Addition of formate dehydrogenase increases the production of renewable alkane from an engineered metabolic pathway

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An engineered metabolic pathway consisting of reactions that convert fatty acids to aldehydes and eventually alkanes would provide a means to produce biofuels from renewable energy sources. The enzyme aldehyde-deformylating oxygenase (ADO) catalyzes the conversion of aldehydes and oxygen to alkanes and formic acid and uses oxygen and a cellular reductant such as ferredoxin (Fd) as co-substrates. In this report, we aimed to increase ADO-mediated alkane production by converting an unused by-product, formate, to a reductant that can be used by ADO. We achieved this by including the gene (fdh), encoding formate dehydrogenase from Xanthobacter sp. 91 (XaFDH), into a metabolic pathway expressed in Escherichia coli. Using this approach, we could increase bacterial alkane production, resulting in a conversion yield of ~50%, the highest yield reported to date. Measuring intracellular nicotinamide concentrations, we found that E. coli cells harboring XaFDH have a significantly higher concentration of NADH and a higher NADH/NAD+ ratio than E. coli cells lacking XaFDH. In vitro analysis disclosed that ferredoxin (flavodoxin):NADP+ oxidoreductase could use NADH to reduce Fd and thus facilitate ADO-mediated alkane production. As formic acid can decrease the cellular pH, the addition of formate dehydrogenase could also maintain the cellular pH in the neutral range, which is more suitable for alkane production. We conclude that this simple, dual-pronged approach of increasing NAD(P)H and removing extra formic acid is efficient for increasing the production of renewable alkanes via synthetic biology-based approaches.

Fossil-derived hydrocarbons in the forms of natural gas and petroleum have been a major source of world energy and have served as material building blocks for decades. However, the shortage of fossil fuels and the tremendous contribution fossil fuel use has on global CO₂ emission is becoming a significant problem (1). The emergence of alternative energy sources has thus become vital to modern energy consumption. One such source is bioenergy, which now accounts for up to a 10% share of global energy consumption (2). Although various alternative energy sources such as solar, wind, and hydro power have shown promise in providing electricity and transportation for urban usage, the demands of hydrocarbon usage, especially that for alkanes for use in long-distance transportation in developing countries, household cooking, and as precursors for material synthesis still remain indispensable (3).

Even though there are several methods available for biofuel production, the efficiency of bioenergy production is hardly sufficient for realistic energy demands in many countries, including the United States (4). Therefore, a sustainable method for the production of alkanes from alternative and renewable resources such as from bio-based products is very important for the security and sustainability of world energy and materials (5, 6).

Synthetic biology approaches have contributed to the construction of a metabolic pathway for the production of alkanes from renewable sources such as from fatty acids and carbohydrates. This method is a green, clean, and sustainable technology for supplying energy and materials for the future (7, 8). Particularly, the pathway targeting the conversion of fatty acids to alkanes provides a means to recycle waste containing fatty acids or triglycerides, such as those derived from oil production industries. In Southeast Asia, the main source of fatty acid or triglyceride waste is derived from palm oil mill effluent (waste from palm oil production) (9). The technology to convert fatty acid to alkane will contribute significantly to the development of a circular economy that focuses on the recycling and conversion of waste into useful materials.

Artificial cascade enzymatic reactions to generate alkanes in microbes can be achieved using the reactions of carboxylic acid
reductase (CAR), fatty acyl-CoA reductase (ACR1), or an acyl-ACP reductase to generate aldehydes from fatty acids, followed by the reaction of aldehyde-deformylating oxygenase (ADO) to generate alkane from aldehyde (Scheme 1) (10–16). This pathway allows host bacteria possessing the genes encoding these enzymes to convert fatty acids to alkanes. Previously, when glucose was used as the starting substrate for cells, the conversion yield of alkane from glucose was quite low, ~0.01–2.8% (Table 1) (10–16), due to the low flux of fatty acid generated from glucose. With the inclusion of genes encoding fatty acid and fatty acyl-CoA esterase/reductase enzymes, the efficiency of fatty acid conversion to alkanes by the engineered pathway was increased significantly (13). In the most recent report, the pathway that can convert fatty acids to alkanes as described in Scheme 1 could achieve an alkane production yield from fatty acid of ~35% (16).

A major bottleneck of the pathway in Scheme 1 for production of alkanes is at the last enzymatic step of ADO to synthesize alkane. ADO is a slow enzyme with a low $k_{\text{cat}}$ value. The $k_{\text{cat}}$ of ADOs from cyanobacteria was reported to be in the range of ~0.1 min$^{-1}$ to 1 min$^{-1}$ (17–21). Under conditions in which the substrate solubility is not optimized or the availability of a reductant to activate the Fe/Fe cofactor is limited, the activity of ADO can be much lower than the reported values (17). One catalytic turnover of ADO theoretically requires four electrons in the form of reduced ferredoxin (Fd) to produce one alkane (using 2 eq of NADPH through reduction of Fd via ferredoxin: NADP$^+$ oxidoreductase (FNR) (14, 19, 22–24)) (Scheme 1). However, previous experiments have shown that more than 2 eq of NADPH were consumed per production of one alkane molecule because electrons in the ADO system also leaked into the pathway of oxygen reduction, leading to the formation of a hydrogen peroxide (H$_2$O$_2$) side product. H$_2$O$_2$ can inhibit the activity of ADO (14, 19), and it has been shown that the inhibitory effect of H$_2$O$_2$ on ADO activity could be alleviated in the in vitro assays using purified catalase (KatE) and ADO or a fusion KatE–ADO enzyme (19). However, there is no significant improvement in terms of alkane production in vivo (12, 14, 19).

To construct a more efficient metabolic pathway to catalyze the conversion of fatty acid to alkane as part of our effort to turn fatty acid waste into renewable alkane in the future, we used enzymology principles to address the improvement of alkane production by ADO. As 1 eq of formate is generated per 1 molecule of alkane produced (25), we aimed to capitalize on the formate as a source of a reducing equivalent to generate...
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NAD(P)H through the reaction of formate dehydrogenase (FDH) (Scheme 2). The reaction of FDH was utilized for two purposes: the first was to produce more NAD(P)H, and the second was to remove a high concentration of formate/formic acid. We hypothesized that a high concentration of formic acid might inhibit the reaction of ADO by ways of decreasing the overall pH of the system as well as by potentially acting as an inhibitor (product inhibition) to the system.

In this study, we constructed an engineered metabolic pathway to convert fatty acids to alkane using genes encoding for ACR1 from Acinetobacter baumannii and ADO from Prochlorococcus marinus MIT9313. Bioinformatic analysis using the Enzyme Function Initiation–Enzyme Similarity Tool (EFI–EST) option C (26) was used to find a novel FDH candidate that can use NADP⁺ and NAD⁺ as substrates for the production of NADPH. Results obtained from these bioinformatic analyses showed that FDH from Xanthobacter sp. 91 (XaFDH) is a novel FDH that has never been characterized and could be a good candidate to be used for improvement of alkane production because it can generate both NADPH and NADH from formate/oxidized formic acid. Therefore, we constructed the alkane production pathway to include genes encoding auxiliary enzymes that might improve the enzymatic reaction of ADO, including XaFDH and reactive oxygen species (ROS) detoxifying enzymes thioredoxin (TrxA) and thioredoxin reductase (TrxB) from Pseudomonas aeruginosa PAO1. The results clearly showed that XaFDH could clearly increase the production yield of alkane to reach a conversion yield of 50%, the best yield to date for alkane production by whole-cell bioconversion.

