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Supplementary Information for

Engineering carboxylic acid reductase for selective synthesis of medium-chain fatty alcohols in yeast

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Supplementary Materials and Methods

Strain construction and cultivation

All the strains and plasmids used in this study are listed in Supplementary Table S2 and S3. The primers for the construction are listed in Supplementary Table S4. The PptA and Sfp1encoding genes were amplified from plasmids derived from a previous study (1) using primers PptA-F/PptA-R and Sfp-F/Sfp-R. The EcAcpS encoding gene was amplified from *E. coli* genomic DNA using primers EcAcpS-F/EcAcpS-R. All the sequences of the codon-optimized heterologous genes are listed in Supplementary Table S5. DNA assembly via homologous recombination was used to establish the mutant library in yeast cells, and a Gibson assembly cloning kit (New England Biolabs) was utilized to combine different mutations. The "MmCAR+NpgA" cassette was amplified from the plasmid pAOH0 derived from a previous study (2), and introduced into pZWM using Gibson. The background strain used for directed evolution was YJZ03H (*pox1Δ hfd1Δ*) derived from a previous study (3). CRISPR/Cas9 was used for genome editing in this study. The strain ZWE243 was derived from ZWE24 by integrating a chimeric acetyl-CoA generation pathway at the *hfd1*Δ locus as described in a previous study (4).*TPO1* was deleted from strain ZWE243 through co-transformation of a repair fragment, and an all-in-one plasmid containing the gRNA and Cas9 with *kanMX* as the marker (5).

A minimal medium with histidine containing 14.4 g/L KH_2PO_4, 7.5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \bullet 7H_2O$, 20 g/L glucose, 2 mL/L trace metal solution (4.5 g/L $ZnSO_4 \cdot 7H_2O$, 0.84 g/L $MnCl_2 \cdot 2H_2O$, 3.0 g/L $FeSO_4 \cdot 7H_2O$, 0.3 g/L $CuSO_4 \cdot 5H_2O$, 4.5 g/L $CaCl_2 \cdot 2H_2O$, 0.4 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.3 g/L $CoCl_2 \cdot 6H_2O$, 0.1 g/L KI, 1.0 g/L H_3BO_3 and 19.0 g/L $Na_2EDTA \cdot 2H_2O$), 1 mL/L vitamin solution (1.0 g/L thiamin–HCl, 0.05 g/L D-biotin, 1.0 g/L pyridoxin–HCl, 1.0 g/L nicotinic acid, 1.0 g/L D-pantothenic acid hemicalcium salt, 0.2 g/L 4-aminobenzoic acid and 25.0 g/L myo-inositol) and 100 mg/L histidine was used for the shake flask cultivations (6). The pH of the medium was adjusted to 6.0 through 4 M KOH. Yeast strains for MCFOH production were cultivated at 200 rpm, 30 °C for 48 h. SD-Ura medium containing 6.7 g/L yeast nitrogen base without amino acids (Formedium™, Norfolk, UK), 0.77 g/L complete supplement mixture without uracil (Formedium) and 20 g/L glucose was used for colony selection. YPD+G418 medium containing 10 g/L yeast extract, 20 g/L peptone, 200 mg/L G418 and 20 g/L glucose was used for selection of colonies containing a *kanMX* cassette. 20 g/L agar (Merck Millipore) was added to make the corresponding solid medium.

Directed evolution of full-length MmCAR

The mutagenesis library of *MmCAR* was constructed by error-prone PCR (GeneMorph II Random Mutagenesis Kit, Agilent Technologies) with a low mutation frequency (0–4.5

mutations/kb) using the primers MmCARwm-F/MmCARwm-R. The product was then co-transformed with two backbone fragments of pZW01 into yeast strain YJZ03 (*pox1Δ hfd1Δ*). The primers used for amplification are shown in Table S4. The plasmid-based libraries were established in yeast. The library sizes for the two rounds of evolution were around 6×10^7 and 4.5×10^7, respectively. All the colonies from the SD-URA plate were all scratched into 20 mL minimal medium with 290 mg/L C8 fatty acid (330 mg/L for the second round). After 24 h, the cells (1:100 diluted) were transferred into 20 mL minimal medium with 310 mg/L C8 fatty acids (350 mg/L for the second round) for 48 h. Then the cells were diluted 1:50, and transferred into 20 mL minimal medium with 330 mg/L C8 fatty acid (370 mg/L for the second round) for 48 h. The enriched cells were plated on SD-URA solid medium, and around 60 single colonies were picked randomly into 96-well plate with minimal medium containing 330 mg/L C8 fatty acids (370 mg/L for the second round). The growth curves were monitored by a Bioscreen C MBR instrument (Growth Profiler 960 was used for the second round). The plasmids from fast-growing colonies were extracted and used to transform strain ZWE243 to determine MCFOH production. The mutant CAR with mutations D241E, H454R, L567M was used as the template for the second round of evolution. The same process was conducted as described above.

Directed evolution of MmCAR A domain

The random mutagenesis library based on the A domain (residues 88–541) of MmCAR was established using error-prone PCR (GeneMorph II Random Mutagenesis Kit,) with the low mutation frequency (0–4.5 mutations/kb) by primer pair CAR-A-F/CAR-A-R. The primers are listed in Table S4. The wild-type MmCAR was the template for the first round of evolution and the mutant CAR with mutations Q182R, Q371R was used for the second round. The sizes of the two libraries were both around 5×10^4. The same workflow for the library enrichment was used as described above, but different concentrations of C8 fatty acid were used, which were 290 mg/L, 300 mg/L and 310 mg/L for the first round and 310 mg/L, 330 mg/L and 350 mg/L for the second round. Around 60 single colonies from first round and second round were cultivated in minimal medium with histidine containing 330 mg/L and 370 mg/L C8 fatty acids, respectively. The growth curves were monitored by a Bioscreen C MBR instrument (Growth Profiler 960 was used for the second round).

Site-directed saturation mutagenesis library

The primers used for establishing the site-directed saturation mutagenesis library based on residues 983–985 are listed in Table S4. The same enrichment method was used as described above with concentrations of 290 mg/L, 310 mg/L, 330 mg/L and 350 mg/L C8 fatty acids. After isolating the variants on an SD-Ura plate, 80 of the colonies were picked to

grow in medium with 380 mg/L of C8 FA. Based on the growth curves generated with the help of a Growth Profiler 960, the clones with the highest growth rates were selected.

Analytical measurements

For MCFOH extraction, 0.5 mL of 10% (w/v) NaCl and 0.5 mL of 2.5 M KOH were added to 4 mL culture broth after 48 h of cultivation. Then, 2 mL of chloroform/methanol mix (1:1, v/v) containing 1-heptanol (60.04 mg/L) as internal standard was added, and vortexed together at 1800 rpm for 10 min. After centrifuging for 10 min at 2000 g, the organic phase was transferred to a GC vial for quantification. For intracellular fatty alcohol extraction, the cell pellets derived from 4 mL of culture broth after centrifugation were collected and freeze dried for 48 h. Then 4 mL of chloroform/methanol (2:1, v/v) containing internal standard (final concentration of 10 mg/L 1-heptanol and 10 mg/L 1-pentadecanol) were added and mixed with freeze-dried cells. A microwave extraction method was used as described in a previous study (7), where the temperature was ramped to 60 ºC from room temperature within 6 min and kept constant for 10 min. 1 mL of 0.73% (w/v) NaCl was added after the sample was cooled down to room temperature, then mixed and centrifuged at 1000 g for 10 min. The organic phase was taken out and concentrated by drying under vacuum, then re-suspend with 100 µL of hexane.

For MCFA extraction, the samples were taken after 48 h of cultivation. 1 mL or 2 mL of culture broth were taken, and MilliQ H_2O was used to adjust the total volume to 4 mL. 0.5 mL of 10% (w/v) NaCl and 0.5 mL acetic acid containing heptanoic acid and pentadecanoic acid as internal standard were added into samples. Then, 2 mL of chloroform/methanol mix (1:1, v/v) were added, the samples were vortexed at 1800 rpm for 30 min. After centrifuging at 3000 rpm for 10 min, 200 µL of the upper chloroform layer were transferred to a new glass tube, and 1 mL 14% BF_3 in methanol was added to methylate the fatty acids overnight. Then, 1 mL MilliQ H_2O and 600 µL hexane were added, and the mixture was vortexed at 2000 rpm for 10 min. After centrifuging for 10 min, the upper hexane layer was taken for GC analysis. The medium chain FAMEs were analyzed by a Focus GC/ISQ (Thermo Fisher Scientific) with a ZB-50 column (30m×0.25 mm×0.25 μm, Phenomenex, Torrance, CA, USA). 1 µL sample was injected (splitless, 240 °C), and helium was used as carrier gas (3 mL/min). The GC program was as follows: The initial temperature of 30 °C was held for 2 min, and increase to 150 °C at a rate of 40 °C/min for 2 min, then increased to 250 °C at a rate of 10 °C/min for 3 min.

The quantification of MCFOHs was performed by gas chromatography (Focus GC, Thermo Fisher Scientific) connected with a Flame Ionization Detector (FID), and a Zebron ZB-5MS GUARDIAN capillary column (30m×0.25 mm×0.25 μm, Phenomenex). The GC program was

as follows: The initial temperature of 45 °C was held for 2 min, and ramped to 220 °C at a rate of 20 °C per min and held for 2 min, then ramped to 300 °C at a rate of 20 °C per min and held for 5 min. The temperatures of inlet and detector were all kept at 300 °C. The flow rate of the air and H_2 were 350 mL/min and 35 mL/min, respectively.

Enzymatic assays

The yeast cells (50 OD) were harvested after 24 h by centrifuging at 4000 g at 4°C for 5 min, then washed twice with PBS buffer. After discarding the supernatant, the cells were resuspended in 0.5 mL extraction buffer (50 mM Tris-Cl, 1 mM EDTA, 1 mM KCl, pH 7.5) containing 10 mM DTT and 1% (v/v) protease inhibitor. The suspensions were vortexed for 20 s ×5 (5 min intervals on ice) with 300 mg 0.2–0.4 mm glass beads. The supernatants of the samples were collected by centrifuging at 20,000 g at 0°C for 20 min. Proteins were quantified using a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). The MmCAR activity assay was performed in 96-well microplates; 90 μL reaction mix containing 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 1mM NADPH, 1 mM ATP and fatty acids (0.5 mM C10, C12, C14, C16:1, C18:1 fatty acid and 5 mM C6, C8 fatty acid) and 10 μL of extracts (5 μg total protein) were mixed to initiate the reaction. Then the reactions were monitored at 340 nm for 30 min at room temperature with a FLUOstar Omega micropate reader (BMG LABTECH GmbH). The MmCAR activity was defined as NADPH oxidation rate (1 μmol/min) of total protein.

