

Directed Evolution of *Methanococcus jannaschii* Citramalate Synthase for Biosynthesis of 1-Propanol and 1-Butanol by *Escherichia coli*[▽]

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Biofuels synthesized from renewable resources are of increasing interest because of global energy and environmental problems. We have previously demonstrated production of higher alcohols from *Escherichia coli* using a 2-keto acid-based pathway. Here, we took advantage of the growth phenotype associated with 2-keto acid deficiency to construct a hyperproducer of 1-propanol and 1-butanol by evolving citramalate synthase (CimA) from *Methanococcus jannaschii*. This new pathway, which directly converts pyruvate to 2-ketobutyrate, bypasses threonine biosynthesis and represents the shortest keto acid-mediated pathway for producing 1-propanol and 1-butanol from glucose. Directed evolution of CimA enhanced the specific activity over a wide temperature range (30 to 70°C). The best CimA variant was found to be insensitive to feedback inhibition by isoleucine in addition to the improved activity. This CimA variant enabled 9- and 22-fold higher production levels of 1-propanol and 1-butanol, respectively, compared to the strain expressing the wild-type CimA. This work demonstrates (i) the first production of 1-propanol and 1-butanol using the citramalate pathway and (ii) the benefit of the 2-keto acid pathway that enables a growth-based evolutionary strategy to improve the production of non-growth-related products.

To meet the increasing energy demand and reduce the negative environmental impact, biofuels from renewable resources represent a promising alternative for reducing the dependence on fossil-derived transportation fuels (15). In particular, longer-chain alcohols are of interest because of their high energy densities and their low hygroscopicities, which reduce problems in storage and distribution. We previously devised a biosynthetic strategy to produce higher alcohols in *Escherichia coli* (2) which takes advantage of the amino acid biosynthesis capability to produce various 2-keto acids and the broad substrate range of 2-keto acid decarboxylases (KDCs) and alcohol dehydrogenases (ADHs).

Because of the growth requirement for amino acids, keto acid biosynthesis is amenable to directed evolution using growth-based selection. Here, we evolved a synthetic pathway for the production of 1-propanol and 1-butanol in *E. coli* by using a 2-keto acid-based selection strategy. No microorganisms have been identified to produce 1-propanol from glucose in industrially relevant quantities, although small amounts have been identified as microbial by-products. 1-Propanol can be esterified to yield diesel fuels and be dehydrated to yield propylene, which is currently derived from petroleum as a monomer for making polypropylene. 1-Butanol has been proposed as a supplement of gasoline as a transportation fuel. It is traditionally produced using *Clostridium* species, and its production using *E. coli* has just begun to be explored (1, 13). 1-Propanol and 1-butanol can be synthesized through 2-ketobutyrate (2) via the KDC and ADH pathway (Fig. 1).

2-Ketobutyrate is a degradation product of threonine and a

precursor of isoleucine. In addition to being a precursor to 1-propanol, 2-ketobutyrate can be converted to 2-ketovalerate and 2-keto-3-methyl-valerate, which are precursors for 1-butanol and 2-methyl-1-butanol, respectively. In most microorganisms, 2-ketobutyrate is synthesized via threonine (Fig. 1). An alternative route to 2-ketobutyrate from pyruvate and acetyl coenzyme A (acetyl-CoA) via citramalate synthase (CimA) has been reported in some organisms (e.g., *Leptospira interrogans* [18, 19] and *Methanococcus jannaschii* [8]). This pathway (designated the citramalate pathway [Fig. 1]) is the most direct route to synthesize 2-ketobutyrate and does not involve transamination followed by deamination. (*R*)-Citramalate synthesized from pyruvate and acetyl-CoA by CimA is then converted to 2-ketobutyrate via LeuCD- and LeuB-mediated reactions, parallel to the similar reactions in leucine biosynthesis. To take advantage of this short keto acid pathway, we expressed (*R*)-citramalate synthase (CimA) of *M. jannaschii* in *E. coli*.

However, heterologous proteins are not always active in foreign hosts and thermophilic enzymes often lose activities at moderate temperatures. To improve the activity of CimA, we employed a directed evolution strategy (10). Because 2-keto acids are precursors of amino acids, these metabolites are essential and can be used as a selection in directed evolution. Here, we have achieved increased production of 1-propanol and 1-butanol by applying a selection based on the requirement of L-isoleucine.

MATERIALS AND METHODS

Reagents. Restriction enzymes and Antarctic phosphatase were from New England Biolabs (Ipswich, MA). The rapid DNA ligation kit was from Roche (Manheim, Germany). KOD DNA polymerase was from EMD Chemicals (San Diego, CA). Oligonucleotides were from Operon (Huntsville, AL).

Strains and plasmids. A list of many of the strains and plasmids used in this study is given in Table 1.

