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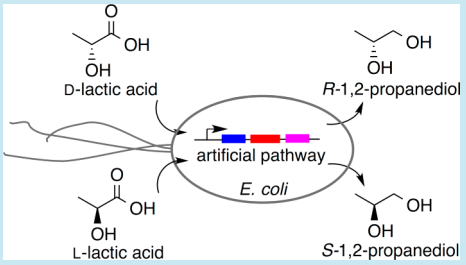
Stereospecific Microbial Conversion of Lactic Acid into 1,2-Propanediol

Wei Niu* and Jiantao Guo*

Department of Chemistry, University of Nebraska−Lincoln, Lincoln, Nebraska 68588, United States

Supporting Information

ABSTRACT: Biocatalytic syntheses are increasingly explored as the alternate platform of chemical production in order to address the sustainable development challenge faced by the current chemical industry. Here, we report the design and implementation of an artificial pathway to convert lactic acid into 1,2-propanediol. It circumvents a highly cytotoxic intermediate that exists in a widely used natural pathway. We identified and characterized a key enzyme that catalyzed the nonnatural step of the pathway. After 72 h of cultivation under shake-flask conditions, an *Escherichia coli* biocatalyst expressing the artificial route synthesized 1.5 g/L of *R*- or 1.7 g/L of *S*-1,2-propanediol from D- or L- lactic acid at high enantiomeric purity, respectively. The bioconversion is part of a novel biosynthetic pathway that can be further incorporated into appropriate microbial hosts for the *de novo* synthesis of optically pure 1,2-propanediol from renewable feedstocks.



KEYWORDS: *1,2-propanediol, lactic acid, artificial pathway, stereospecific, biocatalysis*

Current chemical manufacturing relies heavily on raw materials derived from limited, nonrenewable natural resources. Traditional chemical production processes often cause long-term environmental problems. By using starting materials derived from renewable feedstocks, such as plant cellulosic material, biosyntheses are emerging as indispensable alternatives to address the sustainability issue of the chemical industry.[1] Because commercial chemicals are not well represented by the repertoire of natural products, and natural pathways are often not suitable for large-scale chemical production, efforts are increasingly directed to the discovery and the development of novel biosynthetic pathways for industrial and pharmaceutical chemicals.[2−6]

1,2-Propanediol (1,2-PDO), or propylene glycol, is a bulk industrial chemical with a global demand of around 1.36 million tons/year.[7] It is widely used in the production of unsaturated polyester resin and as a nontoxic replacement of ethylene glycol in deicer and antifreeze products.[8] Current commercial production of 1,2-PDO is mainly by high pressure, high temperature, noncatalytic hydrolysis of propylene oxide, which results in a racemic mixture.[8] 1,2-PDO is a natural metabolite with two well-studied biosynthetic pathways (see Supporting Information, Figure S1): (1) certain bacteria and yeasts produce *S*-1,2-PDO from the catabolism of 6-deoxyhexoses;[9,10] (2) Clostridia strains accumulate the *R*-isomer from glucose via the intermediacy of methylglyoxal.[11,12] Due to limited availability of deoxyhexoses, current studies on microbial production of 1,2-PDO exclusively focus on the methylglyoxal pathway.[13,14] The highest titer of 4.5 g/L from glucose[15] and 5.6 g/L from glycerol[16] were achieved by two engineered *E. coli* strains under fermentor-controlled cultivation conditions, respectively. As *E. coli* is tolerant to over 100 g/L of 1,2-PDO, there is significant room for titer improvement.[17] A major hurdle to achieve higher-titer and high-yield biosynthesis of 1,2-PDO through the methylglyoxal pathway lies in the cytotoxicity of the namesake intermediate. Submillimolar concentrations of methylglyoxal can cause *E. coli* cell to lose viability.[18,19] In this work, we seek to overcome this hurdle by creating a novel 1,2-PDO biosynthetic pathway.

Recent research showed that *Lactobacillus buchneri* strains might encode a third route to 1,2-PDO biosynthesis through the reduction of lactic acid.[20,21] However, details of the pathway are unknown. Based on retrosynthetic analysis and known high-efficiency biosynthetic pathways of alcohols, we filled in this black box with plausible enzymatic reactions and devised an artificial biosynthetic pathway for microbial synthesis of 1,2-PDO from fermentable sugars (e.g., glucose) via the intermediacy of lactic acid (Scheme 1). The lactic acid is first activated by a lactoyl-CoA transferase to form lactoyl-CoA, which is subsequently reduced by a CoA-acylating aldehyde dehydrogenase to form lactaldehyde. The resulting aldehyde is finally reduced to form 1,2-PDO. Because the two consecutive reduction steps do not change the stereochemistry at the C2 position, we expect this novel pathway to be highly stereospecific with the production of *R*-1,2-PDO from D-lactic acid and *S*-1,2-PDO from L-lactic acid as the biosynthetic intermediate, respectively (Scheme 1). Since microbial syntheses of lactic acid are well studied, here we focused efforts on the implementation of the microbial conversion of lactic acid into 1,2-PDO.