Kinetic parameter determination by photometric detection

A single colony of the desired *E. coli* transformant was incubated in 4 mL LB-0.8 G pre-culture medium supplemented with ampicillin (100 μg/mL) at 37 °C and 120 rpm ON. The main culture medium LB-5052 supplemented with ampicillin (50 μg/mL) was inoculated with 0.2% (v/v) of the pre-culture and incubated at 37°C and 120 rpm for 4 h, then lowered to 20 °C and the main culture shaken at 100 rpm for 20 h. Cell pellets were disrupted by a 102 °C converter with a Sonifier 250 (Branson, Danbury, CT), and cell-free extract was obtained by centrifugation at 4°C for 1 h at 20,000g. The supernatants were filtered through 0.4 μm sterile filters. MmCAR variants were purified by nickel affinity chromatography with a linear gradient of 0–100% Buffer B (e.g. 10 mM to 500 mM imidazole) using HisTrap FF Columns on an ÄKTA Pure 100 with a fraction collector F9-C (Unicorn 6.3 software; GE Healthcare) and desalted using a gravity flow protocol using PD10 columns. The protein-containing fractions were pooled, the buffer was exchanged for 50 mM potassium phosphate buffer, pH 7.5, containing 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT and aliquots of the resultant protein solution were shock frozen in liquid nitrogen and stored at -80°C. Protein concentrations were determined using the Pierce™ BCA Protein Assay kit with

BSA as the standard. An NADPH depletion assay was used to determine the specific activity of mutants. Therefore, hexanoic acid (0.01–0.3 M) was dissolved in 0.1 M KOH. The assay composition was as follows: 10 µl of the substrate solution was added to 160 µL of HEPES buffer (100 mM, pH 7.5, containing 10 mM $MgCl_2$). Subsequently, 10 µL of NADPH (10 mM in water), 10 µL of ATP (20 mM in water) and 10 µL of CAR enzyme preparation from Ni-affinity chromatography (1 mg mL^{-1}) were added. The depletion of NADPH was followed on a Synergy Mx Plate-reader (BioTek) at 340 nm and 28 °C for 10 min. Appropriate blank reactions without ATP were carried out in parallel and each reaction was carried out at least in triplicates of 4 technical replicates. Kinetic parameters were determined as described above using increasing substrate concentrations. K_m and V_{max} were calculated based on non-linear regression by using Sigma Plot 14.0.

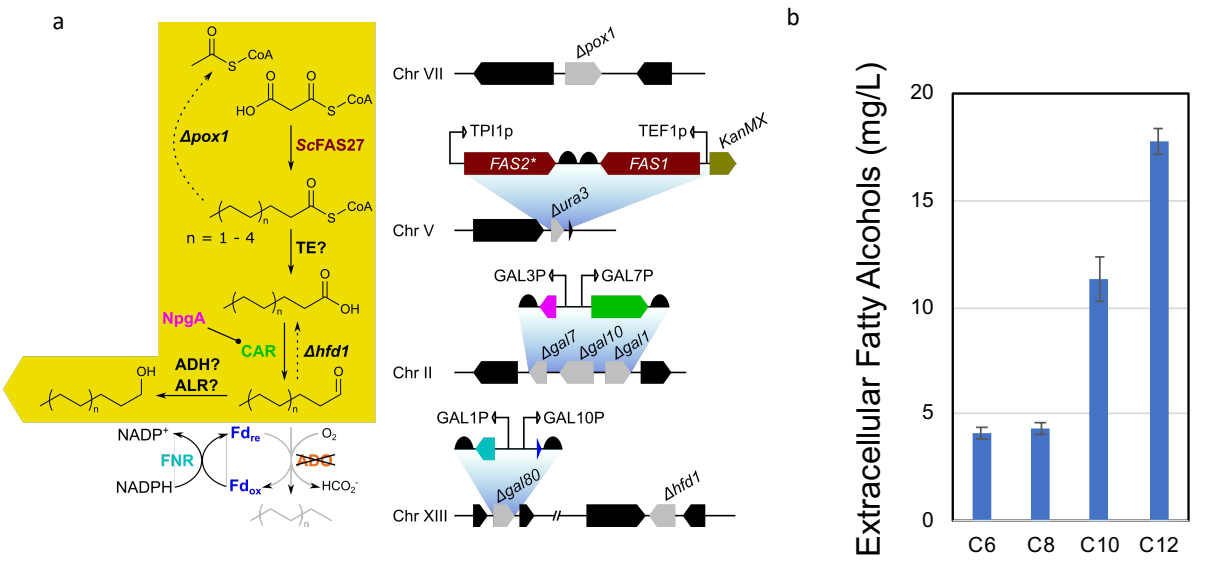

Figure S1. MCFOH production by strain ZW540. (a) Genetic modifications in strain ZW540. The engineered ScFAS27 with G1250S and M1251W mutations in Fas2 was expressed to generate MCFAs. The *POX1* (fatty acyl-CoA oxidase) and *HFD1* (aldehyde dehydrogenase) genes were deleted to avoid reverse reactions. Carboxylic acid reductase (CAR) and its activation protein phosphopantetheinyl transferase NpgA were expressed to reduce fatty acids to aldehydes. The alkane synthesis module including ferredoxin (Fd), ferredoxin-NADP+ reductase (FNR) from *E. coli* and fatty aldehyde deformylating oxygenase (ADO) was not completely deployed, because of a lack of ADO expression. The endogenous alcohol dehydrogenases (ADHs) and aldehyde reductases (ALRs) were supposed to divert fatty aldehydes to alcohols. (b) Extracellular medium-chain fatty alcohols produced by strain ZW540, which was cultivated in minimal medium with 100 mg/L uracil and histidine for 96 h. The mean ± s.d. of three biological replicates in a representative measurement is presented.

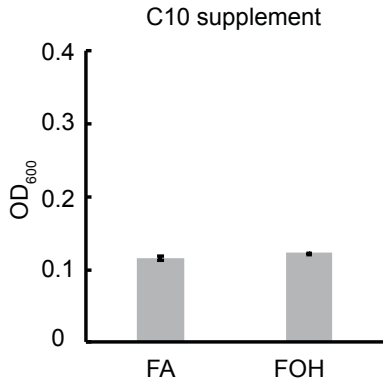

Figure S2. Comprison of C10 fatty acid/fatty alcohol toxicity to yeast cells. Strain YJZ03H was cultivated in minimal medium with 70 mg/L of C10 fatty acid/alcohol for 72 h. The final optical density (OD) was measured at wavelength of 600 nm (OD_{600}). The mean ± s.d. of three biological replicates in a representative measurement is presented.

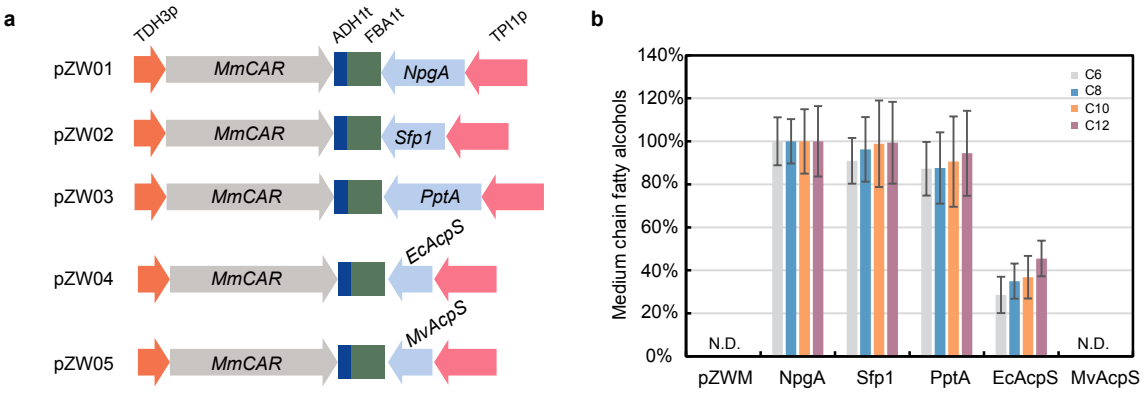

Figure S3. Evaluation of different PPTases for MmCAR. (a) Construction of expression cassettes for MmCAR and PPTases. The MmCAR and PPTase genes were under the control of promoter *TDH3p* and *TPI1p*, respectively. (b) The relative production of MCFOHs in strain ZW2071 containing plasmids for expression of MmCAR with different PPTases, using the empty plasmid pZWM as negative control. Cultivation was performed for 48 h in minimal medium, the mean ± s.d. of three biological replicates in a representative measurement is presented.