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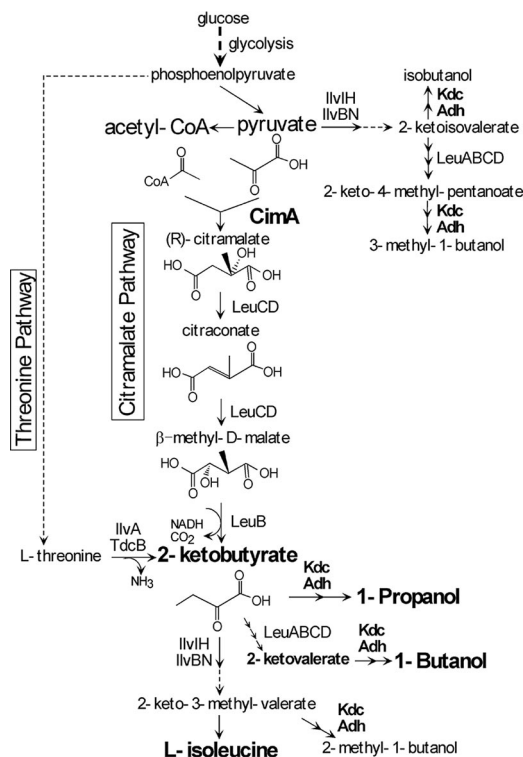


FIG. 1. Schematic representation of the pathway for 1-propanol and 1-butanol production. The engineered citramalate pathway consists of four enzymatic steps from pyruvate to 2-ketobutyrate.

To clone *cimA*, genomic DNA of *M. jannaschii* (ATCC) was used as PCR template with primers A113 (5'-CGAGCGGTACCATGATGGTAAGGATATTTGATACAA-3') and A114 (5'-ACGCGAGTCGACTTAATTCAATAACATATTGATTCTT-3'). PCR products were digested with *Acc65I* and *Sall* and cloned into pSA59 (2) cut with the same enzymes, creating pSA61. To replace replication origin (*ori*) with p15A, pZA31-luc (12) was digested with *SacI* and *AvrII*. The shorter fragment was purified and cloned into plasmid pSA61 cut with the same enzymes, creating pSA63.

To remove the noncoding region in pSA121, pSA121 was used as PCR template with primers A113 and A227 (5'-ACGCGAGTCGACTTACAATTTCCTGTAACCTTCTCTA-3'). PCR products were digested with *Acc65I* and *Sall* and cloned into pSA63 cut with the same enzymes, creating pSA125.

For protein overexpression and purification, *cimA* and *cimA3.7* were amplified with primers A261 (5'-CGGGATCCGGTAAGGATATTTGATACAACACTTA-3') and A114 and A261 and A227, respectively. PCR products were digested with *BamHI* and *Sall* and cloned into pETDuet-1 (Novagen (Madison, WI) cut with the same enzymes, creating pSA153 and pSA154.

Medium and culture conditions for 1-propanol and 1-butanol production. M9 medium containing 72 g/liter glucose, 5 g/liter yeast extract, 100 µg/ml ampicillin, 30 µg/ml kanamycin, and a 1:1,000 dilution of Trace metal mix A5 [2.86 g H₃BO₃, 1.81 g MnCl₂ · 4H₂O, 0.222 g ZnSO₄ · 7H₂O, 0.39 g Na₂MoO₄ · 2H₂O, 0.079 g CuSO₄ · 5H₂O, 49.4 mg Co(NO₃)₂ · 6H₂O per liter of water] was used for cell growth. Preculture in test tubes containing 3 ml of medium was performed at 37°C overnight on a rotary shaker (250 rpm). Overnight culture was diluted 1:100 into 20 ml of fresh medium in a 250-ml screw cap conical flask. Cells were grown at 37°C for 3 h, followed by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultivation was performed at 30°C on a rotary shaker (250 rpm). Gas chromatography-flame ionization detection and high-performance liquid chromatography analyses were carried out as previously described (2).

Directed evolution. Error-prone PCR was carried out as described previously (3) using pSA63 (Table 1) as a template. A plasmid library of *cimA* variants was constructed on pSA63 by ligating the error-prone PCR product digested with *Acc65I* and *Sall*. Ten microliters of the ligation reaction was used to transform 100 µl of XL10 Gold cells (Stratagene, La Jolla, CA). The resultant library size

was calculated (~1 × 10⁶ colonies), and the plasmid library was amplified on LB agar plates containing 30 µg/ml kanamycin. SA405 or SA408 was transformed with the plasmid library. The cells were incubated in 20 ml of M9 medium containing 10 g/liter glucose and 30 µg/ml kanamycin with shaking at 30°C for 3 days. Plasmids were purified from the resulting cultures. DNA shuffling was carried out as described previously (21), except that KOD DNA polymerase was used for fragment amplification. A plasmid library construction and liquid culture selection were carried out as described above. The resulting cultures were spread out on M9 agar plates containing 10 g/liter glucose and 30 µg/ml kanamycin. The plates were incubated at 30°C for 3 days.

Protein purification. The wild-type *CimA* and *CimA3.7* were synthesized from pSA153 and pSA154 in *E. coli* strain BL21 Star (DE3) (Invitrogen, Carlsbad, CA), followed by purification with Ni-nitrilotriacetic acid spin columns (Qiagen, Valencia, CA). Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA).