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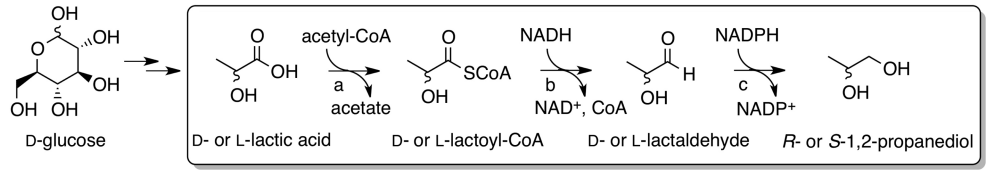
Scheme 1. Artificial Biosynthetic Pathway to 1,2-Propanediol



[a]Lactoyl-CoA transferase (Pct, *Megasphaera elsdenii*). [b]CoA-dependent lactaldehyde dehydrogenase (PduP, *Salmonella enterica*). [c]Lactaldehyde reductase (YahK, *E. coli*). Glucose is used as an example of fermentable sugars.

The key step in the microbial conversion of lactic acid into 1,2-PDO (Scheme 1) is the unprecedented enzymatic reduction of lactoyl-CoA to lactaldehyde. To identify an enzyme that can catalyze the reaction, we cloned three oxygen-tolerant CoA-acylating aldehyde dehydrogenases, including PduP (propionyl-CoA) from *Salmonella enterica*, SauS (sulfoacetyl-CoA) from *Cupriavidus necator* H16 and SucD (succinyl-CoA) from *Clostridium kluyveri*. Each enzyme was expressed and purified as a fusion protein with a C-terminus 6xHis tag from *E. coli* host. Purified proteins were examined in assays that contained either their native CoA substrate or a racemic mixture of D- and L-lactoyl-CoA. The CoA derivatives were chemically synthesized by carbonyldiimidazole-mediated coupling reactions between coenzyme A and free acids.[22] The CoA-acylating aldehyde dehydrogenase activity was monitored by following the oxidation of NADH or NADPH. All three purified proteins were functional on the native substrates, but only the *pduP*-encoded CoA-acylating propionaldehyde dehydrogenase showed significant activity on lactoyl-CoA. We further characterized purified PduP on four CoA substrates (Table 1). The enzyme is active on both D- and L-lactoyl-CoA,

Table 1. Kinetic Properties of PduP

entry	substrates	K_m (μM)[a]	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
1	propionyl-CoA	5.8 ± 0.6	36.6 ± 1.8	6.31×10^3
2	D-lactoyl-CoA	56.6 ± 9.8	2.6 ± 0.5	45.9
3	L-lactoyl-CoA	1205.3 ± 87.2	13.0 ± 1.5	10.8
4	acetyl-CoA	342.5 ± 97.3	12.4 ± 1.1	36.2

[a]Data are the average of three measurements at 37 °C in phosphate buffer (pH 7.0) containing NADH (2 mM).

but it shows different kinetic properties (Table 1). PduP can also catalyze the reduction of acetyl-CoA, which is an abundant CoA derivative in *E. coli* cells with the intracellular concentration ranging from 20 μM to 600 μM at different cell growth stages.[23] The undesirable activity on acetyl-CoA may lead to the formation of ethanol as the reduction product of acetaldehyde. The catalytic efficiency (k_{cat}/K_m; Table 1) of PduP toward the three nonnative substrates are more than 100-fold lower than that of the native substrate, propionyl-CoA. Nevertheless, this is the first time that PduP is shown to be active toward acetyl-CoA, and D- and L-lactoyl-CoA.

We next focused on characterizing the enzyme that catalyzes the first step of the conversion from lactic acid to 1,2-PDO (Scheme 1). The *pct* gene of *Megasphaera elsdenii*[24] was cloned and expressed in *E. coli*. The encoded lactoyl-CoA transferase was purified and examined for its stereospecificity on D- and L-lactic acid (Table 2). Purified Pct enzyme can use both D-lactate and L-lactate as substrates with similar k_{cat} and slightly higher K_m toward the L-isomer. The enzyme showed similar K_m (~50 μM) and k_{cat} (~50 s^{-1}) values for acetyl-CoA when either D- or L-lactic acid isomer was provided (see Supporting Information, Figure S4).

