Applied and Environmental Microbiology, Sept. 2008, p. 5769–5775 Vol. 74, No. 18
0099-2240/08/$08.00+0 doi:10.1128/AEM.00468-08

Engineering of an *Escherichia coli* Strain for the Production of 3-Methyl-1-Butanol[▽]

Michael R. Connor and James C. Liao*

Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, California 90095

Received 27 February 2008/Accepted 23 July 2008

3-Methyl-1-butanol is a potential fuel additive or substitute. Previously this compound was identified in small quantities in yeast fermentation as one of the fusel alcohols. In this work, we engineered an *Escherichia coli* strain to produce 3-methyl-1-butanol from glucose via the host's amino acid biosynthetic pathways. Strain improvement with the removal of feedback inhibition and competing pathways increased the selectivity and productivity of 3-methyl-1-butanol. This work demonstrates the feasibility of production of 3-methyl-1-butanol as a biofuel and shows promise in using *E. coli* as a host for production.

Energy and environmental concerns have resulted in an increased interest in the production of alternative fuels from renewable sources. The primary focus of such research thus far has been on the production of bioethanol, with more than 4.8 billion gallons being produced in the United States in 2006 (22). Ethanol, however, cannot completely replace existing petroleum-based fuels, since the high vapor pressure and water content may present problems in engine performance and the supply infrastructure of the current fuel economy. The use of longer-chain alcohols can compensate for some of these issues. Five-carbon alcohols, such as 3-methyl-1-butanol, possess properties that can increase the potential for a biomass-derived replacement for gasoline. With a vapor pressure more than 20-fold lower than that of ethanol and an energy density calculated from the heat of combustion (16) that is more than 80% (28.2 MJ/liter) of that of gasoline (34.8 MJ/liter) (10), 3-methyl-1-butanol can offer advantages over ethanol as a supplement to or replacement for gasoline.

3-Methyl-1-butanol is a natural, although minor, product in fungus and yeast fermentation. Recent work with yeast has focused on identifying gene targets, mutations, and pathways for increased 3-methyl-1-butanol production (1, 11, 21). 3-Methyl-1-butanol is also used as a precursor for synthesis of various chemicals, such as isoamyl acetate, which has been successfully produced from 3-methyl-1-butanol in *Escherichia coli* (15, 23). Fusel oil, a by-product of ethanol distillation which contains a fraction of 3-methyl-1-butanol, has also been shown to be an effective substrate for the biocatalysis of triolein to biodiesel (20). With a wide variety of uses and its potential role as a fuel, the development of a process for the production of 3-methyl-1-butanol is desirable. Here we demonstrate the first design for the production of 3-methyl-1-butanol in *Escherichia coli* from glucose.

Our laboratory has previously shown that 2-keto acids generated from amino acid biosynthesis can serve as precursors for the Ehrlich degradation pathway to alcohols (4). In order to produce 3-methyl-1-butanol, the valine and leucine biosynthesis pathways can be used to generate 2-ketoisocaproate (KIC), the precursor to leucine. KIC can then be converted to 3-methyl-1-butanol via a decarboxylation and reduction step. The entire pathway to 3-methyl-1-butanol from glucose is diagrammed in Fig. 1.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. JCL16 (*rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$/F′ [*traD36 proAB*+ *lacI*^{q}ZΔM15]) was used as the wild type (WT) (3). XL-1 Blue (Stratagene, La Jolla, CA) was used to propagate all plasmids.

For initial production experiments, strains were grown in a modified M9 medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mg vitamin B_1 per liter of water) containing 20 g/liter of glucose, 5 g/liter of yeast extract, and 1,000× Trace Metals Mix A5 (2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 49.4 mg $Co(NO_3)_2 \cdot 6H_2O$ per liter water), inoculated at 1% from 3 ml overnight cultures in LB into 10 ml of fresh medium in 125-ml screw-cap flasks, and grown at 37°C in a rotary shaker for 4 h. The culture was then induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) and grown at 30°C for 18 h. Antibiotics were added as needed (ampicillin, 100 μg/ml; chloroamphenicol, 35 μg/ml; kanamycin, 50 μg/ml).

For 2-keto acid experiments, 10-ml cultures in 250-ml baffled shake flasks were inoculated at 1% from 3-ml overnight cultures in LB. All cultures were grown at 37°C for 4 h, induced with 1 mM IPTG, and harvested after 18 h of growth at 30°C.

Time course production experiments were conducted as previously described, except that 20 ml modified M9 medium containing 5 g/liter of glucose and 5 g/liter of yeast extract was used in a 250-ml screw-cap flask. The final production experiment (see Fig. 5) was conducted as were the previous experiments except that the medium contained 10 g/liter of glucose at the start. To quantify the 3-methyl-1-butanol produced from yeast extract alone, cultures were induced with 1 mM IPTG and grown at 30°C for 24 h after 4 h of growth at 37°C in modified M9 medium containing 5 g/liter of yeast extract with and without 10 g/liter of glucose.

Reagents. All restriction enzymes and Antarctic phosphatase were purchased from New England Biolabs (Ipswich, MA). The Rapid DNA ligation kit was supplied by Roche (Manheim, Germany). KOD DNA polymerase was purchased from EMD Chemicals (San Diego, CA). Oligonucleotides were ordered from Invitrogen (Carlsbad, CA).