Citramalate synthase assay. The *CimA* enzyme activity was assayed by monitoring the production of CoA over time (8, 14). Purified proteins (0.1 µM) were dissolved in 150 µl of TES buffer (0.1 M [pH 7.5]) containing various concentrations of acetyl-CoA and pyruvate. The production of CoA was confirmed to be linear over 1 h. After incubation at various temperatures for 1 h, 50 µl of 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in 0.1 M Tris-HCl (pH 8.0) was added to measure the appearance of the free SH group of the released CoA SH. The absorbance at 412 nm was recorded. The concentrations of CoASH produced were calculated from a standard curve generated with various concentrations of 2-mercaptoethanol.

RESULTS

***M. jannaschii cimA* partially rescues the isoleucine auxotrophy in *E. coli*.** To construct the citramalate pathway (Fig. 1) in *E. coli*, *cimA* (*M. jannaschii*) and *leuABCD* (*E. coli*) were cloned and expressed under the control of the IPTG-inducible *P_LlacO₁* promoter on a p15A-derived plasmid (pSA63 [Fig. 2A]). To test the activity of the pathway, we used *E. coli* strain SA405, which is deficient in *ilvA* and *tdcB*. This strain is auxotrophic for L-isoleucine as it cannot synthesize 2-ketobutyrate unless the citramalate pathway is active. Thus, the growth rate

TABLE 1. Strains used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
BW25113	<i>rrnB</i> _{T14} <i>ΔlacZ</i> WJ16 <i>hsdR</i> 514 <i>ΔaraBAD</i> _{AH33} <i>ΔrhaBAD</i> _{LD78}	4
JCL16	BW25113/F' [<i>traD36 proAB</i> ⁺ <i>lacI</i> ⁺ ZAM15]	1
KS145	Same as JCL16 but <i>ΔilvI ΔilvB</i>	This work
SA405	Same as JCL16 but <i>ΔilvA ΔtdcB</i>	This work
SA408	Same as SA405 but <i>ΔilvI</i>	This work
Plasmids		
pCS27	p15A ori; Kan ^r ; <i>P_LlacO1::MCS1</i>	13
pSA55	ColE1 ori; Amp ^r ; <i>P_LlacO1::kivd-ADH2</i>	2
pSA63	p15A ori; Kan ^r ; <i>P_LlacO1::cimA-leuABCD</i>	This work
pSA90	p15A ori; Kan ^r ; <i>P_LlacO1::cimA1-leuABCD</i>	This work
pSA121	p15A ori; Kan ^r ; <i>P_LlacO1::cimA2-leuABCD</i>	This work
pSA123	p15A ori; Kan ^r ; <i>P_LlacO1::cimAΔ1117-1473-leuABCD</i>	This work
pSA124	p15A ori; Kan ^r ; <i>P_LlacO1::cimAΔ2Δ1117-1473-leuABCD</i>	This work
pSA142	p15A ori; Kan ^r ; <i>P_LlacO1::cimA3.7-leuABCD</i>	This work
pSA153	Derivative of pETDuet-1 with <i>cimA</i>	This work
pSA154	Derivative of pETDuet-1 with <i>cimA3.7</i>	This work

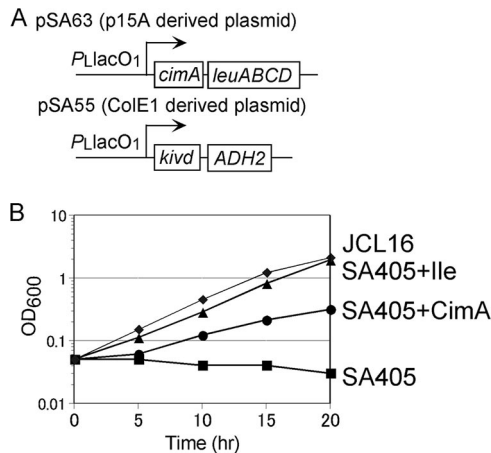


FIG. 2. Transfer of the citramalate pathway to *E. coli*. (A) Schematic representation of the synthetic operons. (B) Time courses for the growth of *E. coli* strain SA405 ($\Delta ilvA \Delta tdcB$) and JCL16. OD₆₀₀, optical density at 600 nm. Cells were incubated in M9 medium containing glucose at 30°C. Diamonds, JCL16; circles, squares, and triangles, SA405 with pSA63 (circles), pCS27 (without *cimA-leuABCD*) (squares), or L-isoleucine (39.5 μ g/ml) (triangles).

of the cell should reflect the activity of the citramalate pathway. Growth rates were compared for SA405 ($\Delta ilvA$ and $\Delta tdcB$) transformed with pSA63 (harboring wild-type *cimA*) (Fig. 2A), pCS27 (without *cimA* and *leuABCD*), or the wild-

type strain (JCL16) (Fig. 2B). SA405 cells not expressing CimA were unable to grow without L-isoleucine (Fig. 2B). The citramalate pathway rescued the growth of SA405 under the same condition, although the growth rate of SA405 with the citramalate pathway was lower than that of JCL16 or SA405 with L-isoleucine (Fig. 2B).

