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Novel Old Yellow Enzyme Subclasses

Christin Peters,[a] David Frasson,[b] Martin Sievers[b] and Rebecca Buller*[a]

Abstract: Many drug candidate molecules contain at least one chiral centre and consequently, the development of biocatalytic strategies to complement existing metal- and organocatalytic approaches is of high interest. However, time is a critical factor in chemical process development and thus, the introduction of biocatalytic steps, even if more suitable, is often prevented by the limited availability of off-the-shelf enzyme libraries. To expand the biocatalytic toolbox with additional ene reductases, we screened 19 bacterial strains for double bond reduction activity using the model substrates cyclohexanone and carvone. Overall, we identified 47 genes coding for putative ene reductases. Bioinformatic analysis of all genes and the biochemical characterization of four representative novel ene reductases led us to propose the existence of two new Old Yellow Enzyme subclasses, which we named OYE class III and class IV. Our results demonstrate that while on a DNA level each new OYE subclass features a distinct combination of sequence motifs previously known from the classical and the thermophilic-like group, their substrate scope more closely resembles the latter subclass.

Introduction

Members of the enzyme families collectively called ene reductases (ER) are effective biocatalysts for stereo-selective *trans*-hydrogenation[1] and - in rare cases - *cis*-hydrogenation[2] of activated alkenes complementing the respective chemical *cis*-hydrogenations via chiral rhodium or ruthenium phosphines (Knowles and Noyori, Nobel prize in chemistry 2001).[3] Recently, the canonical function of ene reductases was further expanded via enzyme engineering to now also include asymmetric reductive carbocyclization yielding chiral cyclopropanes.[4]

Today, members of the ene reductase family are assigned to five enzyme subfamilies.[1b] Most biocatalytic transformations are carried out by members of the Old Yellow Enzyme family of ene reductases. However, the Fe-S cluster depending enoate reductases, the medium-chain dehydrogenases, the short-chain dehydrogenases and the recently identified quinone reductase-like ene reductases[5] (Figure 1) are also being investigated with respect to their industrial potential.[6] Members of the Old Yellow Enzyme family are NAD(P)H-dependent oxidoreductases which catalyze the stereo- and enantioselective reduction of α,β-unsaturated ketones, aldehydes, nitro alkenes, and carboxylic acids.[1a] The first enzyme of this family was discovered by Christian and Warburg in bottom-fermented yeast who named it "yellow enzyme".[7] Subsequently, several other yellow enzymes were found, and the first ene reductase was redefined as the "old yellow enzyme"(OYE1).[8] In many cases the enzymes are important for the detoxification of electrophilic substances, in some cases like enzyme YqjM from *Bacillus subtilis* they play an important role in response to the oxidative stress.[9] Nevertheless, for most enzymes of the OYE family the natural substrates and physiological roles remain unknown.

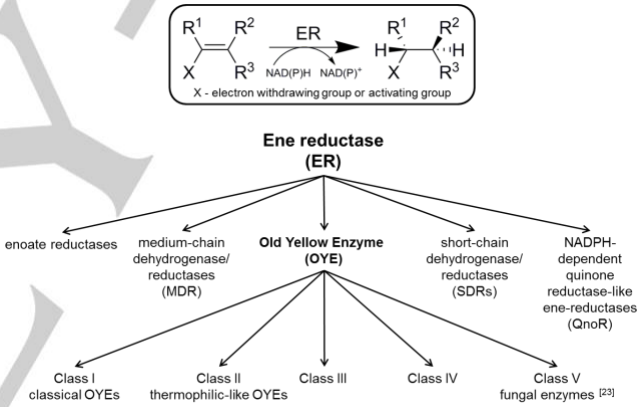

Figure 1. Schematic representation of an asymmetric double bond reduction by Old Yellow Enzymes and an overview of the different ene reductase families.

The catalytic mechanism, which all enzymes of the OYE family follow, is well understood.[10] The enzymes use a bi-bi ping-pong mechanism which can be divided into the reductive half-reaction, where the FMN is reduced through hydride transfer from NAD(P)H, and in the oxidative half-reaction, where the hydride is transferred from the reduced flavin to the Cβ-atom of the activated alkene. To complete the reaction the missing proton for the Cα is provided by a tyrosine residue.[10-11] This specific mechanism leads to an *anti*-addition hydrogenation.[12] To date, the enzymes of OYE family have been divided into two main subclasses, called "classical OYEs" and "thermophilic-like OYEs". All known enzymes of the OYE family have a typical TIM-barrel as basic structure,[13] however the two classes can be distinguished by sequence homology and structural features. The more recently identified thermophilic-like OYE (Class II) was introduced 2010 by the group of N. Scrutton.[1a, 14] The main difference to the classical OYEs is that the thermophilic like OYEs form a functional dimer-dimer interface. An arginine at the

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C-terminus of class II OYEs assist in substrate binding of the adjacent monomer[15] leading to the formation of dimers whereas the classical OYEs are found as monomers or dimers.[16]
The classical OYE subgroup contains yeasts enzyme such as OYE1 from *Saccaromyces carlbergensis*[7] as well as variants from proteobacteria (NCR[17], MR[18], PETNR[19]), flavobacteria (Chr-OYE2[20]) and plants (LeOPR1-3[21]). Recently, it was proposed to further subdivide the classical OYEs into two groups: classical OYEs from plants and bacteria and classical OYEs from fungal sources.[22] In addition, a new OYE subclass containing fungal enzymes was proposed by the group of Verma based on the phylogenetic analysis of a range of fungal strains.[23]

