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Expanding the biocatalytic toolbox of flavoprotein monooxygenases from *Rhodococcus jostii*

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Abstract

With the aim to enlarge the set of available flavoprotein monooxygenases, we have cloned 8 unexplored genes from *Rhodococcus jostii* RHA1 that were predicted to encode class B flavoprotein monooxygenases. Each monooxygenase can be expressed as soluble protein and has been tested for conversion of sulfides and ketones. Not only enantioselective sulfoxidations, but also enantioselective Baeyer-Villiger oxidations could be performed with this set of monooxygenases. Interestingly, in contrast to known class B flavoprotein monooxygenases, all studied biocatalysts showed no nicotinamide coenzyme preference. This feature coincides with the fact that the respective sequences appear to form a discrete group of sequence related proteins, distinct from the known class B flavoprotein monooxygenases subclasses: the so-called flavin-containing monooxygenases (FMOs), N-hydroxylating monooxygenases (NMOs) and Type I Baeyer-Villiger monooxygenases (BVMOs). Taken together, these data reveal the existence of a new subclass of class B flavoprotein monooxygenases, which we coined as Type II FMOs, that can perform Baeyer-Villiger oxidations and accept both NADPH and NADH as coenzyme. The uncovered biocatalytic properties of the studied Type II FMOs make this newly recognized subclass of monooxygenases of potential interest for biocatalytic applications.

Keywords: Enzyme screening, flavoprotein monooxygenases, sulfoxidation, Baeyer-Villiger reaction, enantioselectivity

Abbreviations: CE: cell extract; CCE: cleared cell extract; FMOs: flavin-containing monooxygenases; NMOs: N-hydroxylating monooxygenases; BVMOs: Baeyer-Villiger monooxygenases

1. Introduction

Flavoprotein monooxygenases are attracting attention as selective and oxidative biocatalysts that can be used for the production of high-value chemical building blocks or pharmaceuticals [1]. These biocatalysts efficiently catalyze chemo-, regio-, and/or enantioselective oxygenations using dioxygen as mild oxidant while using NAD(P)H as reductant. Flavoprotein monooxygenases can be divided into 6 distinct classes, with each class containing sequence- and structure-related monooxygenases. Two of these classes (class A and B) are especially appealing when considering biocatalysis. These two classes are typified by being single component enzymes that contain a tightly bound flavin cofactor, while the other monooxygenases rely on multiple protomers and often employ a loosely bound flavin cofactor. The class A of flavoprotein monooxygenases seems to have evolved to catalyse aromatic hydroxylations, as most of the characterized monooxygenases of this class represent hydroxylases, typically acting on a very restricted number of substrates [2]. Class B flavoprotein monooxygenases do not catalyse hydroxylations but perform Baeyer-Villiger oxidations and/or oxygenations of heteroatom-containing compounds. In fact, three class B flavoprotein monooxygenase subclasses have been identified based on specific sequence motifs, which coincide with a preference for specific oxygenation types for each subclass [3]:

1 - Baeyer-Villiger monooxygenases (BVMOs) contain the sequence motif (FxGxxxHxxxWP/D) and primarily catalyse Baeyer-Villiger oxidations, while they are also able to oxygenate heteroatom-containing compounds (N, S, B or Se containing compounds);

2 - the so-called Flavin-containing monooxygenases (FMOs) contain a slightly different sequence motif (FxGxxxHxxxYK/R), and are specialized in oxidizing heteroatom-containing compounds while they are inefficient in catalyzing Baeyer-Villiger oxidations. The FMOs have mainly been studied as xenobiotic degrading enzymes that help the human body to dispose toxic compounds [4, 5]. The human proteome encompasses six FMO isoforms [6], which are able to activate or degrade many drugs. Only very recently FMOs have been considered for their use as biocatalysts, due to the identification and production of a microbial FMO which, in contrast to the human homologs, can be easily expressed as a soluble protein [7].