Directed evolution of CimA. The partial rescue of the isoleucine auxotroph allows the evolution of CimA based on growth improvement. In the first round of mutation, *cimA* variants were generated by error-prone PCR and mutants with increased growth were enriched in liquid media. Plasmids from the pool of fast-growing variants were then purified and used as templates for DNA shuffling in a second round of evolution. After these two rounds of selection, five variants of *cimA* were randomly picked and tested for 1-propanol and 1-butanol production (described in detail below). The *cimA* variant that leads to the highest 1-propanol production, and thus the largest 2-ketobutyrate pool and highest *cimA* activity, was designated *cimA1* (Fig. 3A). This mutant was found to contain three amino acid substitutions (Ile47Val, Lys435Asn, and Val441Ala) and was subjected to the next two generations of mutagenesis, selection, and screening.

The next two rounds of selection were performed using the same scheme as the first two, except that the selection pressure was increased by introducing an *ilvI* knockout in addition to $\Delta ilvA$ and $\Delta tdcB$ in the host (designated as SA408). *IlvI* is the

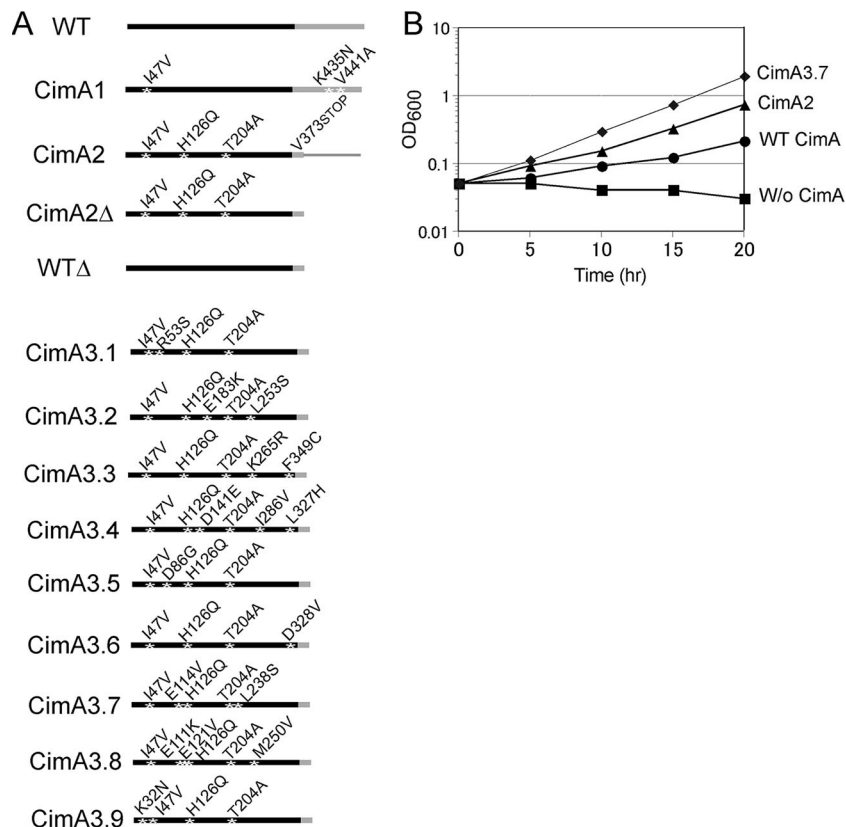


FIG. 3. Progress of the evolution of CimA. (A) Amino acid mutations are shown in the schematic representation of CimA. The gray bar indicates the putative regulator domain. (B) Time courses for the growth of an *E. coli* strain (SA408 [$\Delta ilvA \Delta tdcB \Delta ilvI$]) containing the derivative of pSA63 (*cimA-leuABCD*). Cells were incubated in M9 medium containing glucose at 30°C. Circles, wild-type (WT) CimA; triangles, CimA2; diamonds, CimA3.7; and squares, control (blank plasmid).

TABLE 2. Kinetic parameters of the wild-type CimA and CimA3.7

Protein	K_m (μ M)		k_{cat} (s^{-1})
	Pyruvate	Acetyl-CoA	
Wild type	184 $\pm$ 12	303 $\pm$ 23	0.36 $\pm$ 0.05
CimA3.7	342 $\pm$ 22	105 $\pm$ 11	0.84 $\pm$ 0.08

large subunit of acetohydroxy acid synthase III (AHAS III), which exhibits a higher specificity toward 2-ketobutyrate (6). The remaining isozyme (AHAS I), encoded by the *ilvBN* genes, has higher specificity to pyruvate than to 2-ketobutyrate (6). Since the endogenous concentrations of 2-ketobutyrate ($\sim 10 \mu$ M) are much lower than K_m value for 2-ketobutyrate of AHAS I (~ 5 mM) (5), the deletion of *ilvI* decreases the flux from 2-ketobutyrate to isoleucine and thus requires more 2-ketobutyrate to synthesize isoleucine through the less efficient isozyme.

After the fourth round, eight colonies were randomly picked. We sequenced the *cimA* variant that produced the largest amount of alcohols (denoted *cimA2* [Fig. 3A]). In addition to the amino acid substitutions in CimA1, CimA2 contains two new amino acid substitutions (His126Gln and Thr204Ala) and a frameshift mutation at bp 1117, creating a CimA variant missing the C-terminal domain from the 373rd residue.