Results

Rationale and concept of metabolic pathway construction for the biosynthesis of alkane from exogenous fatty acid

The metabolic pathway designed here (Scheme 2) aimed to use exogenous long-hain fatty acids (LCFA) as substrates to produce long-chain hydrocarbons. In this pathway, two enzymatic steps are involved in the conversion of exogenous fatty acids to alkane. As the first step of fatty acid uptake utilizes an endogenous long-chain fatty outer membrane transporter enzyme to attach CoA to fatty acid (27), the first enzyme that we needed to include in the engineered pathway was ACR1 (encoded by the acr1 gene), which can convert fatty acyl-CoA to fatty aldehyde. We chose the enzyme from A. baumannii, which was isolated from Thai soil (28). The gene encoding for this enzyme (MK492106) was identified, cloned, and used in this study. The sequence of this enzyme shares 84% identity with ACR1 from Acinetobacter calcoaceticus (Fig. S1). We chose ACR1 from Acinetobacter sp. because it was reported that unlike ACR1 from other sources, ACR1 from A. calcoaceticus does not catalyze the further reduction of fatty aldehyde to fatty alcohol (29, 30). In addition, the enzyme can use a wide range of substrates such as fatty acyl-CoA containing 14–22 carbons.

At the second step, fatty aldehyde is further converted to alkane using the enzyme ADO from P. marinus MIT9313. The reaction of ADO (Scheme 1) requires reduced Fd, regenerated by FNR, as a co-substrate in addition to oxygen to convert aldehyde to alkane (14, 15). ADO from P. marinus MIT9313 was chosen for our pathway because it is one of the most characterized ADOs to date. Its known catalytic properties allow more effective usage in metabolic engineering work.

As shown in Fig. 1 for the system demonstration, we were able to produce tridecane from exogenous tetradecanoic acid in Escherichia coli harboring the genes for the enzymes in the basic pathway of alkane biosynthesis (Fig. 2). The constructed system was able to produce tridecane from tetradecanoic acid with ~35% yield (~300 μM tridecane from 850 μM supplied tetradecanoic acid) after 10 h of bioconversion (Fig. 1). This verified the ability to create a basic metabolically-engineered pathway for converting fatty acids to alkanes and demonstrated
that the engineered cells can use exogenous fatty acid as a substrate. Control cells in which the engineered pathway was absent did not show any production of an alkane product.

**Identification, cloning, expression, and biochemical characterizations of a novel FDH suitable for increasing alkane production**

Because the basic engineered metabolic pathway can produce alkanes from fatty acids with ~35% yield, we tried to increase the yield of alkane production by increasing the reducing power of the system using FDH to generate NAD(P)H from formate, a by-product from the reaction of ADO (Scheme 2). The purpose of this reaction is to recycle the reducing power from the formate by-product to increase the level of NAD(P)H inside the cell. NAD(P)H generated from FDH can further reduce Fd to generate reduced Fd that is a co-substrate required for the reaction of ADO. As mentioned previously in the Introduction, the rate-limiting step of the overall metabolic path is at the ADO reaction. The effort to continuously supply reduced Fd should, in principle, increase the amount of alkane product. Addition of FDH as an auxiliary redox reaction for the ADO reaction also gives another advantage in that it also removes excess formate within the cell. High concentrations of formate or formic acid can decrease the overall cellular pH of the system (see later in Fig. 7). In principle, high concentrations of accumulated product do not promote the forward reaction of ADO because the by-product formate may cause product inhibition and does not enhance the thermodynamic driving force of the forward reaction. Therefore, we identified a novel NAD(P)H-dependent FDH based on bioinformatic analysis to obtain the enzyme that can utilize formate and is suitable to be used in the metabolically-engineered pathway.

**Figure 1. Production of alkane from fatty acid by the engineered cells.** A, consumption of tetradecanoic acid by E. coli. B, tridecane production by native E. coli BL21 (DE3) cells is shown as blue circles, and engineered E. coli BL21 (DE3) cells harboring the basic pathway for alkane synthesis are shown as red squares. All data are the mean ± S.D. (n = 3).

**Figure 2. Steady-state kinetics of XaFDH reaction in 50 mM sodium phosphate buffer, pH 7.0.** A, direct plots of initial rates of XaFDH reaction versus NAD⁺ and NADP⁺ concentrations at 200 mM sodium formate. B, direct plots of initial rates of XaFDH reaction versus sodium formate concentrations at 400 μM NAD⁺ and 20 mM NADP⁺. All experiments were done in triplicate, and data represent the mean ± S.D.

Putative genes encoding for NAD(P)H-dependent FDH were identified by NCBI blastp search and were clustered by EFI-EST (26) based on the sequence similarity to the known *Pseudomonas* sp. 101 NAD(P)H-dependent FDH that is widely used in various applications (31). Two putative *fdh* genes from *Cupriavidus necator* (Cufdh, UniprotKB: F8G752) and *Xanthobacter* sp. 91 (Xafdh, GenBank™ ID: WP_029558741.1) were chosen for performing gene expression and activity assays as they are both in the same cluster (Fig. S2) as well-characterized NAD(P)H-dependent FDH in *Burkholderia stabilis* 15516 (32) and *Pseudomonas* sp. 101 (33), respectively.

Results from overexpression of the two selected *fdh* genes indicated that only FDH from *Xanthobacter* sp. 91 could be overexpressed in a soluble form, whereas the gene product from *C. necator* was found in inclusion bodies upon overexpression (Fig. S3A). The FDH from *Xanthobacter* sp. 91 (XaFDH) was successfully expressed in *E. coli* BL21 (DE3) and purified to homogeneity (see “Experimental procedures” and Fig. S3B). As *Xanthobacter* is regularly found in wastewater treatment plants from pharmaceutical and domestic effluents, we expected that XaFDH should be a robust enzyme suitable for future applications.

Steady-state kinetic analysis of XaFDH was carried out to measure $K_m$ and $k_{cat}$ values (see “Experimental procedures”) for the reaction using NADP⁺ and that using NAD⁺ as electron acceptors. Results (Fig. 2, A and B) indicate that XaFDH prefers to use NAD⁺ as an electron acceptor more than NADP⁺ because the enzyme had much lower $K_m$ and higher $k_{cat}$ values when NAD⁺ was used as a substrate (Fig. 2A and Table 2). The $K_m$ values of NAD⁺ and formate for XaFDH were 37 ± 4 and 100 ± 13 mM, respectively, and the $k_{cat}$ was 0.4 ± 0.02 s⁻¹. These kinetic parameters were significantly different from those with NAD⁺, which indicated the $K_m$ values of NAD⁺ and formate for XaFDH as 0.060 ± 0.01 and 9 ± 1 mM, respectively, and a $k_{cat}$ of 30 ± 1 s⁻¹. The preference of XaFDH for NAD⁺ as the electron acceptor over NADP⁺ was also found in other bacterial FDH, e.g. those from *Moraxella* sp. strain C-1 (34) and *Mycobacterium vaccae* N10 (35) but not from *Burkholderia* (32) and *Lactobacillus* (36), in which NADP⁺ is more preferred than NAD⁺.