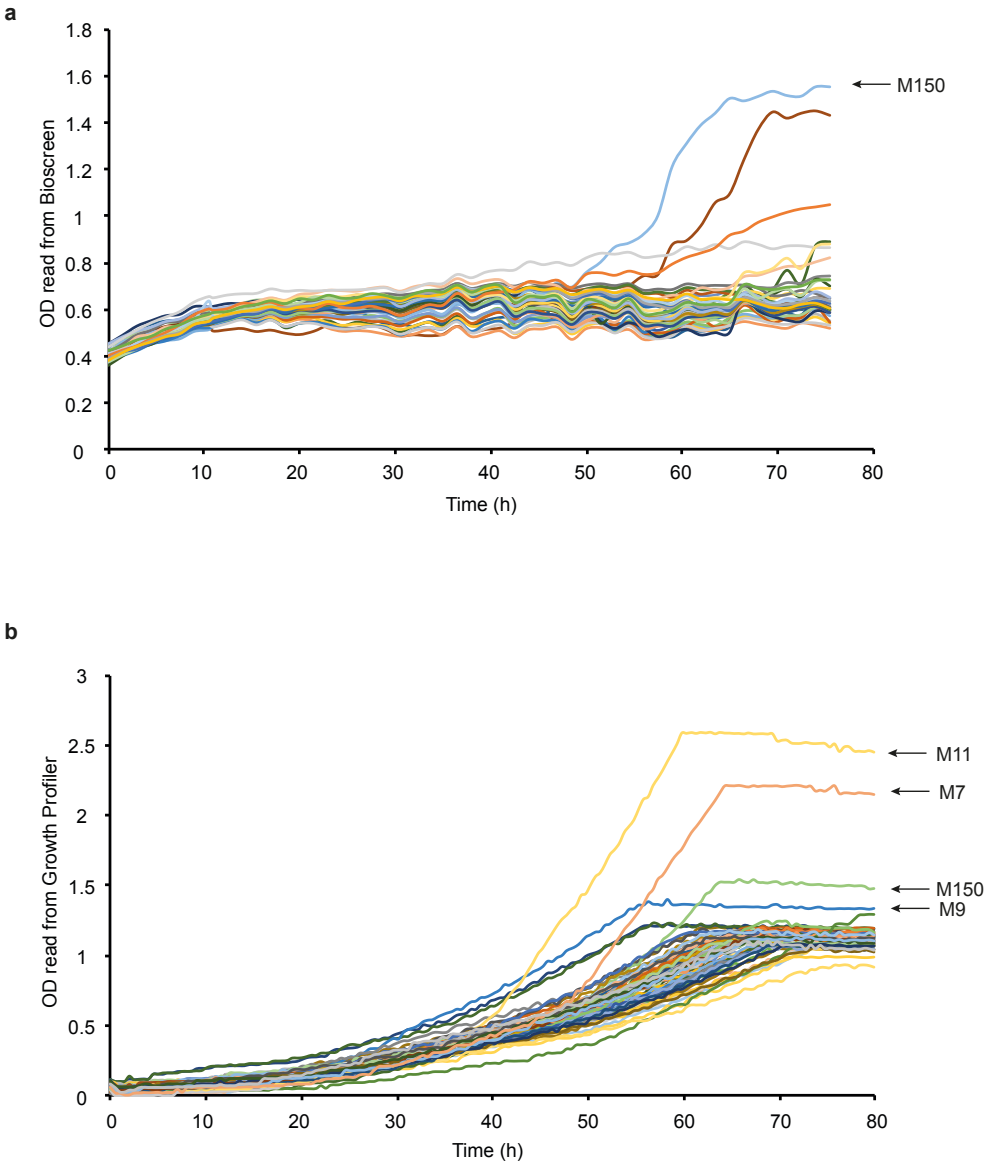

Figure S4. Growth curves of clones expressing enzyme variants from directed evolution based on full-length MmCAR in medium with C8 fatty acids. Around 60 single colonies from the first round (a) and the second round (b) were selected to be cultivated in minimal medium with 330 mg/L (a) and 370 mg/L (b) of C8 FAs, respectively.

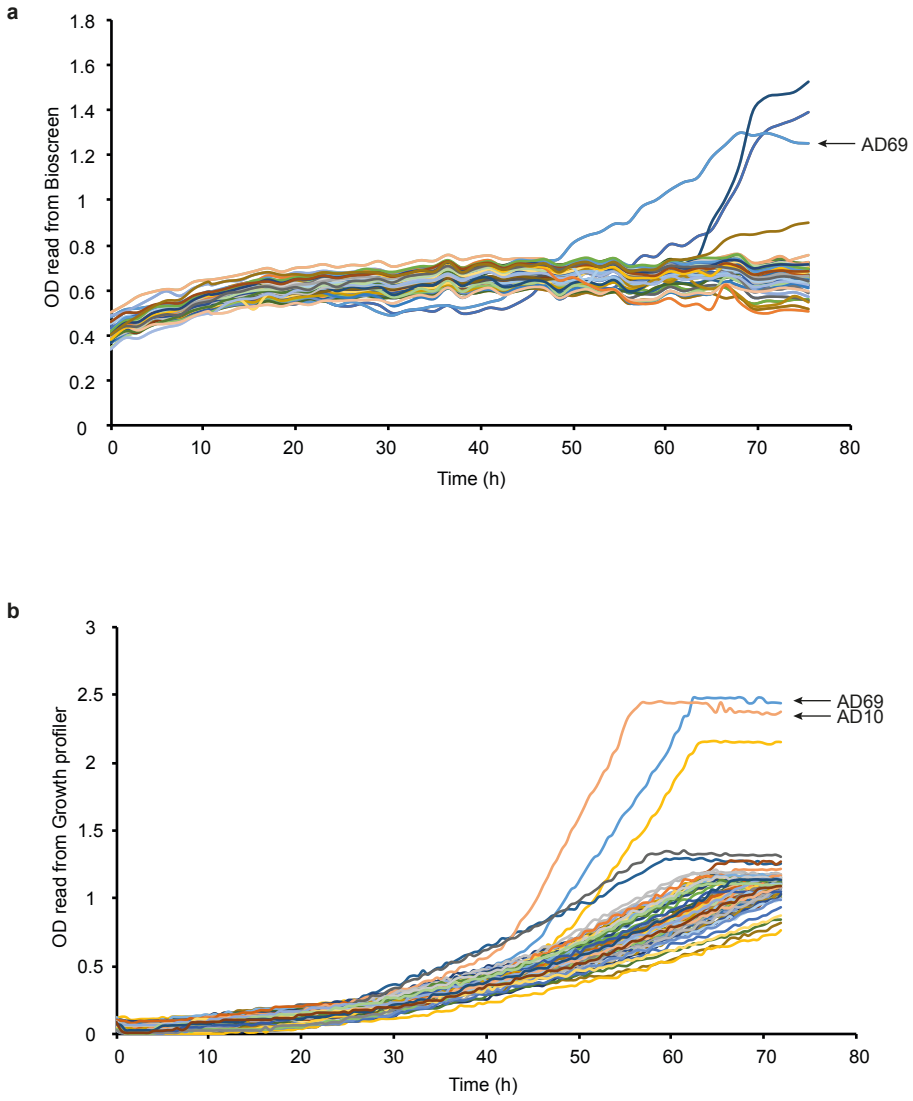

Figure S5. Growth curve of clones expressing enzyme variants from directed evolution based on the A domain of MmCAR in medium with C8 fatty acids. Around 60 single colonies from the first round (a) and the second round (b) were selected to be cultivated in minimal medium with 330 mg/L (a) and 370 mg/L (b) of C8 FAs, respectively.

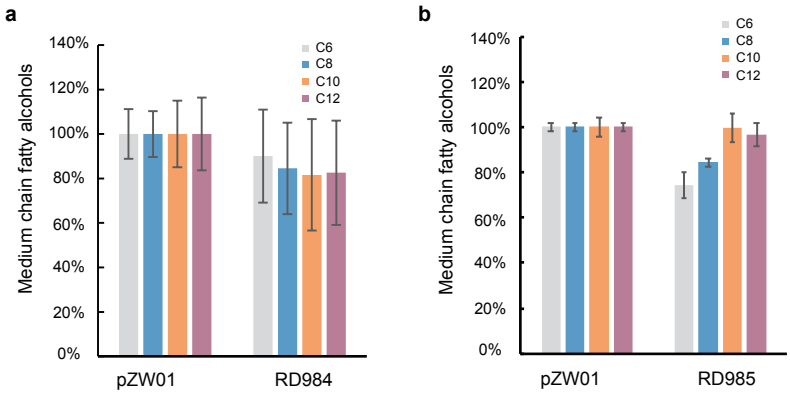

Figure S6. The effect of mutations in MmCAR on the production of MCFOHs. (a) Relative production of MCFOHs in ZWE243 with pZW01 and RD984 (D984G), the wild type MmCAR was used as the reference. (b) Relative production of MCFOHs in strain ZWE243 with the wild type MmCAR and mutant RD985 (M985A), respectively. Cultivation was performed for 48 h in minimal medium, the mean ± s.d. of three biological replicates in a representative measurement is presented.

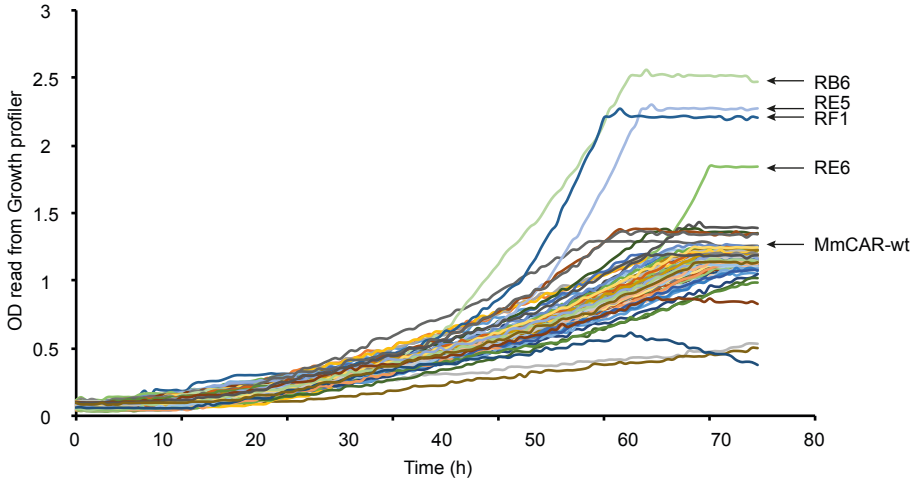

Figure S7. The growth curves of 80 candidates selected from the site-directed saturation mutagenesis library. The colonies were isolated and selected from the SD-Ura plate after the enrichment process, then transferred to minimal medium with 380 mg/L of C8 FA to verify the growth phenotype in microplates using Growth Profiler 960.

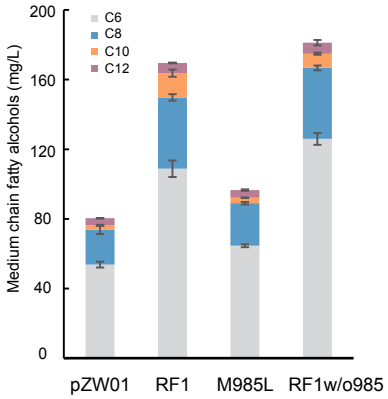

Figure S8. Contribution of the mutations identified in RF1 to MCFOH production. The common mutation (M985L) from the variants derived from site-directed saturation mutagenesis was introduced individually into MmCAR (M985L) and the other two mutations (C983P, D984H) from RF1 (RF1w/o985) were introduced into MmCAR for comparison with M985L. The strain cultivation was performed for 48 h in minimal medium, and the mean ± s.d. of three biological replicates in a representative measurement is presented.

