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Extending Carbon Chain Length of 1-Butanol Pathway for 1-Hexanol Synthesis from Glucose by Engineered *Escherichia coli*

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Supporting Information

ABSTRACT: An *Escherichia coli* strain was engineered to synthesize 1-hexanol from glucose by extending the coenzyme A (CoA)-dependent 1-butanol synthesis reaction sequence catalyzed by exogenous enzymes. The C4-acyl-CoA intermediates were first synthesized via acetyl-CoA acetyltransferase (AtoB), 3-hydroxybutyryl-CoA dehydrogenase (Hbd), crotonase (Crt), and *trans*-enoyl-CoA reductase (Ter) from various organisms. The butyryl-CoA synthesized was further extended to hexanoyl-CoA via β-ketothiolase (BktB), Hbd, Crt, and Ter. Finally, hexanoyl-CoA was reduced to yield 1-hexanol by aldehyde/alcohol dehydrogenase (AdhE2). Enzyme activities for the C6 intermediates were confirmed by assays using HPLC and GC. 1-Hexanol was secreted to the fermentation medium under anaerobic conditions. Furthermore, co-expressing formate dehydrogenase (Fdh) from *Candida boidinii* increased the 1-hexanol titer. This demonstration of 1-hexanol production by extending the 1-butanol pathway provides the possibility to produce other medium chain length alcohols using the same strategy.

In recent years, synthesis of higher chain volatile alcohols by engineered microorganisms has received increasing interest because of energy and sustainability concerns. Naturally produced by *Clostridium* species, 1-butanol has attracted the most attention, and many groups have reported its production in recombinant organisms such as *Escherichia coli*[1,2] by expressing the genes found in *Clostridium* and other organisms to catalyze the 1-butanol synthesis reactions. In this pathway, 1-butanol synthesis starts from acetyl-CoA and is catalyzed by thiolase/acetyl-CoA acetyltransferase (Thl/AtoB), 3-hydroxybutyryl-CoA dehydrogenase (Hbd), crotonase (Crt), butyryl-CoA dehydrogenase (Bcd), and alcohol/aldehyde dehydrogenase (AdhE2). Shen et al.[3] recently achieved high-titer production of 1-butanol (15−30 g/L) using this series of reactions by replacing Bcd with *trans*-enoyl-CoA reductase (Ter), which directly utilizes NADH as the reducing equivalent, and by artificially building up NADH and acetyl-CoA as driving forces. On this basis, here we extend this 1-butanol production pathway to longer chain alcohols such as 1-hexanol. Native organisms, such as *Clostridium kluyveri*, have been reported to ferment ethanol and acetate not only to butyric acid, hexanoic acid, and H2 but also to 1-butanol and 1-hexanol.[4,5] However, the enzyme(s) involved in biosynthesis for 1-hexanol production is not defined.

Previously, Zhang et al.[6] reported 1-hexanol production in *E. coli* from glucose by extending the 2-ketoacid pathway developed by Atsumi et al.[7] The 2-ketoacid pathway for 1-hexanol synthesis is based on a mutated leucine biosynthesis pathway, which employs one acetyl-CoA to extend the chain length by one carbon unit. On the other hand, the CoA-dependent pathway elongates the carbon chain by two carbon units at the expense of one acetyl-CoA. Thus, the latter has a higher carbon yield. Currently, there is no report of 1-hexanol production by the CoA-dependent 1-butanol pathway in engineered microorganisms. In this work, we extended the carbon chain of this pathway to produce 1-hexanol as the first trial of medium chain length alcohol production using the CoA-dependent pathway described in Scheme 1. We confirmed enzyme activities and demonstrated direct 1-hexanol synthesis from glucose in engineered *E. coli*.

Since Shen et al. demonstrated efficient synthesis of 1-butanol in *E. coli*,[3] the first step in constructing the 1-hexanol pathway was to express the potential genes and detect the enzymatic activities for extending the carbon chain length. β-Ketothiolase (BktB) from *Ralstonia eutropha* and *Clostridium acetobutylicum* Hbd and Crt were expressed to catalyze the first three steps after butyryl-CoA. Each enzyme was expressed as the N-terminal fusion protein with His6 and purified by Ni-affinity column chromatography.