Several properties of XaFDH were further characterized, including the enzyme thermostability, pH, and organic solvent tolerance. Results from a ThermoFluor assay revealed that XaFDH has a $T_m$ of 54 °C, which is comparable with the value
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determined for FDH from *Pseudomonas* sp. 101 (58 °C). The optimum temperature based on activity assays of XaFDH also was found to be between 40 and 50 °C (Fig. 3A). We then investigated the stability of purified XaFDH at 40 and 45 °C, and the results showed that XaFDH activity remained the same for about 400 min of incubation (Fig. 3B). After 24 h of incubation at 40 and 45 °C, XaFDH activity slightly dropped but was still retained at 70–80% of the original activity (data not shown). The data suggest that XaFDH is thermostable and suitable for catalyzing the utilization of intracellular formate. Interestingly, activity assays of XaFDH at different pH values indicated that the enzyme was more active under acidic pH (Fig. 3C). The activity was increased to 5.8 ± 0.2 units/mg at pH 5.8, which was 1.5-fold higher than the activity measured at pH 8.0 (4.10 ± 0.05 units/mg). The acid preference of XaFDH is advantageous for its use in engineered metabolic pathways for whole-cell biocatalysts, as our engineered metabolic pathway that generates alkanes can also increase the intracellular acidity (results shown below). The XaFDH identified here is distinctive from most of the reported FDHs that show less activity under acidic conditions (37, 38). XaFDH is also tolerant to organic solvent (which is required when alkane is produced from the cells) because the relative activity stayed the same as the original activity (data not shown) after incubating in 50% v/v hexane in aqueous solution for 1 h. Altogether, the XaFDH identified here has enzymatic properties suitable for metabolic engineered pathways that require high temperatures, low pH values, and organic solvent.

### Table 2

Kinetic parameters of XaFDH using NAD⁺, NADP⁺, and formate as substrates

| Kinetic parameters | XaFDH |
|--------------------|-------|
| NAD⁺ and formate as substrates |  |
| \( k_{cat} \) (s⁻¹) | 0.40 ± 0.02 |
| \( K_m \) (mM) | 37 ± 4⁺ |
| Formate | 100 ± 13⁺ |
| \( k_{cat}/K_m \) (m⁻¹ s⁻¹) for NAD⁺ | 0.011 ± 0.0001 |
| \( k_{cat}/K_m \) (m⁻¹ s⁻¹) for formate | 0.0019 ± 0.0002 |
| NAD⁺ and formate as substrates |  |
| \( k_{cat} \) (s⁻¹) | 31 ± 1 |
| \( K_m \) (mM) | 0.06 ± 0.01⁺ |
| Formate | 9 ± 1⁺ |
| \( k_{cat}/K_m \) (m⁻¹ s⁻¹) for NAD⁺ | 479 ± 30 |
| \( k_{cat}/K_m \) (m⁻¹ s⁻¹) for formate | 3.0 ± 0.2 |

* Data were obtained from experiments varying NAD⁺ concentrations of 0.05–40 mM at a fixed concentration of 200 mM formate.
* Data were obtained from experiments varying formate concentrations of 5–1000 mM at a fixed concentration of 20 mM NAD⁺.
* Data were obtained from experiments varying NAD⁺ concentrations of 0.005–0.800 mM at a fixed concentration of 200 mM formate.
* Data were obtained from experiments varying formate concentrations of 5–1,000 mM at a fixed concentration of 0.4 mM NAD⁺.

### Figure 3

**Effect of temperature and pH on XaFDH activity.** A, specific activity profiles at various temperatures (25–60 °C) indicate that the optimum temperature for the XaFDH reaction was at 45 °C. B, thermostability of XaFDH at 40 °C (blue circles) and 45 °C (red diamonds) was investigated by incubating the enzyme at each temperature and assayed. The data indicate that at 40 °C, XaFDH could retain its original activity up to 400 min and at 45 °C up to 300 min. Activities at both temperatures were at around 80% after 24 h of incubation (data not shown). C, pH-activity profile of XaFDH showed that XaFDH activity decreased upon pH increases. All reactions presented herein were performed in triplicate and represented as the mean ± S.D.
added fdh ("Experimental procedures"). All constructed metabolic pathways are summarized in Scheme 3.

Four types of cells harboring plasmids encoding the different pathways shown in Scheme 3 were grown in Terrific Broth (TB) for 2 h. After the cell culture reached an OD_{600} of 0.6, these gene sets were overexpressed by induction with 1 mM IPTG. After 6 h of protein overexpression at 25°C, cells were harvested by centrifugation at 8000 \times g for 10 min and were used for the biocconversion of tetradecanoic acid to tridecane. Approximately 0.85 mM tetradecanoic acid was used as the starting precursor of tridecane synthesis. The whole-cell bioconversion assay was set up in a closed vessel containing 21% O₂ (air saturation). The concentrations of tridecane and tetradecanoic acid were monitored for 0, 2, 4, 6, 10, and 20 h using a GC-MS–based method (see "Experimental procedures").

Results as shown in Fig. 4A clearly indicate that alkane production was improved significantly in the metabolic pathway that contained XaFDH. The alkane production yield was improved from 35 to 50% (\pm 4\% \mu M) in the E. coli strain harboring XaFDH (cell type 4 and 5). These data indicate that the addition of XaFDH can improve alkane production as we hypothesized. Tridecane production in the E. coli strains expressing TrxA/TrxB (cell type 3) showed no improvement compared with the E. coli strain harboring the basic pathway (cell type 2), with both strains producing the same amount of tridecane (\pm 300 \mu M) (Fig. 4B). The co-overexpression of TrxA/TrxB and XaFDH (cell type 5) did not show any improvement in alkane synthesis compared with the addition of XaFDH alone (cell type 4). The yield in cell type 5 generated a slightly less yield of alkane (\pm 5\%) compared with cell type 4 (Fig. 4B).

The results clearly indicate that only the fdh gene is required for the improvement of alkane production. The addition of ROS scavenging enzymes, TrxA and TrxB, did not improve the yield of tridecane synthesis as shown in Fig. 4A (cell types 3 and 5).

**Measurement of related metabolites to identify factors underlying the improvement seen for the XaFDH-added metabolic pathway**

As the results in Fig. 4A indicated, cells harboring the metabolically-engineered pathway with added XaFDH clearly showed an improved alkane production yield. Further studies were performed to identify the factors underlying the observed improvement of alkane production. Possible explanations for the improvement in alkane yield include: (i) XaFDH increases the cellular level of NADPH/NADH that can be used to reduce Fd to generate more co-substrate for the ADO reaction; (ii) high concentrations of formate inhibit the reaction of ADO; and (iii) high concentrations of formate cause a pH drop that affects the ADO reaction. The addition of XaFDH into the metabolic pathway would help remove excess formate and allow the ADO alkane generation reaction to proceed more efficiently. To explore these possibilities, we used HPLC–UV/MS to measure the relevant metabolites, including NADH, NAD^+, NADPH, and formic acid inside the cells and measured the pH change of the system.