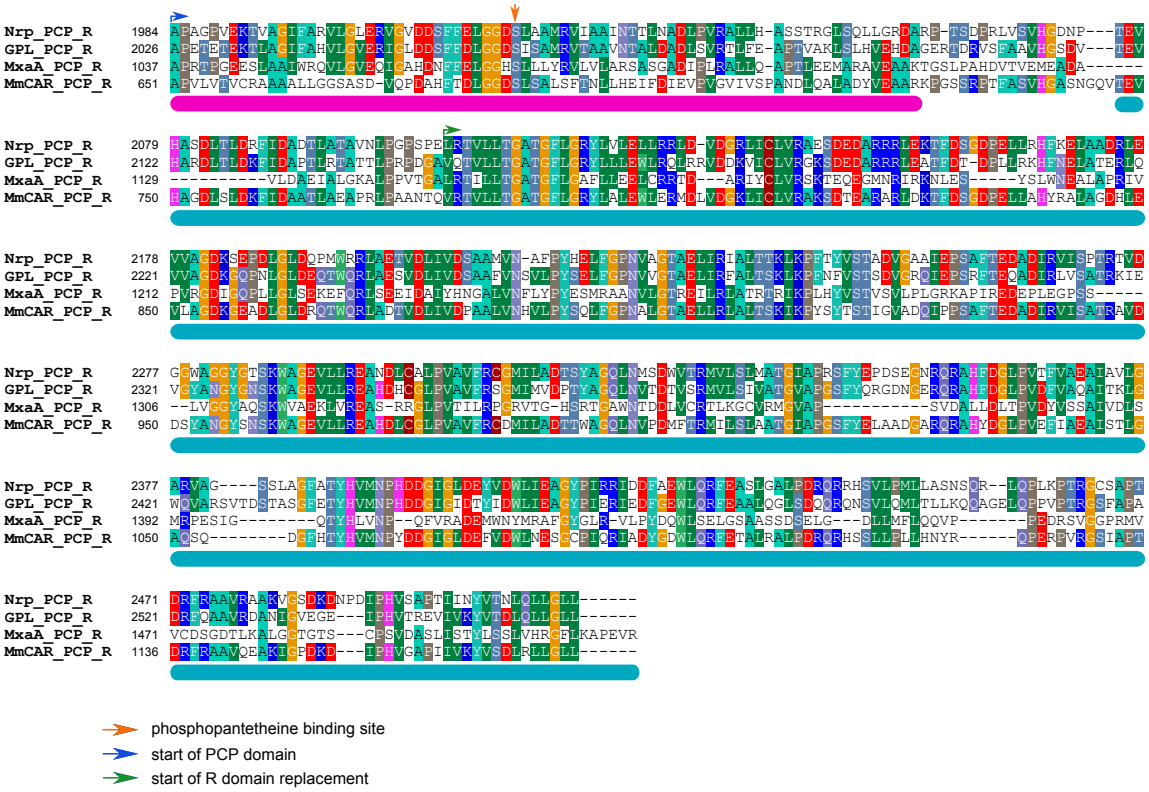

Figure S9. Sequence alignment of PCP-R domains. The PCP-R didomain of MmCAR was aligned with the heterologous PCP-R didomains from the glycopeptidolipid (GPL) biosynthetic protein Mps2 (Uniprot asscession number, Q3L891) of *Mycobacterium smegmatis* (R_{GPL}), the Npr protein (Uniprot asscession number, Q10896) of *Mycobacterium tuberculosis* (R_{NRP}) and the MxaA enzyme (Uniprot asscession number, Q93TX2) of *Stimatella aurantiaca*(R_{MxaA}).

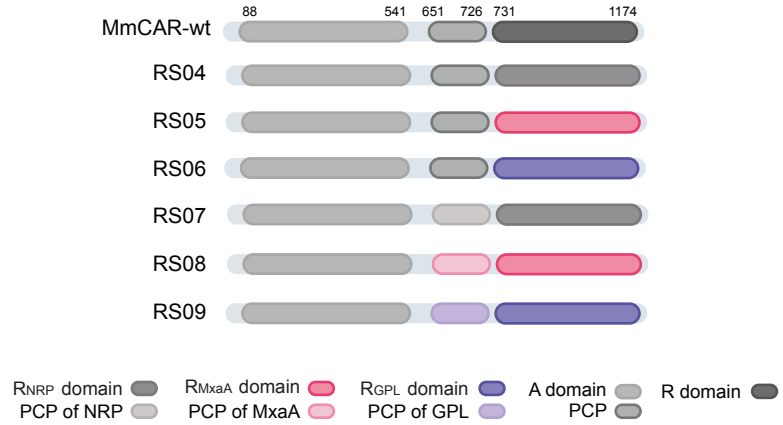

Figure S10. Domain swapping of MmCAR in *S. cerevisiae*. Schematic illustration of MmCAR chimera. The wild-type R domain was replaced in variants RS04, RS05 and RS06. The native PCP-R domain was additionally replaced in variants RS07, RS08 and RS09.

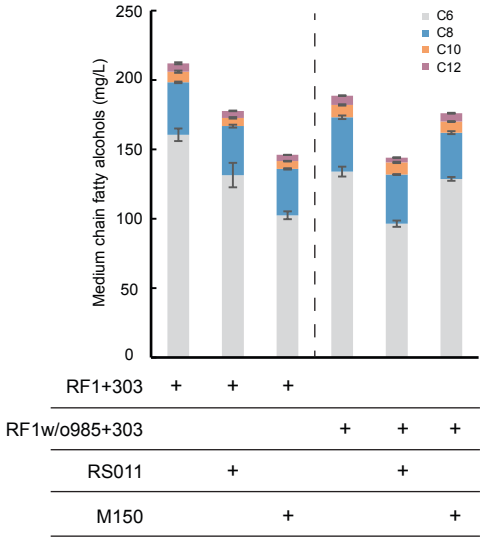

Figure S11. MCFOH synthesis with MmCAR variants generated by the further combination of mutations based on RF1+303 and RF1w/o985+303. The heterologous PCP-R didomain from RS011 and the efficient mutations (D241E, H454R and L567M) from M150 were incorporated into two of the most efficient variants, RF1+303 and RF1w/o985+303, respectively. The tests were performed in ZWE243. The strain cultivation was performed for 48 h in minimal medium, and the mean ± s.d. of three biological replicates in a representative measurement is presented.

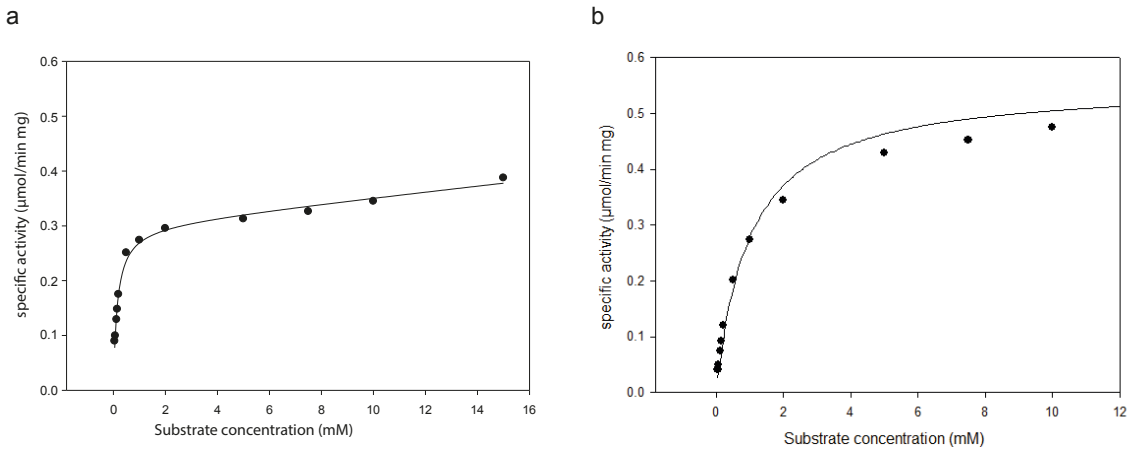

Figure S12. Kinetic characterization of MmCAR wild type (a) and variant I303W (b). The hexanoic acid (C6) was used as substrate. K_M of the wild type was 0.146 ± 0.013 mM, k_{cat} 6.6 ± 0.1 s^{-1} and V_{max} 0.302 ± 0.009 µmol/min mg. K_M of variant I303W was 0.994 ± 0.208 mM, k_{cat} 12.1 ± 0.3 s^{-1} and V_{max} 0.5546 ± 0.0287 µmol/min mg.

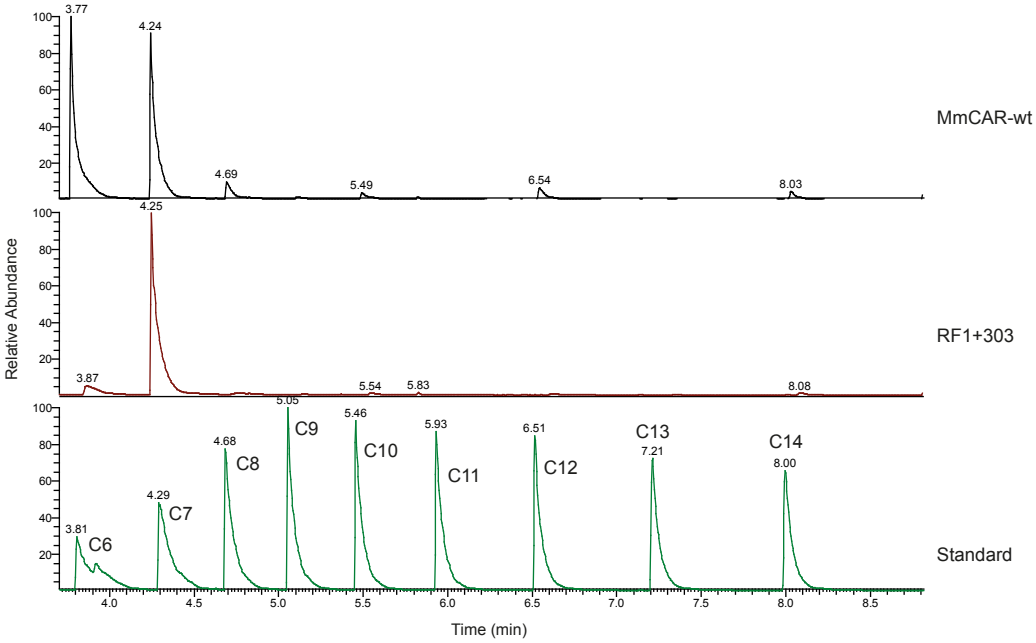

Figure S13. GC spectra of MCFAs derived from yeast strains. Strain ZWE243 containing wild-type MmCAR and RF1+303 were cultivated in minimal medium for 48 h. The extracellular fatty acids of samples were extracted and analyzed by GC-MS.