Applications of ene reductases are manifold and especially Old Yellow Enzymes have been investigated for their synthetic potential. They have been employed, for example, to produce intermediates (*R*)-levodione for the synthesis of carotenoids[24] or methyl (*S*)-2-bromobutanoate which is used for the synthesis of drugs to treat noninsulin-dependent type-2 diabetes mellitus.[25] A remaining challenge for the industrial use of the OYE enzyme family is that their natural stereospecificity is nearly always the same.[26] Initial engineering examples show that the stereopreference can be changed in some cases by point mutations making available stereocomplementary pairs of ene reductases,[5, 27] however, more catalysts of complementing enantioselectivity are needed. In addition, the industrial utility of enzymes is also defined by factors such as enzyme availability, substrate scope, productivity, enzyme stability under process conditions and ultimately enzyme cost.[6]

Thus, to enhance the competitiveness of ene reductase catalyzed reactions we set out to expand the biocatalytic toolbox. By screening a collection of selected bacterial strains with two widely accepted model substrates, we identified strains harboring enzymes capable of double bond reductions. Using a bioinformatic approach, more than 40 genes encoding putative new ene reductases were sourced. Further phylogenetic and biochemical analysis led us to propose two new subclasses in the OYE family. We provide details of this study including our analysis of sequence similarities between the four OYE subclasses, the biochemical characterization of representative members of the novel groups and an assessment of their substrate scope and scalability.

Results and Discussion

Identification of novel Old Yellow Enzymes

Given the broad applicability of ene reductases for synthetic purposes, we set out to identify new members belonging to these enzyme families from microbial sources. In total, 19 bacterial wild type strains from the Culture Collection of Switzerland were screened for their ability to reduce the model substrates carvone (**1**) and 2-cyclohexen-1-one (**2**). As positive controls *Bacillus subtilis* and *Gluconobacter* were used, which are known to harbor ene reductases YqjM[9, 28] and GOX[29], respectively.
Nine of the screened strains exhibited activity against both substrates while six strains were found to be active on only one of the substrates (Table S1). Using the published genome data available for 14 of the positive strains, we searched for the responsible ene reductases using a reported fingerprint motif.[30] This allowed us to identify 47 genes with putative ER activity. Phylogenetic analysis revealed that 22 genes (47%) encoded members of the Old Yellow Enzyme family. As this ER subclass figures most prominently in practical applications, we then concentrated on the characterization of this sub-set of genes. A closer phylogenetic analysis of these 22 sequences revealed that six genes belong to the classic OYE class and six are member of the thermophilic like class (Figure 2). Interestingly, ten of the putative ER genes cluster apart of the two described OYE branches (classical and thermophilic) and build up two distinct new branches in the OYE family phylogenetic tree together with enzymes previously assigned as 'lost proteins'.[22] This finding is in line with the domain family hierarchy of the OYE-like FMN family as classified by NCBI's Conserved Domain Database (CDD)[31] which includes several subfamilies beyond class I and class II enzymes. Using this databank, the

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Christin Peters (born 1987) studied biochemistry at the University of Greifswald and obtained her PhD from University Greifswald (U. T. Bornscheuer) with focus on the establishment of enzyme cascades using ene reductases in whole cell biocatalysis. Then she joined the Biocatalysis group of Prof. Buller at the Zürich University of Applied Science as a postdoc. Since August 2018, she is leading the Biosystems Technology group at the Zürich University of Applied Science which concentrates on enzyme cascade reactions and process optimisation of biotransformations in large scale.

sequences of the here-proposed "class III" and "class IV" enzymes uniquely cluster into subfamily "cd04735 OYE_like_4_FMN" and "cd04747 OYE_like_5_FMN, respectively. Supported by the CDD classification and based on our phylogenetic analysis and the biocatalytic, structural and sequence characterization of the newly discovered ERs we thus suggest an extension of the current OYE classification to henceforth include also class III and IV. Our phylogenetic analysis also gives a first indication that the reported ene reductase Nox[32] and the newly detected Sma-ER1 may build up a putative subclass V together with a set of previously discovered fungal enzymes.[23]

Sequence Analysis

On the sequence level, the proposed new OYE subclasses are characterized by a combination of sequence motifs from the previously described classical and thermophilic groups (Table 1 and Figure S1). Especially in context of the FMN binding environment strictly conserved sequence motifs have been reported for class I and II. These interactions between the protein and the flavin are important as they control the redox potential of the cofactor. Our alignments highlight that in all four OYE classes a glutamine residue (Gln 102 in YqjM) and an arginine residue (Arg 215 in YqjM) known to interact with the pyrimidine ring of the cofactor FMN[15] are strictly conserved.

