Engineering Carboxylic Acid Reductase for Bio-based Chemical Synthesis

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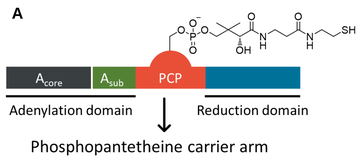
Background

Enzymes are biological catalysts that accelerate chemical transformations for life. Enzymes are also becoming essential in a wide range of industrial applications.[1]

Problem: Kinetic performance of an enzyme may limit its industrial use.

How to overcome the limitation? We can improve the catalytic properties and substrate scope of the enzyme via protein engineering.[3]

Carboxylic Acid Reductase (CAR)s (EC 1.2.1.30) are large multi-domain enzymes that can catalyze the ATP- and NADPH-dependent reduction of carboxylic acids into aldehydes. These enzymes consist of three domains: the adenylation domain (A-domain), the phosphopantetheine carrier protein domain (PCP-domain), and the reduction domain (R-domain).[1, 2]



CARs are **valuable tools for biosynthesis** due to the[1]:

- Broad substrate specificity
- Relaxed reaction conditions
- Domain modularity

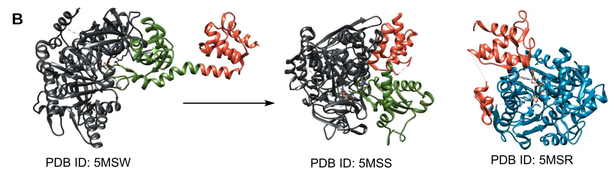
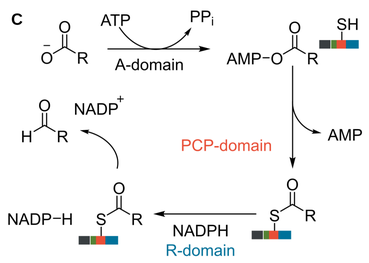


Figure 1. Structure and function of CARs. (A) Schematic representation of the structure of CAR. (B) Crystal structure of *Segniliparus rugosus* CAR (SrCAR). Color code: A-domain A_{core}, grey; A-domain A_{sub}, green; PCP-domain, red; R-domain, blue. (C) Overview of CAR catalytic mechanism.



Methods

We have developed a novel growth-coupled selection method for the engineering of NADPH-dependent enzymes, i.e., CARs.[3]

Host strain engineering: Genes *pgi* (G6P isomerase) and *sthA* (soluble transhydrogenase) were deleted from *E. coli* genome to construct R-Δ2.

CAR mutant library construction: Single-site saturation mutagenesis (SSM) libraries of CAR enzyme from *Mycobacterium avium* (MavCAR) were constructed. Ten sites were randomized with the NNK codon (N = A, C, T, or G, K = T or G, 32 variants at nucleotide level).

Growth-coupled selection of CAR mutants: A single SSM library was transformed into *E. coli* R-Δ2 containing the phosphopantetheinyl transferase-expressing plasmid. Cells were washed and plated on M9 glucose agar plates with carboxylic acid substrate. Colony formation and growth were monitored.

Activity assay: Kinetic assay was performed by monitoring the oxidation of NADPH at 340 nm at 25 °C.