Owing to the unavailability of 3-ketohexanoyl-CoA and 3-hydroxyhexanoyl-CoA, we tested the enzymatic activities of these three reactions in the reverse direction using hexenoyl-CoA as the substrate. Hexenoyl-CoA was chemically synthesized[8] and partially purified by preparative HPLC. The substrate was then incubated with Crt, Hbd, and BktB with appropriate cofactors in a stepwise fashion, and the resulting products were analyzed by HPLC (Figure 1). Hexenoyl-CoA was detected at 10.3 min. In the presence of Crt, hexenoyl-CoA was consumed, and a new peak at 8.7 min appeared (Figure 1B), which was presumably 3-hydroxyhexanoyl-CoA. In the next step, the addition of Hbd and NAD^+ showed a very similar pattern on the chromatogram as the previous reaction (Figure 1C). After the addition of BktB and CoA, acetyl-CoA and butyryl-CoA were detected as two peaks resolving at 4.2 and 8.9 min, respectively (Figure 1D).

The appearance of the expected products (acetyl-CoA and butyryl-CoA) suggested that all enzymes were active toward C6 substrates. These results were consistent with the previous findings that BktB from *R. eutropha* has dissociative activity toward 3-ketohexanoyl-CoA[9] and Crt from *C. acetobutylicum*

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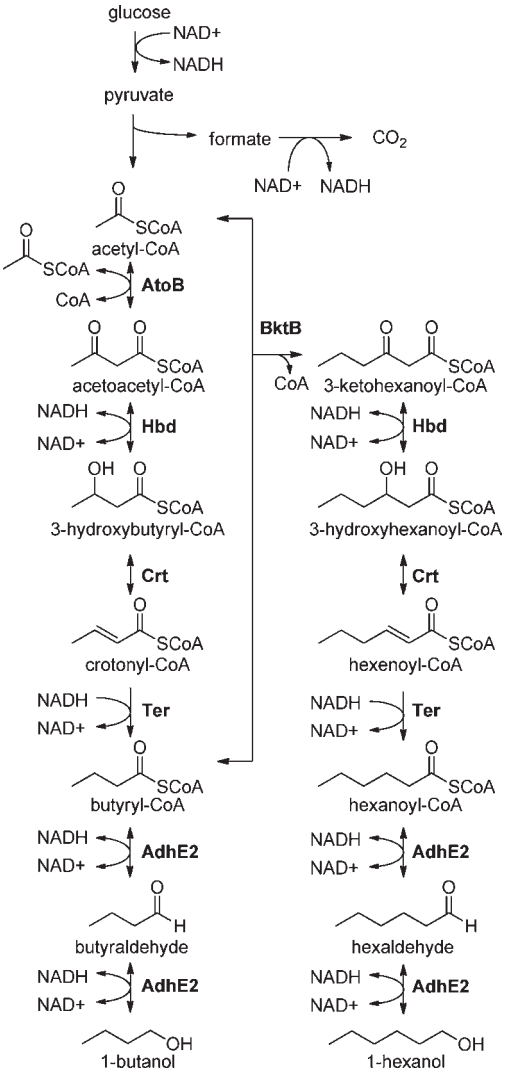
Scheme 1. Artificial Pathway for 1-Hexanol Biosynthesis in *Escherichia coli*[a]



[a] Abbreviations: AtoB, acetyl-CoA acetyltransferase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd-EtfAB, butyryl-CoA dehydrogenase-electron transfer protein; Ter, trans-enoyl-CoA reductase, AdhE2; alcohol/aldehyde dehydrogenase, BktB; β-ketothiolase.

shows activity toward hexenoyl-CoA.[10] Considering that the three-enzyme combination was able to dissociate hexenoyl-CoA into acetyl-CoA and butyryl-CoA and these enzyme reactions are reversible, we expect that these enzymes will be able to produce hexenoyl-CoA using acetyl-CoA and butyryl-CoA under intracellular conditions.

Since *E. coli* AtoB is in the same enzyme family as BktB, we also characterized AtoB activity toward the C6 substrate. However, no significant difference was detected between reactions with and without AtoB (Supporting Information, Figure S1A,B), implying that AtoB could not catalyze the condensation reaction of acetyl-CoA and butyryl-CoA efficiently.

