Fermentation of dihydroxyacetone by engineered *Escherichia coli* and *Klebsiella variicola* to products

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Methane can be converted to triose dihydroxyacetone (DHA) by chemical processes with formaldehyde as an intermediate. Carbon dioxide, a by-product of various industries including ethanol/butanol biorefineries, can also be converted to formaldehyde and then to DHA. DHA, upon entry into a cell and phosphorylation to DHA-3-phosphate, enters the glycolytic pathway and can be fermented to any one of several products. However, DHA is inhibitory to microbes due to its chemical interaction with cellular components. Fermentation of DHA to D-lactate by *Escherichia coli* strain TG113 was inefficient, and growth was inhibited by 30 g·L⁻¹ DHA. An ATP-dependent DHA kinase from *Klebsiella oxytoca* (pDC117d) permitted growth of strain TG113 in a medium with 30 g·L⁻¹ DHA, and in a fed-batch fermentation the D-lactate titer of TG113(pDC117d) was 580 ± 21 mM at a yield of 0.92 g·g⁻¹ DHA fermented. *Klebsiella variicola* strain LW225, with a higher glucose flux than *E. coli*, produced 811 ± 26 mM D-lactic acid at an average volumetric productivity of 2.0 g⁻¹·L⁻¹·h⁻¹. Fermentation of DHA required a balance between transport of the triose and utilization by the microorganism. Using other engineered *E. coli* strains, we also fermented DHA to succinic acid and ethanol, demonstrating the potential of converting CH₄ and CO₂ to value-added chemicals and fuels by a combination of chemical/biological processes.

dihydroxyacetone | fermentation | methane | lactic acid | ethanol

Due to modern technology of extraction, the amount of natural gas produced in 2016 in the United States was 26.5 trillion cubic feet [US Energy Information Administration (US-EIA); https://www.eia.gov/dnav/ng/ng_sum_lsum_a_EPG0_FPD_mmc_f_a.htm]. Due to the high rate of production, the cost of natural gas has fallen to \$3.96 per 1,000 cubic feet (July 2017 industrial price) from a high value of \$13.06 in July 2008 (US-EIA; <https://www.eia.gov/dnav/ng/hist/n3035us3m.htm>). This provides an incentive to upgrade the inexpensive CH₄ to value-added chemicals and liquid fuels that can reach values over \$100 billion. Although biological processes to convert CH₄ to liquid fuels (gas to liquids, GTL) have been discussed (1), these processes are inefficient. An alternative to a technologically complex chemical process (GTL-Fischer-Tropsch) or an inefficient biological process for conversion of CH₄ to chemicals is a hybrid chemical/biological process. The first step in this proposed hybrid process is to generate fermentable sugars, such as dihydroxyacetone (DHA), from natural gas, for which the technology already exists (Fig. 1). The phosphorylated form of this triose (DHA-3-phosphate; DHA-P) is an intermediate of glycolysis. DHA can be catalytically produced from formaldehyde by the formose reaction (2–4) for fermentation by appropriately engineered microbial biocatalysts to any number of chemical and fuel molecules, such as ethanol, butanol, lactate, and succinate, among others. (Fig. 1). Formaldehyde is currently produced industrially from methanol, and methanol itself is produced from CH₄, leading to a chemical process from CH₄ to fermentable sugar DHA (Fig. 1).

Another attractive starting material for the production of DHA is CO₂, and such a process is environmentally friendly. Formaldehyde can be produced chemically from CO₂ via methanol as an intermediate (5, 6). In addition to the chemical

process, formaldehyde can also be produced biologically from CO₂ with formate as an intermediate (Fig. 1) (7). Dickens and Williamson reported as early as 1958 that DHA can be produced biologically by transketolation of hydroxypyruvate and formaldehyde (8). This transketolase is implicated in a unique pentose-phosphate-dependent pathway (DHA cycle) in methanol-utilizing yeast that fixes formaldehyde to xylulose-5-phosphate, yielding DHA as an intermediate in the production of glyceraldehyde-3-phosphate in a cyclic mode (9). DHA in the cytoplasm is phosphorylated by DHA kinase and/or glycerol kinase, and the DHA-P that enters glycolysis provides a route for the utilization of CH₄ and CO₂ by biological systems.

