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Characterization of Carboxylic Acid Reductases for Biocatalytic Synthesis of Industrial Chemicals

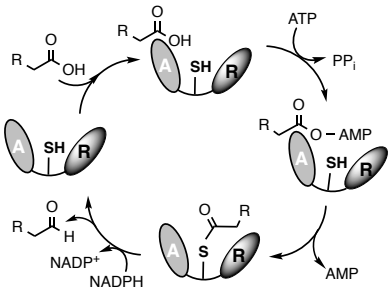
Levi Kramer,[a]† Erome Hankore, [b]† Yilan Liu,[a] Kun Liu,[b] Esteban Jimenez,[c] Jiantao Guo, [b]* and Wei Niu[a]*

Abstract: Carboxylic acid reductases (CARs) catalyze the reduction of a broad range of carboxylic acids into aldehydes, which can serve as common biosynthetic precursors to many industrial chemicals. This work presents a systematic biochemical characterization of five carboxylic acid reductases from different microorganisms, including two known and three new ones, using a panel of short-chain dicarboxylic acids and hydroxyacids, which are common cellular metabolites. All enzymes displayed broad substrate specificities. Higher catalytic efficiencies were observed when the carbon chain length of either the dicarboxylates or the terminal hydroxyacids was increased from C_2 to C_6. In addition, when substrates of the same carbon chain length are compared, carboxylic acid reductases favor hydroxyacids over dicarboxylates as their substrates. Whole-cell bioconversions of twelve carboxylic acid substrates into corresponding alcohols were investigated by coupling the CAR activity with an aldehyde reductase in *E. coli* hosts. Alcohol products were obtained at yields ranging from 0.5% to 71%. The *de novo* stereospecific biosynthesis of 1,2-propanediol isomers was successfully demonstrated with CAR being used as the key pathway enzyme. *E. coli* strains accumulated 7.0 mM *R*-1,2-PDO (1.0% yield) or 9.6 mM *S*-1,2-PDO (1.4% yield) from glucose. This study consolidates carboxylic acid reductases as promising enzymes for sustainable synthesis of industrial chemicals.

Introduction

Chemical industry faces sustainability challenges that arise from both the dwindling starting materials derived from fossil fuels and increasingly unaffordable environmental costs to the human society. As a promising solution to these problems, biocatalytic syntheses, either in the form of enzymatic conversion or microbial whole-cell synthesis, are increasingly explored as the alternate platform of chemical production.[1-2] Besides key advantages over chemical syntheses including reaction selectivity and smaller environmental impact, microbial syntheses use renewable feedstocks, such as plant-based cellulosic material, instead of hydrocarbon as the starting material. Long-term implementations of microbial syntheses therefore will inherently address many sustainability challenges that are facing the chemical industry.

Because commercial chemicals are often 'man-made' molecules, finding suitable enzymes are critical to achieving highly efficient and economical biosynthesis of these non-natural molecules.[3-5] The present work focuses on the use of carboxylic acid reductases (CARs) to catalyze the formation of common biosynthetic precursors for industrial chemical production. More specifically, facile reductions of carboxylic acids by CARs yield aldehydes, a class of common and versatile biosynthetic intermediates that can be readily transformed into target molecules by subsequent enzymatic reactions, such as decarbonylation, reduction, or transamination.[6-7] Aldehydes as biosynthetic intermediates bridge the functional group mismatch between short-chain aliphatic acid/diacid metabolites in naturally high-flux pathways and desirable biosynthetic targets. Current industrial production of ethanol by fermentation is an excellent example where an aldehyde (acetaldehyde) serves as a key intermediate.



Scheme 1. Catalytic mechanism of CAR enzymes. Abbreviations: A, adenylation domain; R, reduction domain; -SH, phosphopantetheinyl group.

Chemical reduction of a carboxylate to an aldehyde group is an energetically demanding reaction, which is also hard to control because of the low energy barrier for the further reduction of the aldehyde product into an alcohol. Three biological routes are currently known to accomplish this challenging transformation: (a) a single enzyme-catalyzed direct reduction;[8-9] (2) a two-enzyme cascade reaction through a coenzyme A thioester intermediate;[10-12] (3) a two-enzyme cascade reaction through a phosphoester intermediate.[13] In comparison to the two-enzyme systems, single enzyme-

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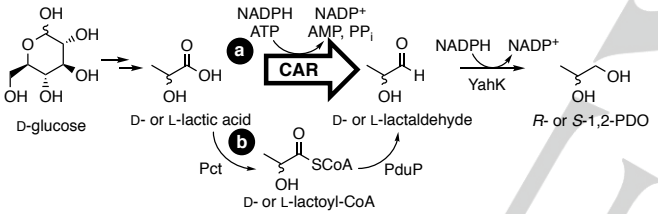
catalyzed reactions present a simpler and more practical option, even though the two-enzyme route (2) has been well explored in microbial production of energy and industrial molecules such as *n*-butanol and1,4-butanediol.[14-15] The CAR enzymes catalyze an irreversible reduction of a carboxylate through *in situ* activation and covalent linkage of the substrate to its posttranslationally added phosphopantetheinyl arm at the expense of an ATP, followed by the reduction of the resulting thioester using NADPH (Scheme 1).[9,16] The overall reaction sequence is orchestrated by three distinct domains of CAR, the adenylation (A) domain, the phosphopantetheinylation (PPT) domain, and the reduction (R) domain (Scheme 1). A recent structural biology study of CARs demonstrated the importance of domain dynamics during the catalytic cycle.[17] Although the native substrate is generally identified as benzoic acid, studies showed that CAR enzymes could tolerate a range of substituents of various properties on the phenyl ring.[18] More interestingly, CARs were reported to be active on aliphatic mono-, di- or tricarboxylic acid metabolites, such as citric acid and malic acid.[18-20] Recent studies also highlighted the use of a CAR from *Mycobacterium marinum* for the biosynthesis of alkanes as biofuel, due to its appreciable activity on C_4-C_{18} fatty acids.[21-22] Additional examples of incorporating CAR activities in (bio)syntheses were recently reviewed.[23-24]



Scheme 2. De novo biosynthesis of 1,2-PDO. a. Direct reduction of lactic acid by CAR; b. Two-enzyme reduction of lactic acid by Pct and PduP. CAR, carboxylic acid reductase; Pct, lactoyl-CoA transferase; PduP, CoA-acylating propionaldehyde dehydrogenase; YahK, lactaldehyde reductase.