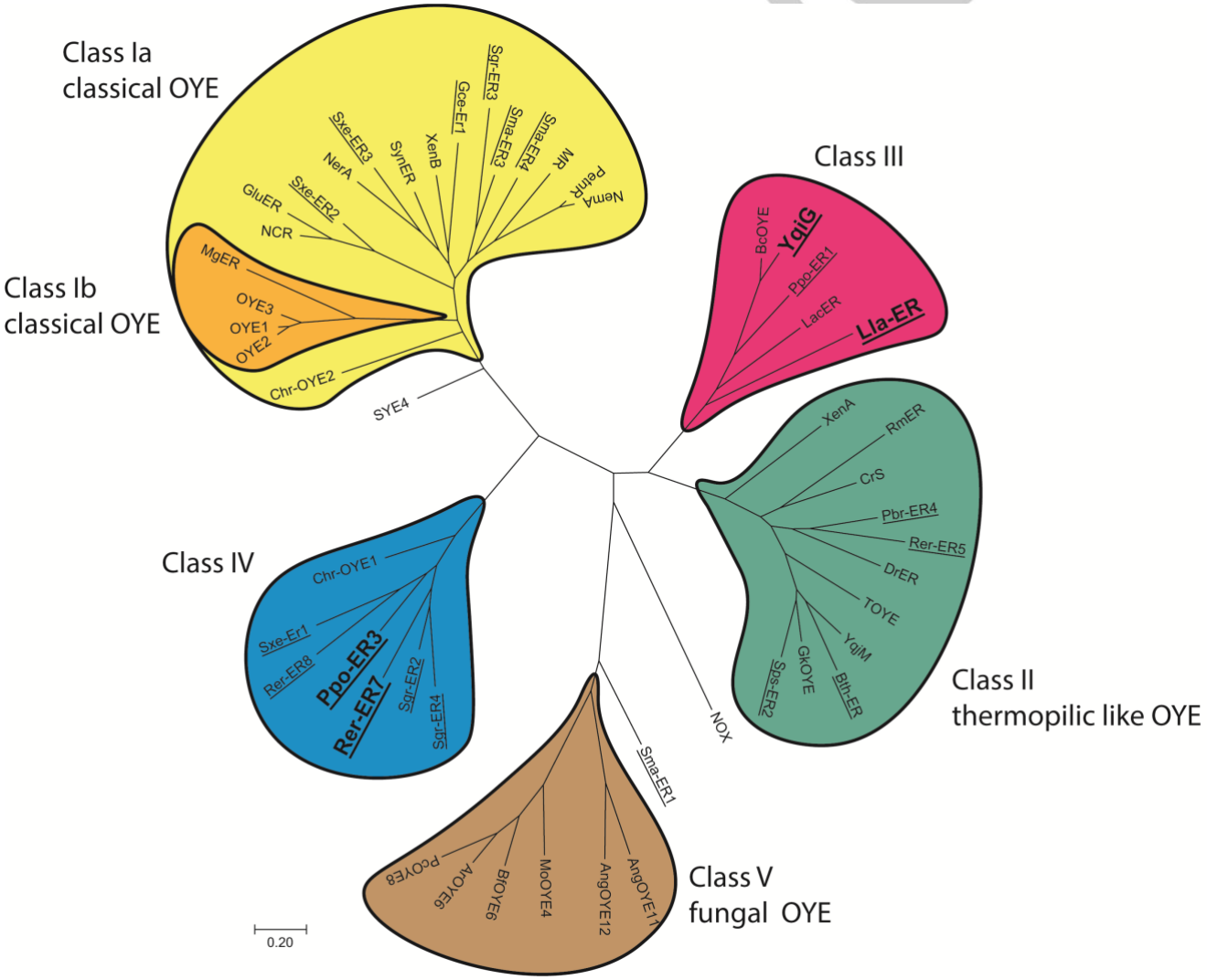


Figure 2. The phylogenetic tree illustrates the integration of the putative novel OYEs (underlined) into the existing OYE family. The maximum likelihood distance tree was constructed using Mega 7.[33] The corresponding alignment was produced via Clustal W.[34] The GenBank codes of the used sequences are deposited in table S4.

In addition to that class II and class IV enzymes possess a conserved histidine pair (His 167, His 164 in YqjM), which in YqjM form hydrogen bonds to the N1 and N3 atom of flavin.[15] Class I and class III enzymes, on the other hand, are commonly characterized by a replacement of the second histidine with an asparagine, for example Asn195 in OYE I.[35] In class I, III and IV a further interaction to the cofactor FMN is provided by a conserved threonine (Thr 37 in OYE1)[36-37] whereas a unique feature of class II enzymes is the presence of a corresponding cysteine residue (Cys 26 in YqjM) for interaction with the flavin O4 atom.[15, 36] The conserved methionine, leucine and arginine residues (Met 25, Leu 311 and Arg 301) in YqjM),[15, 22] which form hydrophobic interactions to the dimethylbenzene moiety of the flavin isoalloaxazine ring and were previously uniquely reported for class II enzymes, are also conserved in the here reported OYE classes III and IV. In class I ERs, on the other hand, a combination of leucine, isoleucine and arginine/tyrosine has been described for this function.[15] A major difference in sequence between all OYE classes can be found in the C-terminal region of their sequence. Class II enzymes feature a combination of two arginines, a glutamine and a tyrosine (Arg 312, Gln 333, Tyr 334 and Arg 336 in YqjM) which are involved in monomer-monomer interactions.[14] This sequence stretch is not present in the other classes. However, we found that class IV enzymes contain a distinct additional amino acid stretch consisting of 17-25 residues which putatively extend the loop between helix 7 and β-sheet 7 based on a comparison with the YqjM sequence and crystal structure.[15] Overall, the sequence alignment highlights that the proposed class III and IV Old Yellow Enzyme feature sequence motifs of both class I and class III enzymes, positioning them in between the classical and thermophilic-like OYEs.

Biochemical characterization of class III and IV enzymes

To understand not only the sequence-based differences of class III and IV enzymes but also obtain an insight into their potential biocatalytic applicability, we set out to biochemically characterize selected candidates. For this study we cloned a total of four genes, two from each new subclass. Our study enzyme set was comprised of YqiG from *Bacillus subtilis* (class III), Rer-ER7 from *Rhodococcus erythropolis* (class III), Ppo-ER3 from *Paenibacillus polymyxa* (class IV) and Lla-ER from *Lactococcus lactis* (class IV). The gene product from *yqig* (*Bacillus subtilis*) has been recently reported for the first time[38] and was assigned as a 'lost protein'[22].

Expression and characterization of the recombinant OYEs

After isolation of the genomic DNA from the selected strains, all genes were successfully amplified and cloned into pET28b by Fastcloning[39] or restriction site cloning and ligation. All plasmids were constructed with a C-terminal His_6-tag for protein purification via affinity chromatography. Soluble recombinant expression in *E. coli* was achieved in all cases. Lla-ER was expressed in *E. coli* Rosetta 2 (DE3), while YqiG, Ppo-ER3 and Rre-ER7 were produced in *E. coli* BL21 (DE3). Rre-ER7 was cultivated in the presence of chaperones GroEL and GroES (Gro7 chaperone system).[40] Target proteins were purified by affinity chromatography (Ni-NTA) and, in the case of Lla-ER and Rer-ER7, the cofactor FMN was reconstituted before further analysis.