3 - *N*-hydroxylating monooxygenases (NMOs) share sequence homology with the above-mentioned class B flavoprotein monooxygenases but lack a typifying sequence motif. Only a conserved histidine can be identified in the region of the BVMO/FMO sequence

motif. So far only a few NMOs from bacteria and fungi have been reported. They typically convert long-chain primary amines by *N*-hydroxylation [8].

The overall sequence homology among all class B flavoprotein monooxygenases reflects the fact that they are all single-component FAD-containing monooxygenases composed of two dinucleotide binding domains (Rossman folds to bind both FAD and NADPH), that allow them to combine flavin reduction and monooxygenation in one polypeptide chain. They often prefer the use of NADPH as electron donor, keeping the $NADP^+$ tightly bound throughout the catalytic cycle [9, 10]. From the three subclasses, mainly the BVMOs have been extensively studied as biocatalysts.

To tap the natural diversity for the discovery of novel oxygenating enzymes *Rhodococcus jostii* RHA1 is a very promising candidate. The proteome of this bacterium is predicted to contain an unusually high variety of oxidative enzymes [11, 12]. Inspired by this observation, we and the Grogan group have recently cloned 22 putative BVMO-encoding genes, and succeeded in producing and exploring the biocatalytic properties of these enzymes [13, 14]. This research has confirmed that all these enzymes indeed act as BVMOs which can be used for a large number of oxygenations. When screening the predicted proteome of *R. jostii* RHA1 for monooxygenases, we also identified a relatively large number of other putative class B monooxygenases that seem to be more closely related to NMOs and FMOs. While BVMOs are relatively rare enzymes that are only found in bacteria and fungi with an average distribution of only one or two BVMO-encoding genes per microbial genome [15], FMOs and NMOs are even more scarce in microbes. FMOs are quite abundantly present in higher eukaryotes (e.g. the 6 isoforms in the human genome and often more than 10 in plant genomes [16]), but these FMOs are often difficult to produce due to their membrane association.

In this paper, we report on the exploration of 8 novel class B flavoprotein monooxygenases obtained from a single microorganism, *R. jostii* RHA1, that share sequence homology with FMOs and NMOs. By optimizing expression vectors and expression conditions, all enzymes were obtained in soluble and holo form. A set of ketones and aromatic sulfides was tested for all enzymes to explore their biocatalytic potential. Also their coenzyme specificity and enantioselective properties were analysed.

2. Experimental

2.1. General materials and methods

Oligonucleotide primers were purchased from Sigma, dNTPs and In-Fusion™ 2.0 CF Dry-Down PCR Cloning Kit from Clontech, Phusion polymerase from Finnzymes. All other chemicals were obtained from Acros Organics, ABCR, Sigma-Aldrich, TCI Europe, and Roche Diagnostics GmbH. The nicotinamide coenzymes were purchased from Codexis.

The NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for DNA sequence retrieval and BLAST searches. The EBI server (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for multiple sequence alignment by CLUSTALW [19]. Treeview software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used for visualization of the sequence relationships.

2.2. Cloning and expression

Escherichia coli TOP10 from Invitrogen was used as a host for DNA manipulations and protein expression. Two expression vectors have been used: (i) a modified pBAD vector (pBAD*N*) in which the NdeI site was replaced by the original NcoI site [17], and (ii) the recently engineered pBAD-based pCRE2 vector which harbors a codon-optimized gene encoding a thermostable mutant of phosphite dehydrogenase (PTDH) with an N-terminal His-tag [18].

The target genes were amplified by PCR using genomic DNA of *R. jostii* RHA1 as template and subsequently cloned into pBAD*N* using the In-Fusion PCR Cloning kit from Clontech, following the recommendations of the manufacturer. Expression of all generated expression constructs was tested using 24-multiwell microtiter plates in the sandwich cover system from Enzyscreen®. Cell cultures of 2.5 ml in LB medium supplemented with 50 µg mL^{-1} ampicillin were grown at four different temperatures (17°C for 48 h; 24°C for 32 h; 30°C and 37°C for 16 h) with four different arabinose concentrations (0.002%, 0.02%, 0.2%, and none) each, with shaking at 200 rpm. Cell extracts (CEs) were obtained using DNase/lysozyme in combination with freezing in liquid nitrogen and thawing at 30°C. CEs as well as cell cleared extracts (CCEs) were analyzed by SDS-PAGE for (soluble) expression of the monooxygenases. For the genes that did not yield expressed soluble protein when cloned into pBAD*N*, the in-house developed pCRE2 expression vector was used.