Shen et al.[3] succeeded in the production of 1-butanol in high concentrations by recruiting *trans*-enoyl-CoA reductase (Ter) in lieu of *C. acetobutylicum* Bcd for the crotonyl-CoA reduction step. Ter from *Euglena gracilis* (EgTer) was previously reported to be able to convert hexenoyl-CoA to hexanoyl-CoA.[11] In contrast,

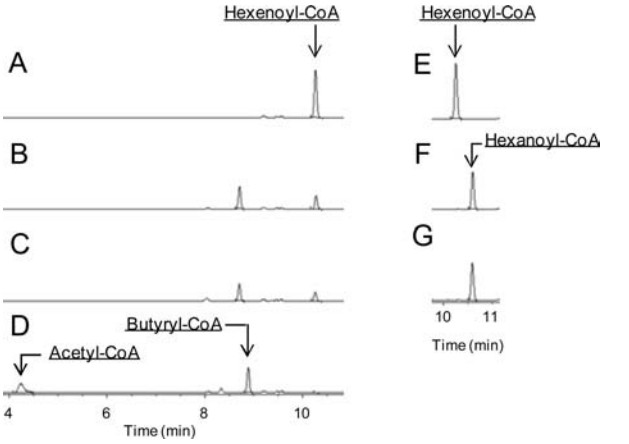


Figure 1. (Left) HPLC analysis of Crt, Hbd, and BktB reaction with hexenoyl-CoA. Hexenoyl-CoA was incubated with (A) no enzymes nor cofactors as a control; (B) Crt; (C) Crt, Hbd, and NAD; or (D) Crt, Hbd, BktB, NAD^+, and CoA. (Right) HPLC analysis of EgTer and TdTer reaction with hexenoyl-CoA. Hexenoyl-CoA was incubated with (E) no enzyme as control; (F) EgTer and NADH; or (G) TdTer and NADH.

Treponema denticola (TdTer), which was used in 1-butanol productions,[3] was reported to not possess reductive activity against C6 substrates.[12] To test these enzymes, we incubated hexenoyl-CoA with purified EgTer or TdTer and analyzed the products. Interestingly, HPLC data showed that both EgTer and TdTer were able to reduce hexenoyl-CoA to hexanoyl-CoA using NADH as the reducing cofactor, suggesting the broad substrate specificity of these enzymes (Figure 1E−G).

The next step is catalyzed by an aldehyde/alcohol dehydrogenase. AdhE2 from *C. acetobutylicum* is known to reduce butyryl-CoA to butyraldehyde and to 1-butanol,[13] but its activity toward longer chain length acyl-CoA substrates was not reported. We tested this activity by a spectrophotometric assay. As shown in Table 1, AdhE2 has a higher activity toward butyryl-CoA compared to acetyl-CoA (consistent with previous data[1]) and also has a significant activity for hexanoyl-CoA (15 ± 9.5 mU/mg). Interestingly, activity toward octanoyl-CoA (36 ± 6.9 mU/mg) is higher than toward hexanoyl-CoA. To confirm that AdhE2 converts hexanoyl-CoA to 1-hexanol (or hexaldehyde) more directly, hexanoyl-CoA was incubated with cell lysate containing AdhE2 and NADH, and the reaction solution was analyzed by GC. As shown in Figure S2, 1-hexanol was detected only in the reaction mixture containing AdhE2.

Having characterized BktB, Hbd, Crt, Ter (TdTer and EgTer), and AdhE2 activities toward C6 substrates, we then tested the production of 1-hexanol by fermentation from glucose. The host strain used was JCL166 or JCL299 (Table S1), both of which have *adhE*, *ldhA*, and *frdAB* genes deleted to increase the NADH availability to drive the reaction. In addition, JCL299 has an additional *pta* gene deletion, which increases the acetyl-CoA availability.[3] The genes for 1-hexanol synthesis were expressed from various plasmids: pEL11 (expressing *atoB*, *adhE2*, *crt*, and *hbd*), pIM8 (expressing Td*ter*), and pEL102 (expressing Td*ter*, *bktB*, and Eg*ter*) (Tables S1 and S2). The strains JCL166/pEL11/pIM8, JCL166/pEL11/pEL102, and JCL299/pEL11/pEL102 were cultivated in TB + 2% glucose media (5 mL) under anaerobic condition for 68 h. Upon centrifugation of broths to exclude the cells, supernatants were analyzed by GC.