Table 2. Kinetic Properties of Pct

entry	substrates	K_m (mM)[a]	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
1	D-lactate	1.3 ± 0.3	51.7 ± 2.1	39.8
2	L-lactate	3.5 ± 0.5	50.4 ± 1.8	14.4

[a]Data are the average of three measurements at 37 °C in phosphate buffer (pH 7.0) containing acetyl-CoA (1 mM).

To enable the bioconversion of lactic acid into 1,2-PDO, we constructed an *E. coli* host strain that lacks the ability to utilize lactate as a carbon source. To this end, the *lldD* gene (encodes the L-lactate dehydrogenase) and the *dld* gene (encodes the D-lactate dehydrogenase) was deleted from the chromosome of K-12 wild-type strain MG1655. We disabled *E. coli* native ability to produce D-lactic acid by the deletion of the *ldhA* gene, which encodes the fermentative D-lactate dehydrogenase. Such deletion is necessary for determining the enantiomeric purity of the bioconversion product by eliminating endogenous source of lactic acid. We also deleted the *adhE* gene, which encodes the fermentative enzyme for ethanol production, so that the installed biocatalytic reactions are the major source of ethanol accumulation. The obtained *E. coli* strain, MGΔ^4 (Δ*lldD*, Δ*dld*, Δ*ldhA*, and Δ*adhE*) showed a significantly longer lag phase than the parent strain MG1655 when they were each cultured in media containing a racemic mixture of sodium lactate as the sole carbon source (see Supporting Information Figure S2). As a consequence, lactate was provided as the starting material in all the bioconversion experiments, while glucose was supplemented as the carbon source for cell growth. We next constructed a gene cassette by sequentially cloning the *pduP* and the *pct* gene behind the P_{tac} promoter of vector pJF118EH. The resulting plasmid, pWN2.094, encoded essential enzyme activities for the reduction of lactate into lactaldehyde. We envisioned that the native alcohol dehydrogenase activities of *E. coli* host strain are sufficient to reduce lactaldehyde to 1,2-PDO.

We examined the bioconversion of lactic acid into 1,2-PDO by culturing strain MGΔ^4/pWN2.094 in glucose minimal media containing 55.5 mM of D- or L-lactate. Samples were taken for cell growth and metabolite accumulation analysis at 24-h time interval. After 72 h of cultivation under aerobic growth conditions in erlenmeyer flasks, the *E. coli* biocatalyst produced 2.0 mM (Figure 1a) and 5.7 mM (Figure 1b) of 1,2-PDO from D- and L-lactate, respectively, which represents 3.7% and 10.2% molar yield from lactate. ^{1}H NMR analysis showed that the residual lactate concentrations are 48 mM for the D- and 45 mM for the L- isomer. In addition to the titer difference between the two bioconversions, we also observed significant growth retardation of the culture for D-lactic acid reduction (Figure 1a). We postulated that the constitutively expressed *E.*