The *cimA2* mutant contains a stop codon at bp 1117, indicating that this operon contains ~ 350 bp of noncoding region between the *cimA* gene and the *leuA* gene located on the synthetic operon. It has been known that large noncoding regions decrease mRNA stability and translational efficiency (11). To eliminate the possibility of an expression deficiency of *leuABCD* downstream of *cimA*, we removed the noncoding region from the plasmid (denoted CimA2 Δ [Fig. 3A]). CimA2 Δ was subjected to the next two generations of mutagenesis, selection, and screening using the same scheme as the last two rounds.

After the sixth round, nine colonies were randomly picked for sequencing (Fig. 3A). The selected *cimA* mutants were recloned into pSA63 to remove the possibility of extra mutations in the plasmid. We did not observe any apparent hot spots of mutations (Fig. 3A), and all mutations were outside of the active site (Fig. 3A).

Growth rates were compared for SA408 ($\Delta ilvA \Delta tdcB \Delta ilvI$) transformed with pSA63 (harboring wild-type *cimA*) (Fig. 2A), pSA121 (containing *cimA2*), pSA142 (containing *cimA3.7*), or pCS27 (without *cimA* and *leuABCD*) (Fig. 3B). The growth rate of strains expressing CimA2 or CimA3.7 was higher than that of the strain expressing wild-type CimA. Cells not expressing CimA were unable to grow under the same conditions (Fig. 3B). The strains with the other CimA3 variants showed growth similar to that of the strain with CimA3.7 (data not shown). These results indicate that growth depends on the activity of CimA to supply precursors for L-isoleucine production.

Purification and characterization of wild-type and evolved CimA. The wild-type CimA and CimA3.7 were expressed from a His tag plasmid (pSA153 or pSA154) and purified as described in Materials and Methods. The kinetic parameters were measured for both of these proteins by monitoring the

production of CoA in the presence of pyruvate and acetyl-CoA at 30°C. The k_{cat} and K_m for pyruvate and for acetyl-CoA of the wild type and CimA3.7 were determined (Table 2). The k_{cat} and K_m for acetyl-CoA of CimA3.7 improved about threefold over the wild-type levels. However, the K_m for pyruvate of CimA3.7 increased over wild-type CimA, although this may not be crucial for the activity in vivo as the cellular concentration of pyruvate (7.5 mM) (20) is much higher than the K_m for pyruvate (0.34 mM) of CimA3.7.

The CimA studied here was isolated from *M. jannaschii*, an extremely thermophilic archaeon. In order to characterize the activity of this thermophilic enzyme under moderate temperatures, the specific activities of the wild type and CimA3.7 were determined over a range of temperatures, from 30 to 70°C (Fig. 4A). The specific activity of the wild type increased at elevated temperatures. Interestingly, CimA3.7 showed higher activity at all temperatures tested relative to wild-type CimA, although the difference of the specific activity was larger at lower temperature, possibly because the mutant was screened at 30°C.

CimA is a homologue of LeuA. The activities of CimA and LeuA are regulated by the corresponding amino acid end product L-isoleucine or L-leucine, respectively (16, 19). It has been shown that L-leucine binds to the C-terminal domain in LeuA (9). CimA3.7 is missing this C-terminal domain shown by homology alignment (Fig. 3A) to be involved in feedback inhibition, suggesting that CimA3.7 may be insensitive to feedback inhibition by L-isoleucine. To test the effects of the deletion in the regulatory domain, the specific activity was assessed in the presence of various concentrations of L-isoleucine. Figure 4B shows the effects of L-isoleucine on the wild type and CimA3.7. The specific activity of the wild type decreased by 64% and 80% with 40 and 80 mM

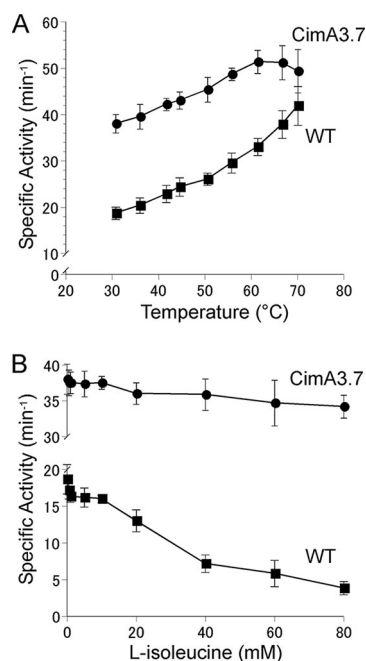


FIG. 4. Enzyme assays. (A) Specific activities (M CoA produced/min/M protein) of the wild type (WT) (squares) and CimA3.7 (circles) at various temperatures. (B) Specific activities of the wild type and CimA3.7 at 30°C in the presence of various concentrations of L-isoleucine.