Growth-Coupled NADPH Recycling Strategy

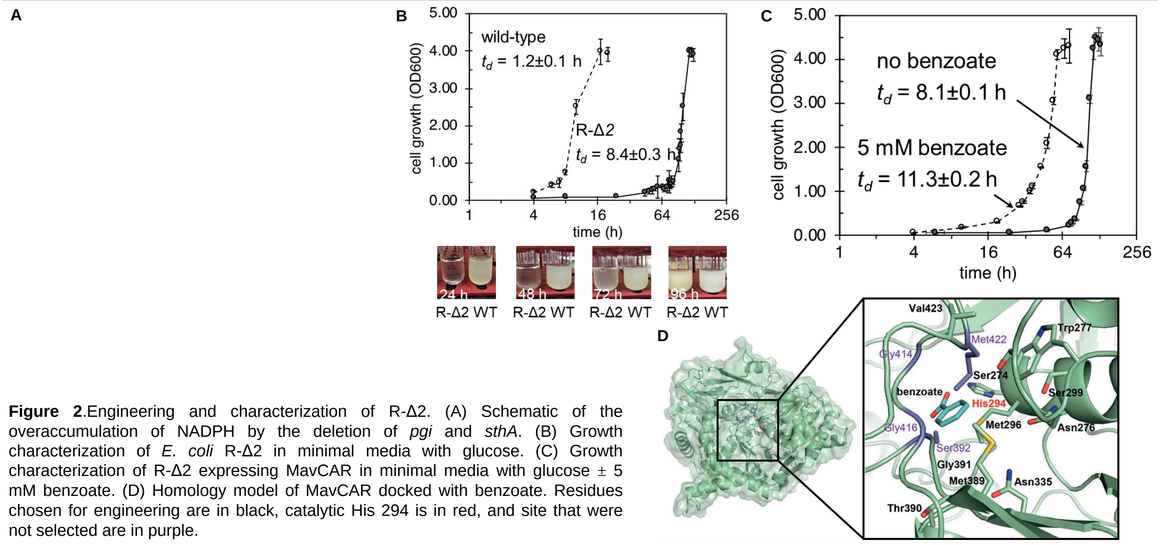


Figure 2.Engineering and characterization of R-Δ2. (A) Schematic of the overaccumulation of NADPH by the deletion of *pgi* and *sthA*. (B) Growth characterization of *E. coli* R-Δ2 in minimal media with glucose. (C) Growth characterization of R-Δ2 expressing MavCAR in minimal media with glucose ± 5 mM benzoate. (D) Homology model of MavCAR docked with benzoate. Residues chosen for engineering are in black, catalytic His 294 is in red, and site that were not selected are in purple.

Engineering of CAR toward Adipate

To demonstrate the feasibility of the selection strategy, the mutant libraries were selected on a less favorable substrate adipate. Six out of the ten libraries (N276, S299, N335, M389, T390, and G391) showed improved growth. All mutants had lower K_m values than wild-type, while N335R showed a 17-fold improvement in catalytic efficiency.[3]

Table 1. Kinetic characterization of MavCAR variants.

	Variant	K_m (mM)	K_{cat} (min^{-1})	K_{cat}/K_m (mM^{-1} min^{-1})
adipate	wild-type	62.0 ± 3.9	59.5 ± 1.6	1.0 ± 0.1
	N335R (8)	2.6 ± 0.1	44.9 ± 0.6	17.0 ± 1.0
	S299K (2)	4.4 ± 0.2	30.1 ± 0.3	6.9 ± 0.3
	M389K (8)	5.4 ± 0.3	32.3 ± 0.4	5.9 ± 0.4
	N276P (2)	11.1 ± 0.5	47.3 ± 0.5	4.2 ± 0.2
	N276R (1)	13.2 ± 0.5	49.3 ± 0.6	3.7 ± 0.2
	S299R (4)	13.3 ± 0.8	45.2 ± 0.9	3.4 ± 0.2
adipate semialdehyde	N335K	9.4 ± 1.3	18.0 ± 0.6	1.9 ± 0.3
	M389R	9.7 ± 0.7	33.2 ± 0.8	3.4 ± 0.2

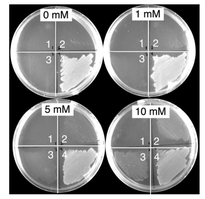


Figure 3. Growth characterization on solid media (1 % glucose) with different concentrations of 6-hydroxyhexanoate in R-Δ2 host. Empty vector, 1; MavCAR, 2; MavCAR-YahK, 3; Wild-type *E. coli*, 4.

In Vivo Bioconversion of Adipate to 1,6-Hexanediol

A whole-cell bioconversion of adipate to 1,6-hexanediol (1,6-HDO) was done using MavCAR variants and *E. coli* aldehyde reductase (EcYahK). After 24 h, different ratios between 6-hydroxyhexanoic acid (6-HHA) and 1,6-HDO were observed. These varying ratios were due to the different activities of the MavCAR variants toward adipate and 6-HHA. Notably, the N335R variant was able to convert nearly all of the adipate to the final product 1,6-HDO.[3]