Results from measuring NADH, NAD^+, NADPH, and NADP^+ using HPLC–UV/MS indicated that the addition of XaFDH increased the NADH concentration and the NADH/NAD^+ redox ratio in E. coli cells significantly (Fig. 5A, cell type 4). The E. coli strain harboring XaFDH (cell type 4) had the highest NADH/NAD^+ redox ratio, \sim 1.5-fold higher than the cell type without XaFDH after 2 h. The results in Fig. 5A also indicated that the NADH/NAD^+ redox ratio in E. coli cells harboring XaFDH (cell types 4 and 5) changed over time, although
the ratio in *E. coli* cells without XaFDH (cell types 1–3) remained at a similar level throughout the whole period of bioconversion. The NADH/NAD\(^+\) redox ratio in the *E. coli* strain harboring XaFDH decreased after 6 h (Fig. 5A), which is around the same period when tridecane production started to reach the maximum point (Fig. 4B). At this stage, the conversion of tetradecanoic acid to tridecane was less, which in turn also stopped the generation of extra formate, and thus NADH from the XaFDH reaction. Despite several attempts, we could only detect cellular NADPH in low amounts, and there was no significant difference in NADPH concentration among the different cell types (data not shown).

It should be noted that the phenomenon of the NADH/NAD\(^+\) ratio initially increasing and then decreasing in Fig. 5A is not an artifact. These results were also observed when similar experiments using independent setups were performed. We noted that the NADH/NAD\(^+\) ratio appeared to be the highest when alkane production reached approximately half of the total yield accumulated.

Results from measuring the formate concentration using HPLC–UV (Fig. 5B) showed that the systems of cell types 2 and 3 have the highest concentrations of formate. Concentrations of formate in the cells expressing XaFDH (cell types 4 and 5) are not significantly different from the control cells (cell type 1). These results showed that cells harboring the basic metabolic pathway shown in Scheme 2 accumulated cellular formate concentrations around 10 mM, whereas the pathway including XaFDH did not show significant accumulation of formate over the time course of the bioconversion process.

We further explored whether the less efficient production of alkane by the basic metabolic pathway is due to product inhibition of ADO by formate. Activity assays of ADO in the presence of various concentrations of tetradecanal (10–80 \(\mu\)M) at 10 mM formate or formic acid at a final pH of 7.0 were carried out (Fig. 6). The results clearly showed that ADO activities were the same in the presence or absence of formate/formic acid. These data clearly indicate that formate or formic acid does not inhibit ADO activity and that the improvement of alkane production by addition of XaFDH is not due to alleviation of product inhibition.

We then measured the pH of the bioconversion reaction to monitor whether accumulation of formate/formic acid caused pH changes. The results (Fig. 7) indicate that in the absence of XaFDH, the pH of the whole-cell bioconversion reactions decreased from the beginning value of 7.5 to pH 5.0 after 6 h. With addition of XaFDH in the metabolic path, the pH drop of the reaction was less severe. Cell type 4 that contained XaFDH retained a final pH of 6.0–6.5 after 6–10 h of bioconversion. We also assayed the activities of ADO as a function of pH and noted that ADO activity at pH 5.0 was only 44% of the activity at pH 7.0 (Fig. S4). These results imply that by having XaFDH co-overexpressed, the cellular pH remained more in the neutral range as compared with the system without XaFDH, which keeps ADO more active.

Altogether, the data indicate that the XaFDH addition pathway generated more NADH, which may lead to more genera-
In turn supports the activity of ADO. In addition, the coupled assay of Fd–NADPH–FNR with ADO was also carried out for comparison. *In vitro* analysis showed that the concentration of tridecane produced from the Fd–NADH–FNR–ADO system is about the same as that produced by the Fd–NADPH–FNR–ADO system (Fig. 8). Although the rate of FNR-catalyzed Fd reduction using NADPH as a reductant is faster than that of NADH (data not shown), the ability of the complete system to produce alkane at similar rates when either NADH or NADPH was used must be due to the slow rate of ADO reaction. Therefore, we have shown for the first time that the FNR–Fd electron transfer system can use NADH to generate reduced Fd and support ADO activity as efficiently as NADPH.

**Discussion**

Our metabolic pathway reported here demonstrates the system that can produce the highest yield of alkane production from fatty acid reported to date. The key improvement of our system resulted from the addition of *XaFDH* that can increase the cellular levels of NADH, which in turn generates more reduced Fd for alkane production. The addition of *XaFDH* also destroys the extra formate accumulated intracellularly that can also maintain the cellular pH in a neutral range.

Alkane biosynthesis in this work utilizes an endogenous pathway of fatty acid uptake and fatty acyl-CoA synthesis in *E. coli* in combination with the engineered pathway containing enzymes for aldehyde synthesis (ACR1) from *A. baumannii* and aldehyde reduction (ADO) from cyanobacteria. Analysis of a starting substrate (fatty acid) and its product (alkane) revealed that the expression of ACR1 and ADO converts fatty acid to alkane in engineered *E. coli* with 35% conversion. Measurements of substrate and product concentrations revealed that fatty acid consumption continued for 20 h while alkane concentrations plateaued at around 6–10 h. The reduced efficiency in alkane biosynthesis observed in *E. coli* over this period was probably caused by a more prominent role of anaerobic fermentation. During the period with low oxygen availability, cellular metabolites such as pyruvate and acetyl-CoA may be accumulated, which can promote the utilization of NADH to produce ethanol and hydroxy acid products such as lactate and acetate (40, 41). Moreover, alcohol synthesis by endogenous aldehyde reductase and alcohol dehydrogenase is another competing pathway for NAD(P)H utilization (42–44). For these reasons, the yield of alkane production by engineered *E. coli* could not reach 100%.

As ADO converts aldehyde into alkane by cleavage of a carbonyl moiety into alkane and formate by-products, accumulation of formate also results in a decrease in pH. The pH of cell type 2, which contained the basic metabolic pathway for alkane production, was measured as pH 5.0 after 6 h.

An improvement of alkane production by our system is derived from the addition of *XaFDH* into the metabolic pathway. *XaFDH* used in this work is a new FDH identified to be thermo- and solvent-tolerant and functions well under acidic conditions. *XaFDH* was expressed to remove formate toxicity and aldehyde reductase and alcohol dehydrogenase is another competing pathway for NAD(P)H utilization (42–44). For these reasons, the yield of alkane production by engineered *E. coli* could not reach 100%.

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Materials and methods

All commercially available chemicals were purchased from Sigma, TCI, or Merck. For molecular biology procedures, XL-Blue competent E. coli (Agilent) was used for plasmid amplification, and BL21 (DE3) competent E. coli (New England Biolabs) was used for protein overexpression. T4 DNA ligase, restriction enzymes, PCR kits, Gibson kits, and other molecular biology reagents were purchased from New England Biolabs. DNA sequencing was performed by Bioneer (South Korea).

Construction of pET17b–acr1 plasmid

The acr1 gene from A. baumannii was amplified with the primers ACR1_F and ACR1_R (Table 3) using the genomic DNA of A. baumannii as a template. The PCR product was digested with XbaI and Xhol and cloned in a pET17b vector at restriction sites XbaI and Xhol, creating pET17b–acr1 plasmid.