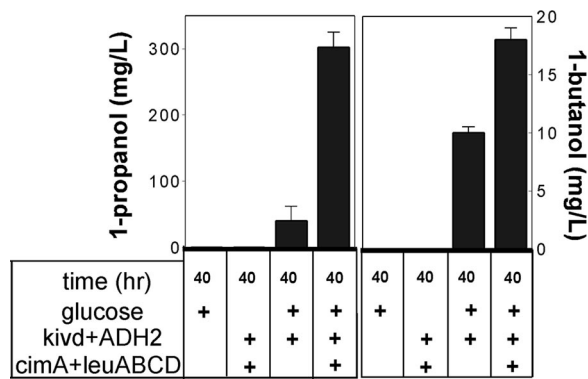


FIG. 5. 1-Propanol and 1-butanol production with the citramalate pathway. (Left panel) 1-Propanol production. (Right panel) 1-Butanol production in the same strain. The host is KS145, and overexpressed genes are indicated below the axis. Cultures were grown at 30°C in M9 medium containing 5 g/liter yeast extract with or without 72 g/liter glucose for 40 h.

L-isoleucine, respectively, suggesting that the wild type is sensitive to feedback inhibition by L-isoleucine, as is CimA of *L. interrogans* (19). In contrast, CimA3.7 activity was unaffected by the addition of isoleucine, demonstrating that CimA3.7 is not sensitive to L-isoleucine. This is most likely due to the deletion of the C-terminal domain.

1-Propanol and 1-butanol production with CimA. The next task was to use the citramalate pathway to enhance the production of 1-propanol and 1-butanol (Fig. 1). An *E. coli* strain (KS145) auxotrophic for L-isoleucine, leucine, and valine ($\Delta ilvI$ and $\Delta ilvB$) was transformed with pSA63 (or other plasmids containing variants of *cimA*) and pSA55 ($P_{lacO1}::kldv-ADH2$ (2)). The deletions of *ilvI* and *ilvB* (Fig. 1) were introduced for two reasons. First, the deletions eliminated the native substrate, 2-ketoisovalerate, for the *leuABCD* pathway, thus reducing the competitive substrate inhibition. Second, these deletions eliminated the production of 2-keto-3-methyl-valerate and 2-keto-4-methyl-pentanoate, which are competing substrates for Kldv. The strain expressing the wild-type *cimA* gene (KS145/pSA63/pSA55) produced 302 mg/liter 1-propanol and 18 mg/liter 1-butanol after 40 h (Fig. 5). KS145 with pSA55 only, where Kldv utilizes endogenous 2-keto acids, produced 40 mg/liter 1-propanol and 10 mg/liter 1-butanol under the same condition. KS145 without pSA55 and pSA63 produced neither 1-propanol nor 1-butanol (Fig. 5). Note that the yeast extract was added to the medium to boost the cell density. However, without glucose added to the medium the cells produce no alcohols (Fig. 5), indicating that these products were derived from glucose, but not from yeast extract.

1-Propanol and 1-butanol production was tested with the selected CimA variants in KS145 by replacing pSA63 with corresponding plasmids (Table 1) containing various *cimA* mutants. As shown in Table 3, the production of 1-propanol from the strain with CimA1 increased 2.3-fold compared to the strain with the wild-type CimA. The production of 1-propanol and 1-butanol from the strain with CimA2 increased 3.9- and 4.3-fold, respectively, compared to the strain with the wild-type CimA. These results indicate that our selection method is effective in isolating CimA variants which lead to higher alcohol production titers.

TABLE 3. 1-Propanol and 1-butanol production with the selected CimA mutants

Protein	Production (mg/liter) of ^a :	
	1-Propanol	1-Butanol
WT	302 (1)	18 (1)
CimA1	695 (2.3)	22 (1.2)
CimA2	1,178 (3.9)	77 (4.3)
CimA2Δ	2,416 (8.0)	106 (5.9)
WTΔ	60 (0.2)	ND ^b
CimA3.1	2,471 (8.2)	373 (20.7)
CimA3.2	2,567 (8.5)	254 (14.1)
CimA3.3	2,392 (7.9)	124 (6.9)
CimA3.4	2,452 (8.1)	114 (6.2)
CimA3.5	2,328 (7.7)	140 (7.8)
CimA3.6	2,353 (7.8)	146 (8.0)
CimA3.7	2,778 (9.2)	393 (21.9)
CimA3.8	2,481 (8.2)	391 (21.7)
CimA3.9	2,446 (8.1)	199 (11.0)

^a Cultures were grown at 30°C for 40 h. The titers of 1-propanol and 1-butanol production from KS145 with pSA55 and pSA63 (*cimA-leuABCD*) and its variations are shown. The change (fold) relative to the strain containing wild-type CimA (WT) is in shown parentheses.

^b ND, not detectable.

To confirm the effect of the noncoding region created by mutation in CimA2, we compared the production from the strain with CimA2 with that from the strain with CimA2Δ (Table 3). The production of 1-propanol and 1-butanol from the strain with CimA2Δ increased 2.4- and 1.6-fold, respectively, compared to the strain with CimA2. As a control, we also constructed a truncated version of the wild-type *cimA* (denoted WTΔ) without the acquired amino acid substitutions