2.3. General procedure for the bioconversions employing the novel FMOs

Conversions were performed essentially as described before [14]. For GC analysis, 500 µl incubations of 50 mM Tris/HCl pH 7.5, 10% glycerol, 1 mM DTT, 1 mM EDTA, 10

μM FAD, 5 mM substrate, 5% cosolvent (1,4-dioxane), 100 μM NADPH, 3.1 μM PTDH, 10 mM phosphite and 5 μM of the corresponding monooxygenase in CCE form (total volume of 0.5 mL), were shaken in glass vials at 24°C for 24 hours. For determining the exact concentration of each enzyme in the respective extract, a recently developed method was used which relies on the decrease in absorbance at 450 nm upon NADPH-mediated reduction of the flavin cofactor [14]. Each conversion was stopped by extracting with ethyl acetate (2 x 0.5 mL containing 0.1% mesitylene as standard), dried with magnesium sulfate and analyzed directly by GC to determine the degree of conversion and the enantioselectivity. The details concerning the (chiral) GC analysis can be found in the Supplemental Information (Table S1). For every tested reaction, control experiments in the absence of enzyme resulted in no conversion.

3. Results

3.1. Identification of putative FMO/NMO-encoding genes

By a PBLAST search of the proteome of *R. jostii* RHA1 [11], not only 23 Type I BVMO-encoding genes could be found [14], but also another 8 genes putatively encoding class B flavoprotein monooxygenases could be identified. All corresponding proteins contain two typical Rossman fold motifs (GxGxxG), clearly distinguishing them from other flavoprotein monooxygenase classes. As for all class B flavoprotein monooxygenases, one Rossman fold motif is close to the N-terminus while the other is in the middle of the sequences. In our previous work [14], we proposed a new conserved sequence motif typical for the Type I BVMO family: [A/G]GxWxxxx[F/Y]P[G/M]xxxD located between the two Rossman fold motifs. This motif entails the conserved active site aspartate and therefore appears a better fingerprint for Type I BVMO sequences. The motif is absent in all sequences used in this study and confirms that they do not represent classical BVMOs. The sequences do also not contain the previously described BVMO-typifying motif. In fact, most of them (all except for monooxygenase H) appear more closely related to FMOs as the FMO-typifying sequence motif can be recognized with only one or two amino acid substitutions (see Table 1). This is also reflected by the fact that most of the sequences have been annotated in the sequence database as putative flavin-containing monooxygenases. When performing a multiple sequence alignment, the clustering becomes apparent. Monooxygenase H is most related to known NMOs, while the other proteins form another distinct group of sequence-related proteins (Figure 1). It also shows that all studied sequences are quite distant from Type I BVMOs. Based on these observations, we annotate

monooxygenase H as an NMO (NMO-H). The other proteins seem to form another isolated cluster of sequence-related class B flavoprotein monooxygenases and therefore we classify them as Type II FMOs (while the known and well-established FMO class can be considered as Type I FMOs). Recently another class B flavoprotein monooxygenase has been described that shows relatively high sequence similarity with these Type II FMOs (FMO-X in Figure 1). The Grogan group has discovered this bacterial monooxygenase that can perform sulfoxidations and can act as BVMO [20]. However, conversion rates described for this monooxygenase seem rather modest (100-1000 fold lower when compared with conversion rates of Type I BVMO).

This sequence analysis hints to a new subclass of class B flavoprotein monooxygenases, Type II FMOs that can be employed as biocatalysts. By cloning, expressing, and testing biocatalytic oxidations, we set out to explore the above-mentioned 8 putative class B flavoprotein monooxygenases.