Here, we report the identification and characterization of three new bacterial CAR enzymes with an emphasis on the potential application of CAR-catalyzed reactions in the biosynthesis of industrial chemicals. A panel of short-chain (C_2-C_6) dicarboxylic acids and hydroxyacids were used as substrates in full kinetic studies of the three new and two known CARs. We found that the carbon chain length of the substrates is the main determinant on the catalytic efficiency of CAR enzymes. A distinct activity difference between the dicarboxylate and the hydroxyacid of the same carbon chain length was also observed. The utility of CAR enzymes was demonstrated by *in vivo* bioconversions of the dicarboxylic acid and hydroxyacid substrates into corresponding alcohols using recombinant *E. coli* strains that overexpressed a CAR and a broad substrate specificity aldehyde reductase. We further integrated the CAR-catalyzed reaction into a *de novo* microbial biosynthesis of 1,2-propanediol (1,2-PDO), which is a bulk industrial chemical with a global demand of around 3 billion lb/year.[25] 1,2-PDO is mainly used in the production of unsaturated polyester resin and as deicer in anti-freeze products. Due to its unique solubility for non-water-soluble molecules and its GRAS statue, 1,2-PDO is also widely used in formulations of food, cosmetics, and medicines. Expression of the CAR enzyme in engineered *E. coli* host strains that are capable of homo-lactate fermentation led to the conversion of lactic acids into corresponding aldehydes, which were further reduced to 1,2-PDOs. The implementation of the CAR enzyme replaced a two-enzyme reaction sequence (path b in Scheme 2) with a single-step direct reaction (path a in Scheme 2) in a previously reported artificial pathway.[26] Under fermenter-controlled microaerobic conditions, *R*-1,2-PDO was accumulated at 7.0 mM and *S*-1,2-PDO was accumulated at 9.6 mM. As a proof-of-concept investigation, this report demonstrates a *de novo* biosynthesis of an industrially important chemical using a CAR enzyme. Further improving CAR activity through protein engineering will likely enable a biocatalytic process that is complementary to the current commercial route of 1,2-PDO production (high pressure, high temperature, noncatalytic hydrolysis of propylene oxide)[27] in feedstock selection.

Results and Discussion

Identification of New CAR Enzymes.

Biochemical and structural characterization of CAR enzymes from *Nocardia iowensis* (NiCAR), *Mycobacterium marinum* (MmCAR), and *Segniliparus rugosus* (SrCAR) have been reported.[17,20-21] Based on their protein sequences, a phylogenetic tree was constructed from potential CAR enzymes that were identified in the GenBank database using BLAST search (Figure S1 of Supporting Information). Three CAR candidates from *Kutzneria albida* (KaCAR), *Mycobacterium aromaticivorans* (MarCAR), and *Mycobacterium avium K-10* (MavCAR), which have no previous literature precedent, were chosen for further biochemical analysis. Phylogenetic analysis shows that the three *Mycobacterium* CARs form one clad, while the KaCAR and NiCAR form another clad (Figure 1). The two clads are evolutionarily more closely related than to the SrCAR.

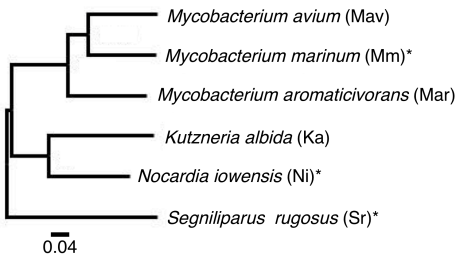


Figure 1. The phylogeny is constructed using MEGA7.0.[28] * The structure of the adenylation and/or the reduction domain of the enzyme is available.

To evaluate their catalytic activities, each of the three potential CAR candidates (KaCAR, MarCAR, and MavCAR) was

expressed as fusion proteins with a C-terminal 6xHis affinity tag in *E. coli.* The phosphopantetheinyl transferase (Sfp) from either *Bacillus subtilis* or *Nocardia iowensis* was co-expressed to ensure efficient activation of CAR enzymes by phosphopantetheinylation. Activities of purified enzymes were assayed on benzoic acid, a commonly accepted CAR substrate (Figure 2). Reduction of the carboxylates were followed by monitoring the oxidation of NADPH at 340 nm.[9] Blank controls either without the carboxylic acid substrate or without the purified enzyme were included for each set of assay. Two known CARs (NiCAR and MmCAR) were also included in the experiment to help gauge the activities of the three candidate enzymes. As shown in Figure 2, all three candidate CARs could indeed catalyze the reduction of benzoic acid. They were confirmed as true carboxylic acid reductases. Among all the enzymes tested, the MavCAR displayed the highest catalytic activity, which was nearly six times more active than the previously characterized MmCAR and slightly more active than the best reported CAR from *Mycobacterium abscessus*.[29]

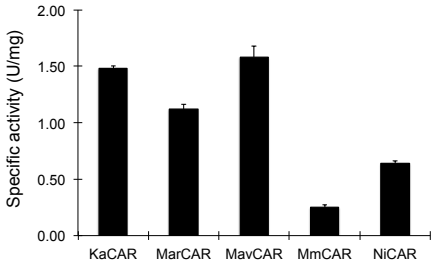


Figure 2. Specific activities of CAR candidates on benzoic acid at 25 °C and pH 7.5. Data are shown as the averages of three measurements. Error bars represent the standard deviations.