Although there are biological, chemical, and hybrid (chemical/biological) processes that can generate DHA from CH₄ and CO₂ (Fig. 1), microbial biocatalysts that ferment DHA to bulk chemicals at high yield and productivity are lacking. A complicating factor in developing microbial biocatalysts for fermentation of DHA to products at the industrial level is that DHA at even moderate concentrations is antimicrobial (10). This growth-inhibitory effect of DHA is apparently due to its propensity to interact with amino groups that induce DNA and protein damage in cells that cannot metabolize DHA rapidly (Maillard reaction) (11). This property of DHA to interact with amino groups has led to the widespread use of DHA as the ingredient in sunless tanning solutions (12). Abiological conversion of DHA to compounds such as lactic acid, a starting material for PLA-based

Significance

World-wide natural gas production in 2016 was 3.55 trillion cubic meters, and the natural gas flared is estimated to contribute about 350 million tons of CO₂. The global warming potential of CH₄ is several orders of magnitude higher than that of CO₂. Upgrading CH₄ to chemicals and liquid fuels converts low-cost natural gas to high-value products and traps it from release into atmosphere. Current chemical technology can produce dihydroxyacetone (DHA) from CH₄ provided a microorganism can ferment this growth-inhibitory sugar. Here we report metabolically engineered microorganisms that ferment DHA to products. Combining the existing technology of chemical conversion of CH₄ to DHA and the fermentation of this sugar is a strategy to transform inexpensive CH₄ to chemicals and liquid fuels.

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Fig. 1. A chemical or biological process for the production of DHA from CO_2 or CH_4 and further fermentation of DHA to product.

plastics, is known (13) and can overcome the inhibitory effect of DHA on microorganisms. However, this process is expected to generate a mixture of D(–) and L(+) isomers of lactic acid that requires expensive purification before use in the biodegradable plastics industry. Since fermentation of sugars by microorganisms is an efficient way of producing optically pure lactic acid, we have evaluated the toxicity of DHA and constructed microbial biocatalysts for production of D-lactic acid from DHA as the feedstock. In addition, once a fermentation process from DHA to D-lactate is developed as a model system, this process can be modified and applied to the production of any one of several metabolic products that can serve as fuels and chemical feedstocks, as demonstrated by fermentation of DHA to ethanol and succinate by appropriately engineered bacterial biocatalysts.

Although a biological pathway for the production of DHA from CH_4 and CO_2 can be designed (Fig. 1), such a pathway is yet to be engineered in a microbial biocatalyst. The next step would be to enhance the rate of production of DHA from these gases to match the fermentation rate of DHA for high productivity of the desired final product. Due to this limitation, at present, an efficient process for converting these gases to value-added products is a hybrid process that couples current chemical technology for production of DHA with fermentation by engineered microorganisms, such as the ones described in this study. As an effective microbial platform for the production of DHA from CH_4 and CO_2 evolves, an integrated biological process can be developed for the rapid conversion of these gases to various fuels and chemicals.

Results and Discussion

During anaerobic growth in glucose-containing medium *Escherichia coli* produces acetate, ethanol, lactate, formate, H_2 , CO_2 , and small amounts of succinate as fermentation products (Fig. S1). Although DHA is not in the glucose fermentation pathway, it is an intermediate of glycerol metabolism in *E. coli*, especially during anaerobic conditions (14). DHA produced by glycerol dehydrogenase (*gldA*) is phosphorylated to DHA-P by a phosphoenolpyruvate (PEP)-dependent kinase encoded by *dhaKLM* that is not associated with transport. The level of DHA kinase was reported to be very low during aerobic growth and increased during O_2 -limitation conditions in glycerol-grown cells (15) and thus limiting the glycerol-DHA-DHA-P pathway to anaerobic growth conditions. DHA-P, an intermediate of glycolysis, is expected to yield the same fermentation products as

seen with glucose fermentation (Fig. S1). In this pathway, DHA added to the medium is transported by a facilitated diffusion channel (glycerol facilitator, GlpF). In *E. coli* and other enteric bacteria, GlpF helps transport glycerol in an energy-independent manner. Since the GlpF channel can also transport glyceraldehyde and, to a lesser extent, erythritol and ribitol (16), it is likely DHA is also transported by this facilitator. Using a cell shrinkage and reswelling assay for glycerol uptake (16), we determined the rate of facilitated diffusion of DHA by a *glpF* mutant, strain LW410, to be about half ($-0.04 \text{ AU} \cdot \text{s}^{-1}$) the value for the parent, strain TG113 ($-0.08 \text{ AU} \cdot \text{s}^{-1}$) at room temperature. In addition to GlpF, additional DHA transport systems also exist in *E. coli* based on the growth and fermentation of DHA by a *glpF* mutant (Fig. 2). The nature of these alternate transport systems is yet to be established, and these could be the same non-GlpF transporters reported for glycerol in *E. coli* (16).