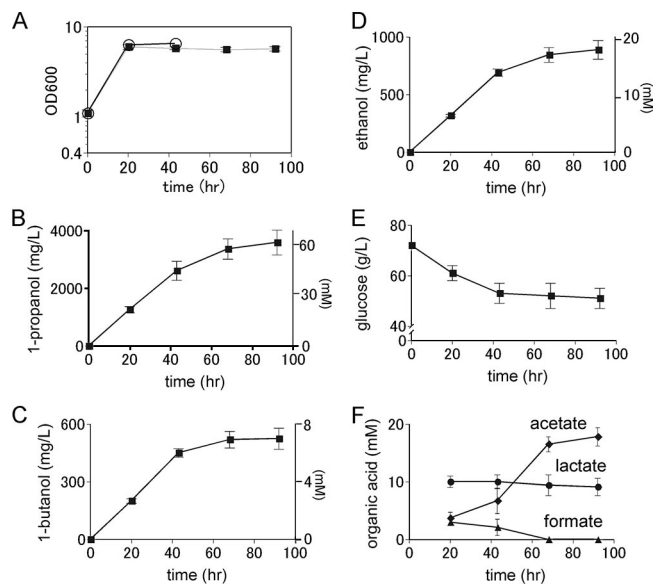


FIG. 6. 1-Propanol and 1-butanol production with CimA3.7. Time profiles of cell growth with IPTG (squares) and without IPTG (open circles) (A); 1-propanol (B), 1-butanol (C), and ethanol (D) production; glucose consumption (E); and organic acid production (acetate [diamonds], lactate [circles], formate [triangles]) (F) from KS145/pSA55/pSA142 (containing *cimA3.7*). Cultures were grown at 30°C in M9 medium containing 72 g/liter glucose and 5 g/liter yeast extract. OD600, optical density at 600 nm.

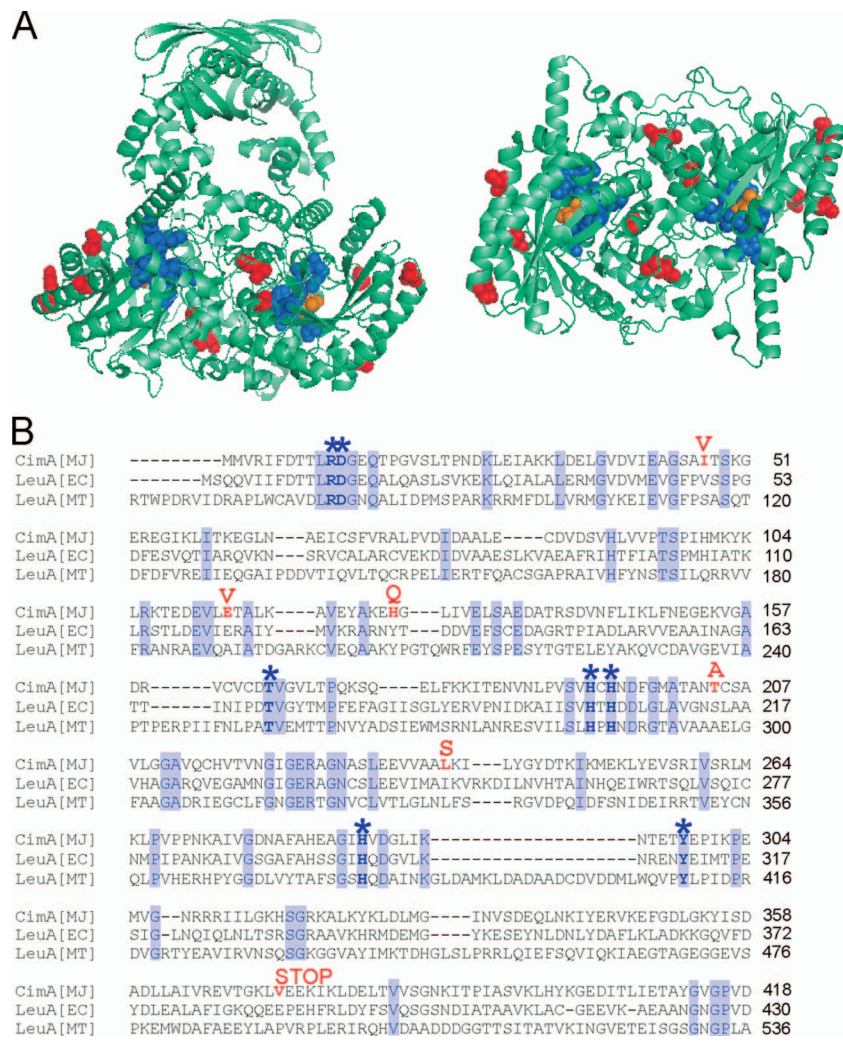


FIG. 7. Sequence analysis of CimA3.7. (A) Structure of *Mycobacterium tuberculosis* LeuA. The residues in the active site and the bound 2-ketoisovalerate are colored blue and orange, respectively. The image on the left contains the regulator domain, while the image on the right does not. The residues corresponding to mutations in CimA3.7 are colored red. (B) Amino acid sequence alignment of CimA (*M. jannaschii*), LeuA (*E. coli*), and LeuA (*M. tuberculosis*). Multiple sequence alignment was carried out using ClustalW (17). Fully conserved residues are shaded. The residues in the active site are shown with asterisks. Residue mutations in CimA3.7 are labeled in red. Gaps in the sequence are shown with dashes.

in CimA2. However, this construct showed diminished 1-propanol and 1-butanol production (Table 3). This result indicates that the truncated version of CimA requires other mutations (Ile47Val, His126Gln, and Thr204Ala) for enhanced activity. The strains expressing the CimA3.1 to CimA3.9 mutants showed production levels of 1-propanol similar to those of the strain expressing CimA2Δ (Table 3). However, 1-butanol production increased compared to that in the strain with CimA2Δ (Table 3). The production of 1-propanol and 1-butanol from the strain expressing the CimA3.7 variant increased 9.2- and 21.9-fold, respectively, compared to that in the strain with wild-type CimA. In addition to the amino acid substitutions in CimA2Δ, CimA3.7 contains two new amino acid substitutions (Glu114Val and Leu238Ser).

