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Rapid Report

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Construction of a Part of a 3-Hydroxypropionate Cycle for Heterologous Polyketide Biosynthesis in *Escherichia coli*

Satoshi Yuzawa,[†,§] Naoki Chiba,[†,§] Leonard Katz,[†,∥] and Jay D. Keasling*[,†,‡,§,∥,⊥]

[†]QB3 Institute, University of California, Berkeley, California 94270, United States
[‡]Department of Chemical and Biomolecular Engineering, University of California, Berkeley, California 94270, United States
[§]Joint BioEnergy Institute, 5885 Hollis Street, Emeryville, California 94608, United States
[∥]Synthetic Biology Engineering Research Center, 5885 Hollis Street, Emeryville, California 94608, United States
[⊥]Physical Bioscience Division, Lawrence Berkeley National Laboratory, Berkeley, California 94270, United States

Supporting Information

ABSTRACT: Polyketides, an important class of natural products with complex chemical structures, are widely used as antibiotics and other pharmaceutical agents. A clear barrier to heterologous polyketide biosynthesis in *Escherichia coli* is the lack of (2*S*)-methylmalonyl-CoA, a common substrate of multimodular polyketide synthases. Here we report a route for synthesizing (2*S*)-methylmalonyl-CoA from malonyl-CoA with a 3-hydroxypropionate cycle in thermoacidophilic crenarchaeon. The engineered *E. coli* strain produced both propionyl-CoA and methylmalonyl-CoA at intracellular levels similar to those of acetyl-CoA and succinyl-CoA, respectively. This approach may open a way to produce a variety of polyketide drugs in *E. coli* from renewable carbon sources.

Heterologous natural product biosynthesis has emerged as a strategy for overproducing clinically important compounds that are too complex to be synthesized chemically. To this end, organisms like *Escherichia coli* provide key features of an ideal host in terms of the growth rate, culture conditions, and available recombinant DNA technologies. In fact, *E. coli* has been used for industrial-scale production of a variety of compounds for many years.[1]

Polyketide compounds, an important class of natural products with complex chemical structures, are widely used as antibacterial, immunosuppressant, antitumor, antifungal, and antiparasitic agents. However, many polyketides are produced by complex, slow-growing microorganisms such as actinobacteria and myxobacteria, making their production and isolation difficult. In addition, there is a growing body of evidence from genomic studies that many polyketides remain undiscovered because of an inability to cultivate the producing organisms.[2] Moving genes that are predicted to encode polyketide synthases from such hosts into *E. coli* can be a promising way to accelerate drug discovery.

Multimodular polyketide synthases (PKSs) conduct a programmed, stepwise process that leads to the generation of poly-β-ketones with varying degrees of reduction at the β-carbonyl centers. Multimodular PKSs frequently use (2*S*)-methylmalonyl coenzyme A (CoA) as a substrate. For example, the 6-deoxyerythronolide B synthase (DEBS) assembles one propionyl-CoA and six (2*S*)-methylmalonyl-CoAs to produce 6-deoxyerythronolide B (6dEB), the polyketide backbone of the antibacterial agent erythromycin.[3] Native *E. coli* metabolism, however, does not produce methylmalonyl-CoA at appreciable levels, which is a clear barrier to the production of polyketides in this host.

One biosynthetic route to methylmalonyl-CoA is the carboxylation of propionyl-CoA. In the first report of heterologous production of 6dEB in *E. coli*, exogenously added propionate was converted into propionyl-CoA by the native propionyl-CoA ligase. Propionyl-CoA was then carboxylated by propionyl-CoA carboxylase from *Streptomyces coelicolor* to produce (2*S*)-methylmalonyl-CoA.[4] This pathway was also demonstrated in *Saccharomyces cerevisiae*.[5] The other route to (2*S*)-methylmalonyl-CoA is the two-step isomerization of TCA cycle-derived succinyl-CoA by adenosylcobalamin-dependent methylmalonyl-CoA mutase and methylmalonyl-CoA epimerase.[6]

Here, we report another route to the biosynthesis of (2*S*)-methylmalonyl-CoA in *E. coli* using a 3-hydroxypropionate (3HP) cycle from thermoacidophilic crenarchae *Metallosphaera sedula* and *Sulfolobus tokodaii*.[7] The pathway (Figure 1) is part of the 3HP cycle and converts malonyl-CoA into propionyl-CoA with nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP). Propionyl-CoA can then be converted into (2*S*)-methylmalonyl-CoA as previously reported.[4]