Upon phosphorylation, DHA-P enters the glycolysis pathway and is converted to pyruvate with associated ATP and NADH production. Thus, only two steps are unique for DHA metabolism in *E. coli*: transport and phosphorylation. Fermentation of two DHA molecules to one each of acetate and ethanol would yield a net of three ATPs, while fermentation to two lactates results in a net yield of two ATPs (Fig. S1). These ATP yields (two DHA equivalents) are the same as in glucose fermentation by this bacterium. This shows that the anaerobic growth of *E. coli* with DHA as a fermentable carbon source is not constrained energetically or by redox balance.

Lack of Growth of *E. coli* in DHA-Minimal Medium. Wild-type *E. coli* (strains B, ATCC11303; C, ATCC8739; K-12, W3110; and W, ATCC9637) did not grow with DHA as a carbon source in mineral salts medium under aerobic conditions. This is expected due to the very low level of DHA kinase in aerobically grown cells (15). However, similar results were also obtained under anaerobic growth conditions in DHA-mineral salts medium. It is known that DHA can interact with medium components such as phosphate and generate methylglyoxal, a highly reactive growth inhibitor (17, 18). Lowering the phosphate concentration to 1 mM (19) did not overcome this inhibition of *E. coli* growth by DHA

Fig. 2. Inhibition of the growth of *E. coli* by DHA. Strain TG113(pDC117d) or LW416 (TG113, ΔglpF , pDC117d) was inoculated into pH-controlled (7.0) fermentations (37°C) with the indicated concentration of DHA in LB. Cell density and lactate concentration were determined during a 48-h period, and the highest values are presented. Reported values for LW416 with 456 mM DHA are from 96-h incubation. Dashed lines indicate TG113 (pDC117d); solid lines indicate LW416; circles indicate cell density; squares indicate D-lactate titer.

(111 mM). Rapid conversion of DHA to nontoxic products, such as DHA-P, is expected to minimize the inhibitory effect of DHA on microbial biocatalysts. To understand the physiological constraints in the anaerobic metabolism of DHA in *E. coli*, a lactate-producing derivative of *E. coli*, strain TG113 (20), was used in this study. The reported average specific and volumetric productivities of D-lactate for strain TG113 in mineral salts medium with glucose are 1.0 g·h⁻¹·g cells⁻¹ and 1.92 g·L⁻¹·h⁻¹, respectively, over a 24-h period. The high glucose flux to D-lactate in this strain is expected to minimize potential rate-limiting step(s) from the glycolytic pathway in DHA fermentation.

DHA Fermentation by *E. coli* Strain TG113. Due to the interaction of DHA with phosphate in mineral salts medium and the generation of inhibitory compounds, *E. coli* strain TG113 was grown in rich medium with DHA (111 mM; 10 g·L⁻¹). Strain TG113 grew in this medium utilizing the nutrients in LB and fermented DHA at a very low level (Table 1 and Fig. S24). About 40 mM DHA was consumed during the first 24 h, and the D-LA yield was 0.66 g·g⁻¹ of consumed DHA. Since there are only two steps between medium DHA and the glycolytic intermediate DHA-P, and the DHA is transported by facilitated diffusion, the rate-limiting step is apparently DHA kinase. Strain TG113 grown in LB with DHA and harvested at the late-exponential phase of fermentative growth did contain DHA kinase activity but at a very low level (about 0.1 unit; μmol·min⁻¹·mg protein⁻¹) (Table 2). However, this DHA kinase activity is higher than the previously reported values for glycerol-grown *E. coli* culture, apparently due to a higher level of DHA, an inducer of *dhaKLM*, in the cytoplasm (15, 21). Due to the inability of strain TG113 to ferment DHA, the copy number of the *dhaKLM* operon with its native promoter was increased by introducing plasmid pDC4. Under similar conditions, strain TG113(pDC4) grew to a higher cell density and fermented almost all of the 111 mM DHA added to the LB medium (Table 1 and Fig. S24). The reason for this difference in fermentation of DHA (111 mM) by the two cultures with comparable in vitro DHA kinase activity (Table 2) is not apparent.