Table 1. Sequence motifs specific for the four Old Yellow Enzyme subclasses. Predicted interactions between FMN and residues of class III and class IV enzymes are based on sequence alignments and docking studies using homology models of of YqiG and Ppo-ER3 as representative members of class III and class, respectively (Figure S11 and S112). All residue numbers refer to an arbitrarily selected example enzyme of the respective class. The enzyme name is given in brackets.

Associated role of sequence motif	Class I (OYE I)	Class II (YqjM)	Class III (YqiG)	Class IV (Ppo-ER3)
Interaction with pyrimidine ring of FMN[15]	Gln 115 Arg 244	Gln 102 Arg 215	Gln 104 Arg 226	Gln 122 Arg 244
Interaction to N1 and N3 of FMN[15, 35]		His 164 His 167		His 191 His 194
	His 192 Asn195		His 169 Asn172	
Interaction with isoalloxazine ring O4 atom of FMN[36]	Thr 38		Thr 30	Thr 48
		Cys 26		
Interaction with the dimethyl benzene moiety of FMN[15, 22]		Met 25 Leu 311 Arg 308	Met 29 Leu 322 Arg 319	Met 47 Leu 366 Arg 363
	Leu 37 Ile 352 Arg 348			
C-terminal sequence for monomer-monomer interaction[14]		Arg 312 Gln 333 Tyr 334 Arg 336		

All four purified ene reductases were obtained as active enzymes, capable of converting cyclohexenone **2**. As is the case for class I and class II OYEs,[22] we found that NADPH is the preferred physiological coenzyme of the novel ERs (Table S3). This cofactor is commercially expensive, however, the dependency on stoichiometric amounts of this compound can be circumvented by established recycling systems such as the coupled enzyme approach[41] or alternative hydride sources.[42] Further relevant parameters for application such as pH optimum, temperature optimum and long-term temperature-stability were determined for YqiG (class III) and Ppo-ER3 (class IV) using substrate **2** or **7**.

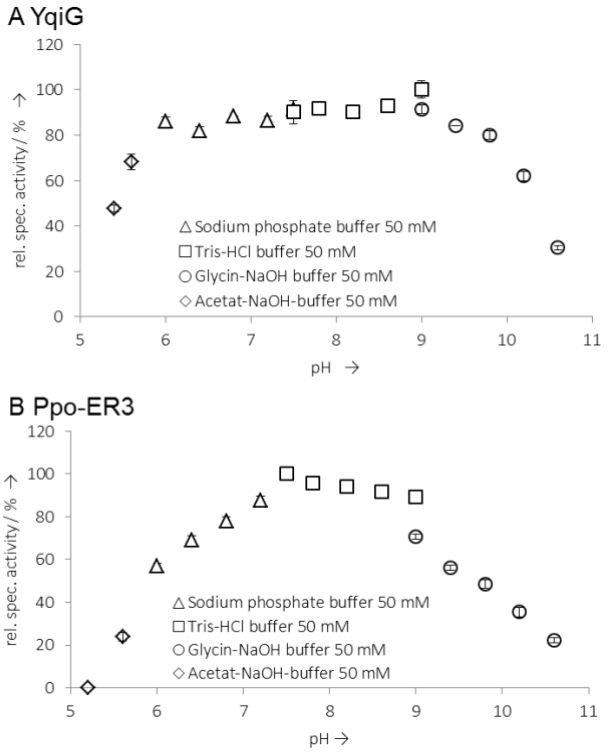


Figure 3. Activity profile of YqiG and Ppo-ER3 correlated to pH, measured with NADPH-Assay in different buffers and with 1 mM substrate **2** in case of YqiG and with **7** in case of Ppo-ER3.

As reported, enzyme YqiG exhibits a broad pH optimum, preferably operating between pH 6 and 9 (Figure 3A).[38] YqiG's pH profile is thus similar to the one of LacER[43] (pH_{opt} = 7 - 9) which, judging from our phylogenetic analysis, also classifies as a group III enzyme. The pH profile of Ppo-ER3 is more bell-shaped with the pH-optimum at 7.5 (Figure 3B). Overall, the observed pH profiles for the class III and IV OYEs are in line with reported values for class I and II enzymes, which mostly exhibit pH optima between 6 - 7.5 (class I examples: XenB[44] and NemA[44]) and 6 - 8 (class II: YqjM[45] and Chr-OYE3[46]).

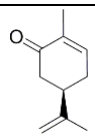
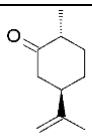
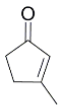
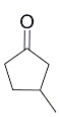
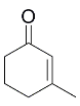
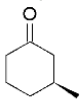
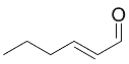
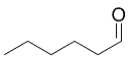
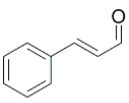
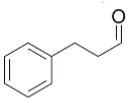
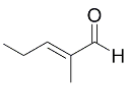
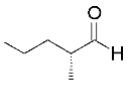
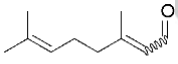
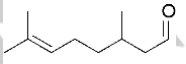
In terms of thermal robustness, class III enzyme YqiG was shown by us and others to possess interesting long-term stability up to 30°C. After 26 h incubation at 20 °C, the enzyme activity toward substrate **2** remained virtually unchanged while a residual activity of 90 % was detected after an identical incubation at 30 °C consistent with the results of Sheng *et. al.*[38] Furthermore, short-term exposure (up to 6 hours) of YqiG to 40°C led to an only marginal activity loss of 10 % opening the door to applications that require elevated temperatures (Figure S6).
Class IV enzyme Ppo-ER3 exhibited similar long-term stabilities at 20°C and 30°C as YqiG and residual activities for the conversion of substrate **7** after 24 h of incubation were 95 % and 83 %, respectively (Figure S7). Ppo-ER3's temperature optimum for biocatalysis reactions was shown to be between 25 °C and 40 °C as determined with substrate **7** (Figure S8).

