.1 – 3 mM for NADH.

Substrate scope: Assays were performed as above monitoring activity based on NADPH consumption at 340 nm. Reactions were performed with 0.04 μ M enzyme, 0.2 mM NADPH and 0.2 mM substrate dissolved in DMSO and used at a constant DMSO concentration of 5 % (v/v).

Determination of product distribution and coupling efficiency: Biotransformations were prepared largely as below with the exception of direct supply of NADPH (1.2 mM) instead of a cofactor recycling system. After 6 h samples were acid extracted into MTBE and derivatised with BSTFA + 1% TMCS as described in the supporting information (Page S13, Supporting Information). Derivatised samples were analysed by GC-MS and products were identified by the distinctive MS fragmentation of the trimethylsilyl derivatised acids and hydroxyacids. Coupling efficiency was determined as previously reported,^[10] using 0.2 μ M NADPH and 0.25 μ M **4** with reactions monitored as described above.

Biotransformations: Reactions were performed using purified protein or clarified cell lysate as specified. Cells expressing the appropriate P450 were harvested, resuspended in 0.1 M TRIS pH 7.5 and lysed by sonication then centrifuged to obtain clarified cell lysate. Biotransformations were conducted using clarified cell lysates containing 4 μ M P450 or purified protein at 0.5 or 0.05 μ M. Fatty acid substrates were dissolved in DMSO (used at 10 % (v/v) final concentration) and used at concentrations between 1 – 50 mM. A cofactor recycling system consisting of glucose, glucose dehydrogenase (Codexis) and 0.5 mM NADP⁺ was added to facilitate generation of the more expensive NADPH. Reactions were performed for 16 h, then extracted and analysed by GC-FID as specified in the Supporting Information (Page S14).