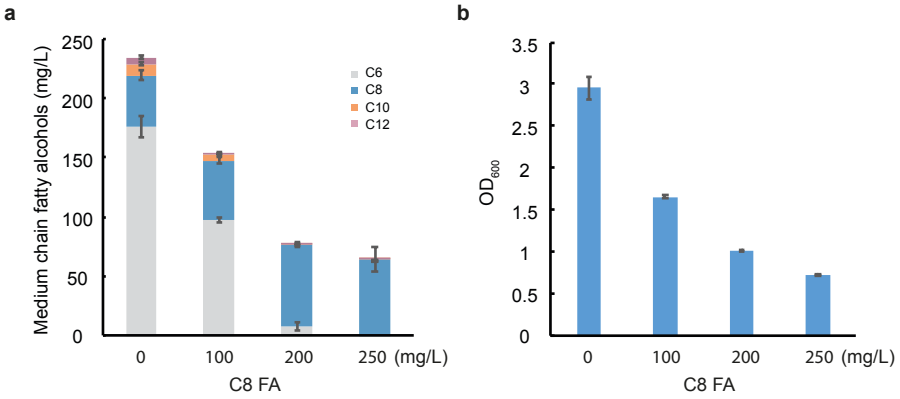

Figure S14. Effect of feeding C8 FA on MCFOH production and cell growth in yeast. (a) Production of MCFOHs in medium with different concentrations of C8 FA. (b) The final OD of strains grown with C8 FA. The strain ZWE243 containing RF1+303 was cultivated with 0, 100, 200, 250 mg/L C8 FA, MCFOH and the final OD was extracted and measured for 48 h. The mean ± s.d. of three biological replicates is presented.

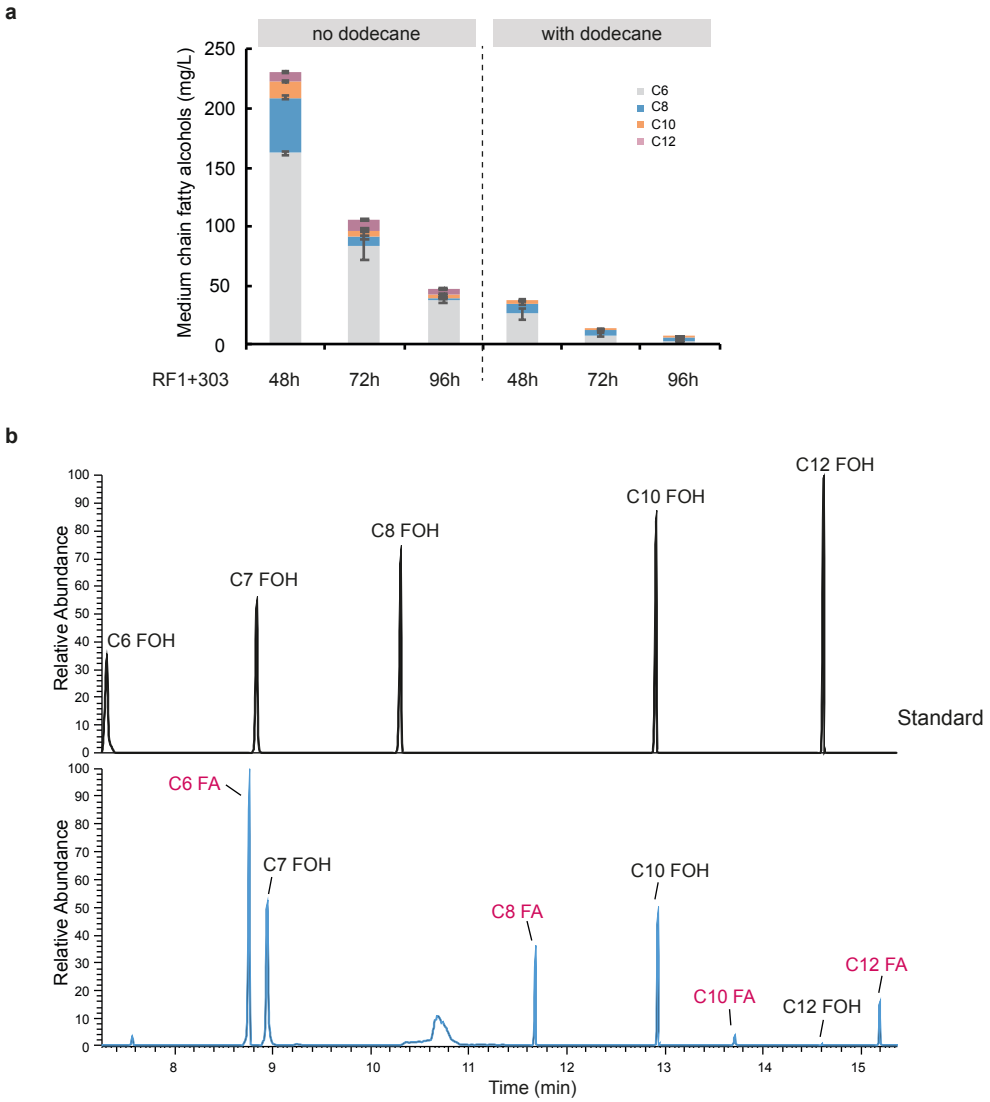

Figure S15. The synthesis of MCFOHs through bi-layer cultivation in yeast. (a) Production of MCFOHs in yeast cultivated with or without dodecan layer. The MCFOHs were extracted from the aqueous layer at 48 h, 72 h and 96 h, respectively. (b) GC spectra of samples taken from dodecane layer. The samples were extracted and analyzed by GC-MS with BSTFA derivatization at 48 h. The yeast strain ZWE243 with RF1+303 was cultivated in minimal medium with either no dodecane or 10% (v/v) dodecane. The mean ± s.d. of three biological replicates is presented.

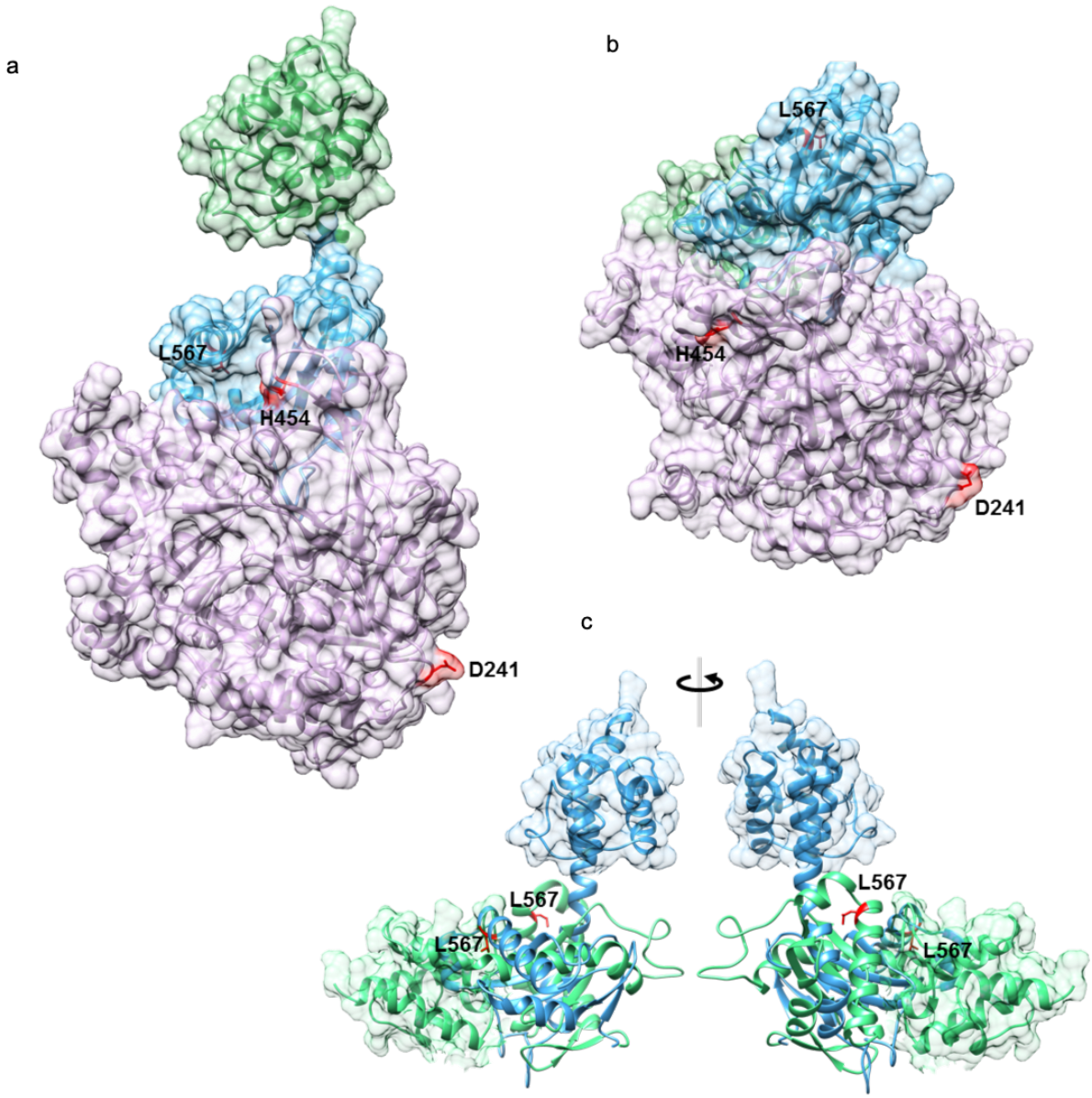

Fig S16. Position of all mutations in M150. (a-b). Mapping of the mutated residues (red sticks) on the structure models of MmCAR A-PCP didomain in adenylation state (a) and thiolation state (b). A_{core}, A_{sub} and PCP domains are shown in purple, blue and green, respectively. D241 and H454 residues are located on the surface of the A_{core} domain, while L567 is buried in the A_{sub} domain. (c) Comparison of the location of L567 between adenylation state (blue) and thiolation state (green), whose structures were superpositioned based on the A_{core} domain.