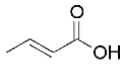
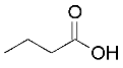
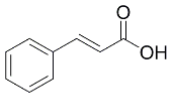
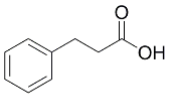
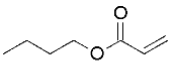
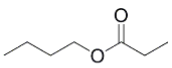
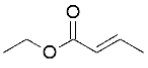
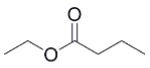
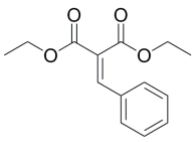
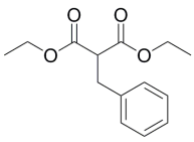
Thus, temperature optima of the investigated class III and IV enzymes are more similar to those of their mesophilic counterparts from class I, e.g. 25-40 °C for OYE2p[47] or 30-50 °C for NemA.[44] In contrast, thermophilic-like class II OYEs, which have an average increased thermal stability compared to class I enzymes, partly exhibit higher temperature optima as observed for CrS (T_{opt} = 65°C)[48] GeoER (T_{opt} = 70°C)[49] and FOYE1 (T_{opt} = 50°C).[50] However, it must be noted that the thermophilic-like subclass displays a wide variety of temperature profiles and also includes non-thermostable OYE relatives such as YqjM [45] and XenA.[51]

Substrate scope

To obtain an impression of the substrate scope, activity of all four enzymes toward a series of structurally diverse aliphatic and cyclic alkenes bearing ketone, aldehyde, carboxylic acid or ester function as electron-withdrawing groups was investigated (Table 2). In class III, YqiG showed medium to good conversions for most substrates within one hour and accepted both cyclic and linear structures. Lla-ER, on the other hand, demonstrated to be a poorer catalyst for the investigated substrates and incubation times of 24 hours were required to obtain meaningful results. Lla-ER preferentially accepted unsubstituted cyclic substrates or small aldehydes. In class IV, Ppo-ER3 readily accepted most substrates showing very good conversion especially for substrate **2**, **7**, **8** and **9** while Rer-ER7 only reduced linear aldehydes **9** and **10** with conversions > 10 % while all other substrates were not or only very poorly accepted. Interestingly, class III and IV enzymes were found to have no or extremely low activity for cyclic β-methylated substrates such as 3-methylcyclopentenone **4** and 3-methylcyclohexenone **5** while α-substituted substrates (*R*)-carvone **1** and 2-methylcyclohexenone **6** were accepted. This result clusters the new ER classes with class II enzymes which are similarly reported to not accept cyclic β -methylated unsaturated substrates, while class I enzymes typically convert these compounds.[22] Also for the aliphatic dienal substrate citral, class III and IV enzymes exhibit a similar conversion behavior as class II enzymes and show only low to modest conversions (class III: YqiG 8.7% conversion, Lla-ER 1.3 % conversion; class IV: Ppo-ER 3 12.4 % conversion, Rer-ER 7 12.3 % conversion). Class I enzymes, on the other hand, are known to accept citral well leading to conversion yields of up to 67 % for OYE 2.6 and 69 % for NemA.[52] Based on the results obtained with our substrate series, the investigated class III and IV enzymes seem to display a similar stereo-preference as class I and II enzymes.[22] Enzymes from the new groups preferentially form the expected (*R*)-product in the reduction of α-substituted cyclohexenone **6** and yield the (*S*)-product for conversion of the β-substituted cyclohexenone **5** (conversions for all four enzymes were < 1 %). The diastereoselectivity for the conversion of substrate **1** by YqiG and Ppo-ER3 reached 89% de and 91% de, respectively. Thus, the two enzyme operate in the same range as other well-performing OYEs such as for example LacER (class III) from *Lactococcus casei* (86 % de)[43] or OYE1 (class I) from *Sacchromyces cerevisiae* (88 % de).[53]

Table 2. Conversion and enantiomeric excess of various substrates with purified enzymes as determined by GC after 1h [a]

Substrate	Product	YqiG		Lla-Er [b]		Ppo-Er3		Rer-Er7[b]	
		Conv. [%]	ee [%]	Conv. [%]	ee [%]	Conv. [%]	ee [%]	Conv. [%]	ee [%]
 **1**	 (2*R*,5*R*)-**1a**	22.8 ± 3.1	89	2.6 ± 0.1	>99	24.3 ± 1.2	91	1.7 ± 0.1	>99
 **2**	 **2a**	54.9 ± 6.1		34.9 ± 4.2		85.8 ± 6.8		2.5 ± 0.3	
 **3**	 **3a**	10.3 ± 0.8		3.2 ± 0.8		51.4 ± 2.9		n.d.	
 **4**	 **4a**	n.d.		n.d.		n.d.		n.d.	
 **5**	 (*S*)-**5a**	< 1	> 99	< 1	> 99	<1	>99	<1	>99
 **6**	 (*R*)-**6a**	8.7 ± 0.3	83	2.1 ± 0.2	11	21.4 ± 0.2	86	2 ± 0.1	16
 **7**	 **7a**	37.0 ± 0.2		52.4 ± 0.9		68.2 ± 6.0		3.0 ± 0.1	
 **8**	 **8a**	58.2 ± 2.4		65.8 ± 4.1		72.9 ± 2.6		n.d.	
 **9**	 (*S*)-**9a**	53.3 ± 2.4	33	14.9 ± 0.6	5	74.5 ± 0.1	66	13.5 ± 0.6	1
 **10**	 **10a**	8.7 ± 0.3		1.4 ± 0.2		12.4 ± 0.1		12.3 ± 0.1	