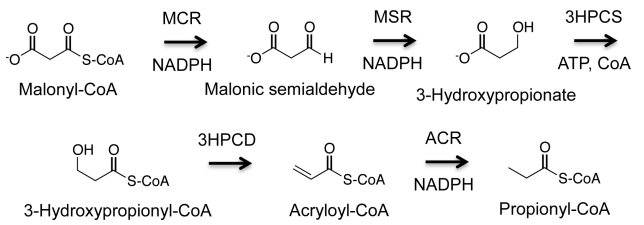

Figure 1. Part of a 3-hydroxypropionate cycle.

Received: October 17, 2012
Revised: November 12, 2012
Published: November 26, 2012

dx.doi.org/10.1021/bi301414q | *Biochemistry* 2012, 51, 9779−9781

Conversion of malonyl-CoA into propionyl-CoA in *E. coli* requires five enzymatic activities: malonyl-CoA reductase (MCR), malonic semialdehyde reductase (MSR), 3-hydroxypropionyl-CoA synthase (3HPCS), 3-hydroxypropionyl-CoA dehydratase (3HPCD), and acryloyl-CoA reductase (ACR). The optimal temperatures of these enzymes, however, were reported to be ≥65 °C.[8−11] To analyze the activities at temperatures at which *E. coli* can grow, we synthesized these genes with codons optimized for expression in *E. coli* and constructed expression vectors to produce the corresponding N-terminal hexahistidine-tagged proteins. We used *E. coli* BL21(DE3) as a host to express the synthetic genes. The proteins were purified by nickel affinity chromatography at yields ranging from 2 to 10 mg/L. To reconstitute the system in vitro, we mixed the purified enzymes in equimolar ratios, incubated them for 1 h at 23 °C in the presence of malonyl-CoA, ATP, and NADPH, and analyzed the resulting reaction mixture by liquid chromatography and mass spectrometry (LC−MS). We monitored molecular ions for CoA, malonyl-CoA, 3-hydroxypropionyl-CoA, acryloyl-CoA, and propionyl-CoA, the proposed products from the reaction. As shown in Figure 2, malonyl-CoA was almost completely converted into

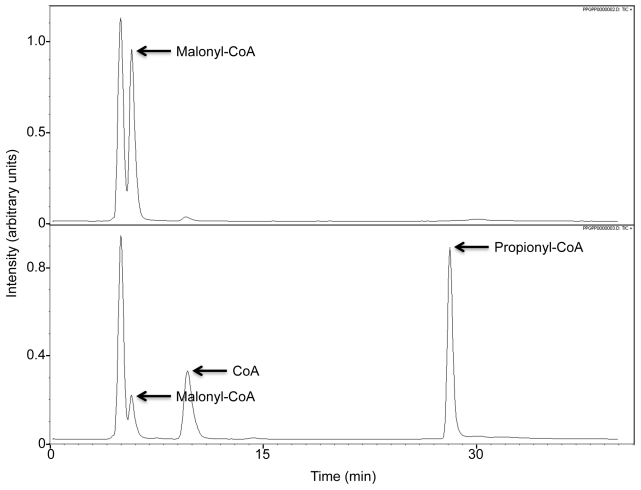

Figure 2. In vitro biosynthesis of propionyl-CoA from malonyl-CoA. Malonyl-CoA (250 μM) was incubated for 1 h at 23 °C with 1 mM NADPH and 1 mM ATP in the absence (top) or presence (bottom) of MCR, MSR, 3HPCS, 3HPCD, and ACR (1 μM each). The resulting reaction mixtures were analyzed by LC−MS.

propionyl-CoA. No other acyl-CoA was detected (except for a small amount of CoA), demonstrating the robustness of the pathway even at 23 °C. We calculated the turnover rate of the entire cycle to be >4.2 min^{-1} at 23 °C. We also attempted to produce acryloyl-CoA from 3-hydroxypropionate in vitro by adding 3HPCS and 3HPCD. Interestingly, 3-hydroxypropionyl-CoA and propionyl-CoA were the major products observed, rather than acryloyl-CoA (Figure 1 of the Supporting Information). Via addition of ACR to the system, 3-hydroxypropionyl-CoA was completely consumed to produce propionyl-CoA as a sole product (Figure 1 of the Supporting Information).