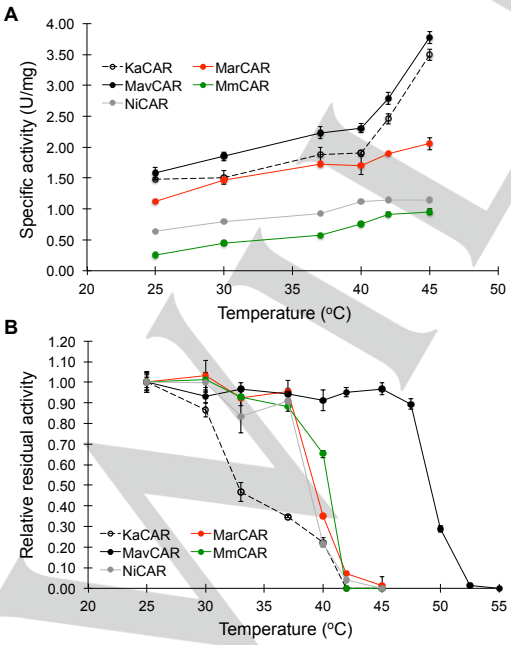


Figure 3. Effect of temperature on CAR variants. A. The specific activities of CARs at various reaction temperatures; B. The thermostability of CAR enzymes. The relative residual activity is calculated using specific activity of enzymes stored at 4 °C as the benchmark. Data are shown as the averages of three measurements. Error bars represent standard deviations.

Next, we examined how the temperature and the pH affected the activities of all five CAR enzymes with benzoic acid as the substrate. The temperature screen was performed at 25, 30, 37, 40, 42, and 45 °C in phosphate buffer (pH 7.5; Figure 3A). A general trend of temperature-dependent increase of activities was observed for all CARs. A more pronounced activity increase was detected for MavCAR and KaCAR between 40 and 45 °C. The thermostability of CARs was tested using enzymes that were incubated at indicated temperatures for 30 min (Figure 3B) followed by enzyme assays at room temperature under standard conditions. After treatment at 45 °C, all CAR enzymes except the MavCAR were completely inactivated, while KaCAR is the least thermally stable enzyme. The MavCAR retained over 90% of the activity following heating at 45 °C. It rapidly lost activity between 47.5 °C and 50 °C, and became inactive at 52.5 °C. The higher specific activity at elevated reaction temperature and the good thermostability make the MavCAR an attractive choice as a biocatalyst. The pH profiles of the CAR enzymes were tested between pH 5.5 and 9.0 at 25 °C. All enzymes were most active when the pH was near neutral. While MarCAR showed an optimum activity at pH 6.5, KaCAR and MmCAR displayed an optimum activity at pH 7.5 (Figure 4). Both MavCAR and NiCAR showed a broad optimum pH range between 6.5 and 7.5. Significant reductions in catalytic activity were observed at lower or higher pH values (Figure 4).

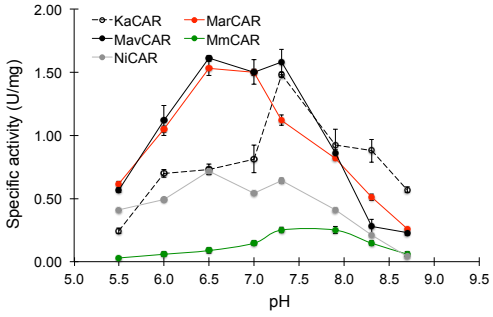


Figure 4. Effect of reaction pH on the activity of CAR variants. Data are shown as the averages of three measurements. Error bars represent standard deviations.

Kinetic Studies of CAR Enzymes on C_2-C_6 Dicarboxylic Acids and Hydroxyacids.

Short chain carboxylic acids, such as lactic acid and succinic acid, are common metabolites that can be accumulated as bioproducts. Further enzymatic reduction of the carboxylates leads to aldehyde products that can serve as precursors to various industrial chemicals, such as 1,2-propanediol and 1,4-butanediol. We therefore examined the kinetic properties of

NiCAR, MmCAR, KaCAR, MarCAR, and MavCAR by using a panel of C_2-C_6 dicarboxylic acid (Table 1) and hydroxyacid (Table 2) substrates. The straight-chain carbon skeleton of above molecules deviates significantly from the aromatic scaffold of benzoic acid, the native CAR substrate. In addition, a second hydrophilic functional group, carboxylate in dicarboxylic acids or hydroxyl group in hydroxyacids, exists in these substrates. Nevertheless, all the five CAR enzymes were able to tolerate the structural changes and showed broad substrate promiscuity to different extents, while benzoic acid remained as the preferred substrate.

Table 1. Kinetic studies of CARs on dicarboxylate substrates (C_2-C_6). [a.] CARs, 1, KaCAR; 2, NiCAR; 3, MmCAR; 4, MarCAR; 5, MavCAR. [b.] *n.d.*, Activity was not detected. Data are the average of three measurements with standard deviation.