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Table 1. Specific Activities of AdhE2 for Acetyl-CoA, Butyryl-CoA, Hexanoyl-CoA, and Octanoyl-CoA[a]

	specific activities (mU/mg protein)			
strain/plasmid	acetyl-CoA	butyryl-CoA	hexanoyl-CoA	octanoyl-CoA
JCL166	1.1	5.1	ND	3.7
JCL166/ pCS38_*adhE2*	30 ± 7.2	41 ± 6.9	15 ± 9.5	36 ± 6.9

[a] Cells were cultured under anaerobic condition. JCL166 was tested as a control. U = μmol/min. ND = not detectable.

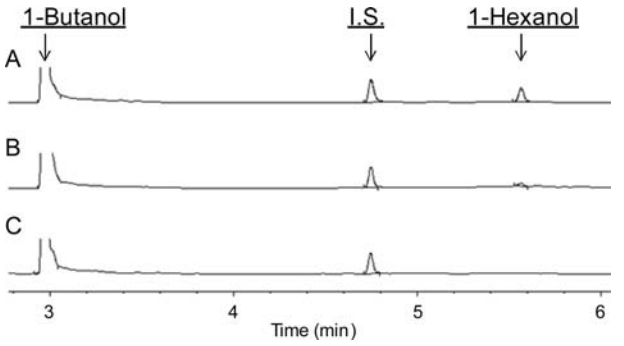

Figure 2. GC analysis of 1-hexanol production in engineered *E. coli*: (A) JCL299/pEL11/pEL102, (B) JCL166/pEL11/pEL102, and (C) JCL166/pEL11/pIM8 cultures. I.S. = internal standard (2-methyl-1-pentanol).

In the culture of JCL166/pEL11/pIM8, there was no detectable amount of 1-hexanol, but in cultures of JCL166/pEL11/pEL102 and JCL299/pEL11/pEL102, 23 ± 11 and 27 ± 15 mg/L of 1-hexanol were detected in 68 h, respectively (Figure 2). In order to confirm the chemical identity of 1-hexanol, we further analyzed these samples by GC-MS. The retention time and MS spectra of the samples were identical to those of the 1-hexanol standard (Figure S4).

Nielsen et al.[14] and Shen et al.[3] reported that overexpression of *fdh* gene enhanced 1-butanol production by increasing the intercellular NADH pool. So in the next step, we overexpressed *fdh* using plasmid pCS138 in addition to the 1-hexanol synthesis genes, and the resulting strain JCL299/pEL11/pEL102/pCS138 was examined. Furthermore, in order to replenish the carbon supply and maintain neutral pH during fermentation, we refreshed the media every 24 h by extracting 10% of broth and feeding same amount of fresh TB media containing adequate amounts of glucose and NaOH. During the 7 h of aerobic culturing, the cells grew up to 2.4 of OD600, but no 1-hexanol production was detected. After switching to anaerobic conditions, production of 1-hexanol started and reached at 47 mg/L, 48 h (Figure S5). This strain also produced 5.1 and 6.5 g/L of 1-butanol at 48 and 68 h, respectively.

Further improvement of 1-hexanol production requires improving the enzymatic activities toward C6 substrates. Currently, 1-butanol is still the main product, indicating that BktB (and/or Hbd, Crt, Ter) is not active enough to produce hexanoyl-CoA. Interestingly, AdhE2 has 70−80% activity toward acetyl-CoA compared to butyryl-CoA; however, ethanol production is much lower (~0.2 g/L) than that of 1-butanol. This result implies that the intercellular concentration of acetyl-CoA is much lower than that of butyryl-CoA. Therefore, it would be important to increase the intercellular hexanoyl-CoA concentration to enhance 1-hexanol production. Nevertheless, the strategy described in this study may be extended further for the production of other even-number longer chain alcohols.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedure, strains and plasmids, HPLC analysis, MS spectrum, and time course of 1-hexanol production. This material is available free of charge via the Internet at http://pubs.acs.org.

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