Increasing the concentration of DHA to 333 mM (30 g·L⁻¹) abolished the growth of strain TG113 with or without plasmid pDC4 (Fig. S2B). The medium also turned brown over time, possibly the result of an interaction between DHA and cells. The culture density of strain TG113 or TG113(pDC4) reached an OD₄₂₀ of about 0.3 at about 5 h before a sufficient quantity of growth-inhibitory compounds were generated. Less than 10 mM D-lactate was produced by these cultures (Table 1). It is possible that at the 333-mM DHA concentration the rate of transport of DHA is higher than the rate of conversion to DHA-P by the low DHA kinase activity (about 0.1 unit). This imbalance in transport and phosphorylation could lead to a higher intracellular DHA pool, triggering production of inhibitory compounds, as seen by the accumulation of brown-colored compounds in the medium, as well as to a potential direct interaction of DHA with cellular components (Maillard reaction) (11). The accumulation of the brown-colored compounds required the presence of cells. An

Table 1. Growth and DHA fermentation of *E. coli*

Plasmid	[DHA], mM	D-LA, mM	Yield	Cells
[DHA], 111 mM				
None	40	25	0.63	2.0
+pDC4	100	90	0.90	4.8
+pDC117d	110	100	0.91	3.6
[DHA], 333 mM				
None	33	8	0.23	0.4
+pDC4	40	7	0.18	0.3
+pDC117d	310	275	0.89	6.9

Cells, highest cell density (OD₄₂₀) in 24-h fermentations in LB with the indicated amount of DHA; DHA, amount of DHA consumed; D-LA, highest amount of D-lactate produced; yield, g D-LA/g of DHA consumed⁻¹.

Table 2. DHA kinase activities of *E. coli* and *K. variicola*

Plasmid	DHA kinase activity, μmol·min ⁻¹ ·mg protein ⁻¹			
	Anaerobic		Aerobic	
	ATP	PEP	ATP	PEP
<i>E. coli</i> TG113				
None	UD	0.10	UD	UD
pDC4	UD	0.07	0.02	UD
pDC117d	1.00	0.03	0.15	UD
<i>K. variicola</i> LW225				
None	1.67	1.81	0.48	0.47
pDC117d	0.79	0.74	0.26	UD
pLW63	1.33	0.91	0.22	0.04
pLW63*	0.93	0.02	0.54	0.05

UD, undetectable activity (less than 0.01 unit).

**dhaK* was induced from a *trc* promoter with 25 μM IPTG.

alternative possibility, that DHA-P is produced at a higher rate than glycolytic flux and the accumulating DHA-P is converted to methylglyoxal by methylglyoxal synthase, can be ruled out since strain TG113 carries a deletion of *mgsA* encoding this enzyme.

Fermentation of DHA by *E. coli* Strain TG113 with ATP-Dependent DHA Kinase.

Since strain TG113(pDC4) with PEP-dependent DHA kinase failed to grow at 30 g·L⁻¹ DHA, a gene encoding ATP-dependent DHA kinase was cloned with its native promoter from *Klebsiella oxytoca* strain M5A1 (*dhaK*; plasmid pDC117d) and was introduced into strain TG113. Strain TG113(pDC117d) grew to an OD of 6.9 and fermented 333 mM DHA in less than 24 h with no detectable brown-colored compound in the medium (Table 1 and Fig. S2B). In this strain, the ATP-dependent DHA kinase activity was about 1 unit, about 10 times higher than the level of PEP-dependent activity of strain TG113 (Table 2). Apparently, a level of DHA kinase activity higher than that of the native PEP-dependent activity is needed to support the growth of *E. coli* in a medium with 30 g·L⁻¹ DHA. It should be noted that, in addition to the enzyme, a higher level of a phosphate donor, PEP or ATP, is also required to rapidly remove DHA as DHA-P and to mitigate its inhibitory effect on cells. ATP-dependent DHA kinase is better suited here, since conversion of DHA to pyruvate generates two ATPs, while the same set of reactions can generate only one PEP. This twofold-higher level of phosphate donors (ATPs) in the cytoplasm can support higher DHA kinase activity and offset the inhibitory effect of the higher DHA concentration in the medium.