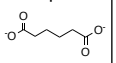
	CAR[a]	K_m (mM)[b]	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1}min^{-1})
oxalate 	1-5		*n.d.*	
malonate 	1-4		*n.d.*	
	5	160.5±49.7	1.5±0.2	0.009±0.003
succinate 	1-3		*n.d.*	
	4	281.3±84.3	5.9±0.8	0.02±0.01
	5	13.2±3.3	3.4±0.2	0.26±0.07
glutarate 	1	44.3±15.2	6.3±0.6	0.14±0.05
	2	314.2±172.3	16.8±4.4	0.05±0.03
	3	273.3±43.0	7.3±0.5	0.03±0.01
	4		*n.d.*	
	5	55.1±4.0	13.5±0.4	0.24±0.02
adipate 	1	46.4±10.7	15.6±1.3	0.34±0.08
	2	36.2±7.0	14.1±0.9	0.39±0.08
	3	31.6±2.2	10.9±0.2	0.34±0.02
	4	44.4±13.0	39.7±4.2	0.89±0.28
	5	3.7±0.6	13.5±0.4	3.64±0.6
benzoate 	1	0.24±0.06	173.6±7.9	731.7±188.8
	2	0.78±0.08	104.1±3.1	133.2±14.6
	3	0.30±0.06	35.5±1.4	118.0±25.7
	4	4.2±0.8	209.6±18.1	50.3±10.6
	5	1.4±0.2	242.1±14.0	162.1±26.8

Table 2. Kinetic studies of CARs on hydroxyacid substrates (C_2-C_6). [a.] CARs, 1, KaCAR; 2, NiCAR; 3, MmCAR; 4, MarCAR; 5, MavCAR. [b.] *n.d.*, Activity was not detected. Data are the average of three measurements with standard deviation.

	CAR[a]	K_m (mM)[b]	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1}min^{-1})
glycolate 	1	133.4±13.4	13.5±0.6	0.10±0.01
	2	163.7±48.7	4.5±0.5	0.03±0.01
	3	76.8±11.9	6.0±0.3	0.08±0.01
	4		*n.d.*	
	5	227.9±31.4	21.1±1.4	0.09±0.01
3-hydroxy propionate 	1	267.3±29.0	13.8±0.6	0.05±0.01
	2	143.6±22.4	19.7±1.1	0.14±0.02
	3	245.3±38.5	15.5±1.3	0.06±0.01
	4	111.5±19.1	34.4±2.4	0.31±0.06
	5	67.3±11.8	46.3±3.3	0.69±0.13
4-hydroxy butyrate 	1	61.0±6.1	66.7±2.6	1.1±0.1
	2	31.0±4.3	34.8±1.6	1.1±0.2
	3	66.9±6.5	23.9±0.9	0.36±0.04
	4	128.2±18.5	61.7±4.1	0.48±0.08
	5	16.0±2.1	42.6±1.7	2.7±0.4
5-hydroxy pantanoate 	1	26.6±4.9	132.5±10.0	5.0±1.0
	2	10.1±1.7	43.3±1.8	4.3±0.8
	3	39.9±4.0	33.8±1.3	0.85±0.09
	4	36.0±3.8	65.8±2.8	1.8±0.2
	5	12.8±1.8	59.3±2.7	4.6±0.7
6-hydroxy hexanoate 	1	11.7±1.2	109.2±4.1	9.3±1.0
	2	4.6±0.7	46.2±2.5	10.1±1.6
	3	22.9±3.3	27.3±1.7	1.2±0.2
	4	10.6±2.0	79.9±6.0	7.5±1.5
	5	10.0±1.4	93.5±3.3	9.3±1.4
L-lactate 	1	100.4±20.2	17.9±1.4	0.18±0.04
	2	165.6±64.3	5.5±0.9	0.03±0.01
	3	229.5±23.5	14.8±0.8	0.06±0.01
	4	340.2±71.8	30.0±3.7	0.09±0.02
	5	217.3±26.6	65.3±3.9	0.30±0.04
D-lactate 	1	100.3±21.7	4.6±0.3	0.05±0.01
	2	142.0±41.3	5.8±0.6	0.04±0.01
	3	240.9±51.8	5.6±0.6	0.02±0.01
	4	310.4±48.1	17.0±1.3	0.05±0.01
	5	162.9±23.3	23.4±1.5	0.14±0.02

Two general trends of CAR activity were observed: (1) When the carbon chain length of either the dicarboxylates (Table 1) or the terminal hydroxyacids (Table 2) was increased from C_2 to C_6, all CARs showed lower K_m and higher k_{cat} values, which correlates with an increase in catalytic efficiency. In an extreme case of C_2 dicarboxylate, oxalate, none of the CAR enzymes showed any detectable activity. The observed preference for substrates of longer carbon chain length resonates with previous report where fatty acids were examined as substrates of MmCAR.[21] (2) When substrates of the same carbon chain length were compared, all CARs favored hydroxyacids over

dicarboxylates as their substrates. This trend was more prominent for KaCAR, NiCAR, and MmCAR, which showed no detectable activity on any of the C_3 and C_4 dicarboxylic acids, but were still active on the corresponding hydroxyacids. Among all the examined enzymes, MavCAR showed the best activities on dicarboxylic acids and was the only CAR that could utilize malonate as the substrate. The MavCAR was also highly active on hydroxyacids, while KaCAR and NiCAR displayed appreciable activities on this group of substrates as well. We speculate that varied activities on dicarboxylate and hydroxyacid of the same chain length are resulted from the active site properties of CAR enzymes. With benzoic acid as the native substrate, a highly hydrophobic active site with limited ability to engage hydrogen bond or ionic interaction was observed for selected CARs.[17] In comparison to hydroxyl group, the negatively charged carboxylate group may lead to even greater unfavorable interaction(s). We also tested two non-terminal hydroxyacids, stereoisomers of lactic acid, and observed that all the examined CAR enzymes were active with slight preference to the L-isomer. The activities were generally lower than when 3-hydroxy propionate was used as the substrate.