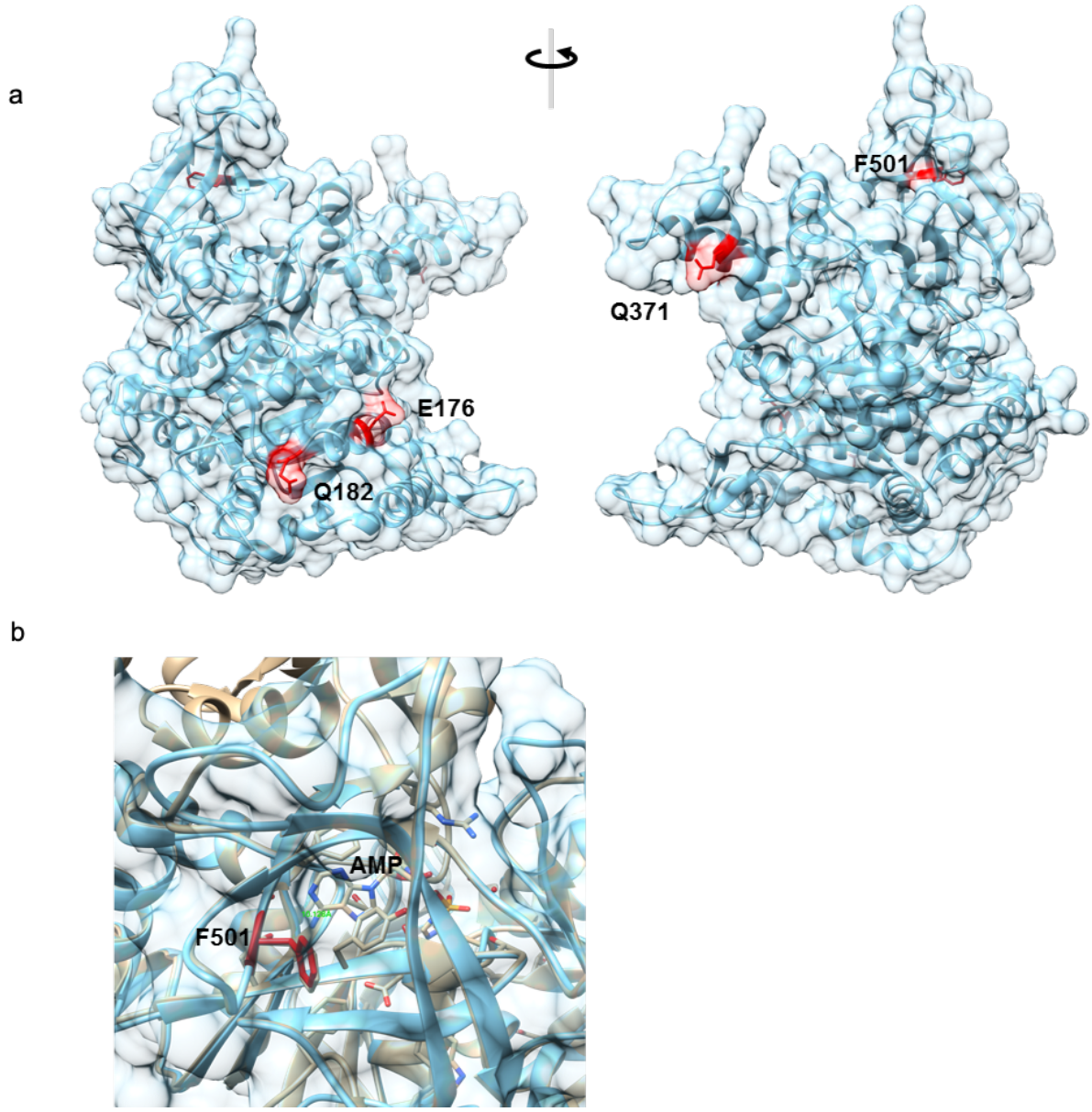

Fig S17. Position of all mutations in AD10. (a). Mapping of the mutated residues (red sticks) in the A_{core} domain of MmCAR. All four mutated residues (red) are located on or proximate to the surface. (b) Structure detail of F501 residue. The A-PCP didomain of SrCAR with AMP ligand (PDB ID: 5MST) is shown in brown, and the modeled structure of MmCAR A_{core} domain is shown in blue. The shortest distance between F517 (equivalent to F501 in MmCAR) and the adenine base of AMP is 11 Å .

Supplementary Table S1. Comparison of fatty alcohol production from different engineered microbial cells

Host	Titer (g/L)	Yield (g/g)[a]	Media	Carbon source	Cultivation	Ref.
S. cerevisiae	0.252 (C6-C12)	0.0126	Minimal medium	20 g/L glucose	Shake flask	This study
S. cerevisiae	0.050 (C8)	0.0025	Rich YPD medium	20 g/L glucose	Shake flask	(8)
S. cerevisiae	0.447 (C10, C12)	0.022	Modified Synthetic complete medium[b]	~20 g/L glucose	Shake flask, dodecane overlay	(9)
Y. lipolytica	0.550 (C10)	0.011	Rich YPD medium with low nitrogen	50 g/L glucose	Shake flask	(10)
E. coli	0.091 (C6, C8)	0.0046	M9-based medium	20 g/L glucose	Shake flask	(11)
E. coli	1.8 (C6-C16)[c]	0.18	LB medium	10 g/L glucose	Shake flask, dodecane overlay	(12)

a. No carbon sources from peptone and yeast extract were considered; **b.** SC medium with nitrogen limitation; **c.** The titer and yield were calculated for total fatty alcohols (C6-C16).

Supplementary Table S2. List of strains used in this study.

Strain (*S. cerevisiae*)	Description	References
YJZ03H	*MATα MAL2-8c SUC2 ura3-52 pox1Δ hfd1Δ*	Lab collection
YJZ03	*MATα MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ hfd1Δ*	(3)
ZW540	***YJZ03*** *gal1/7/10Δ::GAL3p-NpgA-FBA1t+GAL7p-MmCAR-ADH1t gal80Δ::GAL10p-EcFd-TDH2t+GAL1p-EcFNR-CYC1t ura3Δ::loxP-kanMX-loxP+TEF1p-ScFAS1-ADH1t+TPI1p-ScFAS2(G1250S, M1251W, ScACP-'AcTesA)-FBA1t*	(5)
ZW2071	*MATα MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ ura3Δ::loxP-kanMX-loxP+TEF1p-ScFAS1-ADH1t+TPI1p-ScFAS2(G1250S, M1251W, ScACP-'AcTesA)-FBA1t*	(5)
ZWE24	***Evolved strain with higher tolerance to octanoic acid,*** *MATa MAL2-8c SUC2 his3Δ1 ura3-52 pox1Δ XI-3::TEF1p-eCAS9-ADH1t X2:GAL1p-AnACLa-CYC1t+GAL10p-AnACLb-ADH1t gal80Δ::TDH3p-RPS25Ai-FAS2(ACP-AcTesA, KS*)-FBA1t FAS1pΔ::eTDH3p-RPS25Ai*	(4)
ZWE243	***ZWE24*** *hfd1Δ::TPI1p-MmACL-FBA1t+TDH3p-RtME-CYC1t+tHXT7p-'MDH3-TDH2t+PGK1p-CTP1-ADH1t*	This study
YH28	***ZWE243*** *tpo1Δ*	This study

Supplementary Table S3. List of plasmids used in this study.

Plasmids	Description	References
pZWM	*2μ, Amp^R, KlURA3*	(13)
pZW01	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-ADH1t, TPI1p-NpgA-FBA1t*	This study
pZW02	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-ADH1t, TPI1p-Sfp1-FBA1t*	This study
pZW03	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-ADH1t, TPI1p-PptA-FBA1t*	This study
pZW04	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-ADH1t, TPI1p-EcAcpS-FBA1t*	This study
pZW05	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-ADH1t, TPI1p-MvAcpS-FBA1t*	This study
M150	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (D241E, H454R, L567M)-ADH1t, TPI1p-NpgA-FBA1t*	This study
M7	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (Q151R, D241E, M293L, H454R, L567M, F933Y)-ADH1t, TPI1p-NpgA-FBA1t*	This study
M9	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (D241E, H454R, D482V, E566D, L567M, D879N)-ADH1t, TPI1p-NpgA-FBA1t*	This study
M11	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (P161H, A184S, D241E, H454R, L567M, S643T, H750R, K808N, P834L)-ADH1t, TPI1p-NpgA-FBA1t*	This study
AD69	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (Q182R, Q371R)-ADH1t, TPI1p-NpgA-FBA1t*	This study
AD10	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (E176G, Q182R, Q371R, F501Y)-ADH1t, TPI1p-NpgA-FBA1t*	This study
AD302	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (Q302W)-ADH1t, TPI1p-NpgA-FBA1t*	This study
AD303	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (I303W)-ADH1t, TPI1p-NpgA-FBA1t*	This study
RS01	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-ADH1t, TPI1p-NpgA-FBA1t, GPD1p-PCP-R_{NRP}-CYC1t*	This study
RS02	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-ADH1t, TPI1p-NpgA-FBA1t, GPD1p-PCP-R_{MxaA}-CYC1t*	This study
RS03	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-ADH1t, TPI1p-NpgA-FBA1t, GPD1p-PCP-R_{GPL}-CYC1t*	This study
RS011	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-GGGGS-PCPR_{NRP} -ADH1t, TPI1p-NpgA-FBA1t*	This study
RS021	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-GGGGS-PCPR_{MxaA} -ADH1t, TPI1p-NpgA-FBA1t*	This study
RS031	*2μ, Amp^R, KlURA3, TDH3p-MmCAR-GGGGS-PCPR_{GPL} -ADH1t, TPI1p-NpgA-FBA1t*	This study
RF1	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (C983P, D984H, M985L)-ADH1t, TPI1p-NpgA-FBA1t*	This study
RE5	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (C983A, D984N, M985L)-ADH1t, TPI1p-NpgA-FBA1t*	This study
RE6	*2μ, Amp^R, KlURA3, TDH3p-MmCAR (C983I, D984A, M985I)-ADH1t, TPI1p-NpgA-FBA1t*	This study

RB6	*2µ, Amp^R, KlURA3, TDH3p-MmCAR (C983F, D984T, M985L)-ADH1t, TPI1p-NpgA-FBA1t*	This study
M985L	*2µ, Amp^R, KlURA3, TDH3p-MmCAR (M985L)-ADH1t, TPI1p-NpgA-FBA1t*	This study
RF1w/o985	*2µ, Amp^R, KlURA3, TDH3p-MmCAR (C983P, D984H)-ADH1t, TPI1p-NpgA-FBA1t*	This study
M150+303	*2µ, Amp^R, KlURA3, TDH3p-MmCAR (D241E, H454R, L567M, I303W)-ADH1t, TPI1p-NpgA-FBA1t*	This study
RS011+303	*2µ, Amp^R, KlURA3, TDH3p-MmCAR (I303W)-GGGGS-PCPR$_{NRP}$ -ADH1t, TPI1p-NpgA-FBA1t*	This study
RF1+303	*2µ, Amp^R, KlURA3, TDH3p-MmCAR (C983P, D984H, M985L, I303W)-ADH1t, TPI1p-NpgA-FBA1t*	This study

Supplementary Table S4. List of the primers used in this study.