In a fed-batch fermentation, strain TG113(pDC117d) produced 580 ± 21 mM D-lactate (52 ± 1.9 g·L⁻¹) in 55 h after an initial lag of about 10 h (Fig. 3). The average volumetric productivity of D-lactate for this culture over a 34-h period was 1.24 g·L⁻¹·h⁻¹. This value is about 70% of the volumetric productivity reported for strain TG113 with glucose in mineral salts medium (20). The lactate yield was 0.94 g·g⁻¹ DHA fermented. These results show that the DHA kinase and not the glycolytic flux is apparently the rate-limiting reaction in the conversion of DHA to fermentation products. Strain TG113(pDC117d) also produced very low but detectable amounts of glycerol (27 ± 5 mM), especially during the late stationary phase of growth, catalyzed by glycerol dehydrogenase operating in the reverse direction. Deletion of *gldA* (strain LW290) eliminated glycerol production during DHA fermentation.

Increasing the DHA concentration in the medium above 30 g·L⁻¹ resulted in lack of growth of all *E. coli* cultures (Fig. 2). This suggests that phosphorylation of DHA, either by DHA kinase or ATP availability, is unable to keep up with the rate of entry of DHA, resulting in the accumulation of DHA in the cytoplasm that is growth inhibitory. The highest amount of DHA fermented by strain TG113(pDC117d) in a fed-batch mode was about 0.62 ± 0.02 M in 55 h to produce 0.58 ± 0.02 M D-lactate (yield of

Fig. 3. Fed-batch fermentation of DHA by *E. coli* strain TG113(pDC117d). Fermentation was started with 333 mM DHA in LB medium at 37 °C. At 29 h an additional 333 mM DHA was added to the cultures. The pH of the cultures was controlled at 7.0 with 2M KOH.

0.94 g·g⁻¹) (Fig. 3), although strain TG113 is known to produce higher than 1 M D-lactate from 0.67 M glucose in mineral salts medium in about 48 h (20). It is possible that the declining specific productivity of the aging culture accounts for this low titer. A further increase in the DHA kinase level and/or glycolytic flux to raise the ATP level in the cell to support higher kinase activity is needed to reach the D-lactate titer of strain TG113 on glucose.

Deleting *glpF* Increased Tolerance of *E. coli* to DHA. The results presented suggest that a balance between the rate of transport and the conversion of DHA to DHA-P is needed for effective fermentation of DHA to products (Fig. S3). Any deviation from this leads to the accumulation of DHA in the cytoplasm and inhibition of growth. As discussed above, with a kinase that uses ATP as the phosphate donor, the optimum concentration of DHA for fermentation by *E. coli* strain TG113(pDC117d) was shifted to 333 mM from 111 mM DHA for a strain with PEP-dependent DHA kinase (Table 1 and Fig. S2). An alternate way of shifting the balance toward DHA kinase and DHA-P is to lower DHA transport. Toward this objective, *glpF* was deleted from the chromosome of TG113(pDC117d). Deleting *glpF* (strain LW416), and thus eliminating one of the DHA transporters, increased DHA tolerance to about 450 mM, compared with a tolerance of 333 mM DHA for the *glpF*⁺ parent with the ATP-dependent DHA kinase (Fig. 2). Both the parent and *glpF* mutant, strain LW416, grew and fermented DHA to D-lactate at about the same rate up to about 350 mM DHA. At about 450 mM DHA, TG113 (pDC117d) did not grow, while the *glpF* mutant grew but at a rate that was about 30% of the value of the 333-mM DHA culture. The final cell density of the 450-mM DHA culture was about 60% of the 333-mM DHA culture. Due to the lower cell density, the average volumetric productivity of D-lactate of the 450-mM DHA culture was about 30% of the same culture with 350 mM DHA (1.4 g·L⁻¹·h⁻¹). These results show that by lowering DHA transport, the internal DHA concentration can be set in balance with the ATP-dependent DHA kinase activity at 450 mM DHA. The lower growth rate of this culture suggests that the rate of transport at DHA concentrations ≥450 mM is still higher than the in vivo kinase activity that detoxifies DHA. Additional mutations in the yet to be identified transporter(s) can help establish a DHA pool that is in balance with the kinase activity.

These results are in agreement with the working model that a balance between transport and conversion of DHA to DHA-P is critical to sustain growth and fermentation of DHA to products

(Fig. S3). This can be achieved by manipulating the kinase and transport. An alternative process-based approach to fed-batch fermentation is a continuous feed of DHA at the optimum concentration, and this is expected to support the fermentation of this triose that can be derived from CH_4 to a higher concentration of the desired product by engineered microbial biocatalysts.