To further analyze the kinetic parameters of the system, an NADPH consumption assay was conducted at 25 and 37 °C. The pathway consumes three NADPH molecules to produce one molecule of propionyl-CoA from one molecule of malonyl-CoA. We measured the turnover rate of the system using an equimolar mixture of MCR, MSR, 3HPCS, 3HPCD, and ACR. The observed rates were 22 min^{-1} at 25 °C and 37 min^{-1} at 37 °C (Figure 2 of the Supporting Information). As expected, the system showed a faster rate at a higher temperature. To identify the bottleneck enzyme in the pathway at temperatures at which *E. coli* can grow, we measured the rates by reducing each enzyme concentration by 5-fold. The results indicated that MCR is the rate-limiting enzyme at both 25 and 37 °C (Figure 3 of the Supporting Information).

To construct the pathway in *E. coli*, we used the BglBrick cloning strategy to express several genes simultaneously from a single plasmid.[12] We subcloned the genes encoding 3HPCS, ACR, MSR, and 3HPCD in this order into a single BglBrick vector that contained the lacUV5 promoter and the p15A origin of replication (pNC015). The order of the genes were determined by the soluble expression levels of individual genes in *E. coli* BL21(DE3): the gene that showed the weakest expression was placed in the first position. We subcloned MCR into another BioBrick vector that contained the tetracycline promoter and the ColE1 origin of replication (pNC011). We transformed *E. coli* BL21(DE3) with the two plasmids and examined soluble expression levels by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. All proteins were produced at levels similar to each other (Figure 4a of the Supporting Information). We also constructed an expression vector containing all of the genes but found that balanced expression was lost employing this plasmid (Figure 4b of the Supporting Information).

E. coli K207-3 is an engineered *E. coli* strain whose genome contains *accA1* and *pccB*, which encode the two-component propionyl-CoA carboxylase that converts propionyl-CoA to (2*S*)-methylmalonyl-CoA, from *S. coelicolor* under control of the T7 promoter.[13] The genome also lacks several genes that are responsible for propionyl-CoA and methylmalonyl-CoA catabolism: *prpR*, *prpB*, *prpC*, and *prpD* (all of which are involved in propionyl-CoA degradation) and *ygfG* (which is a putative methylmalonyl-CoA decarboxylase).[13] In addition, the genome encodes *sfp* from *Bacillus subtilis* that encodes the substrate promiscuous surfactin phosphopantetheinyl transferase that converts the expressed apo PKS proteins to their corresponding holo forms.[4]

We transformed *E. coli* K207-3 with pNC011 and pNC015 or the control plasmids lacking the pathway genes, and the cultures were grown at 37 °C in LB medium until the OD_{600} reached 0.4−0.5. Following induction with isopropyl β-D-galactopyranoside (IPTG) and anhydrous tetracycline (aTc), the cultures were grown for additional 3 h at 37 °C. The cells were extracted with an acetonitrile/methanol/water mixture (2:2:1) with 0.1% formic acid at 4 °C. The resulting solution was lyophilized and then dissolved in a methanol/water mixture (1:1) to analyze propionyl-CoA and methylmalonyl-CoA production in *E. coli* by LC−MS. As shown in Figure 3, the propionyl-CoA level was significantly higher in the host with the pathway than in the control strain. The concentration of propionyl-CoA was determined to be 1.9-fold greater than that of acetyl-CoA (Figure 5 of the Supporting Information). Acetyl-CoA is the most abundant acyl-CoA in *E. coli*; the absolute concentration in the cell was reported to be 610 μM.[14] Methylmalonyl-CoA was also observed in the presence of the pathway. The level was 1.3-fold lower than that of succinyl-CoA (Figure 5 of the Supporting Information), the second most abundant acyl-CoA in *E. coli*, whose intracellular concentration was previously determined to be 230 μM.[14] These values