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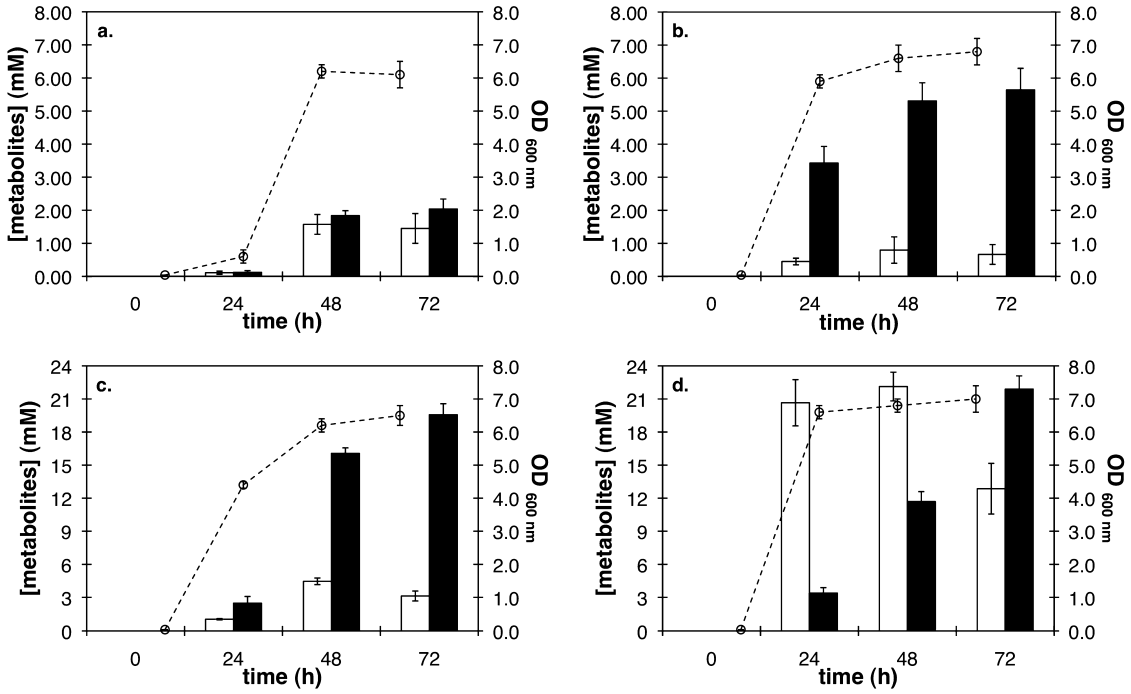

Figure 1. Bioconversion of lactate into 1,2-PDO by *E. coli* strains. (a) MGΔ[4]/pWN2.094, D-lactate; (b) MGΔ[4]/pWN2.094, L-lactate; (c) MGΔ[4]/pWN2.094/pWN2.096, D-lactate; (d) MGΔ[4]/pWN2.094/pWN2.096, L-lactate. Open circles, $OD_{600\ nm}$; open bars, ethanol; filled bars, 1,2-PDO. Data are the average of three experiments.

coli alcohol dehydrogenase(s) has sufficient activity toward the L-lactaldehyde but not the D-isomer. To remedy this problem, we screened several *E. coli* short-chain and medium-chain alcohol dehydrogenases, and identified that YahK[25] is active on both the D- and the L-lactaldehyde. We constructed plasmid pWN2.096 by inserting the *E. coli yahK* gene after the P_{lac} promoter on vector pSU18. Strain MGΔ[4]/pWN2.094/pWN2.096 was examined for 1,2-PDO synthesis from lactate. At 72 h, a total of 19.6 mM (1.5 g/L, Figure 1c) and 21.9 mM (1.7 g/L, Figure 1d) 1,2-PDO were accumulated from D- or L-lactate, which corresponds to 35.3% and 39.4% molar yield. The residual lactate concentrations are 29.4 mM and 33.6 mM, respectively. The overexpression of the YahK protein significantly improved the initial growth rate for the D-lactic acid bioconversion culture and improved the titer and yield for both D- and L- bioconversions. On the other hand, significantly higher concentrations of ethanol were produced by *E. coli* strains that overexpressed YahK. This effect is more prominent for the culture with L-lactate (Figure 1d). We attribute this result to the kinetics property of *pduP*-encoded lactoyl-CoA dehydrogenase, which has the highest catalytic efficiency on D-lactoyl-CoA, followed by acetyl-CoA and L-lactoyl-CoA (Table 1).

Biosynthesized 1,2-PDO was partially purified from the bacterial culture media and derivatized using *p*-bromobenzoyl imidazole.[26] The enantiomeric purity of the resulting 1,2-PDO benzyl diesters were analyzed by HPLC equipped with a Chiralcel OD column. When D-lactate (≥98% ee) was used in the bioconversion, *R*-1,2-PDO was produced at 99% ee. When L-lactate (≥98% ee) was used, *S*-1,2-PDO was accumulated at 98% ee (see Supporting Information, Figure S6). The bioconversions retained the stereochemistry at the C2 position. This work is the first demonstration of *S*-1,2-PDO production at significant level with engineered microbial catalysts. A single *E. coli* strain allows one to access both 1,2-PDO enantiomers by simply switching to corresponding starting material.

In summary, we designed and successfully installed a highly stereospecific artificial microbial conversion of lactic acid into 1,2-PDO in *E. coli*. Our current research focuses on *de novo* biosynthesis of 1,2-PDO from renewable feedstocks following the devised novel biosynthetic pathway (Scheme 1) in host strains with lactic acid-producing ability[27,28] through protein engineering and metabolic engineering approaches. In addition, 1,2-PDO can be (bio)catalytically converted into other C3 industrial compounds, such as 1-propanol, 2-propanol, and propylene, which are currently and mainly derived from petroleum.

