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Directed Evolution of P450 Fatty Acid Decarboxylases via High-Throughput Screening towards Improved Catalytic Activity

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P450 fatty acid decarboxylases (FADCs) have recently been attracting considerable attention owing to their one-step direct production of industrially important 1-alkenes from biologically abundant feedstock free fatty acids under mild conditions. However, attempts to improve the catalytic activity of FADCs have met with little success. Protein engineering has been limited to selected residues and small mutant libraries due to lack of an effective high-throughput screening (HTS) method. Here, we devise a catalase-deficient *Escherichia coli* host strain and report an HTS approach based on colorimetric detection of H_2O_2-consumption activity of FADCs. Directed evolution enabled by this method has led to effective identification for the first time of improved FADC variants for medium-chain 1-alkene production from both DNA shuffling and random mutagenesis libraries. Advantageously, this screening method can be extended to other enzymes that stoichiometrically utilize H_2O_2 as co-substrate.

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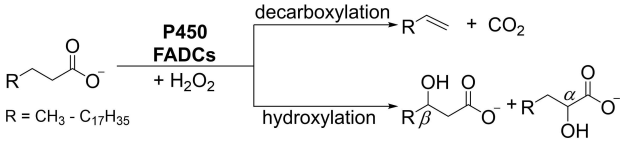
Biocatalyst-based reactions can provide environmentally friendly alternatives to many chemistry-based solutions. However, enzymes found from natural resources usually have moderate activities, unsatisfactory stabilities, and/or unfavorable selectivity. Directed evolution (DE) of enzymes as recognized by the 2018 Nobel Prize in Chemistry represents an invaluable biotechnological approach to improving enzymes' activities and stabilities, and to tuning their chemo-, regio-, and stereoselectivity for better application in synthetic biology designs and assemblies.[1] In all cases, a fast and accurate screening method for a specific catalytic trait is the crucial prerequisite of obtaining desired enzymes from numerous variants.[2] While notable progress has been made in improving library quality,[3] lack of efficient high-throughput screening (HTS) methods can still hamper the application of DE, particularly, in large library screening of the reactions where substrates, products, or cofactors are not readily detectable unless using highly specified and sophisticated approaches such as GC or HPLC.[1e,4]

P450 fatty acid decarboxylases (FADCs) are recently identified members of the CYP152 peroxygenase family, cytochrome P450 enzyme superfamily.[5] These enzymes, via a unique one-step oxidative decarboxylation process using H_2O_2 as sole oxygen and electron donor, effectively convert the biologically abundant feedstock free fatty acids (FFAs) to terminal olefins (*i.e.*, 1-alkenes) which are important biofuel components and biomaterial building blocks (Scheme 1).[6] P450 FADCs have shown broad substrate scope. Particularly, the first identified FADC $OleT_{JE}$ can convert C_4–C_{20} straight-chain saturated FFAs into their corresponding one carbon less 1-alkenes.[7] It also produces small amounts of α- and β-hydroxylated FFAs as minor products.[5a] The great application potential in sustainable production of biohydrocarbons and the unique (oxidative decarboxylation) catalytic properties of $OleT_{JE}$ have inspired a



Scheme 1. Free fatty acid decarboxylation and hydroxylation catalyzed by CYP152 peroxygenases.

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growing number of studies on its catalytic mechanisms and mutagenetic engineering to expand its substrate scope and to modulate its activity and chemoselectivity.[4,6,8]

These studies on $OleT_{JE}$ have led to mechanistic understanding of the decarboxylation/hydroxylation bifurcation,[6b,c,8b,f,9] and identification of the key residues in regulating decarboxylation activity and substrate positioning.[6a,8b] However, engineering P450 FADCs towards improved alkene production remains a major challenge. Almost all protein engineering attempts on $OleT_{JE}$ at the substrate-binding-site residues have only resulted in neutral or even compromised decarboxylation activity.[4,8c–e] Owing to its unique catalytic mechanism and elaborate reaction bifurcation pathways (chemo-selectivity), subtle changes in the position or orientation of FFA substrate could be detrimental to $OleT_{JE}$ decarboxylation/catalytic activity,[8c–e] highlighting the complexity of $OleT_{JE}$ catalytic behaviors and the difficulty to (semi-)rationally design an improved decarboxylation activity of P450 FADCs.