Primers	Sequences (5´-3´)
Primers for the site-mutation plasmids construction	
CAR-Q302W-F	CCATACAAGATCCATCTACCCATAAC
CAR-Q302W-R	TGGATCTTGTATGGTACTTTATGCAAC
CAR-I303W-F	CCATACAACCATTGTCTACCCATAAC
CAR-I303W-R	GACAATGGTTGTATGGTACTTTATGC
CAR-M985A-F	GTATCTGCCAAAATAGCGTCGCATCTAAAGACAGCAACTGG
CAR-M985A-R	CTTTAGATGCGACGCTATTTTGGCAG
CAR-M985L-F	AAAATTAAGTCGCATCTAAAGACAGCAACTGGTAAAC
CAR-M985L-R	GATGCGACTTAATTTTGGCAG
MmCAR-F2	ATTCATCAGCTTGACCGTGGGC
MmCAR-R2	AAGCCTTGGATGCTGTTCCTG
Primers for didomain expression	
FBA1t-R	AGTAAGCTACTATGAAAGACTTTAC
FBA1t-GPD-F	GTCTTTCATAGTAGCTTACTAGTTTATCATTATCAATACTCGCC
GPD-R	ATCCGTCGAAACTAAGTTCTGG
GPD-4DQVPCP-F	AGAACTTAGTTTCGACGGATATGGCTCCAGCAGGTCCAGTTG
CYC1t-4DQV-R	GCGTGACATAACTAATTACATGATTATAACAAACCTAACAATTGC
CYC1t-F	TCATGTAATTAGTTATGTCACGC
CYC1-ADH1t-F	GAAGGCTTTAATTTGCGGCCGCATATCTACAATTGGGTGAAATGG
CYC1t-R	GGCCGCAAATTAAAGCCTTC
GPD-4U7WPCP-F	CAGAACTTAGTTTCGACGGATATGGCACCAAGAACTCCAGG
CYC1t-4U7W-R	GCGTGACATAACTAATTACATGATTATTCTGGAGCTTTCAAGAAACC
GPD-GPLPCP-F	GAACTTAGTTTCGACGGATATGGCTCCAGAAACTGAAACAG
CYC1t-GPL-R	GCGTGACATAACTAATTACATGATTACAACAAACCTAATAATTGC
KlURA3-R	CGTGGATCTATATCACGTGATTTGC
KlURA3-F	GCAAATCACGTGATATAGATCCACG
ADH1t-CYC1t-R	TCACCCAATTGTAGATATGCGGCCGCAAATTAAAGCCTTCG
ADH1t-F2	GCATATCTACAATTGGGTGAAATG

ADH1t-R	GCGAATTTCTTATGATTTATG
ADH1t-4DQV-F	TAAATCATAAGAAATTCGCTTATAACAAACCTAACAATTGC
CAR-G4S-PCP4DQV-R	GATTTGAGATTGTTGGGTTTGTTGGGTGGCGGTGGATCTGCTCCAGCAGGTCCAGTTG
fusionCAR-F	CAACAAACCCAACAATCTCAAATCTG
ADH1t-4U7W-F	CATAAATCATAAGAAATTCGCTTATTCTGGAGCTTTCAAGAAACC
CAR-G4S-PCP4U7W-R	GAGATTGTTGGGTTTGTTGGGTGGCGGTGGATCTGCACCAAGAACTCCAGGTGAAG
ADH1t-GPL-F	ATAAATCATAAGAAATTCGCTTACAACAAACCTAATAATTGC
CAR-G4S-PCPGPL-R	GAGATTGTTGGGTTTGTTGGGTGGCGGTGGATCTGCTCCAGAAACTGAAACAG
Random mutagenesis library for the full-length MmCAR (round I and II)	
MmCARwm-F	CTTATTTTTTTTATAACTTATTTAATAATAAAAATCATAAATCATAAGAAATTCGCTTACAACAAACCCAACAATCTC
MmCARwm-R	GTTTTAAAACACCAAGAACTTAGTTTCGAATAAACACACATAAACAAACAAAATGTCACCTATCACCAGAGAAG
pZWM-TPI1p-F	CATCGTCCTCTCGAAAGGTGGATCTACGTATGGTCATTCT
ADH1t-R-CAR	GCGAATTTCTTATGATTTATG
CAR-F-TDH3	TTTGTTTGTTTATGTGTGTTTATTCGAAAC
pZWM-PR	CACCTTTCGAGAGGACGATG
Random mutagenesis for A domain of MmCAR (round I and II)	
CAR-A-F	CAATCTGGCTTTCAATTCTTCGACAGGAACAGCATCCAAGGCTTCTTGTGTTGGTACTATGACTGCTAACAAATAAGCTCTGGCTGAATTACCGTATATATATATTTGTCTAACTAATGG
CAR-A-F	ATGCTGATAGACCTGCTTTGGCACAAAGATCAGTAGAATTTGTTACAGATGCAGGTACTGGTCATACTACATTGAGATTGTTACCACACTTCGAAACTATCTCTTACGGTGAATTATGGG
CAR-12712-F	AGTTTCGAAGTGTGGTAACAATC
pZWM-PR	CACCTTTCGAGAGGACGATG
pZWM-TPI1p-F	CATCGTCCTCTCGAAAGGTGGATCTACGTATGGTCATTCT
CAR-11349-R	AATTCAGCCAGAGCTTATTTGTTAGCAGTC

Primers for saturation site-directed mutagenesis library	
983-SaMu-F	CCAGGTTGTATCTGCCAAAATNNNNNNNNNTCTAAAGACAGCAACTGG
pZWM-TDH3p-R	CATCAGGTGGTCATGGCCCTTCTCGAGTTTATCATTATCAA
pZWM-TPI1p-F	CATCGTCCTCTCGAAAGGTGGATCTACGTATGGTCATTCT
983-SaMu-R	ATTTTGGCAGATACAACCTGGGCCGG
pZWM-PR	CACCTTTCGAGAGGACGATG
pZWM-PF	AAGGGCCATGACCACCTGATG

Supplementary Table S5. The codon-optimized sequences used in this study.