 **11**	 **11a**	n.d.	n.d.	n.d.	n.d.
 **12**	 **12a**	n.d.	n.d.	n.d.	n.d.
 **13**	 **13a**	4.5 ± 0.3	n.d.	15.0 ± 1.2	<1
 **14**	 **14a**	n.d.	n.d.	n.d.	n.d.
 **15**	 **15a**	10.5 ± 1.3 [c]	<1 [c]	n.d [c]	n.d. [c]

[a] All reactions were performed in triplicates with 5 mM substrate, 6 mM NADPH and 100 µg ml^{-1} purified enzyme. In all cases, biotransformation results were verified by control reactions omitting the enzyme. [b] t = 24 h. [c] in a plastic Eppendorf tube.

Only very few Old Yellow Enzymes (e.g LyngbyaER1[54] or XenB[44]) have been reported to yield a better diastereomeric excess for the conversion of substrate **1**.

The only weakly activated acid and ester substrates **11**, **12** and **14** were not converted by any of the four enzymes. This is in line with results obtained for other OYE members such as OYE1 (class I),[55] PETNR (class I)[56] and YqjM (class II),[45] which also do not reduce monocarboxylic acid or esters like **12.** Interestingly, class III enzymes Ppo-ER3 and Lla-ER reduce the bulky substrate **15.** Ppo-ER3 even achieves conversions of up to 10% in one hour, thus outperforming all members of a library of 23 ene reductases reported in a recent substrate scope evaluation by Reβ *et al.*[57] In this study, only enzyme DBVPG from *Kazachstania lodderae* and OPR I from *Lycopersicon esculentum* showed activity for this substrate and conversions of 3% and 4%, respectively, were detected after 24 hours.

Preparative biocatalysis

Finally, we set out to demonstrate the practical applicability of the novel Old Yellow Enzymes for the synthesis of added-value compounds. Enzymatic reactions at an increased scale give insights into process robustness and therefore produce valuable information for further process optimization work. We decided for the reduction of cinnamaldehyde in a substrate concentration of 5.6 mM (0.75 g L^{-1}, total substrate load 150 mg) using the previously well-performing Ppo-ER3 as the biocatalyst. In the preparative-scale experiment the starting compound **8** was converted by the class IV enzyme to the desired product in 1 h and the resulting product **8a** was isolated in 88% yield. Notably, this reaction proceeded with complete conversion underlining the compatibility of the ene reductase Ppo-ER3 with higher substrate loadings.

Conclusions

We successfully screened 19 bacterial wild type strains to identify novel, synthetically useful ene reductases. Genome analysis of active strains allowed us to identify 47 genes with putative ER activity, 42 of which had to our knowledge not been described in literature before. Our phylogenetic and

sequence analysis of the 22 genes belonging to the Old Yellow Enzyme family led us to propose the introduction of two new OYE subclasses, named OYE class III and IV, complementing the existing classical and the thermophilic like OYE groups. Both new subclasses contain a mixture of sequence motifs previously associated uniquely with class I or class II enzymes.

To investigate the biochemical characteristics of the novel groups, we solubly produced two enzymes for each class, YqiG and Lla-ER for class III and Rre-ER7 and Ppo-ER3 for class IV in *E. coli*. Cofactor preference, pH and temperature optimum were found to be similar to known OYEs. Substrate scope for the four enzymes was investigated with a panel of 15 compounds and especially enzymes YqiG and Ppo-ER3 accepted a wide range of structures ranging from aliphatic aldehydes and ketones, to cyclic ketones and even a bulky ester (compound **15**, only accepted by YqiG and Lla-ER). Elucidated substrate preference of class III and IV enzymes based on the tested compound panel showed a higher similarity to the substrate scope of class II OYE as β-substituted cyclic alkenes were not accepted and citral conversion was low.

Finally, applicability of the here-introduced enzymes to preparative scale synthesis was demonstrated using class IV enzyme Ppo-ER3 for the conversion of cinnamaldehyde at an elevated substrate concentration.

Experimental Section

Materials: All chemicals of the highest available purity or of analytical grade were purchased from Merck (Darmstadt, Germany), VWR (Hannover, Germany), or Carl Roth (Karlsruhe, Germany) and were used without further purification unless otherwise specified. NADPH tetrasodium salt was ordered from Oriental Yeast Co. Ltd (Tokyo, Japan). Restriction enzymes, polymerases and T4 ligase were obtained from New England Biolabs GmbH (Beverly, MA, USA) and primers were ordered from Microsynth (Balgach, Switzerland). The sequencing was performed by Eurofins Genomics GmbH (Ebersberg, Germany) or Microsynth (Balgach, Switzerland). The His-Trap FF and the HiTrap desalting columns were ordered from GE Healthcare (Uppsala, Sweden).

Bacterial strains, culture conditions, and plasmids: All bacterial screening strains were purchased from the Culture Collection of Switzerland (CCOS, Wädenswil, Switzerland). *E. coli* BL21 (DE3) [*fhuA2 [lon] ompT gal (λ DE3) [dcm]* Δ*hsdS*] was purchased from New England Biolabs (Beverly, MA, USA), *E. coli* XL-1 Blue *[recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* F´ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tet^{r})] was ordered from Agilent Technologies (Stevens Creek Blvd., CA, US). The *E.coli* strain Rosetta 2 (DE3) [F^{-} *ompT hsdS*$_{B}$(r_{B^-} m_{B^-}) *gal dcm* (DE3) pLysSRARE2 (Cam^{R})] and the empty plasmid pet28b(+) were obtained from Merck (Darmstadt, Germany). *E. coli* strains were cultured routinely in LB or TB medium and if necessary were supplemented with kanamycin (50 mg mL^{-1}). Bacterial cultures were incubated in baffled Erlenmeyer flasks in a New Brunswick™ Innova® 42 orbital shaker at 200 rpm at 37 °C. Bacteria on agar plates were incubated in a HERATherm Thermo Scientific incubator in air. All materials and biotransformation media were sterilized by autoclaving at 121°C for 20 min. Aqueous stock solutions were sterilized by filtration through 0.20 µm syringe filters. Agar plates were prepared using LB medium supplemented with 1.5% (w/v) agar.