Time profiles of alcohol production with CimA3.7. Since CimA3.7 is the best alcohol producer, the production profiles of KS145/pSA55/pSA142 (containing cimA3.7) were

characterized in shake flasks. Cell growth stopped after 10 h and remained stationary during alcohol production (Fig. 6A). The growth with IPTG was similar to that without IPTG, indicating that overexpression of this pathway had almost no effect on cell growth. Both 1-propanol and 1-butanol production increased in a linear fashion up to 40 h, after which the production rate appeared to decrease (Fig. 6B and C). This strain produced more than 3.5 g/liter 1-propanol and 524 mg/liter 1-butanol after 92 h. The formation of ethanol may be due to the native production by *adhE* or by the decarboxylation of pyruvate by *Kivd* (Fig. 6D). This result indicates that overexpression of the citramalate pathway coupled with 1-propanol and 1-butanol production can be tolerated by *E. coli*. The rate of glucose consumption decreased after 40 h, which is consistent with the alcohol production rates (Fig. 6E). Acetate and lactate are the major organic acids produced at a significant level (Fig. 6F).

DISCUSSION

The success in improving 1-propanol and 1-butanol production by directed evolution coupled with L-isoleucine biosynthesis demonstrates an important strategy for the production of biofuels. Since the production of these alcohols is not required for growth, it is difficult to devise a selection scheme for directed evolution. Here we demonstrated that the 2-keto acid-based pathway for alcohol production enables a growth-based selection. The selected CimA variants are missing the C-terminal domain (Fig. 3A and 7) involved in feedback inhibition, as shown by homology alignment, suggesting that the variants may be insensitive to feedback inhibition by L-isoleucine. The *in vitro* enzyme assay showed that CimA3.7 was indeed insensitive to L-isoleucine (Fig. 4B). However, this truncated form needed additional mutations to exhibit high activity (Fig. 3A and Table 3). This led to the speculation that the structural stability or the dimer formation rate of the truncated form was improved by some of these mutations. Removing negative feedback is a straightforward strategy to increase L-isoleucine production. However, it is difficult to improve activity while removing an entire regulator domain. Directed evolution enables us to isolate variants which could not be constructed readily by rational design.

We employed CimA from *M. jannaschii*, an extreme thermophilic archaeon (8). Many thermophilic enzymes lose activity at moderate temperatures. Thus, the dependence of activity of CimA variants on temperature is interesting. The specific activity at 30°C of the wild type and CimA3.7 decreased 2.2- and 1.3-fold, respectively, over that at 70°C. Apparently, mutations in CimA3.7 increase both its activity and stability at moderate temperatures. However, the relative superiority of CimA3.7 decreases with increasing temperature (Fig. 4A), presumably because CimA mutants were screened solely for improvements in activity in *E. coli* at 30°C. The final product of the directed evolution, CimA3.7 (Fig. 3A), exhibits a k_{cat} 2.3 times greater and a k_{cat}/K_m for acetyl-CoA 6.7 times greater than those of the wild type at 30°C (Table 2). Only five amino acid substitutions brought about this increase in *E. coli*, showing that a thermophilic protein can rapidly adapt to a mesophile when strong selective pressure is applied. Further analysis is required to explain how the acquired mutations could help the activity of CimA in *E. coli*.

In the absence of actual crystallographic data, we cannot determine the specific mechanisms responsible for the observed increase activity of CimA3.7. However, the acquired mutations are not located near the catalytic center shown by homology alignment (Fig. 7B), suggesting these mutations may stabilize its active structure. It has been shown that thermophilic proteins have larger amino acid side chains, higher residue hydrophobicity, more charged amino acids, and fewer uncharged polar residues than mesophilic proteins (7). Twelve out of 18 substitutions identified in CimA3s result in the substitution of larger amino acids with smaller ones. Eight substitutions resulted in the substitution by amino acid residues with lower hydrophobicity. The replacement of native residues with uncharged polar residues was observed six times. Additionally, eight substitutions resulted in the replacement of a charged residue with a noncharged residue. Continued analysis of these mutations should provide further insight into the mechanism which leads to higher activity of CimA.

For further improvement of the alcohol production, the next step would be the strain modification by using a metabolic engineering approach. It is important to remove side products for achieving high yield. Ethanol, acetate, and lactate are the major side products in this strain (Fig. 6D and F). Obviation of the side products while maintaining metabolic balance is one key metabolic engineering objective for biofuel applications of this pathway.

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