3.2. Expression of the putative class B flavoprotein monooxygenases

The targeted genes were cloned in a pBAD-based expression vector (pBAD*N*, [17]) by ligation free cloning. By varying the temperature and arabinose concentration, optimal expression conditions were determined. By this approach, 6 proteins could be obtained in soluble form with good expression levels (Table 1). Only for 2 genes, expression did not yield satisfying amounts of soluble protein. We solved this expression problem by cloning the respective genes into the recently developed pCRE2 expression vector [18]. With these constructs we were able to achieve soluble expression of all targeted proteins that were predicted to represent class B flavoprotein monooxygenases.

Interestingly, in two cases, FMO-E and FMO-G, it was found that during expression the growth medium developed a bluish colour, as shown in Table 1. We have previously observed this phenomenon when expressing a phenylacetone monooxygenase mutant capable of converting indole into indigo blue [21]. Also the expression of several type I BVMOs from *R. jostii* RHA1 led to blue pigment formation [14]. The same phenomenon has been observed when expressing a bacterial FMO in *E. coli* [22]. Apparently, the respective monooxygenases are able to form indigo blue by oxidizing the endogenous indole. The fact that indigo blue formation is only observed upon expression of distinct enzymes, confirms the functional expression of these monooxygenases, in our case of FMO-E and FMO-G.

3.3. Substrate profile of the overexpressed monooxygenases

Even though using CCE for the reactivity tests, we used the same concentration of recombinant enzyme in all assays to allow a direct comparison of the results. For this, the amount of active enzyme in the CCEs was first quantified with the NADPH-mediated flavin reduction method established and described before [14]. This revealed that all extracts contained significant amounts of NADPH-dependent flavoproteins, which is in agreement with the SDS-PAGE analysis of CCEs, and confirms that all enzymes contain a flavin cofactor upon expression and accept NADPH as coenzyme.

To determine the biocatalytic potential of the studied monooxygenases, each monooxygenase was tested for activity on several ketones and aromatic sulfides: 2-indanone, cyclopentadecanone, phenylacetone, bicyclo[3.2.0]hept-2-en-6-one, thioanisole, 4-methylthioanisole, ethyl benzyl sulfide, and benzyl phenyl sulfide.

None of the monooxygenases was found to convert 2-indanone or cyclopentadecanone. In contrast, intriguingly, FMO-E, FMO-F, and FMO-G were found to be able to convert phenylacetone and bicyclo[3.2.0]hept-2-en-6-one (Scheme 1). Product analysis by GC revealed that phenylacetone was converted into the corresponding ester, benzylacetate (Table 2). Enantiodivergent oxidation of bicyclo[3.2.0]hept-2-en-6-one was analyzed by chiral GC. This revealed that the monooxygenases have a preference for the formation of the normal lactone, while still a significant amount of the abnormal lactone is formed (Table 2). While the biocatalysts are not very enantioselective in formation of the normal lactone, the FMO-F and FMO-G yield >65% ee for the abnormal (1*S*,5*R*) enantiomer. Taken together, these data show that FMO-E, FMOF and FMO-G act as *bona vide* BVMOs.

Next, four prochiral aromatic sulfides with varying substituents (thioanisole, 4-methylthioanisole, ethyl benzyl sulfide, and benzyl phenyl sulfide) were tested as substrates. The relatively small substrate thioanisole was the only sulfide that was converted by most of the monooxygenases in detectable amounts, as indicated in Table 3. Only NMO-H did not convert significant amounts of thioanisole or any other tested sulfide, strengthening the classification of this particular enzyme as a NMO. For the majority of the biocatalysts, mainly the (*R*)-methyl phenyl was preferentially formed, with the exception of FMO-G, which led to the (S)-enantiomer. The enantioselectivity of the thioanisole sulfoxidation varied from low to moderate, achieving the highest optical purity (*ee* = 57%), when employing FMO-G. The conversion of 4-methylthioanisole could only be detected in very low conversions. This is in line with the observation of Grogan that FMO-X only showed a

poor conversion rate with this aromatic sulfide [20]. The enantioselective outcome with this substrate when using the Type II FMOs from *R. jostii* RHA1 was similar to that achieved with thioanisole. The more bulky substrate benzyl ethyl sulfide was only converted by FMO-E, FMO-F and FMO-G, but with poor conversion. Only the corresponding methyl phenyl sulfone was detected as product, indicating that these biocatalysts are able to oxygenate the initially formed sulfoxide to the overoxidized product. The bulky benzyl phenyl sulfide was not converted by any of the tested monooxygenases.