Whole-Cell Reduction of Short-Chain Carboxylic Acids using MavCAR.

Activities of CAR enzymes on short-chain carboxylic acids were further investigated using *E. coli* strains that expressed MavCAR, which was shown to be the most promiscuous CAR variant. *E. coli* cells were cultured in media (pH 7.0) spiked with an indicated short-chain carboxylic acid of interest to the final concentration of 100 mM. Glucose was included in the media as the carbon source for cell growth and to supply ATP and NADPH as co-substrates for CAR activities.

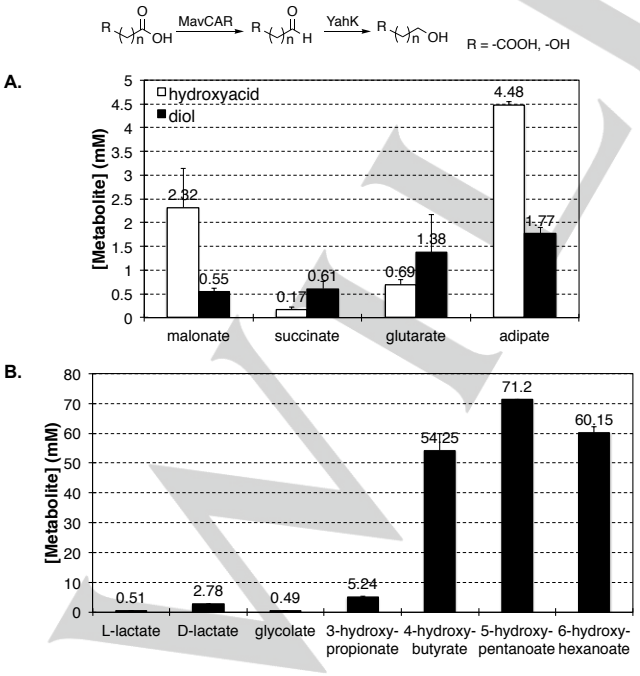


Figure 5. Reduction of short-chain carboxylates using *E. coli* cells expressing MavCAR and YahK. The x-axis represents the substrates spiked in the culture media. (A) Dicarboxylic acid substrates. (B) Hydroxyacid substrates. Cells were cultured in media (pH 7.0) containing 100 mM of indicated carboxylate. Data are the average of three measurements with standard deviation.

As an initial test, we examined the *in vivo* bioconversion of lactic acids into 1,2-PDO. A previously constructed *E. coli* strain PDO-Δ^4 was used.[26] This strain lacks the activities of D- and L-lactate dehydrogenases, the fermentative D-lactate dehydrogenase, and an alcohol dehydrogenase due to chromosomal deletions of *dld*, *lldD, ldhA*, and *adhE* genes. As a result, PDO-Δ^4 cannot use lactic acid isomers as the carbon source and is also incapable of producing lactic acid. It was used in previous report for the bioconversion of lactic acids into 1,2-propanediol through a CoA acylating pathway.[26] Since the genetic deletions of *ldhA* and *adhE* eliminated reactions that compete for reducing equivalent and reduced accumulation of byproducts, the PDO-Δ^4 strain is generally beneficial to the reduction reaction of other carboxylic acids. Therefore, the PDO-Δ^4 strain was employed in all of our *in vivo* bioconversion studies except for glycolic acid. The consumption of glycolic acid by *E. coli* significantly affected the bioconversion efficiency. To minimize the loss of glycolic acid to cellular metabolism, *E. coli* strain EG-Δ^4 was constructed and used in the bioconversion of glycolic acid to ethylene glycol. It was derived from MG1655 through the deletion of *glcD*, *gcl*, *aceB*, and *glcB* genes, which encodes the glycolate oxidase, glyoxylate carboligase, malate synthase A, and malate synthase G, respectively. Above manipulations abolish cells' ability to use glycolic acid as the carbon source. Preliminary experiment for 1,2-PDO synthesis from isomers of lactic acid also showed that the expression of a *yahK*-encoded broad substrate range aldehyde reductase was beneficial for the reduction of aldehydes into alcohols (Figure S5 of Supporting Information).[30] This enzyme was therefore overexpressed in all the whole-cell reduction experiments (Figure 5).

Suitable *E. coli* hosts were transformed with an F plasmid that encoded the Sfp protein and a pSC101-based plasmid, which contained a *MavCAR-yahK* gene cassette. Following 72 h of aerobic cultivation, metabolites in the media were analyzed by HPLC. For each of the C_3-C_6 dicarboxylic acid substrate, both the hydroxyacid, the product from the reduction of a single carboxylate, and the diol, the fully reduced products, were observed (Figure 5A). For each of the C_2-C_6 hydroxyacid substrate, the corresponding diol product was detected (Figure 5B). By using the formation of the alcohol product as a measure of the *in vivo* activity of MavCAR, a similar trend of substrate preference as that observed in the kinetic characterization was detected. With the increase of carbon chain length, higher yields of the corresponding alcohol products were obtained with either the dicarboxylic acid or the hydroxyacid substrates. In addition, when substrates of the same carbon chain length were used, significantly higher yields were obtained with hydroxyacid substrates. The only deviation from this general trend was the

higher bioconversion yield of malonate than those of its C_4 and C_5 analogs. It is possible that higher activity of YahK enzyme on malonate semialdehyde leads to faster overall kinetics of the bioconversion from malonate to 3-hydroxypropionate. Possible toxicity caused by the carboxylate substrates was evaluated by monitoring the cell growth (Figure S4 and Table S4 of Supporting Information). Although extended lag phase was observed in the presence of several compounds, minimal effect on the doubling time was observed (less than 32%) for all substrates except 3-hydroxypropionate. Products formed in the above whole-cell experiments are all industrial chemicals with varied market volumes and values. In particular, ethylene glycol (production of glycolate), 1,3-propanediol (product of malonate/3-hydroxypropionate), 1,4-butanediol (product of succinate/4-hydroxybutyrate), and 1,2-propanediol (product of lactates) are important monomers in polymer industry. The whole-cell bioconversion results further emphasized the great biosynthetic potential of CAR enzymes.