Construction of pCDF–fd–fnr–ado plasmid

For construction of pCDF–fd–fnr–ado plasmid, the ado gene from P. marinus MIT9313 was first cloned into a pCDF-Duet-1 vector. The ado gene was amplified with the primers ADO_F and ADO_R (Table 3) using the ado gene that was codon-optimized, synthesized, and supplied on pUC57 by Genescript as a template. The PCR product was digested with NdeI and Xhol and cloned into MCS2 of pCDFDuet-1 vector at the restriction sites NdeI and Xhol, creating the pCDF–ado plasmid. The pCDF–ado was cut with NcoI and EcoRI and ligated into the NcoI/EcoRI-digested PCR product of the fd gene from Synechocystis sp. PCC 6803, resulting in pCDF–fd. Primers Fd_F and Fd_R (Table 3) were used to amplify the fd gene that was codon-optimized and synthesized by Genescript. The fd gene used in this study encodes 2Fe–2S Fd. The PCR product was digested with NdeI and Xhol and ligated into pCDFDuet-1 vector at the restriction sites NdeI and Xhol, creating the pCDF–ado plasmid.

Construction of pRSF–fdh, pRSF–trxA–trxB, and pRSF–trxA–trxB–fdh plasmids

For construction of pRSF–fdh plasmid, the fdh gene from Xanthobacter sp. 91 was amplified with the primers FDH_F and FDH_R (Table 3) using the fdh gene that was codon-optimized, synthesized, and supplied on pUC57 plasmid by Genescript as a template. The resulting PCR product was digested with NdeI and Xhol and ligated with NdeI/Xhol cut pRSFDuet-1 vector to yield the pRSF–fdh plasmid.
trxA–trxB plasmid, pRSFDuet-1 plasmid was digested with Ncol and Xhol and ligated with the PCR product of trxA and trxB genes using Gibson assembly kit. The PCR products of trxA and trxB genes were amplified with primers trxA_F and trxA_R and primers trxB_F and trxB_R, respectively, using genomic DNA of P. aeruginosa PA01 as template. For construction of pRSF–trxA–trxB–fdh plasmid, pRSFDuet-fdh plasmid was digested with Ncol and EcoRI and ligated with the PCR product of trxA and trxB genes using the Gibson assembly kit.

**E. coli whole-cell biocatalyst preparation**

The selected gene cassettes in expression plasmids were transformed into competent *E. coli* BL21 (DE3) with 15 ng of each plasmid, and the transformed cells were selected by growing on LB with ampicillin, streptomycin, and kanamycin. A single colony was selected and grown in LB with three antibiotics as a starting liquid culture overnight at 37 °C. The corresponding variant was then subcultured into TB media with 1% inoculant and grown at 37 °C until the OD600 reached 0.6. The culture was then induced with 1 mM IPTG and incubated further at 25 °C for 6 h. Bacterial cells were harvested by centrifugation at 4 °C and used for biocatalytic reactions.

**Whole-cell biocatalysis and product extraction**

The pelleted *E. coli* paste, with expressed proteins, was resuspended and adjusted cell density to OD600 of 20 in 100 mM potassium phosphate buffer, pH 7.5, supplemented with 2.5% glucose, and 0.85 mM tetradecanoic acid. The biocatalytic reaction (1 ml) was incubated at 25 °C in a 20-ml gas tight vial for designated periods (0, 2, 4, 6, 10, and 20 h) and quenched by adding 2 ml of ethyl acetate solvent containing internal standards (10 μM dodecane and 250 μM tetradecanoic) with vigorous mixing. Upon addition of ethyl acetate, the mixture was centrifuged (4 °C, 3900 × g, 20 min) to enhance phase separation, and the upper organic layer was analyzed by GC-MS.

**XaFDH expression and purification**

The overexpression of the XaFDH in *E. coli* BL21 (DE3) was carried out at 25 °C in ZY auto-induction medium system. In brief, *E. coli* BL21 (DE3) cells harboring pET11a–XaFDH plasmid were grown at 37 °C with shaking at 250 rpm to obtain OD600 ~1.0, and then the culture was adjusted to a temperature of 25 °C for protein expression. Protein purification was achieved by resuspending cell pellets in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM DTT, 60 μM PMSF, and 0.5 mM EDTA, disrupting cells by sonication, clearing cell lysates by centrifugation at 28,700 × g for 1 h, and precipitating the protein with 40–60% (w/v) ammonium sulfate. The precipitated proteins were dissolved and dialyzed in 4 liters of 50 mM sodium phosphate buffer, pH 7.0, at 4 °C for 16 h prior to further purification by DEAE-Sepharose anion-exchange chromatography. A linear gradient of 50 mM sodium phosphate buffer, pH 7.0, containing 50–400 mM NaCl was used for protein elution. The fractions of eluted XaFDH were pooled and concentrated using stirred cell equipment with a Millipore YM-10 membrane. The purity of XaFDH after all steps of purification was analyzed by 12% (w/v) SDS-PAGE.

**ADO expression and purification**

The overexpression of the ADO was carried out in *E. coli* BL21 (DE3) at 25 °C in ZY auto-induction medium system as mentioned above. *E. coli* BL2 (DE3) harboring pET17b-ado plasmid was cultivated for protein expression. The protein purification was achieved by resuspending cell pellets in a lysis buffer (50 mM sodium phosphate buffer, pH 7.0, 1 mM DTT and 100 μM PMSF), disrupting cells by sonication, clearing cell lysates by centrifugation at 28,700 × g for 1 h, removing nucleic acid by using 0.5% (w/v) polyethyleneimine, and precipitating proteins with 40–60% ammonium sulfate. DEAE-Sepharose anion-exchange chromatography was used for further purification. A linear gradient of 50 mM sodium phosphate buffer, pH 7.0, containing 50–400 mM NaCl was used for protein elution. The fractions of eluted ADO were pooled and concentrated using stirred cell equipment with a Millipore YM-10 membrane. The purity of ADO after all steps of purification was analyzed by 12% (w/v) SDS-PAGE.
Determination of XaFDH steady-state kinetics

The steady-state kinetics parameters for XaFDH were determined using a spectrophotometric method. In this assay, the $K_m$ and $k_{cat}$ values were determined through initial velocity measurement by varying the concentrations of NAD$^+$ (0.005–0.800 mm), NADP$^+$ (0.05–40 mm), or formate (5–1000 mm). The XaFDH concentration was fixed at 10 nM and 10 $\mu$M when assayed with NAD$^+$ and NADP$^+$, respectively. The production of NADH and NADPH was monitored by following the UV absorption at 340 nm in 50 mm phosphate buffer at pH 7.0 at 25 °C. The reaction was initiated by adding XaFDH. Data were analyzed using the Michaelis-Menten equation to obtain the kinetic parameter $k_{cat}$ and $K_m$ values for all substrates.