FMO-X was found to be indifferent towards NADPH or NADH [20]. This is in sharp contrast with the known Type I BVMOs which only accept NADPH as coenzyme. It also is different from the Type I FMOs, that typically prefer NADPH as a coenzyme. Conversion of several of the previously identified substrates where therefore tested via GC with both nicotinamide cofactors. When we replaced NADPH by NADH, almost identical degrees of conversion and enantioselectivities were found for the conversions for all tested Type II FMOs. This is a highly interesting and relevant finding as it suggests that the Type II FMOs have as general characteristic a relaxed coenzyme specificity. This is attractive for biocatalytic applications, as NADH is a cheaper source than it phosphorylated analogue (when considering use as isolated enzyme) while NADH is also present at higher level in cells (when considering use of whole cells). This shows that the Type II FMOs described in this paper, together with the previously described FMO-X [20], represent a newly discovered subclass of class B flavoprotein monooxygenases that can accept both NADPH and NADH.

4. Discussion

Eight class B flavoprotein monooxygenases from *R. jostii* RHA1 have been expressed in soluble form and studied for their biocatalytic potential. From the sequence alignment study, one of the targeted monooxygenases (NMO-H) was found to belong to the group of NMOs. The prototype enzymes for the subclass of *N*-hydroxylating flavoprotein monooxygenases (NMOs) are L-ornithine N5-hydroxylase and L-lysine N6-hydroxylase [25]. An L-ornithine hydroxylase had already been identified in the *R. jostii* RHA1 proteome (Rmo_Orn in Figure 1, [26]). When comparing NMO-H with known NMO sequences it shows highest sequence similarity with the lysine hydroxylase IucD from *E. coli* (Figure 1). This suggests that the NMO-H described in this study probably represents a lysine hydroxylase present in *R. jostii* RHA1. The restricted substrate scope of lysine hydroxylases also is in line with the observation that NMO-H is not converting any aromatic compound

tested in this study [27]. This confirms that, in contrast to BVMOs and FMOs, NMOs are rather restricted in substrate acceptance and appear to be of little value as biocatalysts.

The FMOs described in this paper do not seem to belong to the typical FMO subclass as (i) they do not contain a fully conserved FMO-typifying motif (Table 1), and (ii) they form another cluster of sequence-related proteins distinct from the three other subclasses, FMOs, NMOs, and BVMOs (see Figure 1). Also their catalytic properties deviate from the other subclasses by accepting both nicotinamide coenzymes, NADH and NADPH, without significant difference. Furthermore, several of these FMOs were shown to perform Baeyer-Villiger oxidations, a reaction for which typical FMOs display hardly any activity. We coined this newly recognized subclass of class B flavoprotein monooxygenases Type II FMOs.

This study reveals that of the tested Type II FMOs, FMO-E, FMO-F, and FMO-G may represent valuable new oxidative biocatalysts. These three enzymes were found to be active in both sulfoxidations and Baeyer-Villiger reactions. Multiple sequence alignment revealed that they form a separate cluster of sequences (Figure 1). Except for showing a relative high sequence homology with each other, these three monooxygenases also share an *N*-terminal extension of about 160 residues (see Figure S1). This may suggest that this newly identified subclass of monooxygenases has evolved as separate subclass of class B flavoprotein monooxygenases, distinct from the Type I BVMOs, towards a new subclass of potent Baeyer-Villiger monooxygenases. Therefore, these three FMOs or close homologs thereof may represent interesting alternative biocatalysts (for instance, due to their NADH acceptance) when compared with the known Type I BVMOs. With merely using the N-terminal sequence of one of three Type II FMOs, it is possible to identify hundreds of homologous putative Type II FMOs. It will be exciting to see whether these homologs indeed display similar biocatalytic properties. Future research will reveal whether monooxygenases from this newly identified subclass of class B flavoprotein monooxygenases (Type II FMOs) are indeed valuable as biocatalysts; e.g. their operational stability and substrate scope has still to be explored.