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- [1] D. R. Nelson, *Biochim. Biophys. Acta, Proteins Proteomics* **2017**.
- [2] D. R. Nelson, *Hum. Genomics* **2009**, *4*, 59-65.
- [3] R. Bernhardt, *J. Biotechnol.* **2006**, *124*, 128-145.
- [4] R. Fasan, *ACS Catal.* **2012**, *2*, 647-666.
- [5] H. M. Girvan, A. W. Munro, *Curr. Opin. Chem. Biol.* **2016**, *31*, 136-145.
- [6] F. P. Guengerich, *J. Biochem. Mol. Toxicol.* **2007**, *21*, 163-168.
- [7] F. Hannemann, A. Bichet, K. M. Ewen, R. Bernhardt, *Biochim. Biophys. Acta, Gen. Subj.* **2007**, *1770*, 330-344.
- [8] A. W. Munro, H. M. Girvan, K. J. McLean, *Biochim. Biophys. Acta* **2007**, *1770*, 345-359.
- [9] G. A. Roberts, A. Celik, D. J. B. Hunter, T. W. B. Ost, J. H. White, S. K. Chapman, N. J. Turner, S. L. Flitsch, *J. Biol. Chem.* **2003**, *278*, 48914-48920.
- [10] M. Tavanti, J. L. Porter, S. Sabatini, N. J. Turner, S. Flitsch, *ChemCatChem* **2017**, DOI:10.1002/cctc.201701510.
- [11] E. O'Reilly, V. Kohler, S. L. Flitsch, N. J. Turner, *Chem. Commun.* **2011**, *47*, 2490-2501.
- [12] A. W. Munro, D. G. Leys, K. J. McLean, K. R. Marshall, T. W. B. Ost, S. Daff, C. S. Miles, S. K. Chapman, D. A. Lysek, C. C. Moser, C. C. Page, P. L. Dutton, *Trends Biochem. Sci.* **2002**, *27*, 250-257.
- [13] L. O. Narhi, A. J. Fulco, *J. Biol. Chem.* **1986**, *261*, 7160-7169.
- [14] S. Modi, M. J. Sutcliffe, W. U. Primrose, L.-Y. Lian, G. C. K. Roberts, *Nat. Struct. Mol. Biol.* **1996**, *3*, 414-417.
- [15] A. J. Warman, O. Roitel, R. Neeli, H. M. Girvan, H. E. Seward, S. A. Murray, K. J. McLean, M. G. Joyce, H. Toogood, R. A. Holt, D. Leys, N. S. Scrutton, A. W. Munro, *Biochem. Soc. Trans.* **2005**, *33*, 747-753.
- [16] J. O. Metzger, U. Bornscheuer, *Appl. Microbiol. Biotechnol.* **2006**, *71*, 13-22.
- [17] M. A. Noble, C. S. Miles, S. K. Chapman, D. A. Lysek, A. C. MacKay, G. A. Reid, R. P. Hanzlik, A. W. Munro, *Biochem. J* **1999**, *339*, 371-379.
- [18] A. W. Munro, J. G. Lindsay, J. R. Coggins, S. M. Kelly, N. C. Price, *Biochim. Biophys. Acta, Bioenerg.* **1995**, *1231*, 255-264.
- [19] S. M. Hoffmann, M. J. Weissenborn, L. Gricman, S. Notonier, J. Pleiss, B. Hauer, *ChemCatChem* **2016**, *8*, 1591-1597.
- [20] A. Robin, G. A. Roberts, J. Kisch, F. Sabbadin, G. Grogan, N. Bruce, N. J. Turner, S. L. Flitsch, *Chem. Commun.* **2009**, 2478-2480.
- [21] X. Ren, J. A. O'Hanlon, M. Morris, J. Robertson, L. L. Wong, *ACS Catal.* **2016**, *6*, 6833-6837.
- [22] J.-B. Wang, A. Ilie, M. T. Reetz, *Adv. Synth. Catal.* **2017**, *359*, 2056-2060.
- [23] J. C. Lewis, S. Bastian, C. S. Bennett, Y. Fu, Y. Mitsuda, M. M. Chen, W. A. Greenberg, C.-H. Wong, F. H. Arnold, *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 16550-16555.
- [24] V. B. Urlacher, A. Makhsumkhanov, R. D. Schmid, *Appl. Microbiol. Biotechnol.* **2006**, *70*, 53-59.
- [25] S. Schulz, M. Girhard, S. K. Gassmeyer, V. D. Jaeger, D. Schwarze, A. Vogel, V. B. Urlacher, *ChemCatChem* **2015**, *7*, 601-604.
- [26] X. Ren, J. A. Yorke, E. Taylor, T. Zhang, W. Zhou, L. L. Wong, *Chem. - Eur. J.* **2015**, *21*, 15039-15047.
- [27] P. Meinhold, M. W. Peters, A. Hartwick, A. R. Hernandez, F. H. Arnold, *Adv. Synth. Catal.* **2006**, *348*, 763-772.
- [28] A. Ilie, R. Agudo, G.-D. Roiban, M. T. Reetz, *Tetrahedron* **2015**, *71*, 470-475.
- [29] S. D. Munday, O. Shoji, Y. Watanabe, L.-L. Wong, S. G. Bell, *Chem. Commun.* **2016**, *52*, 1036-1039.
- [30] R. Fasan, M. M. Chen, N. C. Crook, F. H. Arnold, *Angew. Chem. Int. Ed.* **2007**, *46*, 8414-8418.
- [31] M. C. U. Gustafsson, O. Roitel, K. R. Marshall, M. A. Noble, S. K. Chapman, A. Pessegueiro, A. J. Fulco, M. R. a. Cheesman, *Biochemistry* **2004**, *43*, 5474-5487.
- [32] P. K. Chowdhary, M. Alemseghed, D. C. Haines, *Arch. Biochem. Biophys.* **2007**, *468*, 32-43.
- [33] M. Dietrich, S. Eiben, C. Asta, T. A. Do, J. Pleiss, V. B. Urlacher, *Appl. Microbiol. Biotechnol.* **2008**, *79*, 931-940.
- [34] K. Y. Choi, E. Jung, D. H. Jung, B. P. Pandey, H. Yun, H. Y. Park, R. J. Kazlauskas, B. G. Kim, *FEBS J.* **2012**, *279*, 1650-1662.
- [35] N. Nakayama, A. Takemae, H. Shoun, *J. Biochem.* **1996**, *119*, 435-440.
- [36] B. M. Nestl, S. C. Hammer, B. A. Nebel, B. Hauer, *Angew. Chem. Int. Ed.* **2014**, *53*, 3070-3095.
- [37] H. Li, T. L. Poulos, *Nat. Struct. Biol.* **1997**, *4*, 1870-1874.
- [38] P. Bracco, D. B. Janssen, A. Schallmeyer, *Microb. Cell Fact.* **2013**, *12*, 95-95.
- [39] M. Theurer, Y. El Baz, K. Koschorreck, V. B. Urlacher, G. Rauhut, A. Baro, S. Laschat, *Eur. J. Org. Chem.* **2011**, *2011*, 4241-4249.
- [40] T. Omura, R. Sato, *J. Biol. Chem.* **1964**, *239*, 2370-2378.

Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPER

A new twist: Here new alternatives to the well-studied prototype self-sufficient CYP102A1 (P450 BM3) are presented. The new BM3 homologues, CYP102A25 (BMar) and CYP102A26 (PHal), have different substrate profiles encompassing saturated and unsaturated fatty acids. PHal also displays higher regioselectivity for production of the ω -2 hydroxy acid than previously reported.

Dr. Joanne L. Porter, Jack Manning, Selina Sabatini, Michele Tavanti, Prof. Dr. Nicholas J. Turner and Prof. Dr. Sabine L. Flitsch*

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Characterisation of CYP102A25 from *B. marmarensis* and CYP102A26 from *P. halophilus*: P450 homologues of BM3 with preference towards hydroxylation of medium chain fatty acids