De novo Biosynthesis of 1,2-Propanediol (1,2-PDO) using MavCAR

We further demonstrated the utility of CAR enzymes in the *de novo* biosynthesis of 1,2-PDOs through the reduction of lactates. Two previously constructed host strains were used in the synthesis of each stereoisomer.[31] *E. coli*, PDO-Δ^7, which has chromosomal deletions of *dld*, *lldD*, *adhE*, *frdA*, *pflB*, *mgsA*, and *aldA* genes,[32-33] was used in the production of *R*-1,2-PDO through the reduction of D-lactic acid. *E. coli*, PDO-Δ^7*lldh*, which has an additional chromosomal modification by replacing the native *ldhA* gene with *lldh* gene from *Pediococcus acidilactici*, was used in the production of *S*-1,2-PDO through the reduction of L-lactic acid.[34] Each host was transformed with plasmids encoding MavCAR, YahK, and Sfp. Resulting strains were examined for 1,2-PDO synthesis from glucose under a fermentor-controlled two-phase cultivation scheme.[14] Following the initial aerobic phase of cell mass accumulation, expression of MavCAR and YahK was induced by IPTG addition. After an additional hour of growth, the airflow was reduced for microaerobic cultivation and the glucose feed was initiated. This point of transition was defined as t = 0 h. The microaerobic condition was chosen to enable the production of ATP through oxidative phosphorylation, while still allows the *de novo* biosynthesis of lactic acid as the substrate for the CAR enzyme. Sampling was done at indicated time points to monitor the cell growth and metabolites accumulation until the glucose concentration in the media was below 5 mM. The two *E. coli* strains produced 7.0 mM *R*-1,2-PDO from D-lactate at 22 h and 9.6 mM *S*-1,2-PDO from L-lactate at 48 h, which corresponds to 1.0% and 1.4% yield from glucose, respectively (Figure 6). Optical purity analysis showed that each isomer was produced at over 99% *ee* (Figure S6 of Supporting Information). In addition, the PDO-Δ^7 strain also accumulated 695 mM D-lactate, while the PDO-Δ^7*lldh* strain produced 407 mM L-lactate (Figure S7 and S8 of Supporting Information). Other metabolites, including pyruvate, acetate, and ethanol, were also detected (Figure S7 and S8 of Supporting Information). Since the two *E. coli* hosts lost the native pathway of ethanol biosynthesis because of the *adhE* deletion, a possible source of ethanol production is the reduction of acetate by CAR and YahK.

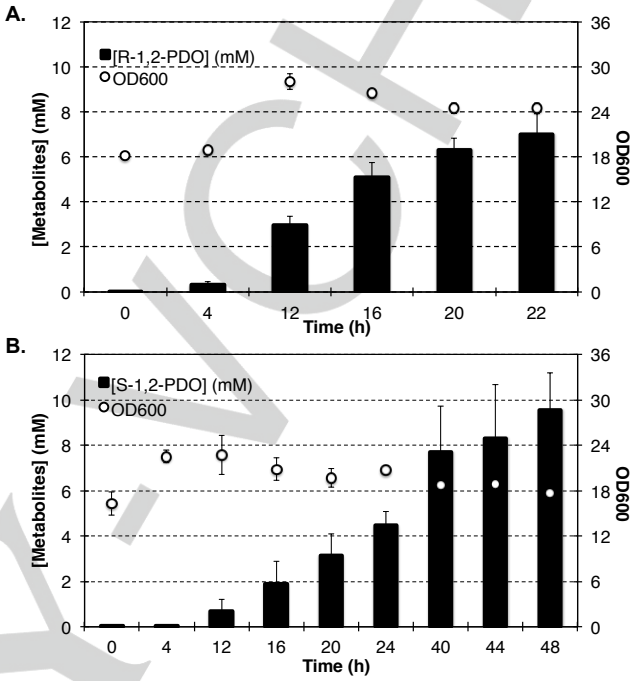


Figure 6. *De novo* biosynthesis of 1,2-PDO isomers by *E. coli*. (A) *R*-1,2-PDO accumulation by PDO-Δ^7 strain. (B) *S*-1,2-PDO accumulation by PDO-Δ^7*lldh* strain. Open circles, $OD_{600\ nm}$; filled bars, 1,2-PDO. Data are the average of two measurements with standard deviation.