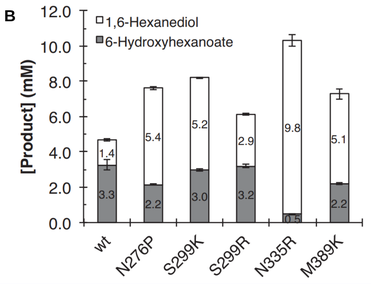


Figure 4. Whole-cell bioconversion of adipate to 6-hydroxyhexanoate and 1,6-hexanediol using MavCAR and EcYahK. (A) *In vivo* biocatalytic cascade for the synthesis of 1,6-HDO. (B) Bioconversion of 1,6-HDO from 10 mM adipate after 24 h. Reaction was performed in M9 minimal media (pH 7.0) containing 10 g/L glucose, 0.25 mM IPTG, 10 mM adipic acid, and appropriated antibiotics. Conversion of 6-HHA and 1,6-HDO was analyzed and quantified by HPLC.

Lactamization using MavCAR

Lactams are cyclic amides that possess significant industrial relevance for the synthesis of nylon, various plastics, and the amino acid lysine. However, their chemical production processes are intricate and fraught with hazardous working conditions, leading to the generation of substantial quantities of waste materials. The use of enzyme catalysis in lactam synthesis could potentially address these challenges, offering a greener and safer alternative.[4]

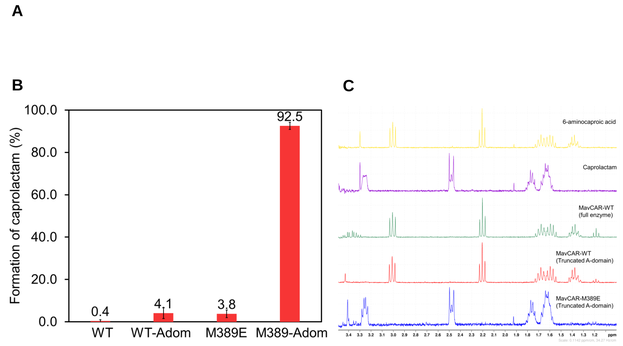


Figure 5. Biosynthesis of caprolactam by MavCAR. (A) Overview of the synthesis of caprolactam by CAR adenylation domain. (B) Biosynthesis of caprolactam by MavCAR-WT (WT), MavCAR-WT A-domain (WT-Adom), MavCAR-M389E (M389E), and MavCAR-M389E A-domain (M389E-Adom). (C) ^{1}H-NMR of the biosynthesis of caprolactam. Reaction was performed with 12 μM CAR, 5 mM 6-aminocaproic acid, 15 mM ATP, 10 mM $MgCl_2$, 1 μL *E. coli* Pyrophosphatase, in 50 mM Tris buffer pH 9.0 at 45 °C for 24 h.

Conclusion and Future Work

- The growth-coupled NADPH recycling strategy allows the identification of MavCAR variants with enhanced activity toward less favorable substrates.
- Selection results showed that five positions N276, M296, S299, N335, and M389 have important roles in substrate recognition.
- This method allows a high-throughput and accessible selection with a low rate of false positive variants.
- Truncated A domain of MavCAR-M389E catalyzed the formation of caprolactam from 6-aminocaproic acid.
- Future work will focus on improving the selection scheme and further engineering CARs to produce C6 industrial chemicals.

Acknowledgements

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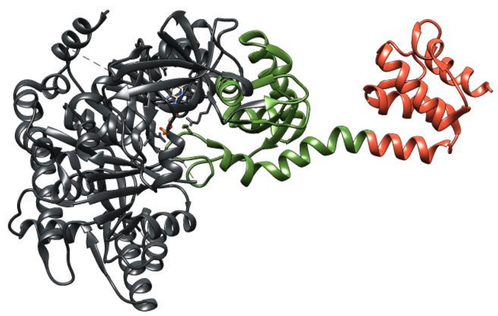

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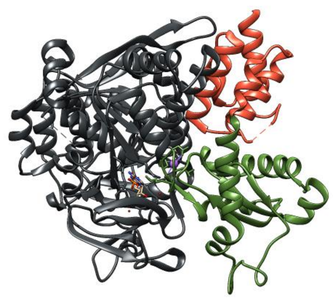

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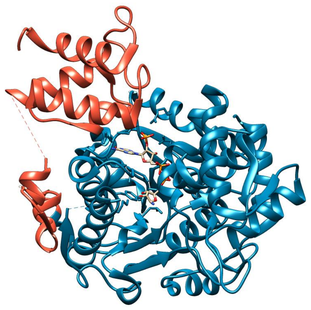

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