Fermentation of DHA by *Klebsiella variicola*. The experiments with *E. coli* suggested that fermentation of DHA can be limited at two steps: DHA kinase or glycolytic flux that generates the phosphate donor for enzyme activity. To distinguish between the two *K. variicola* (strain AC1), which has a higher glucose flux than *E. coli*, was selected for DHA fermentation. The specific rate of glucose consumption by strain AC1 (wild type) in rich medium was determined to be $6.2 \pm 0.35 \text{ g} \cdot \text{h}^{-1} \cdot \text{g dry weight of cells}^{-1}$, and in glucose mineral salts medium this value declined only slightly to $5.09 \pm 1.22 \text{ g} \cdot \text{h}^{-1} \cdot \text{g cells}^{-1}$. Glucose flux of a homolactate producing a derivative of strain AC1, strain MR902, was calculated to be $5.9 \pm 1.6 \text{ g} \cdot \text{h}^{-1} \cdot \text{g cells}^{-1}$ when grown in LB + glucose medium, and this value increased when strain MR902 was grown in mineral salts medium ($7.2 \pm 0.9 \text{ g} \cdot \text{h}^{-1} \cdot \text{g cells}^{-1}$). The average volumetric productivity of D-lactate for strain MR902 was $4.4 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ in rich medium. These values are about twice the productivity for a lactate-producing *E. coli* strain grown under similar conditions with glucose (22, 23). Due to the higher glucose flux and lactate productivity, glycolytic flux is not expected to limit DHA fermentation in *K. variicola*. In addition, strain LW225 also produces an ATP-dependent DHA kinase from the chromosomal *dhaK* (Table 2).

Although strain LW225 had a higher glucose flux, the growth rate of this strain in DHA-containing medium was lower than that of an LB-glucose culture (Fig. S4). The fermentative growth rate of the culture with glucose was 0.84 h^{-1} , compared with a μ value of 0.25 h^{-1} for a DHA culture. The specific productivity of lactate with glucose was $5.4 \text{ g h}^{-1} \text{ g cells}^{-1}$, while the specific productivity with DHA as the carbon source was about 35% of the glucose value ($1.9 \text{ g h}^{-1} \text{ g cells}^{-1}$). These results suggest that strain LW225 also has a limiting step in DHA utilization, most probably at the DHA kinase activity, as seen with the *E. coli* strain TG113. Under fermentative conditions, the native ATP- and PEP-dependent DHA kinase activities of strain LW225 were 1.7 and 1.8 units, respectively (Table 2), suggesting that in *K. varicola* DHA kinase activity is limited not by the enzyme level but by the availability of the cosubstrate ATP and PEP.

In contrast to *E. coli*, *K. variicola* fermented 333 mM (30 g·L⁻¹) DHA using native DHA kinase(s) (Fig. S4). Even with its higher level of native DHA kinase activity (Table 2), *K. variicola* was unable to grow when the DHA concentration was increased above 350 mM (Fig. S5) as seen with *E. coli* TG113(pDC117d) (Fig. 2 and Fig. S5). This inhibition is apparently due to an imbalance between the transport of DHA into the cytoplasm and the ability of the cell to provide ATP/PEP at a rate needed to detoxify DHA by conversion to DHA-P. Due to this growth inhibition by higher concentrations of DHA, strain LW225 fermentations were run in fed-batch mode (Fig. 4). Under this fermentation condition, a D-lactate titer of 811 ± 26 mM (72 g·L⁻¹) was reached in about 60 h. The average volumetric productivity of lactate over a 20-h period was 2.3 g·L⁻¹·h⁻¹. This is about 40% of the D-lactate productivity (5.6 g·L⁻¹·h⁻¹) of *K. variicola* with glucose as the carbon source under similar fermentation conditions. During the late stationary phase, glycerol was also detected as a coproduct, possibly as a result of energy imbalance. Introducing plasmid pDC117d encoding ATP-dependent DHA kinase into strain LW225 to increase the copy number of *dhaK* did not significantly alter the fermentation profile of *K. variicola*, suggesting that the native chromosomal copy of DHA kinase is sufficient to support growth and fermentation of DHA by strain LW225. Unexpectedly, and for reasons that are not clear, the presence of plasmid pDC117d in strain LW225 lowered the measured in vitro DHA kinase activity with either ATP or PEP as substrate (Table 2). Plasmid vector pBR322 without *dhaK* had no effect on the in vitro DHA kinase