Screening for ene reductase activity in bacterial strains: The different bacterial strains were cultivated in triplicates in 48 deep well plates using 1.5 ml TSB media without glucose for 24 h at 25 °C with 600 rpm in a Eppendorf Thermomixer C. All screening plates contained two positive control strains and blanks with no strain. After harvesting by centrifugation (15 min 3500 g HERAEUS Multifuge 4KR centrifuge), the cell pellets were resuspended in 1 ml lysis buffer (50 mM sodium phosphate buffer pH 7.5, 1 mg ml^{-1} lysozyme) and incubated for 1.5 h at 30 °C and 600 rpm. The supernatant of the crude cell extract was used after 20 min centrifugation (3500 g) for a NADPH-assay and biocatalysis reactions with substrates **1** and **2**. The NADPH-assay was performed during 3 min using 20 µl or 100 µl supernatant in the presence of 0.6 mM NADPH and 2 mM substrate in 200 µl 50 mM sodium phosphate buffer (pH 7.5) monitoring at 340 nm with the Tecan Spark 10M plate reader. For the 500 µl biocatalysis reactions in sodium phosphate buffer (50 mM, pH 7.5) with 2 mM substrate, 3 mM NADPH and 491 µl supernatant were used and incubated 16 h at 30 °C and 600 rpm. 500 µl sample volume was extracted twice with 200 µl and 100 µl methyl *tert*-butyl ether and dried over sodium sulphate. The extracted solution was then subjected to GC analysis.

Cloning: Total genomic DNA from all strains was extracted by using the DNeasy Blood and Tissue Kit from Qiagen (Hilden, Germany). Plasmid isolation, PCR purification, and gel extraction (Macherery Nagel, Düren, Germany) were performed according to the manufacturers' instructions. All genes were cloned into pet28b with a C-terminal His-tag.

Cloning of the lla-er and yqig gene into pET28b(+) was performed applying the FastCloning method[39]. Primers for amplification of the lla-er gene from genomic DNA were: Fw-5'-GAG TGC GGC CGC AAG CTT GAT ATC ATA CTT TCC TG-3', Rev-5'-GAA GGA GAT ATA CCA TGC CAA ATC AAT TAA C-3'

Corresponding primers for amplification of the plasmid pET28b(+): Fw-5'-CAG GAA AGT ATG ATA TCA AGC TTG CGG CCG C-3'; Rev-5'-GTT AAT TGA TTT GGC ATG GTA TAT CTC CTT CTT AAA GTT AAA C-3'

Primers for amplification of the yqig gene from genomic DNA: Fw-5'-GAA GGA GAT ATA CCA TGA ATC CTA AGT ATA AGC C-3'; Rev-5'- GTG CGG CCG CAA GCT TAT CTT TAT AAG GCA CCC AG-3';

Corresponding primers for amplification of the plasmid pET28b(+): Fw-5'- GCT TAT ACT TAG GAT TCA TGG TAT ATC TCC TTC TTA AAG-3'; Rev-5'- GTG CCT TAT AAA GAT AAG CTT GCG GCC GCA CTC-3'

Cloning of the ppo-er3 gene and rer-er7 gene was done by PCR amplification, restriction enzyme digestion and ligation into the pet28b plasmid. For Rer-ER7 the *Nco*I and *Hind*III restriction sites were used and the following primers were used for amplification: Fw-5'-GGG CCC CCA TGG GCT TAA TAG TTA ATG CAC-3' and Rev-5'-GGG CCC AAG CTT GTT CAG GTG CGT GCG ATG TTC-3'

For Ppo-ER3 the *Nco*I and *Xho*I restriction sites were used and the following primers were utilized for amplification: Fw-5'- GGG CCC

CCA TGG GCG AAT TGT ATA ACA GAA -3' and Rev-5'- GGG CCC CTC GAG ATT CAA CGT TTT TAA TAC TTC AT-3'

Sequences have the following GenBank accession numbers YqiG (MK257766), Ppo-ER3 (MK257767), Lla-ER (MK257768) and Rer-ER7 (MK257769).

Expression: Expression of Ppo-ER3 and YqiG in *E. coli* BL21 (DE3) was performed by inoculation of LB media (400 mL) supplemented with kanamycin (100 µg mL^{-1}) with an overnight culture (4 mL; 1:100) of the recombinant strains. The main culture was incubated at 37 °C and 180 rpm until OD_{600} = 0.6–0.8 was reached. At this point enzyme expression was induced with 0.1 mM IPTG, and incubation was continued for 16 h at 25 °C. The cultivation of Lla-ER in *E.coli* Rosetta 2 (DE3) was performed in the same way except that TB medium was used and temperature after induction was lowered to 20°C. Rer-ER7 was produced in an identical manner as YqiG except that the *E. coli* BL21 (DE3) strain co-expressed chaperones GroEL and GroES from plasmid Gro7 (Takara Bio Inc., Tokyo, Japan). Consequently, chaperone production was induced previous to enzyme production at OD_{600} = 0.4 with 0.1 mg ml^{-1} *L*-arabinose. Cells were harvested by centrifugation at 4500×g for 10 min at 4 °C and used directly or cell pellets were stored at -20 °C.