Genes	Sequences (5´-3´)
PCP-R_{GPL}	GCTCCAGAAACTGAAACAGAAAAGACTTTAGCTGGTATTTTTGCACATGTTTTGGGTGTTG AAAGAATCGGTTTGGATGATTCTTTCTTTGATTTGGGTGGTGACTCTATTTCAGCAATGAGA GTTACTGCTGCAGTTAATACAGCTTTGGATGCAGATTTGTCTGTTAGAACTTTGTTTGAAGC TCCAACAGTTGCAAAATTGTCATTACATGTTGAACATGATGCTGGTGAAAGAACAGATAGA GTTTCTTTTGCTGCAGTTCATGGTTCAGATGTTACTGAAGTTCATGCAAGAGATTTGACATT GGATAAGTTTATTGATGCTCCAACTTTGAGAACAGCAACTACATTACCAAGACCAGATGGT GCTGTACAAACAGTTTTATTAACAGGTGCCACAGGTTTCTTGGGTAGATACTTATTATTGGA ATGGTTGAGACAATTGAGAAGAGTTGACGATAAAGTTATTTGTTTGGTTAGAGGTAAATCT GATGAAGATGCTAGAAGAAGATTAGAAGCAACTTTCGATACAGATCCATTGTTGAGAAAGC ATTTCAACGAATTGGCTACTGAAAGATTACAAGTTGTTGCAGGTGACAAAGGTCAACCAAA TTTGGGTTTAGATGAACAAACATGGCAAAGATTGGCTGAATCTGTTGATTTGATTGTTGATT CAGCTGCATTTGTTAATTCTGTTTTGCCATACTCAGAATTGTTCGGTCCAAACGTTGTTGGT ACTGCTGAATTGATTAGATTTGCATTGACATCAAAGTTGAAGCCTTTTAATTTTGTTTCTACT TCAGATGTTGGTAGACAAATTGAACCATCTAGATTCACTGAACAAGCTGATATCAGATTGG TTTCAGCAACAAGAAAGATCGAAGTTGGTTACGCTAACGGTTACGGTAATTCTAAATGGGC TGGTGAAGTTTTGTTAAGAGAAGCACATGATCATTGTGGTTTACCAGTTGCTGTTTTTAGAT CTGGTATGATTATGGTTGATCCAACTTATGCAGGTCAATTGAACGTTACTGATACAGTTTCT AGAATGGTTTTGTCAATCGTTGCTACAGGTGTTGCACCAGGTTCTTTTTACCAAAGAGGTG ACAATGGTGAAAGACAAAGAGCTCATTTTGATGGTTTGCCAGTTGATTTCGTTGCTCAAGC AATCACAAAGTTAGGTTGGCAAGTTGCTAGATCAGTTACTGATTCTACAGCATCAGGTTTT GAAACTTACCATGTTATGAATCCACATGATGATGGTATCGGTATCGATACATACATCGATT GGTTGATTGAAGCTGGTTACCCAATCGAAAGAATCGAAGATTTCGGTGAATGGTTACAAAG ATTTGAAGCTGCATTGCAAGGTTTATCTGATCAACAAAGACAAAATTCAGTTTTGCAAATGT TGACTTTGTTGAAGCAACAAGCTGGTGAATTACAACCACCAGTTCCAACAAGAGGTTCTTT TGCTCCAGCAGATAGATTTCAAGCTGCAGTTAGAGATGCAAATATTGGTGTTGAAGGTGAA ATTCCACATGTCACCAGAGAAGTCATCGTCAAGTATGTCACAGATTTGCAATTATTAGGTTT GTTG
PCP-R_{MxaA}	GCACCAAGAACTCCAGGTGAAGAATCTTTAGCTGCAATTTGGAGACAAGTTTTGGGTGTTG AACAAATCGGTGCTCATGATAATTTCTTTGAATTAGGTGGTCATTCATTGTTGTTGTACAGA GTTTTGGTTTTGGCTAGATCTGCATCAGGTGCTGATATTCCATTAAGAGCATTATTGCAAG CTCCAACTTTGGAAGAAATGGCTAGAGCAGTTGAAGCTGCAAAAACTGGTTCTTTACCAGC TCATGATGTTACAGTTGAAATGGAAGCTGATGCAGTTTTGGATGCAGAAATTGCTTTGGGT AAAGCATTGCCACCAGTTACAGGTGCTTTGAGAACAATTTTGTTAACTGGTGCTACAGGTT TCTTGGGTGCATTTTTGTTGGAAGAATTGTGTAGAAGAACAGATGCTAGAATCTATTGTTTA GTTAGATCTAAGACTGAACAAGAGGGTATGAACAGAATCAGAAAGAATTTGGAATCTTACT CATTGTGGAACGAAGCTTTGGCACCAAGAATTGTTCCAGTTAGAGGTGACATTGGTCAAC CATTGTTGGGTTTGTCTGAAAAGGAATTCCAAAGATTGTCAGAAGAAATCGATGCTATCTA TCATAACGGTGCATTGGTTAATTTCTTGTACCCATACGAATCTATGAGAGCTGCAAATGTTT TGGGTACAAGAGAAATCTTGAGATTGGCTACTAGAACAAGAATTAAACCATTGCATTACGT TTCTACTGTTTCAGTTTTGCCATTAGGTAGAAAAGCACCAATTAGAGAAGATGAACCATTAG AAGGTCCATCTTCATTGGTTGGTGGTTACGCTCAATCTAAATGGGTTGCAGAAAAATTGGT TAGAGAAGCTTCAAGAAGAGGTTTACCAGTTACAATTTTGAGACCAGGTAGAGTTACTGGT CATTCTAGAACAGGTGCATGGAATACTGATGATTTGGTTTGTAGAACATTGAAAGGTTGTG TTAGAATGGGTGTTGCTCCATCAGTTGATGCATTGTTAGATTTGACTCCAGTTGATTACGTT TCTTCAGCTATCGTTGATTTGTCTATGAGACCAGAATCAATTGGTCAAACTTACCATTTGGT TAATCCACAATTTGTTAGAGCTGATGAAATGTGGAATTATATGAGAGCATTTGGTTACGGTT TGAGAGTTTTACCATATGATCAATGGTTGTCTGAATTAGGTTCAGCTGCATCTTCAGATTCT GAATTGGGTGACTTGTTGATGTTCTTGCAACAAGTTCCACCAGAAGATAGATCTGTTGGTG GTCCAAGAATGGTTGTTTGTGATTCAGGTGACACATTGAAAGCTTTAGGTGGTACTGGTAC ATCTTGTCCATCAGTTGATGCATCTTTGATCTCAACTTACTTGTCTTCATTGGTTCATAGAG GTTTCTTGAAAGCTCCAGAA
PCP-R_{NRP}	GCTCCAGCAGGTCCAGTTGAAAAGACTGTTGCTGGTATTTTTGCAAGAGTTTTGGGTTTAG AAAGAGTTGGTGTTGATGATTCTTTCTTTGAATTGGGTGGTGACTCATTAGCTGCAATGAG AGTTATTGCTGCAATTAATACTACATTGAACGCTGATTTGCCAGTTAGAGCTTTGTTACATG CATCTTCAACTAGAGGTTTGTCTCAATTGTTAGGTAGAGATGCTAGACCAACATCTGATCC AAGATTAGTTTCAGTTCATGGTGACAATCCAACTGAAGTTCATGCTTCTGATTTGACATTGG ATAGATTCATTGATGCAGATACTTTGGCTACAGCAGTTAATTTGCCAGGTCCATCACCAGA

	ATTGAGAACTGTTTTGTTAACTGGTGCTACAGGTTTCTTGGGTAGATATTTGGTTTTGGAAT TGTTGAGAAGATTGGATGTTGATGGTAGATTGATCTGTTTAGTTAGAGCAGAATCTGATGA AGATGCTAGAAGAAGATTGGAAAAGACTTTCGATTCAGGTGACCCAGAATTGTTGAGACAT TTCAAGGAATTGGCTGCAGATAGATTAGAAGTTGTTGCTGGTGACAAATCTGAACCAGATT TGGGTTTAGATCAACCAATGTGGAGAAGATTGGCAGAAACTGTTGATTTGATTGTTGATTC AGCTGCAATGGTTAACGCTTTCCCATACCATGAATTGTTCGGTCCAAATGTTGCAGGTACA GCTGAATTGATCAGAATCGCATTGACTACAAAGTTAAAACCTTTTACTTACGTTTCTACAGC TGATGTTGGTGCTGCAATTGAACCATCAGCTTTTACTGAAGATGCTGATATCAGAGTTATTT CTCCAACTAGAACAGTTGATGGTGGTTGGGCTGGTGGTTATGGTACATCAAAATGGGCAG GTGAAGTTTTGTTGAGAGAAGCTAACGATTTGTGTGCATTACCAGTTGCTGTTTTTAGATGT GGTATGATTTTGGCAGATACTTCTTACGCTGGTCAATTGAACATGTCAGATTGGGTTACTA GAATGGTTTTGTCTTTAATGGCAACAGGTATTGCTCCAAGATCTTTTTACGAACCAGATTCA GAAGGTAATAGACAAAGAGCTCATTTTGATGGTTTACCAGTTACTTTTGTTGCTGAAGCAAT TGCTGTTTTGGGTGCAAGAGTTGCTGGTTCTTCATTAGCAGGTTTTGCTACATACCATGTT ATGAATCCACATGATGATGGTATTGGTTTGGATGAATACGTTGATTGGTTGATCGAAGCAG GTTACCCAATCAGAAGAATCGATGATTTCGCTGAATGGTTACAAAGATTTGAAGCATCTTT GGGTGCTTTACCAGATAGACAAAGAAGACATTCAGTTTTGCCAATGTTGTTGGCATCTAAC TCACAAAGATTGCAACCATTGAAACCAACTAGAGGTTGTTCTGCTCCAACAGATAGATTCA GAGCTGCAGTTAGAGCTGCAAAAGTTGGTTCTGATAAGGATAACCCAGATATCCCACATGT TTCAGCTCCAACTATTATTAACTACGTTACAAATTTGCAATTGTTAGGTTTGTTA
NpgA	ATGGTGCAAGACACATCAAGCGCAAGCACTTCGCCAATTTTAACAAGATGGTACATCGACA CCCGCCCTCTAACCGCCTCAACAGCAGCCCTTCCTCTCCTTGAAACCCTCCAGCCCGCTG ATCAAATCTCCGTCCAAAAATACTACCATCTGAAGGATAAACACATGTCTCTCGCCTCTAAT CTGCTCAAATACCTCTTCGTCCACCGAAACTGTCGCATCCCCTGGTCTTCAATCGTGATCT CTCGAACCCCAGATCCGCACAGACGACCATGCTATATTCCACCCTCAGGCTCACAGGAAG ACAGCTTCAAAGACGGATATACCGGCATCAACGTTGAGTTCAACGTCAGCCACCAAGCCT CAATGGTCGCGATCGCGGGAACAGCTTTTACTCCCAATAGTGGTGGGGACAGCAAACTCA AACCCGAAGTCGGAATTGATATTACGTGCGTAAACGAGCGGCAGGGACGGAACGGGGAA GAGCGGAGCCTGGAATCGCTACGTCAATATATTGATATATTCTCGGAAGTGTTTTCCACTG CAGAGATGGCCAATATAAGGAGGTTAGATGGAGTCTCATCATCCTCACTGTCTGCTGATC GTCTTGTGGACTACGGGTACAGACTCTTCTACACTTACTGGGCGCTCAAAGAGGCGTATA TAAAAATGACTGGGGAGGCCCTCTTAGCACCGTGGTTACGGGAACTGGAATTCAGTAATG TCGTCGCCCCGGCCGCTGTTGCGGAGAGTGGGGATTCGGCTGGGGATTTCGGGGAGCC GTATACGGGTGTCAGGACGACTTTATATAAAAATCTCGTTGAGGATGTGAGGATTGAAGTT GCTGCTCTGGGCGGTGATTACCTATTTGCAACGGCTGCGAGGGGTGGTGGGATTGGAGC TAGTTCTAGACCAGGAGGTGGTCCAGACGGAAGTGGCATCCGAAGCCAGGATCCCTGGA GGCCTTTCAAGAAGTTAGATATAGAGCGAGATATCCAGCCCTGTGCGACTGGGGTGTGTA ATTGCCTATCCTAA
MvAcpS	ATGTTGCCAGTCGGTATAGTTGGTATTGGTATTGATTTGGTTTCTATCCCAGAATTTGCAGA ACAAGTAGACAGACCTGGTACTGTCTTTGCTGAAACTTTCACACCTGGTGAAAGAAGAGAT GCTGCTGATAAATCTTCATCCGCCGCTAGACATTTGGCAGCCAGATGGGCTGCAAAAGAA GCAGTAATTAAGGCCTGGAGTGGTTCAAGATTCGCCAAGAGACCAGTCTTACCTGAAGCT ATCCATAGAGATATCGAAGTTATTACAGACATGTGGGGTAGACCAAGAGTTAGATTGTCAG GTGCCGTAGCTGAACACTTAAAGGAAGTTACCATCCATTTGTCCTTAACCCACGAAGCTGA TACTGCTGCTGCT GTTGCAGTATTGGAAGAAAGATAA

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