Fig. 4. Fed-batch fermentation of DHA in LB by *K. variicola* strain LW225. Fermentation was started with 333 mM DHA in LB at 37 °C and pH 7.0. At 23, 34, and 46 h, an additional 333 mM DHA was added to a total of 1.33 M ($120 \text{ g} \cdot \text{L}^{-1}$). All the added DHA was fermented at 60 h.

activity of strain LW225. Replacing the native promoter in plasmid pDC117d with an inducible *trc* promoter (LW225 with plasmid pLW63) did not overcome the negative effect of plasmid-borne *dhaK* on measured *in vitro* DHA kinase activity in either the absence or presence of the inducer isopropyl β -D-1-thiogalactopyranoside (IPTG) (25 μM). It is possible that production of DHA kinase above a critical level drains the ATP pool, triggering a yet to be identified control system that regulates the level of kinase in the cell.

These results show that DHA can be readily fermented to D-lactate by either *E. coli* strain TG113(pDC117d) or *K. variicola* strain LW225 under appropriate fermentation conditions. A reduction in the rate of transport of DHA to match the flux rate of intermediary metabolism and the supply of ATP/PEP could overcome the toxicity of DHA while also improving energy balance and D-lactate productivity (Fig. S3).

Fermentation of DHA to Succinic Acid by Strain KJ122(pDC117d). Fermentation of DHA to D-lactic acid raised the possibility that DHA produced from CH_4 can be fermented to any one of several products that are currently produced from hexoses and pentoses by various microbial biocatalysts. To demonstrate this potential, strain KJ122, an *E. coli* strain that produces succinic acid as the major fermentation product (24), was transformed with plasmid pDC117d, and the transformants fermented DHA to succinate (Fig. 5A). In this fed-batch fermentation, about 0.5 M DHA was converted to about 0.3 M succinate ($38 \text{ g} \cdot \text{L}^{-1}$), and the succinate yield was $0.86 \text{ g} \cdot \text{g}^{-1}$ DHA consumed (Fig. 5A). The conversion efficiency was 66% of the theoretical yield. About 85 mM acetate was also produced by this culture, mostly during the growth phase. Further metabolic engineering to minimize acetate is expected to increase the succinate titer and productivity.

Fermentation of DHA to Ethanol by *E. coli* Strain SE2378(pDC117d). The abundance of natural gas and its current low price in the United States raised the possibility of converting CH_4 to liquid fuels (GTL) that can be more readily used as a transportation fuel. As discussed earlier, the technology exists for the conversion of natural gas to DHA provided an efficient microbial biocatalyst can be developed for the fermentation of DHA to ethanol. We have previously constructed ethanologenic strains of *E. coli* and *K. oxytoca* (25). One of these ethanologens, *E. coli* strain SE2378, was transformed with plasmid pDC117d to ferment DHA to ethanol. The results presented in Fig. 5B show

that DHA can be effectively fermented to ethanol at a yield of $0.39 \text{ g} \cdot \text{g}^{-1}$ (77% of the theoretical yield). This is comparable to the yield of $0.41 \text{ g} \cdot \text{g}^{-1}$ glucose fermented by strain SE2378 (26). Other ethanologenic bacterial and yeast strains, appropriately engineered for DHA fermentation, have the potential to increase the titer and yield of ethanol from natural gas with DHA as an intermediate.

Conclusion

Although DHA is inhibitory to the growth and fermentation of *E. coli*, the activity of DHA kinase was identified as the rate-limiting step. By introducing an ATP-dependent DHA kinase, the inhibitory effect of DHA was partially mitigated in *E. coli*. A fed-batch fermentation process was used to mitigate the toxicity of DHA. With these modifications, DHA was fermented by *E. coli* and *K. variicola* to D-lactic acid. Using appropriate engineered *E. coli* derivatives, DHA was also fermented to succinic acid or ethanol (Table S4). Further metabolic evolution of these microbial biocatalysts is anticipated to increase product titer, yield, and productivity. These results show that DHA produced from CO_2 or natural gas is a valuable feedstock for fermentation to desired product(s).

Fig. 5. Fermentation of DHA to succinate or ethanol by engineered *E. coli* derivatives. (A) Fermentation of DHA in LB by *E. coli* strain KJ122(pDC117d) to succinate was started with 87 mM DHA at 37 °C and pH 7.0. At various times (24, 42.5, 71.5, 93.5, and 145 h), 100 mM DHA was added to the fermentation to a total of 590 mM. (B) Fermentation of DHA in LB by an ethanologenic *E. coli* strain SE2378(pDC117d) was started with 99 mM DHA at 37 °C and pH 7.0. At 21.5 h, an additional 181 mM DHA was added to the fermentation for a total of 280 mM.