METHODS

Molecular Biology and Microbiology. Bacteria strains *Clostridium kluyveri* (ATCC 8527), *Cupriavidus necator* H16 (ATCC 17699), *Megasphaera elsdenii* (ATCC 17753), and *Salmonella enterica* (ATCC 700720) were purchased from American Type Culture Collection. The *E. coli* chromosomal deletion was carried out using the PCR product-mediated method.[29] Table S1 (see Supporting Information) lists the sequences of all the primers that are used in this study. For *E. coli* bioconversion of lactic acid, a 100 μL of an overnight culture was transferred into 10 mL of M9 minimal salts media containing glucose (10 g/L) and sodium lactate (55.5 mM) in 50 mL erlenmeyer flask, which were then covered with aluminum foil. Triplicate cultures of each strain were cultivated at 37 °C with shaking at 250 rpm. Samples were removed at 24-h intervals starting at 0 h. Cell densities were determined by measurement of absorption at 600 nm (OD_{600}). Solute concentrations in cell-free broth were determined by GC and ^{1}H NMR.

Syntheses of CoA Derivatives. For the initial screen of CoA-acylating aldehyde dehydrogenases, the CoA derivatives

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were synthesized by following reported procedure[22] with modifications (see Supporting Information). For the kinetics analysis of PduP, propionyl-CoA, D- and L-lactoyl-CoA were synthesized enzymatically. A typical enzymatic reaction contained purified Pct (5 U), sodium salt of carboxylic acid (0.25 mmol) and acetyl-CoA (0.005 mmol) in potassium phosphate buffer (50 mM, pH 7.0) at a final volume of 2.5 mL. The reaction was carried out at 37 °C for 1 h with gentle mixing, then quenched by the addition of formic acid. The quenched reaction was centrifuged to remove precipitates. After adjusting the pH to neutral, the CoA derivatives were purified by HPLC equipped with a semipreparative C18 column and eluted with acetonitrile in water (0.1% TFA). The fraction of CoA derivative was collected and lyophilized. Concentrations of CoA solutions were determined by absorbance at 260 nm using the molar extinction coefficient value of acetyl-CoA (15 400 $M^{-1}cm^{-1}$).

Enzymology. Activities of CoA-acylating aldehyde dehydrogenases were determined by measuring the acyl-CoA dependent oxidation of NADH at 37 °C. Activities of the lactoyl-CoA transferase were assayed by monitoring the lactoyl-CoA formation from acetyl-CoA and lactate at 37 °C using HPLC method. Quantification of lactoyl-CoA was done using the calibration curve of acetyl-CoA. Enzyme kinetics was analyzed using the Graphpad Prism software. Data was fitted to Michaelis–Menten equation using nonlinear regression algorithm.

Analytical Methods. Samples analyzed by gas chromatography were prepared in methanol containing *n*-propanol as the internal standard. Gas chromatography was performed on a Varian CP-3800 GC-FID equipped with an Agilent VF-WAXms capillary column (30 m × 0.25 mm × 0.25 μm). Temperature programming began with an initial temperature of 40 °C for 4 min, then was increased to 80 °C at a rate of 20 °C/min, and finally increased to 250 °C at a rate of 50 °C/min, and held for 5 min. Ethanol is detected at 3.18 min, *n*-propanol at 5.05 min, and 1,2-PDO at 10.04 min. HPLC was performed on an Agilent 1260 infinity LC system equipped with quaternary pump and variable wavelength detector. Analysis of CoA derivatives used a Poroshell 120 EC-C18 (0.46 × 50 mm, 2.7 μm) column eluted with acetonitrile in potassium phosphate buffer (25 mM, pH 7.0) at a gradient of 5% to 30% in 20 min. Enantiomeric purity analysis used a Chiralcel OD (0.46 mm x 25 cm) column eluted with hexane/isopropanol = 99:1 (v/v). For ^{1}H NMR quantification of solute concentrations, solutions were concentrated to dryness under reduced pressure and redissolved in D_2O containing 0.75% of the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid. Concentrations were determined by comparing integrals of each compound with the integral corresponding to TSP (δ = 0.0 ppm) in the ^{1}H NMR.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details for enzyme assays, enzyme kinetics analysis, syntheses of CoA derivatives and 1,2-PDO enantiomeric purity analysis. This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*Email: wniu2@unl.edu.
*Email: jguo4@unl.edu.

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Notes

The authors declare no competing financial interest.

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