Enzyme purification: For cell disruption, the cell pellet was resuspended in 25 mL buffer (100 mM sodium phosphate buffer pH 7.5, 300 mM NaCl) supplemented with 30 mM imidazole. Cell disruption was performed by a single passage through a French pressure cell at 2000 psi. By centrifugation at 4500× g for 30 min cell debris was separated from the crude cell extract. Purification was performed by affinity chromatography by the C-terminal His-Tag with an automated Äkta purifier system. A 5 mL HisTrap FF column was equilibrated with buffer. After the crude cell extract was applied on the column, unbound protein was eluted with five column volumes of buffer supplied with 39 mM of imidazole. The elution of the desired enzyme was accomplished by three column volumes of buffer containing 300 mM imidazole. Washing, flow through, and elution fractions were analyzed by SDS-PAGE. Desired fractions were collected for desalting using the Äkta purifier to remove the imidazole and to change the buffer to 50 mM sodium phosphate (pH 7.5). This step was performed by using three coupled 5 mL HiTrap desalting columns. After the column was equilibrated with sodium phosphate buffer (50 mM, pH 7.5), the collected enzyme solution was loaded. Protein fractions were analyzed using the Äkta purifier system by online absorption measurement at 280 nm. In the case of enzyme Rer-ER7 Tris-HCl buffer at pH 9.0 supplemented with 0.1 mM FMN was used instead to avoid precipitation. The protein content of the crude cell extracts as well as of the purified and desalted fractions was determined by using the BCA kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Standard curves were recorded with bovine serum albumin (BSA) at 0.02–2 mg mL^{-1}. Samples were measured in triplicate at suitable dilutions. The NanoDrop was used to determine the protein content of purified protein samples based on the corresponding extinction coefficients.

Activity measurement: Activity measurements were performed spectrophotometrically by observing NADPH consumption for 180 s at 340 nm in 96 well plates with the Tecan Spark 10M. Biocatalytic experiments to obtain pH, temperature and stability profiles were measured in sodium phosphate buffer (50 mM, pH 7.5) at room temperature in the presence of 1 mM substrate and 0.6 mM NADPH. Purified and desalted enzyme solutions (1–20 µL) were added to give a final enzyme concentration of 0.01 to 0.05 before the reaction mixture was adjusted to a final volume of 200 µL with buffer. The pH profile was generated using appropriate buffer systems at a concentration of 50 mM each (Tris-HCl, sodium phosphate, glycine-NaOH or acetate-NaOH buffer).

Biocatalysis reaction: For *in vitro* biocatalysis reactions desalted enzyme solution (final concentration of 100 µg mL^{-1}), 5 mM substrate (in DMF) supplemented with 6 mM NADPH were combined in a sodium phosphate buffer (50 mM, pH 7.5) and adjusted to a final volume of 1 mL. Reactions were incubated in closed GC-vials at 30°C and 1000 rpm. All biocatalysis reactions were done in triplicates and samples for analysis (500 µl) were taken after 1 h and 24 h. In all cases, biotransformation results were verified by control reactions omitting the enzyme.

Preparative scale biocatalysis reaction: For the preparative biocatalysis purified and desalted Ppo-ER3 (final concentration 75 µg mL^{-1} enzyme solutions) in 200 ml sodium phosphate buffer (50 mM, pH 7.5) with 150 mg substrate (5.6 mM; 1.12 mmol) **8**, 400 mg glucose, 1 mM NADPH and 3 U/ml GDH were used. After 1h an analytical sample was taken for GC analysis (full conversion) before the product was extracted three times with diethyl ether, dried over Na_2SO_4 and the solvent was evaporated to obtain the final product. Yield: 132 mg (0.98 mmol, 88%) Purity: 95 % (GC).

NMR data of the isolated product:
1H NMR (500 MHz, chloroform-d): δ [ppm] 9.86-9.85 (m, 1H), 7.37 – 7.31 (m, 3H), 7.26 – 7.21 (m, 2H), 2.99 (t, J = 7.6 Hz, 2H), 2.84 – 2.80 (m, 2H).
13C-NMR (500 MHz, chloroform-d): δ [ppm] = 28.14 (t, 1-C); 45.30 (t, 1-C); 126,32 (d, 1-C); 128.29 (d, 2-C); 128,61 (d, 2-C); 140.33 (s, 1-C); 201.55 (d, 1-C)

GC-Analysis: 500 µl samples were extracted twice with 200 µl and 100 µl methyl *tert*-butyl ether and dried over sodium sulfate. The extract was then subjected to GC analysis (see supplement for detailed GC methods).

Keywords: ene reductase • biocatalytic toolbox • novel Old Yellow Enzyme subclasses • phylogenetic analysis • enzyme sourcing

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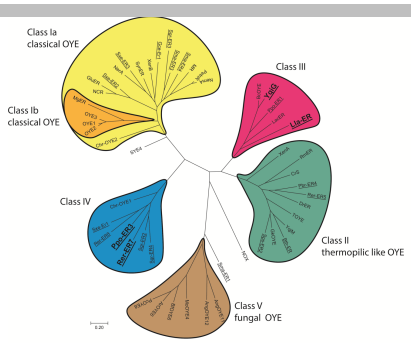
To expand the biocatalytic toolbox of available ene reductases we screened selected bacterial strains for double bond reduction activity. The genomes of strains exhibiting activity were analysed bio-informatically and 47 genes encoding putative ene reductases were identified. Interestingly, our combi-natorial approach of phylogenetic and biochemical analysis allowed us to identify two novel Old Yellow Enzyme subclasses.



Christin Peters, David Frasson, Martin Sievers and Rebecca Buller

Page No. – Page No.
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Page No. – Page No.

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