Thus, to explore the fitness landscape of the protein sequence space and to effectively tune the P450 FADC activity towards different FFA substrates, DE at whole gene sequence level combined with an efficient HTS approach is compelling. Previously, a small combinatorial active-site saturation testing (CASTing) library of $OleT_{JE}$ was built to screen for improved activities against structurally different aromatic carboxylic acid substrates by growing cells in 50 ml bottles in order to collect enough enzymes for GC-based activity determination, as the activity levels of $OleT_{JE}$ were too low in the usual 2 mL-format 96-well plates.[4] To circumvent the low-throughput complicated GC-based screening, we developed the first 96-well plate HTS system for quick assessment of P450 peroxygenase activities by using a H_2O_2 consumption based colorimetric assay combined with an efficient enzyme recovery in cell lysates. With this novel method solving the two major issues that hampered the DE of this class of enzymes, a number of mutants towards improved substrate conversion and/or 1-alkene production were successfully obtained from both a DNA shuffling library and a random mutagenesis library, providing valuable candidates for mechanistic analysis and further protein engineering.

In view that P450 FADCs catalyze FFA substrates to react with H_2O_2 at a 1:1 stoichiometry to generate oxidative products, we reasoned that H_2O_2 consumption could be used as a means to indirectly evaluate enzyme activity. H_2O_2 levels can be readily measured in a high-throughput manner via colorimetric or spectrophotometric assays with high sensitivity and reproducibility using commercially available H_2O_2 assay kits (*e.g.*, Amplex Red, see Supporting Information). Indeed, a good correlation between the Amplex Red H_2O_2 assay and the GC analysis of catalytic activity was first observed when using purified $OleT_{JE}$ enzyme *in vitro* (Figure S1). Next, in order to eliminate background H_2O_2 consumption in host cells, a catalase-deficient strain HXΔEG was constructed by deleting the two primary endogenous H_2O_2 scavenger genes, *katE* and *katG*, in the *Escherichia coli* BL21(DE3) host strain using a λRED recombination approach (Scheme S1). Thus, we expected that the H_2O_2 consumption would specifically relate to the P450 peroxygenase activity in HXΔEG (Figure 1). In fact, a dramatic reduction of background H_2O_2-consumption was seen in the HXΔEG cell lysate compared to its wild-type counterpart, and an excellent correlation between H_2O_2 consumption and the GC-based FFA conversion was also observed from the P450 FADC-supplemented cell lysates (Figure S2). Of note, the trace base-line H_2O_2 consumption in HXΔEG was likely due to low degradation activities of endogenous alkyl hydroperoxide reductase (Ahp) which functions at low H_2O_2 level (<10 μM) in *E. coli,* and other unknown H_2O_2 scavenging enzymes if exist. However, triple deletion of *ahp*, *katE*, and *katG* resulted in severe cell growth defect.[10]

Then, to address the issue of inadequate enzyme content in cell lysates of 96-well plate cultures encounter by Wang *et al.*,[4] we optimized the conventional cell lysis system by supplementing nonionic surfactant Triton X-100 at 0.1% (v/v) final concentration, which dramatically improved the efficiency of cell membrane disruption and the release of soluble enzymes (Figure S3). Subsequent preliminary assay development using HXΔEG as host strain for the P450 FADC ($OleT_{JE}$ or CYP-Sm46Δ29) expression showed good assay reproducibility and dynamic ranges (with a coefficient of variation (% CV) between

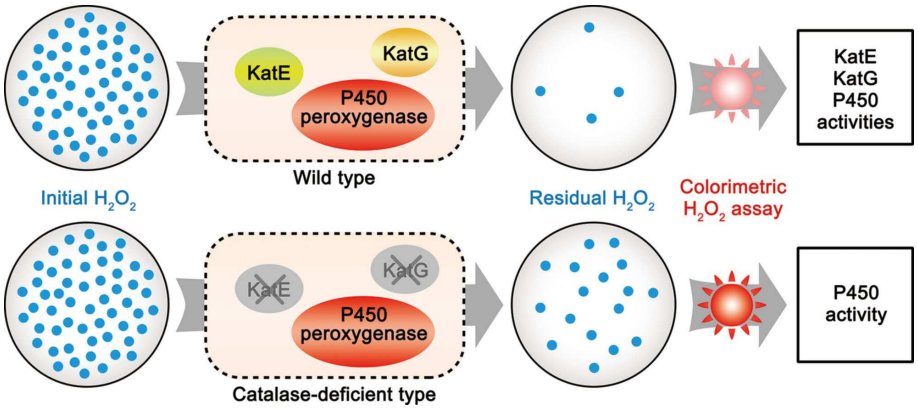

Figure 1. Schematics of the colorimetric Amplex Red H_2O_2 consumption assay coupled to the P450 peroxygenase activity in different *E. coli* host strains: wild type *v.s.* the catalase-deficient type.