5. Conclusions

In the present paper, a set of eight new flavoprotein monooxygenases from *R. jostii* RHA1 has been expressed and obtained in soluble form by employing two expression vectors and optimizing expression conditions. Sequence analysis established that one of these enzymes represents a NMO, while the other cluster as a separate class B flavoprotein

monooxygenase subclass: Type II FMOs. The monooxygenases have been tested for their ability to perform sulfoxidations and Baeyer-Villiger oxidations. Interestingly, three of the Type II FMOs are able to catalyze both reaction types and accept both NADPH or NADH as coenzyme, presenting a different behavior with respect to classical FMOs and BVMOs. Type II FMOs might develop as an interesting alternative to BVMOs, as they are able to catalyze the similar reaction with a relaxed nicotinamide cofactor acceptance.

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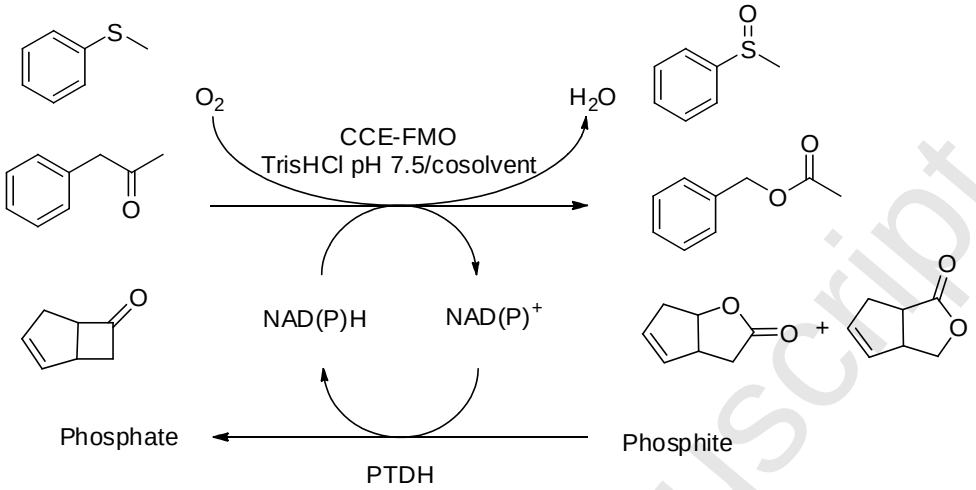

Scheme 1. Oxidations of thioanisole and phenylacetone catalyzed by the novel flavoprotein monooxygenases using NADH or NADPH as coenzymes.

Table 1. All identified class B monooxygenases with their sequence characteristics and expression performance in *E. coli*.

monooxygenase	accession number	Rossman motif *GxGxxG*	FMO motif *FxGxxxHxxx*$^{Y}/_{F}{}^{K}/_{R}$	Rossman motif *GxGxxG*	length (aa)	soluble [a] expression
FMO-A	RHA1_ro00740	GxGxxG	WxGxxxHxxxYR	GxGxxG	375	++[b]
FMO-B	RHA1_ro03334	GxGxxG	AxIxxxHxxxYR	GxGxxG	418	++
FMO-C	RHA1_ro04494	GxGxxG	PxIxxxHxxxYR	GxSxxG	365	++
FMO-D	RHA1_ro05032	GxGxxG	FxGxxxHxxxYS	GxGxxA	369	++[b]
FMO-E	RHA1_ro00824	GxGxxG	FxGxxxHxxxYD	GxGxxA	580	++
FMO-F	RHA1_ro04244	GxGxxG	FxGxxxHxxxHP	GxNxxA	602	+++
FMO-G	RHA1_ro05696	GxGxxG	FxGxxxHxxxFV	GxCxxG	595	+++
NMO-H	RHA1_ro08654	GxGxxN	H	GxGxxG	447	+

[a] + indicates a clear visible protein band using SDS-PAGE, while +++ indicates a great overexpression was observed; [b] indicates that the respective monooxygenase has been expressed using the pCRE2 expression vector while all others have been expressed using the pBAD*N*-vector [16].