Investigation of XaFDH stability by ThermoFluor assay

The ThermoFluor assay is a temperature-based assay to determine the stability of proteins using a real-time PCR machine. The assay reaction consisted of 50 mm sodium phosphate buffer, 40 $\mu$M XaFDH, and 400 $\mu$M SYPRO Orange dye. The temperature was set from 25 to 90 °C with an increment rate of 1 °C per min. The stability curves and the midpoint values (melting temperature, $T_m$) were obtained by plotting fluorescence against reaction temperatures. The derivative plot was performed for an easy interpretation of the $T_m$ of protein. The temperature at which the derivative is the lowest point is the $T_m$.

Investigation of the optimum temperature on XaFDH

The optimum temperature of XaFDH was investigated by pre-incubating the reaction mixture (50 mm phosphate buffer, pH 7.0, 0.05 mm NAD$^+$, 200 mm sodium formate) without enzyme at various temperatures (25, 30, 35, 40, 45, 50, 55, and 60 °C) for 15 min to obtain temperature equilibration. The activity assays were initiated by adding XaFDH (2.5 $\mu$M) into the incubated mixture. The absorbance at 340 nm was monitored for NADH production. XaFDH activity versus temperatures were plotted to examine the optimum temperature of the XaFDH reaction.

Investigation of the pH effect on XaFDH activity

Activities of XaFDH at various pH values were measured at 25 °C in 50 mm sodium phosphate and sodium pyrophosphate buffers for pH ranges of 6–8.5 and 8.5–10, respectively. The assay reactions consisted of 0.05 mm NAD$^+$, 200 mm sodium formate, and 2.5 $\mu$M XaFDH. The absorbance at 340 nm was monitored for NADH production. Enzyme activities were plotted as a function of final pH values.

Formate inhibition assay

Inhibition of ADO by formate was investigated by measuring initial velocities at various concentrations of tetradecanoic acid (10, 20, 40, and 80 $\mu$m) with a fixed concentration of formate (10 mm) and formic acid (10 mm). The final pH of the reactions was 7.0. The reactions were performed in 100 mm HEPES buffer, pH 7.2, containing 100 mm KCl, 1 mm ADO, 100 mm PMS, 1 mm NADH and 40 $\mu$m ferrous ammonium sulfate. Assays were initiated by adding various concentrations of tetradecanoic solution containing 10 mm formate or 10 mm formic acid. The reactions (0.5 ml) were incubated at 37 °C for 1.5 min and quenched by adding 1 ml of ethyl acetate solvent containing internal standards (10 $\mu$m dodecanate and 250 $\mu$m tetradecanate) with vigorous mixing by a vortex mixer, followed by centrifugation at 18,534 $\times$ g for 10 min to separate the organic phase, and analyzed by GC-MS.

Tridecane and tetradecanoic acid analyses

GC-MS using HP5-MS capillary column attached to a mass spectrometer (Agilent 7890B) in electron ionization mode (EI-MS 5977B) was used for analysis of tridecane and tetradecanoic acid. Fragmentation patterns of individual compounds were validated using combined Wiley MS and NIST 2014 databases for known compounds. Quantitative analysis was performed on Agilent ChemStation Quantitative Analysis software to obtain a correlation of known concentrations of each compound versus GC-MS signals. The GC condition used was 250 °C at an inlet. Temperature program started at 60 °C for 3 min and ramped to 200 °C with a rate of 10 °C/min, and then stayed at 200 °C for 2 min and ramped to 260 °C at a rate of 20 °C/min, and finally stayed at 260 °C for 3 min. Samples (5 $\mu$l) were used for analysis in each run. Standard curves with known concentrations of standard compounds (1–500 $\mu$m) versus GC-MS signals were constructed for analysis of substrate consumed and product formed.

Analysis of cell metabolites

For analyzing formate that was produced during the alkane production process, bioconversion reactions (0.5 ml) were centrifuged for 2 min at 18,500 $\times$ g at 4 °C, and the supernatant was then filtered through a 0.2-$\mu$m syringe filter. The resulting filtrate was analyzed by HPLC–UV detection (Agilent) using a cation-exchange column (Agilent Hi-Plex H, 7.7 × 300 mm, 8 $\mu$m) operated at 50 °C. The analytes were eluted with 0.01 M H$_2$SO$_4$ at a flow rate of 0.3 ml/min. The detection wavelength was set at 210 nm. The identity of formate was confirmed by comparison of HPLC profiles with that of the standard formate.

For measuring the intracellular concentration of NADH, NAD$, NADP^+$, and NADPH, bacterial cell pellet was resuspended in 250 $\mu$l of 5 mm ammonium acetate buffer, pH 11, containing 10 $\mu$m L-arabinose as internal standard. The bacterial cells were lysed on ice by sonication. The resulting cell lysate was centrifuged at 18,500 $\times$ g at 4 °C for 10 min for separation of supernatant and cell debris. Proteins in the supernatant were removed by Amicon 3-kDa cutoff centrifugal filter device (Millipore). The resulting flow-through was then analyzed by HPLC using ZORBAX SB-Aq RRHD column (2.1-mm inner diameter × 50 mm, 1.8-$\mu$m particle size). The mobile phase used was 20 mM ammonium acetate buffer, pH 5.0. The flow rate was set to 0.2 ml/min, and the column temperature at 25 °C. The HPLC was directly coupled to HPLC–UV/MS (Agilent 1260 infinity) with an electrospray ionization source operated in a negative ion mode. The mass spectrometer was operated in selected ion monitoring mode to detect and quantify NAD(P)H and NAD(P)$^+$ based on their retention times, UV absorption characteristics, and $m/z$ of the ion. The identities of
NAD(P)H and NAD(P)⁺ were confirmed by comparison with the standard compounds.

**In vitro assay of Fd reduction with NADH by FNR**

Reduction of Fd with NADH by FNR was assayed in an anaerobic glove box, and the reduction of Fd was measured spectrophotometrically as a decrease in the absorbance of NADH at 340 nm. FNR (10 μM final concentration) was mixed with Fd (10 and 20 μM final) and NADH (100 μM NADH final) in 100 mM HEPES buffer, pH 7.2, containing 100 mM KCl. The reactions were incubated at 25 °C, and the depletion of NADH was monitored at various time points (0, 30, and 60 min).

**Activity assay of ADO in the presence of Fd–NADH–FNR and Fd–NADPH–FNR**

To compare the efficiency of Fd–NADH–FNR or Fd–NADPH–FNR reducing systems in supporting the activity of ADO, the product of the ADO reaction using Fd–NADH–FNR and Fd–NADPH–FNR as reductants was measured. The enzyme reactions were set up under anaerobic conditions. The reaction contained 20 μM ADO, 200 μM tetradecanal, 40 μM Fd, 20 μM FNR, and 1 mM NADH or 1 mM NADPH in a total volume of 500 μl of 100 mM HEPES buffer, pH 7.2, in the presence of 100 mM KCl. Reactions with the same concentrations of ADO, FNR–NADH, FNR–NADPH, and FNR–Fd–FNR were also incubated with 200 μM tetradecanal in 100 mM HEPES buffer, pH 7.2, in the presence of 100 mM KCl to serve as control reactions. All reactions were initiated by adding 1 ml of ambient air (21% O₂) and were incubated at 37 °C for 60 min and quenched by adding 1 ml of 10% trichloroacetic acid containing internal standard (10 μM dodecane and 250 μM tetradecane). The organic phase was separated by centrifugation at 18,534 × g for 10 min and subjected to product analysis by GC-MS.