Materials and Methods

Strains, Media, and Growth Conditions. The bacterial strains, plasmids, and primers used in this study are listed in [Tables S1–S3](#), respectively, in [Supporting Information](#). Bacterial cultures were grown in LB medium as described previously (27). The mineral salts medium was AM1 medium (28). Anaerobic cultures were grown in 13 × 100 mm screw-cap tubes filled to the top. Fermentations were in 500-mL vessels with 250 mL of medium as described previously with pH control at 37 °C (29). Fermentations started aerobically due to the air in the gas phase and gentle mixing of the liquid by a magnetic stirrer (200 rpm) for base addition. As the cell density increased to an OD₄₂₀ of about 0.5, the limitation of O₂ resulted in anaerobic conditions (severe O₂ limitation) and the initiation of fermentative growth.

Strain Constructions.

E. coli mutants. *E. coli* strains TG113, KJ122, and SE2378 were described previously (20, 24, 26). Strain LW290 is a derivative of strain TG113 with a deletion of *gldA* that eliminated glycerol dehydrogenase. This strain was constructed using the method described by Datsenko and Wanner (30). Strain LW410 was derived from strain TG113 and carries a 572-bp internal deletion of *glpF* and was constructed using the method of Datsenko and Wanner (30). Details of construction of strains LW290 and LW410 are presented in [Supporting Information](#).

K. variicola strain LW225. *K. variicola* strain LW225, a mutant of wild-type strain AC1 lacking butanediol synthesis pathway enzymes, α -acetolactate decarboxylase (*budA*), acetolactate synthase (*alsS*), and pyruvate formate-lyase (*pfhA*), was constructed to eliminate side reactions at the pyruvate node for production of D-lactate. Details of strain construction are presented in [Supporting Information](#).

Construction of plasmids pDC4, pDC117d, and pLW63. Plasmid pDC4 carries the *dhaKLM* of *E. coli* encoding PEP-dependent DHA kinase in the pCR2.1-TOPO vector. Plasmid pDC117d carries the gene encoding an ATP-dependent DHA kinase (*dhaK*) from *K. oxytoca* M5A1 in the plasmid vector pBR322. Plasmid pLW63 includes the *K. oxytoca dhaK* with a lactose-inducible *trc* promoter in

the plasmid vector pTrc99a. Details of construction of these plasmids are in [Supporting Information](#).

Enzyme Assays. DHA kinase activity was determined in crude extracts of cultures grown in 250 mL of LB + DHA (30 g·L⁻¹) in fermenters with pH control (7.0) or under aerobic conditions (a 2.8-L Fernbach flask in an Eppendorf shaker at 200 rpm) at 37 °C to the midexponential phase of growth. Cells harvested by centrifugation at 4,200 × *g* for 10 min at 4 °C were washed once with 20 mL of Hepes buffer (50 mM; pH 7.5). Cells collected by centrifugation (5,900 × *g*; 5 min) were resuspended in 2 mL of Hepes buffer. Cells were passed through a French pressure cell operating at 20,000 psi, and the extract was centrifuged at 17,500 × *g* for 10 min to remove cell debris. The supernatant was centrifuged again at 39,000 × *g* for 20 min to remove large vesicles. This supernatant served as the extract for the enzyme assay. Protein concentration was determined by the Bradford method (31).

DHA kinase was assayed using ATP or PEP as the phosphate donor in a coupled assay as described previously (32, 33). One milliliter of assay mixture for ATP-dependent activity contained Hepes buffer (50 mM; pH 7.5), MgCl₂ (2.55 mM), NADH (0.25 mM), ATP or PEP (1 mM), glycerol-3-phosphate dehydrogenase (rabbit muscle, 1.7 units; Sigma-Aldrich), and cell extract. The initial rate of oxidation of NADH after the addition of DHA (1 mM) was determined in the coupled reaction at 340 nm. One unit of enzyme activity is 1 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$.

Analytical Methods. Organic acids, ethanol, and DHA were determined using an Agilent (1200) HPLC system equipped with dual detectors (UV and refractive index, in series) and a Bio-Rad Aminex HPX-87H column (34). The optical purity of lactic acid was determined as before (35).

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