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wells at 5.6%). Again, the background H_2O_2-consumption in HXΔEG host cells was dramatically decreased compared to that in wild-type *E. coli* host cells (Figure S4).

To demonstrate the effectiveness of the HTS method (Figure 2), we first elected to build a DNA shuffling library of $OleT_{JE}$ and CYP-Sm46Δ29, which are two representative P450 FADCs among the fast growing number of CYP152 family peroxygenases and share a DNA/protein sequence identity of 66/65%,[5c] with the intention of integrating the beneficial amino acid residues from these two homologous enzymes (Figure S5 & S6). The library showed an average recombination efficiency of approximately 1.3 crossover plus about 2 mutations per gene (~1.3 kb), which is similar to reported optimal values (1.0–2.5 crossovers/gene).[11] This library was used to screen for improved variants against the C_{12} FFA substrate lauric acid (LA) using the established HTS method. Initial screening of ~4,000 colonies showed 9.5% mutants of the library (368/3872) outperformed the less active parental enzyme CYP-Sm46Δ29. Among these, 44.3% (163/368; 4.2% of the total 3,872) outperformed the higher-performing parental enzyme $OleT_{JE}$ (Figure S7). Ranked according to their H_2O_2-consumption indexes normalized to P450 levels in cell lysates (*i.e.*, $\Delta A_{560}/A_{420-490}$, see Supporting Information), 80 variants that showed at least 1.2-fold higher index values over $OleT_{JE}$ were chosen for a second round screening in a triplicate format (Figure S8). Subsequently, 20 top scored variants were selected for protein purification and *in vitro* enzyme assays. Sequence analysis revealed that 8 out of the 20 variants are CYP-Sm46Δ29-backbone mutants (*i.e.*, the majority of coding sequence originates from the *CYP-Sm46Δ29* gene), while 12 are $OleT_{JE}$-backbone mutants (Table S2). Their substrate conversion and alkene production activities were then evaluated by GC analysis and compared with those of CYP-Sm46Δ29 and $OleT_{JE}$ parent enzymes (Figure S9). Mutants with the highest fold-increase in catalytic activity over their corresponding backbone-parent were selected for further analysis.

Among those CYP-Sm46Δ29-backbone mutants, P13G11 was verified to have the highest LA conversion activity (Table 1).

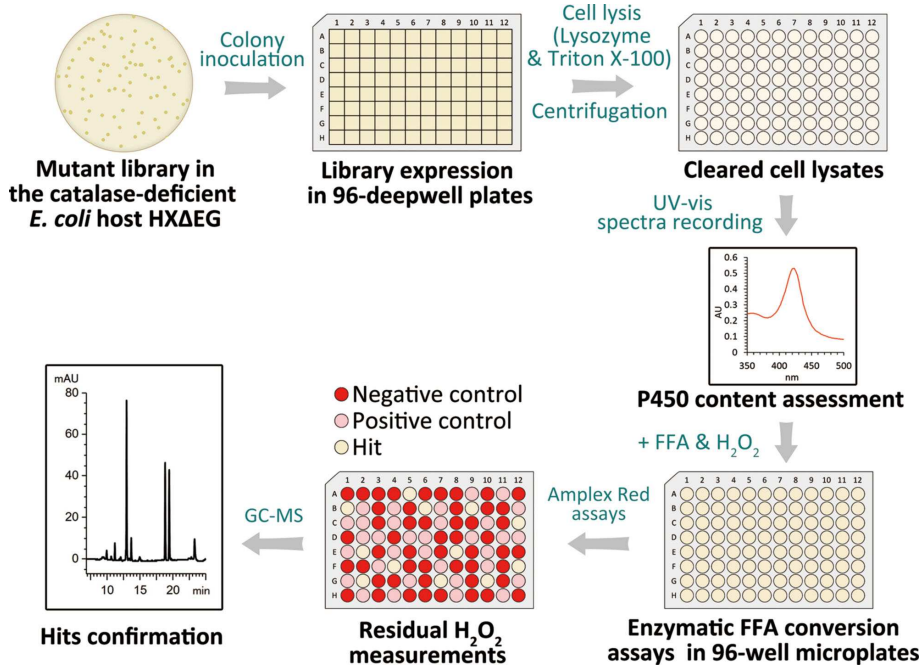

Figure 2. Flowchart of the 96-well plate format high-throughput screening.