Conclusions

Biocatalytic synthesis is becoming an indispensible choice for chemical productions. Fulfilment of its full potential requires relentless efforts into the discovery and the characterization of enzymes that can catalyze the transformation of common cellular metabolites into desirable industrial products. In this study, we characterized five CAR enzymes on a panel of C_2-C_6 short chain dicarboxylic acids and hydroxyacids, which can be synthesized by microbial hosts through endogenous or engineered pathways. The data showed that CAR enzymes were active on a broad range of substrates and could tolerate the presence of additional functional groups (i.e., hydroxyl and carboxyl) in the substrates. The carbon chain length is a major factor that influences the activity of CARs. Using the most promiscuous CAR variant from our study, we completed whole-cell bioconversions of dicarboxylic acid and hydroxyacid substrates into corresponding alcohols, including industrially important platform chemicals ethylene glycol, 1,2-propanediol, 1,3-propanediol, 1,4-butanediol, and 1,6-hexanediol. The utility of CAR enzyme in the *de novo* biosynthesis from a renewable starting material was further demonstrated through the production of *R*- and *S*-1,2-PDO stereoisomers from glucose by

engineered *E. coli* strains. The present work lays a solid foundation to employ CAR enzymes in biocatalytic syntheses. In light of recent advance in the structural studies of CARs, our efforts currently focus on protein engineering and pathway engineering to enable and optimize the *de novo* biosynthesis of industrial chemicals (e.g., ethylene glycol and propanediols). Overall, CARs' ability to catalyze a single-step reduction of acid metabolites positions them as promising enzymes for the sustainable synthesis of chemicals.

Experimental Section

General.

All chemicals are of reagent grade or higher. All solutions were prepared in deionized water that was further treated by Barnstead Nanopure® ultrapure water purification system (Thermo Fisher Scientific Inc). LB medium[35] (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). M9 salts (1 L) contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g), and NaCl (0.5 g). M9 glucose medium contained glucose (10 g), $MgSO_4$ (0.12 g), and thiamine hydrochloride (0.001 g) in 1 L of M9 salts. For whole-cell bioconversion experiments, dicarboxylic acid or hydroxyacid substrates were included in M9 glucose medium at a final concentration of 100 mM. The pH of the media was adjusted to 7.0 using KOH prior to inoculation. Fed-batch fermentation media (850 mL) contained Na_2HPO_4 (6.78 g), KH_2PO_4 (3 g), NH_4Cl (2 g), NaCl (0.5 g), and $(NH_4)_2SO_4$ (1 g). Antibiotics were added where appropriate to the following final concentrations: chloramphenicol (in methanol), 17 mg/L; kanamycin, 50 mg/L; ampicillin, 100 mg/L. Protein concentrations were determined using the Bio-Rad Bradford protein assay kit (Bio-Rad Laboratories Inc). A Biotek Synergy HTX platereader was used to record UV-vis spectra.

CAR Cloning and Protein Purification.

Standard protocols were used for the construction, purification, and analysis of plasmid DNA.[36] PCR amplifications were carried out using KOD HotStart DNA polymerase by following manufacture's protocol. Primer synthesis and DNA sequencing services were provided by Eurofins MWG Operon. Table S1 (see Supporting Information) lists the sequences of all the primers that were used in this study. Bacteria strains *Kutzneria albida* (ATCC25243), *Mycobacterium aromaticivorans* (ATCCBAA-1378), and *Bacillus subtilis* (ATCC6051) were purchased from American Type Culture Collection (ATCC). Strain *Nocardia iowensis* (DSM45197) was purchased from the German Collection of Microorganisms and Cell Cultures GmbH. All strains were cultured by following the venders' instructions. Genomic DNAs were isolated using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific Inc). The genomic DNAs of *Mycobacterium avium K-10* (ATCCBAA-968D) and *Mycobacterium Marinum* (ATCCBAA535D) were purchased from ATCC. The Sfp-encoding gene of *B. subtilis* or *N. iowensis* was cloned together with the PA1 promoter[37] into the *Xba*I site of plasmid pET30b (Novagen). Genes encoding CARs were amplified without the stop codon and cloned between the *Nde*I and *Xho*I sites of the modified pET30b vector. Table S2 of Supporting Information lists the all plasmids that were used in this study. Sequence-verified plasmids were transformed into protein expression host *E. coli* BL21(DE3). Following cultivation in LB media to the exponential growth phase, protein expression was induced with IPTG at 0.5 mM. CAR proteins with C-terminal Hisx6 tags were purified using Ni Sepharose 6 Fast Flow resin (GE Healthcare) by following manufacture's protocol. Purified proteins were dialyzed against buffer containing potassium phosphate (25 mM, pH 7.5), NaCl (150 mM), glycerol (5%) and DTT (1 mM) prior to biochemical assays. Purity of proteins was analyzed by SDS-PAGE (Figure S2 of Supporting Information).

Biochemical Assays.

All carboxylic acid substrates were purchased either as free acid or salt except 5-hydroxypentanoic acid and 4-hydroxybutyric acid, which were obtained from the hydrolysis of the commercially available corresponding lactones. CAR enzyme assays followed reported procedures at indicated temperature, pH, and substrate concentration.[9] The enzyme assay solution (0.15 mL) contained NADPH (0.15 mM), ATP (1 mM), $MgCl_2$ (10 mM), NaCl (150 mM), glycerol (5%), DTT (1 mM), appropriate amount of purified protein, and a substrate of interest. In 2-(N-morpholino)ethanesulfonic acid buffer (MES, 50 mM) with pH range of 5.5-6.5 and Tris-HCl buffer (50 mM) with pH range of 7.0-9.0 were used in the experiments to study the pH effects on CAR enzymes. All other assays were carried out in phosphate buffer (25 mM, pH 7.5). Sodium benzoate at a final concentration of 5 mM was used in assays where the specific activities (Figure 2), the temperature profiles (Figure 3A), the thermostability (Figure 3B), and the pH profiles (Figure 4) of CAR enzymes were examined. In thermostability studies, aliquots of an CAR enzyme were incubated at the indicated temperature for 30 min, then stored on ice for 5 min prior to experiments. Enzyme assays were initiated upon the addition of a substrate of interest. CAR activity was measured spectrophotometrically by following the oxidation of NADPH at 340 nm. A molar extinction coefficient of 6,220 $M^{-1}cm^{-1}$ (340 nm) was used for NADPH. Blank controls either without the carboxylic acid substrate or without the purified enzyme were included for each set of assay. Triplicate experiments were performed for each condition. Data of kinetics assays were analyzed using Prism 7 (GraphPad Software).