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**References**

1. Lu, K. (2014) Materials in Energy Conversion, Harvesting, and Storage, pp 1–10, John Wiley & Sons, Inc., Hoboken, NJ
2. Ho, D. P., Ngo, H. H., and Guo, W. (2014) A minireview on renewable sources for biofuel. Bioresour. Technol. 169, 742–749 CrossRef Medline
3. Lynd, L. R. (2017) The grand challenge of cellulosic biofuels. Nat. Biotechnol. 35, 912–915 CrossRef Medline
4. Hill, J., Nelson, E., Tilman, D., Polasky, S., and Tiffany, D. (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc. Natl. Acad. Sci. U.S.A. 103, 11206–11210 CrossRef Medline
5. Peralta-Yahya, P. P., Zhang, F., del Cardayre, S. B., and Keasling, J. D. (2012) Microbial engineering for the production of advanced biofuels. Nature 488, 320–328 CrossRef Medline
6. Lee, S. Y., Kim, H. U., Chae, T. U., Cho, J. S., Kim, J. W., Shin, J. H., Kim, D. I., Ko, Y.-S., Jang, W. D., and Jang, Y.-S. (2019) A comprehensive metabolic map for production of bio-based chemicals. Nat. Catal. 2, 18–33 CrossRef
7. Liao, J. C., Mi, L., Pontrelli, S., and Luo, S. (2016) Fuelling the future: microbial engineering for the production of sustainable biofuels. Nat. Rev. Microbiol. 14, 288–304 CrossRef Medline
8. Zhou, Y. J., Kerkhoven, E. J., and Nielsen, J. (2018) Barriers and opportunities in bio-based production of hydrocarbons. Nat. Energy 3, 925–935 CrossRef
9. Sheil, D. (2009) The impacts and opportunities of oil palm in Southeast Asia: what do we know and what do we need to know? Center for International Forestry Research, Bogor, Indonesia CrossRef
10. Akhtar, M. K., Turner, N. J., and Jones, P. R. (2013) Carboxylic acid reduction is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities. Proc. Natl. Acad. Sci. U.S.A. 110, 87–92 CrossRef Medline
11. Choi, Y. J., and Lee, S. Y. (2013) Microbial production of short-chain alkanes. Nature 502, 571–574 CrossRef Medline
12. Coursolle, D., Lian, J., Shanklin, J., and Zhao, H. (2015) Production of long chain alcohols and alkanes upon coexpression of an acyl-ACP reductase and aldehyde-deformylating oxygenase with a bacterial type-I fatty acid synthase in E. coli. Mol. Bioloryst. 11, 2464–2472 CrossRef Medline
13. Harger, M., Zheng, L., Moon, A., Ager, C., An, J. H., Choe, C., Lai, Y. L., Mo, B., Zong, D., Smith, M. D., Egbert, R. G., Mills, J. H., Baker, D., Pultz, I. S., and Siegel, J. B. et al. (2013) Expanding the product profile of a microbial alkane biosynthetic pathway. ACS Synth. Biol. 2, 59–62 CrossRef Medline
14. Kallio, P., Pásztor, A., Thiel, K., Akhtar, M. K., and Jones, P. R. (2014) An engineered pathway for the biosynthesis of renewable propane. Nat. Commun. 5, 4731 CrossRef Medline
15. Menon, N., Pásztor, A., Menon, B. R., Kallio, P., Fisher, K., Akhtar, M. K., Leys, D., Jones, P. R., and Scrutton, N. S. (2015) A microbial platform for renewable propane synthesis based on a fermentative butanol pathway. Biotechnol. Biofuels 8, 115 CrossRef Medline
16. Patrikainen, P., Carbonell, V., Thiel, K., Aro, E. M., and Kallio, P. (2017) Comparison of orthologous cyanobacterial aldehyde deformylating oxygenases in the production of volatile C3–C7 alkanes in engineered E. coli. Metab. Eng. Commun. 5, 9–18 CrossRef Medline
17. Eser, B. E., Das, D., Han, J., Jones, P. R., and Marsh, E. N. (2011) Oxygen-independent alkane formation by non-heme iron-dependent cyanobacterial aldehyde dehydrogenase: investigation of kinetics and requirement for an external electron donor. Biochemistry 50, 10743–10750 CrossRef Medline
18. Khara, B., Menon, N., Levy, C., Mansell, D., Das, D., Marsh, E. N., Leys, D., and Scrutton, N. S. (2013) Production of propane and other short-chain alkanes by structure-based engineering of ligand specificity in aldehyde-deformylating oxygenase. Chembiochem 14, 1204–1208 CrossRef Medline
19. Andre, C., Kim, S. W., Yu, X. H., and Shanklin, J. (2013) Fusing catalase to an alkane-producing enzyme maintains enzymatic activity by converting the inhibitory by-product H₂O₂ to the cosubstrate O₂. Proc. Natl. Acad. Sci. U.S.A. 110, 3191–3196 CrossRef Medline
20. Bao, L., Li, J. J., Jia, C., Li, M., and Lu, X. (2016) Structure-oriented substrate specificity engineering of aldehyde-deformylating oxygenase towards aldehydes carbon chain length. Biotechnol. Biofuels 9, 185 CrossRef Medline
21. Paul, B., Das, D., Ellington, B., and Marsh, E. N. (2013) Probing the mechanism of cyanobacterial aldehyde dehydrogenase using a cyclopropyl aldehyde. J. Am. Chem. Soc. 135, 5234–5237 CrossRef Medline
22. Li, N., Norgaard, H., Warui, D. M., Booker, S. J., Krebs, C., and Bollinger, J. M. J. (2011) Conversion of fatty aldehydes to alka(enes) and formate by a cyanobacterial aldehyde dehydrogenase: cryptic redox by an unusual di-metal oxygenase. J. Am. Chem. Soc. 133, 6158–6161 CrossRef Medline
23. Rajakovitch, L. J., Norgaard, H., Warui, D. M., Chang, W.-C., Li, N., Booker, S. J., Krebs, C., Bollinger, J. M. Jr., and Pandela, M.-E. (2015) Rapid reductase of the dифференцированный interafaciate intermediate in aldehyde-deformylating oxygenase by a cyanobacterial ferredoxin: evidence for a free-radical mechanism. J. Am. Chem. Soc. 137, 11695–11709 CrossRef Medline
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24. Pandelia, M. E., Li, N., Norgaard, H., Warui, D. M., Rajakovich, L. I., Chang, W.-C., Booker, S. J., Krebs, C., and Bollinger, J. M., Jr. (2013) Substrate-triggered addition of dioxygen to the differic cofactor of aldehyde-deformylating oxygenase to form a differic-peroxide intermediate. J. Am. Chem. Soc. 135, 15801–15812 CrossRef Medline

25. Warui, D. M., Li, N., Norgaard, H., Krebs, C., Bollinger, J. M., Jr., and Booker, S. J. (2011) Detection of formate, rather than carbon monoxide, as the stoichiometric coproduct in conversion of fatty aldehydes to alkanes by a cyanobacterial aldehyde decarboxylase. J. Am. Chem. Soc. 133, 3316–3319 CrossRef Medline