Table 1. Catalytic activities of the wild-type and mutant P450 FADCs against different carbon-chain length FFA substrates.

Enzyme + Substrate	Substrate conversion ratio [%]	1-alkene formation [%]	DC/HD ratio[c]	TON[d]	k_{catapp} [min^{-1}]	K_{Mapp} [μM]	k_{cat}/K_M [e] [$\mu M^{-1} min^{-1}$]
CYP-Sm46Δ29 + LA [a]	33.6 ± 2.7	19.1 ± 1.7	1.3	269	186.1 ± 47.2	23.7 ± 8.1	8.1
P13G11 + LA[a]	80.3 ± 2.3	65.9 ± 2.4	4.6	495	591.0 ± 20.9	22.9 ± 2.8	25.8
$OleT_{JE}$ + LA[a]	98.2 ± 0.6	83.8 ± 2.9	5.8	769	856.8 ± 27.8	40.5 ± 3.6	21.2
$OleT_{JE}$ + OA[b]	45.5 ± 1.6	36.8 ± 4.9	3.8	234	75.6 ± 12.4	181.6 ± 64.9	0.42
3-10F12 + OA[b]	55.2 ± 1.8	43.8 ± 15.2	3.8	368	56.7 ± 4.6	117.5 ± 26.3	0.48

[a] 0.5 μM enzyme, 200 μM lauric acid (LA) and 220 μM H_2O_2 in 200 μl Na_2HPO_4 buffer (pH 8.0) for 2 h at 30 °C. [b] 0.2 μM enzyme, 200 μM octanoic acid (OA) and 220 μM H_2O_2 in 200 μl Na_2HPO_4 buffer (pH 8.0) for 40 min at 30 °C. [c] DC/HD ratio refers to the decarboxylation (DC) over hydroxylation (HD) chemoselectivity. The hydroxylation activity was estimated by subtracting the alkene production from the total substrate conversion. [d] Maximum turnover numbers of FFA conversion: 1 μM enzyme, 1 mM substrate, fed-batch addition of H_2O_2 (10 × 100 μM every 30 min) at 30 °C. [e] Initial rates were measured by the amount of substrate converted per μM enzyme per min. Data (shown as mean ± SE) were derived from Michaelis-Menten equation using OriginPro 8.0.

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Its substrate conversion and alkene production ratios were 2.4-fold and 3.5-fold higher than its parent CYP-Sm46Δ29, respectively, which resulted in a 3.5-fold increase in its decarboxylation over hydroxylation chemoselectivity (DC/HD) (Table 1 & Figure S10). Moreover, P13G11 exhibited a 1.8-fold higher maximum turnover number (TON) than CYP-Sm46Δ29. However, its conversion activity and TON were still lower than those of $OleT_{JE}$ (Table 1). Steady-state kinetic analysis showed that P13G11 exhibited a 3.2-fold increase in k_{cat}/K_M value over CYP-Sm46Δ29. When compared with $OleT_{JE}$, P13G11 displayed a reduced k_{catapp} but a 2-fold decreased K_{Mapp} value, which together resulted in a slightly better catalytic efficiency (k_{cat}/K_M) than $OleT_{JE}$ (Table 1). Typical time-course reactions of P13G11 in parallel with its parent enzymes against C_{12} LA are depicted in Figure S11.

Protein sequence analysis of P13G11 revealed that the fragment of residues D105-E212 in CYP-Sm46Δ29 was replaced by the corresponding segment in $OleT_{JE}$ (E105- P212). The crossover happened mainly in the low identity region with most of the consecutive identical stretches less than 5 residues (Figure S12). Some key residues previously identified to be important for substrate positioning and product distribution, such as I170 and L176, are included in this region.[8c–e] Our finding that substitutions in this region resulted in an improved mutant is consistent with the notion that precise substrate positioning and elaborate coordination within the binding pocket is pivotal to the P450 FADC's activity. Moreover, the key catalytic residue R245 in the modelled P13G11 structure was found to adopt an orientation as in $OleT_{JE}$ (Figure S13), providing a valuable mutant for future mechanistic study using computational methods.