Whole-Cell Bioconversions.

E. coli EG-Δ^4 and PDO-Δ^4 were constructed following the PCR product-mediated gene deletion method using primers in Table S1 of Supporting Information.[38] The appropriate host was transformed with plasmid pZF-Sfp(Ni) and pZSC101-MavCAR-YahK (Table S2 of Supporting Information). For bioconversions, a single colony was inoculated in 5 mL LB media with appropriate antibiotics and cultured at 37 ºC overnight. A 100 μL of the overnight culture was transferred into 5 mL of M9 minimal salts media (pH 7.0) containing glucose (10 g/L), IPTG (0.25 mM), and a carboxylic acid substrate of interest (100 mM) in 30 mL culture tubes. Triplicate cultures of each strain were cultivated at 37 ºC with shaking at 250 rpm for 72 h. Products in cell-free broth were identified and quantified by HPLC based on previously established retention times (Table S3 of Supporting Information) and calibration curves using authentic standards. For products with low concentrations, including ethylene glycol and 1,4-butanediol, ^{1}H NMR was also used to confirm their formation.

***De novo* Biosynthesis.**

Fermentations were carried out in a 2.0 L working capacity UniVessel® controlled by a Biostat® B controller (Sartorius AG). Temperature and pH were controlled with proportional-integral-derivative (PID) control loops. The temperature was controlled at 37 °C. The pH was controlled at 7.0 by the addition of acid (2 N H_2SO_4) and base (concentrated NH_4OH at the first stage or 20% $NaHCO_3$ at the second stage). Dissolved oxygen was measured using an OxyFerm probe. Fed-batch fermentations were run in duplicate. Results were reported as the average of the two runs. A seed culture was started by the introduction of a single colony into 5 mL of M9 glucose medium. Culture was grown at 37 °C with shaking for 16 h and subsequently transferred into 95 mL of M9 glucose medium. The culture was grown under the same condition for an additional 10 h. A fermentation was initiated by transferring the seed culture (100 mL) into the fermentation vessel, which contained the fed-batch fermentation medium (850 mL) and 20 g of glucose (50 mL). The total initial volume of the fermentation culture was 1 L. A two-stage cultivation scheme was used. In the first stage, the D.O. was maintained at 10% air saturation through sequential ramping of impeller speed and airflow rate to the preset values of 1100 rpm and 1.0 cubic liter per minute, respectively. At this point, IPTG was added to a final concentration of 0.25 mM. Following an additional 1 h of cultivation, the microaerobic stage was initiated by reducing the airflow rate to 50 cubic centimeter per minute. At the same time, glucose solution (600 g/L) was fed into the fermenter at a rate of 4.17 g/h for 24 h. Throughout the second stage, the D.O. value registered as 0% air saturation. Samples were taken at indicated time points. Cell densities were determined by measurement of absorption at 600 nm (OD_{600}). Glucose concentration was monitored by HPLC. Cell-free broth was obtained for metabolite analysis. Cell pellets were lysed for protein expression analysis.

Analytical Methods.

An Agilent 1260 Infinity HPLC instrument (Agilent Technologies) equipped with an Aminex HPX-87H column (300x7.8 mm) (Bio-Rad Laboratories) was used for metabolite identification and quantification. To prepare samples for analysis, cell-free broth was mixed with internal standard (100 mM oxalic acid) at 95:5 (v/v) ratio, then filtered through a 0.2 μm PTFE syringe filter. A 10 μL of the prepared sample was injected. Samples were eluted at 45 °C with 5 mM sulfuric acid (0.6 mL/min). All the carboxylic acids were detected at 210 nm using a diode array detector. Alcohols and glucose were detected by a refractive index detector. Retention times of all analytes are shown in Table S3 of Supporting Information. ^{1}H NMR spectra were recorded on a Bruker Avance III-HD NMR Spectrometer (300 MHz) instrument. Samples were prepared by mixing cell-free broth with D_2O at a 9:1 (v/v) ratio. The sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP) was included in the D_2O as the internal standard for calibration. A solvent suppression program was applied to suppress the signal of water. The optical purity analysis of 1,2-PDO from the *de novo* biosynthesis was carried out following a previously reported procedure.[26]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: carboxylic acid reductase • biocatalysis • carboxylic acid • aldehyde • industrial chemical

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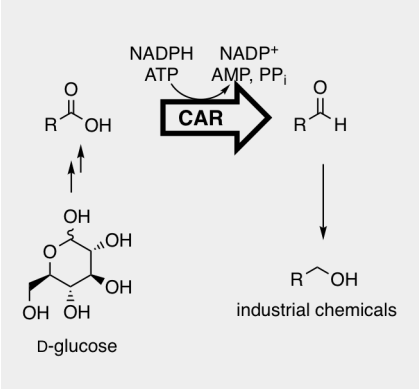
Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPER

Find a CAR: Carboxylic acid reductases (CARs) from different sources were fully characterized using a panel of short-chain dicarboxylic acids and hydroxyacids. Whole-cell bioconversions of twelve carboxylic acid substrates into corresponding alcohols were investigated by coupling the CAR activity with an aldehyde reductase in *E. coli* hosts. The de novo stereospecific biosynthesis of 1,2-propanediol isomers was successfully demonstrated.



Levi Kramer, Erome Hankore, Yilan Liu, Kun Liu, Esteban Jimenez, Jiantao Guo, and Wei Niu

Page No. – Page No.

Characterization of Carboxylic Acid Reductases for Biocatalytic Synthesis of Industrial Chemicals