dx.doi.org/10.1021/bi301414q | *Biochemistry* 2012, 51, 9779−9781

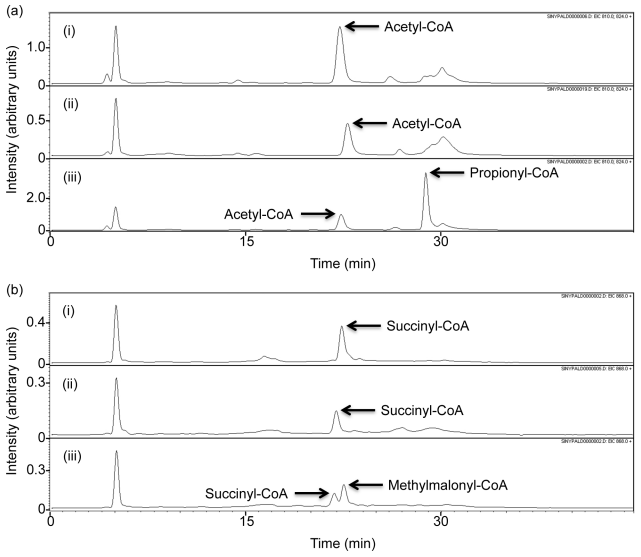

Figure 3. Propionyl-CoA and methylmalonyl-CoA biosynthesis in *E. coli*. *E. coli* K207-3 carrying plasmids encoding MCR, MSR, 3HPCS, 3HPCD, and ACR (pNC011 and pNC015) or the control plasmids (pNC016 and pNC017) were grown with or without inducers (IPTG and aTc) and extracted, and the intracellular contents were analyzed by LC−MS. (a) Acetyl-CoA and propionyl-CoA analyses. (b) Succinyl-CoA and methylmalonyl-CoA analyses. (i) pNC016 and pNC017 with inducers. (ii) pNC011 and pNC015 without inducers. (iii) pNC011 and pNC015 with inducers.

suggest that levels of both propionyl-CoA and methylmalonyl-CoA in the engineered *E. coli* strain should be sufficient to support polyketide biosynthesis; K_M values of DEBS acyltransferase domains that accept propionyl-CoA and (2*S*)-methylmalonyl-CoA were previously determined to be 13 and 24 μM, respectively.[15,16] However, the growth rate was reduced upon pathway expression, indicating that fatty acid biosynthesis was attenuated by malonyl-CoA consumption in the engineered strain (Figure 7 of the Supporting Information).

We also tested the pathway in *E. coli* BL21(DE3) and BAP1, the parental *E. coli* strain of K207-3.[4] In both cases, we observed a pathway-dependent production of propionyl-CoA. The levels of propionyl-CoA, however, were almost 100-fold lower than that observed in the engineered *E. coli* K207-3 strain (Figure 6 of the Supporting Information). The reason is unclear, because the genomes of BAP1 and K207-3 should be identical except for the presence of *accA1* and *pccB*, the absence of *ygfG*, and a *panD* mutation in the K207-3 strain. One possibility is that propionyl-CoA carboxylase from *S. coelicolor* can also carboxylate acetyl-CoA in the K207-3 host to increase the level of malonyl-CoA, which is the starter substrate of the pathway.

In summary, overproducing polyketide drugs and novel candidate compounds is important for meeting future clinical demands. One such way is to use heterologous hosts like *E. coli* to express PKS proteins because many polyketides are produced by complex, slow-growing microorganisms or organisms that cannot currently be cultivated. However, native *E. coli* metabolism lacks several important substrates, including (2*S*)-methylmalonyl-CoA. Two pathways have been reported to produce (2*S*)-methylmalonyl-CoA in *E. coli*.[4,6] The mutase-epimerase pathway was shown to be 10-fold less efficient than the propionate-dependent pathway in terms of producing 6dEB.[6] The propionate-dependent pathway is a promising alternative, but propionate is currently derived from petroleum sources, whose costs are expected to increase in the future. The pathway described here to produce (2*S*)-methylmalonyl-CoA uses malonyl-CoA, ATP, NADPH, and bicarbonate, all of which can be produced from glucose as a sole carbon source. Thus, this strategy may open a way to produce a variety of polyketide drugs in *E. coli* from renewable carbon sources.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (510) 495-2764. E-mail: keasling@berkeley.edu.

Funding

This work was funded by the Advanced Research Projects Agency-Energy (ARPA-E), U.S. Department of Energy, via Grant DE-AR0000091, by the National Science Foundation (Grant EEC-0540879 to the Synthetic Biology Research Center), and by the Joint BioEnergy Institute, which is funded by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, under Contract DE-AC02-05CH11231.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We also thank Edward Baidoo of the Joint BioEnergy Institute for LC−MS analysis.

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