26. Gerlt, J. A., Bouvier, J. T., Davidson, D. B., Imker, H. J., Sadkhin, B., Slater, D. R., and Whalen, K. L. (2015) Enzyme function initiative-enzyme similarity tool (EFI–EST): a web tool for generating protein sequence similarity networks. Biochim. Biophys. Acta 1854, 1019–1037 CrossRef Medline

27. Wiener, M. C., and Horanyi, P. S. (2011) How hydrophobic molecules traverse the outer membranes of Gram-negative bacteria. Proc. Natl. Acad. Sci. U.S.A. 108, 10929–10930 CrossRef Medline

28. Chaiyen, P., Suadee, C., and Wilairat, P. (2001) A novel two-protein component flavoprotein hydroxylase. Eur. J. Biochem. 268, 5550–5561 CrossRef Medline

29. Reiser, S., and Somerville, C. (1997) Isolation of mutants of Acinetobacter calcoaceticus deficient in wax ester synthesis and complementation of one mutation with a gene encoding a fatty acyl coenzyme A reductase. J. Bacteriol. 179, 2969–2975 CrossRef Medline

30. Willis, R. M., Wahlen, B. D., Seefeldt, L. C., and Barney, B. M. (2011) Characterization of a fatty acyl-CoA reductase from Marinobacter aquaeolei VT8: a bacterial enzyme catalyzing the reduction of fatty acyl-CoA to fatty alcohol. Biochemistry 50, 10550–10558 CrossRef Medline

31. Bommarius, A. S., Schwarm, M., Stingl, K., Kottenhahn, M., Huthmacher, K., and Drauz, K. (1995) Synthesis and use of enantiomerically pure tert-leucine. Tetrahedron: Asymmetry 6, 2851–2888 CrossRef

32. Hatrongjit, R., and Packdibamrung, K. (2010) A novel NADP⁺-dependent formate dehydrogenase from Burkholderia stabilis 15516: screening, purification and characterization. Enzyme Microb. Technol. 46, 557–561 CrossRef

33. Tishkov, V. I., Galkin, A. G., Marchenko, G. N., Egorova, O. A., Sheluhov, D. V., Kulakova, L. B., Dementieva, L. A., and Egorov, A. M. (1993) Catalytic properties and stability of a Pseudomonas sp. 101 formate dehydrogenase mutants containing Cys-255–Ser and Cys-255–Met replacements. Biochim. Biophys. Res. Commun. 192, 976–981 CrossRef Medline

34. Asano, Y., Sekigawa, T., Inukai, H., and Nakazawa, A. (1988) Purification and properties of formate dehydrogenase from Moraxella sp. strain C-1. J. Bacteriol. 170, 3189–3193 CrossRef Medline

35. Galkin, A., Kulakova, L., Tishkov, V., Eski, N., and Soda, K. (1995) Cloning of formate dehydrogenase gene from a methanol-utilizing bacterium Mycobacterium vaccae N10. Appl. Microbiol. Biotechnol. 44, 479–483 CrossRef Medline

36. Alpdagtas, S., Yücel, S., Kapkaç, H. A., Liu, S., and Binay, B. (2018) Discovery of an acidic, thermostable and highly NADP⁺-dependent formate dehydrogenase from Lactobacillus buchneri NRRL B-30929. Biotechnol. Lett. 40, 1135–1147 CrossRef Medline

37. Mesentsev, A. V., Laman, V. S., Tishkov, V. I., Ustinnikova, T. B., and Popov, V. O. (1997) Effect of pH on kinetic parameters of NAD⁺- dependent formate dehydrogenase. Biochem. J. 321, 475–480 CrossRef Medline

38. Tishkov, V. I., and Popov, V. O. (2004) Catalytic mechanism and application of formate dehydrogenase. Biochemistry 69, 1252–1267 Medline

39. Leadbeater, C., McIver, L., Campopiano, D. J., Webster, S. P., Baxter, R. L., Kelly, S. M., Price, N. C., Lysek, D. A., Noble, M. A., Chapman, S. K., and Munro, A. W. (2000) Probing the NADPH-binding site of Escherichia coli flavodoxin oxidoreductase. Biochem. J. 352, 257–266 CrossRef Medline

40. Clark, D. P. (1989) The fermentation pathways of Escherichia coli. FEMS Microbiol. Rev. 5, 223–234 CrossRef Medline

41. Unden, G., and Bongaerts, J. (1997) Alternative respiratory pathways of Escherichia coli: energetics and transcriptional regulation in response to electron acceptors. Biochim. Biophys. Acta 1320, 217–234 CrossRef Medline

42. Cao, Y. X., Xiao, W. H., Liu, D., Zhang, J. L., Ding, M. Z., and Yuan, Y. J. (2015) Biosynthesis of odd-chain fatty alcohols in Escherichia coli. Metab. Eng. 29, 113–123 CrossRef Medline

43. Fillet, S., and Adrio, J. L. (2016) Microbial production of fatty alcohols. World J. Microbiol. Biotechnol. 32, 152 CrossRef Medline

44. Nozzi, N. E., Desai, S. H., Case, A. E., and Atsumi, S. (2014) Metabolic engineering for higher alcohol production. Metab. Eng. 25, 174–182 CrossRef Medline

45. Waugh, M. W., and Marsh, E. N. (2014) Solvent isocon effects on alkane formation by cyanobacterial aldehyde deformylating oxygenase and their mechanistic implications. Biochemistry 53, 5537–5543 CrossRef Medline

46. Yeam, J., and Park, W. (2012) Biochemical characterization of ferredoxin-NADP⁺ reductase interaction with flavodoxin in Pseudomonas putida. BMB Rep. 45, 476–481 CrossRef Medline

47. Lee, Y. H., Tamura, K., Maeda, M., Hoshino, M., Sakurai, K., Takahashi, S., Ikegami, T., Hase, T., and Goto, Y. (2007) Cores and pH-dependent dynamics of ferredoxin-NADP⁺ reductase revealed by hydrogen/deuterium exchange. J. Biol. Chem. 282, 5959–5967 CrossRef Medline

48. Bhatt, A. N., Shukla, N., Alverti, A., Zanetti, G., and Bhakuni, V. (2005) Modulation of cooperativity in Mycobacterium tuberculosis NADPH-ferredoxin reductase: cation- and pH-induced alterations in native conformation and destabilization of the NADP⁺-binding domain. Protein Sci. 14, 980–992 CrossRef Medline

49. Jang, S., and Imlay, I. A. (2007) Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. J. Biol. Chem. 282, 929–937 CrossRef Medline

50. Seaver, L. C., and Imlay, J. A. (2004) Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? J. Biol. Chem. 279, 48742–48750 CrossRef Medline

51. Bottin, H., and Lagoutte, B. (1992) Ferredoxin and flavodoxin from the cyanobacterium Synechocystis sp. PCC 6803. Biochim. Biophys. Acta 1101, 48–56 CrossRef Medline

52. van den Heuvel, R. H., Svergun, D. I., Petrovskov, M. V., Coda, A., Curti, B., Ravasio, S., Vanoni, M. A., and Mattevi, A. (2003) The active conformation of glutamate synthase and its binding to ferredoxin. J. Mol. Biol. 330, 113–128 CrossRef Medline