Table 2. Baeyer-Villiger oxidation of phenylacetone and (±)-bicyclo[3.2.0]hept-2-en-6-one.

	phenylacetone	(±)-bicyclo[3.2.0]hept-2-en-6-one			
	conv. (%)	conv. (%)	ratio normal:abnormal	ee normal (%)	ee abnormal (%)
FMO-E	20	100	79:21	9 (1*S*,5*R*)	7 (1*R*,5*S*)
FMO-F	19	88	78:22	< 1 (1*S*,5*R*)	70 (1*S*,5*R*)
FMO-G	5	100	78:22	7 (1*R*,5*S*)	66 (1*S*,5*R*)

Table 3. Sulfoxidation of thioanisole.

	thioanisole		
	conv. (%)	ee (%)	configuration
FMO-A	5	11	*R*
FMO-B	4	7	*R*
FMO-C	4	33	*R*
FMO-D	16	25	*R*
FMO-E	56	8	*R*
FMO-F	9	≤5	-
FMO-G	21	57	*S*

Figure Captions.

Figure 1. An unrooted phylogenetic tree of the studied monooxygenases and known class B flavoprotein monooxygenases. The included sequences are: type I BVMOs [EtaA, Mycobacterium tuberculosis H37Rv (NP_218371.1); HAPMO, *Pseudomonas fluorescens* ACB (Q93TJ5.1); CDMO, *Rhodococcus ruber* SCI (AAL14233.1); CPDMO, *Pseudomonas sp*. strain HI-70 (BAE93346.1); CHMO1, *Acinetobacter calcoaceticus* NCIMB 9871 (BAA86293.1); PAMO, *Thermobifida fusca* (YP_289549.1); STMO, *Rhodococcus rhodochrous* (BAA24454.1); ACMO, *Gordania sp*. strain TY-5 (BAF43791.1); MEKMO, Pseudomonas veronii MEK700 (ABI15711.1); CPMO, *Comamonas sp*. strain NCIMB 9872 (Q8GAW0)], [BVMOs 1-23 from *Rhodococcus jostii* RHA1 (see [13] for numbering)], type I FMOs [*Arabidopsis thaliana*: FMO1Ara(Q9LMA1); FMO2Ara (Q9FKE7); *Homo sapiens*: FMO1hum(Q01740); FMO2hum (Q99518); FMO3hum (P31513); FMO4hum (P31512); FMO5hum (P49326); *Drosophila melanogaster*: FMO1Dro (Q9W1E9); FMO2Dro (Q7K3U4); FMO1dog, *Canis familiaris* (Q95LA2); FMOMeth, *Methylophaga sp*. strain SK1 (JC7986); FMOTyr, *Tyria jacobaeae* (D6CHF7); FMOFish, *Oncorhynchus mykiss* (B2LGF9)], NMOs [NRho_Orn, *Rhodococcus jostii* RHA1 (YP_704660.1); PVDA_Orn, *Pseudomonas aeruginosa* (NP_251076); IucD_Lys, *Escherichia coli* (YP_444061.1); SidA_Orn, *Aspergillus fumigatus* (XP_755103.1)], type II FMOs [FMO_X, *Stenotrophomonas maltophilia* (B2FLR2); the type II FMOs A-G from *Rhodococcus jostii* RHA1 described in this study].