Nevertheless, none of the $OleT_{JE}$-backbone mutants exhibited significantly improved decarboxylation activity over the parent $OleT_{JE}$ (Figure S9), stressing the difficulty in fine-tuning the FADC activity. It has been noted that some proteins are much more evolvable than others and *vice versa*.[12] An (directed) evolution, which was compared to a hill-climbing exercise in the 'fitness landscape' of protein sequence space, is always easier from a less favorable starting point than from an already superior parent.[13] Previous engineering efforts had witnessed that subtle changes in the binding pocket of $OleT_{JE}$ could overturn the enzyme's chemoselectivity or even abolish its decarboxylation activity, let alone LA is already an optimal substrate[8i] and the evolution space to further increase activities against it could be relatively and reasonably narrow.[12] In fact, there are multiple reasons for a given enzyme becoming difficult to improve. One of those could be a local optimum or dead-end has been reached.[12,13b] In such cases, taking a sideway or downhill step from neutral or even inferior mutants usually help to escape the so-called dead-end.[14] In this sense, the mutant P13G11 could open a new adaptive pathway and provide a good starting point for an alternative evolution path uphill.

To further explore the fitness landscape of the sequence space of $OleT_{JE}$, another logical strategy appeared to be constructing and screening a randomized mutant library of $OleT_{JE}$ for improved activities towards a suboptimal FFA substrate. To this end, a shorter-chain C_8 octanoic acid (OA) was chosen as the screening substrate due to its moderate conversion by wild-type $OleT_{JE}$ (< 50%). An error-prone PCR randomized mutant library was constructed (Figure S14). Again, ~4,000 colonies were screened using the established HTS method. 74 variants that showed higher H_2O_2-consumption index values in the initial screening were subjected to a second-round screening in triplicate (Figure S15 & S16). Then 10 top-scored variants were selected for *in vitro* enzymatic assays with purified enzymes to verify the activity improvement. As a result, clone 3-10F12 showed the highest OA conversion and 1-heptene production which are both 1.2-fold higher than those of $OleT_{JE}$ (Table 1, Figure S17). Statistical analysis using the Student's *t*-test showed a *p* value of 0.03 ($p < 0.05$), representing a statistically significant difference. Further kinetic analysis indicated an increased substrate binding affinity (a 1.5-fold decreased K_{Mapp}) of the mutant, albeit a reduced k_{catapp}, resulting in a net catalytic efficiency (k_{cat}/K_M) 14% higher than that of the parental $OleT_{JE}$. Furthermore, 3-10F12 exhibited a 1.8-fold higher TON compared to $OleT_{JE}$ at 0.1% enzyme loading (Table 1). Typical time-course reactions of 3-10F12 in comparison with its parent $OleT_{JE}$ against C_8 OA substrate are depicted in Figure S11.

Protein sequence analysis of 3-10F12 revealed that this mutant contains several mutations including L10S, K52N, K81Q, D345E and I386V. Structure-wise, these mutations are mostly located on the outer region of the enzyme, distant from the substrate binding pocket (Figure S18). The closest residue K81Q is 14.2 Å away from the carboxyl group of the FFA substrate. Other mutations are more than 20 Å away from the carboxyl moiety. Although contribution of individual mutations on the activity improvement and whether synergistic or antagonistic effects exist is to be delineated, this finding that distal mutations improve catalytic traits highlights the importance of the unpredictable allosteric effects of distal residues on enzyme activity,[15] and also underline the difficulty to rationally design a better mutant for this family of P450 FADCs.

In summary, we have developed an efficient HTS approach to facilitate rapid evaluation of large mutant libraries and acquisition of improved P450 peroxygenases. In the present proof-of-concept demonstration studies, ~8,000 clones were efficiently screened from two mutant libraries built upon different strategies. With only one round of evolution, P450 FADC variants were successfully captured with improved catalytic traits including higher TONs against different chain-length FFA substrates, demonstrating the robustness and effectiveness of this novel screening method. Although the HTS mainly detects the improvement in overall substrate conversion activity, improved variants were also proved to possess higher alkene yields. Despite that the activity enhancement over $OleT_{JE}$ was not dramatic (at this initial evolution stage), the method holds its excellence and generality to be broadly applied for P450 peroxygenases and other enzymes with H_2O_2 as co-substrate. With the established HTS method, we therefore solve the inherent problems that limit fast screening of P450 FADCs and envision broader applications of this method in the future

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in 1-alkene production, enzyme toolbox enrichment, and in-depth investigation of catalytic mechanisms.

Experimental Section

Experimental details are provided in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: 1-alkene · directed evolution · high-throughput screening · H_2O_2 · P450 fatty acid decarboxylases

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