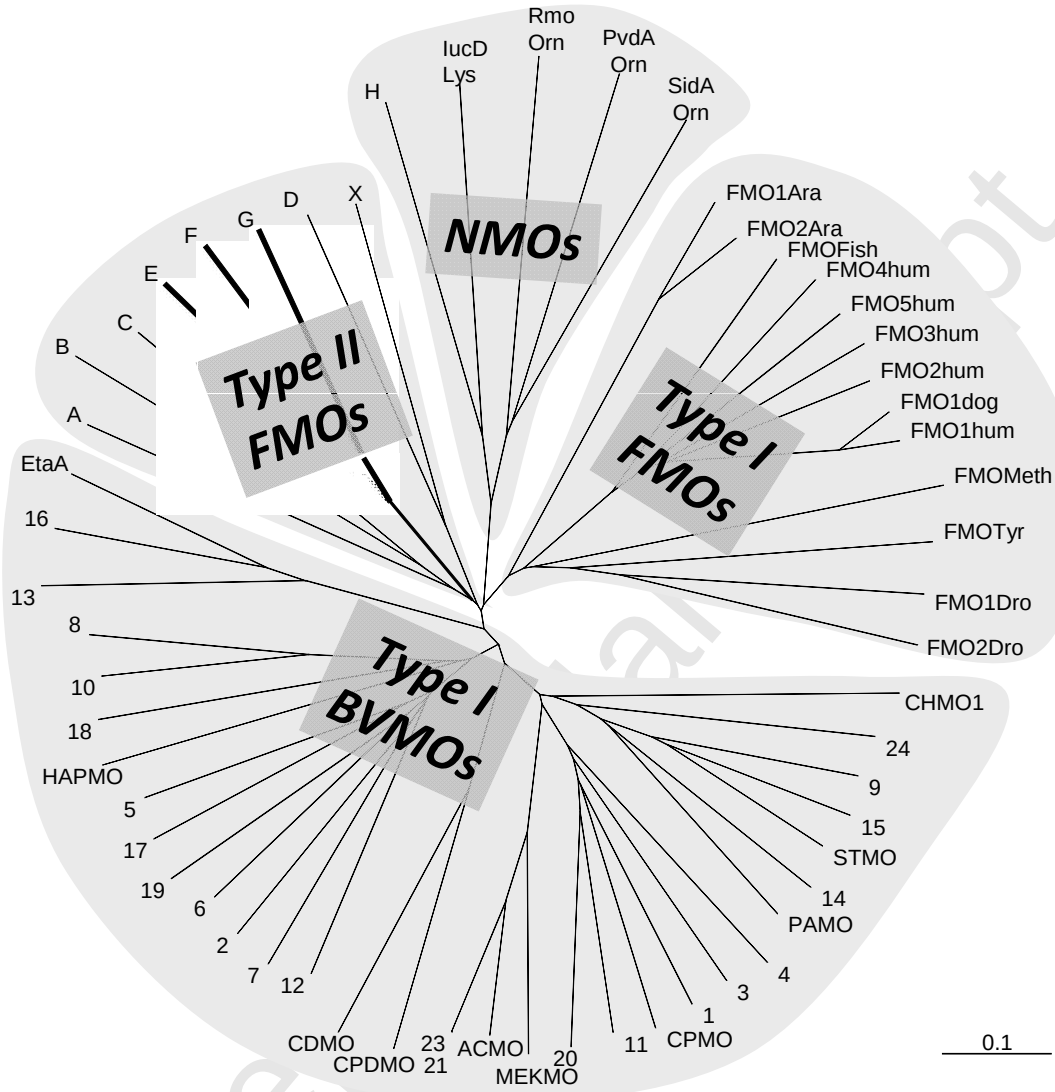

Figure 1

Research highlights

- 8 newly identified flavoprotein monooxygenases were identified and produced in recombinant form
- Several of the discovered monooxygenases were shown to catalyse enantioselective sulfoxidations and Baeyer-Villiger oxidations
- Sequence and biocatalytic analyses have revealed a new sequence-related group of flavoprotein monooxygenases that can catalyse Baeyer-Villiger oxidations and sulfoxidation and that shown no coenzyme preference.