DNA techniques. *E. coli* strain JCL260 was constructed as described previously (3). Branched-chain-amino-acid aminotransferase (encoded by *ilvE*) and tyrosine aminotransferase (encoded by *tyrB*) were deleted by P1 transduction from strains JW5606 and JW4014 (5), respectively. All strains and plasmids are listed in Table 1. Oligonucleotides are listed in Table 2.

To clone the valine biosynthesis genes *ilvIHCD* (*E. coli*) and *alsS* (*Bacillus subtilis*), along with *ilvCD* (*E. coli*), the low-copy-number origin of replication (ori) from pZS24-MCS1 (17) was removed by digestion with SacI and AvrII and

* Corresponding author. Mailing address: University of California, Department of Chemical and Biomolecular Engineering, 5531 Boelter Hall, Los Angeles, CA 90095. Phone: (310) 825-1656. Fax: (310) 206-4107. E-mail: liaoj@ucla.edu.

▽ Published ahead of print on 1 August 2008.

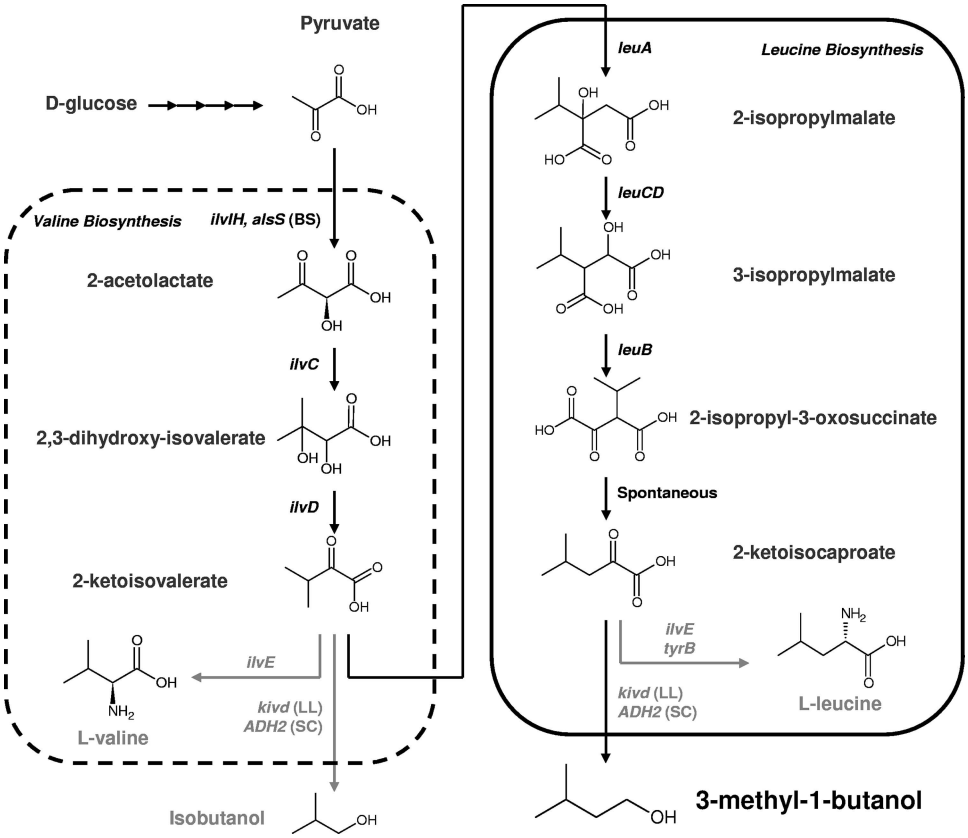


FIG. 1. Metabolic pathway from glucose to 3-methyl-1-butanol. All genes are from *E. coli* unless otherwise noted. BS, *B. subtilis*; LL, *L. lactis*; SC, *S. cerevisiae*.

then ligated into the corresponding sites of pSA54 and pSA69 to create plasmids pIAA1 and pIAA11, respectively.

To clone *kivd* from *Lactococcus lactis* and *ADH2* from *Saccharomyces cerevisiae*, the ColE1 ori of pSA55 was removed by digestion with SacI and AvrII and replaced with the p15A ori of pSA54 digested with the same restriction enzymes to create pIAA13. To better control the expression of these genes, *lacI* was amplified from *E. coli* MG1655 genomic DNA with KOD polymerase using the primers lacISacIf and lacISacIr and ligated into the SacI site of pSA55 to be

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant genotype[a]	Reference or source
Strains		
XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB* $lacI^qZ\Delta M15$ Tn*10* (Tet^r)]	Stratagene
JCL16	BW25113/F′ [*traD36 proAB*$^+$ $lacI^qZ\Delta M15$]	3
JCL260	As JCL16, but Δ*adhE* Δ*ldhA* Δ*frdBC* Δ*fnr* Δ*pta* Δ*pflB*	3
IAA6	As JCL260, but Δ*ilvE*	This study
IAA10	As JCL16, with P_{lacO-1} integrated upstream of *leuABCD*	This study
IAA69	As JCL260, but Δ*ilvE* Δ*tyrB*	This study
Plasmids		
pZS24-MCS1	pSC101 ori; Kan^r; $P_{lac-ara-1}$:MCS1	17
pSA54	p15A ori; Kan^r; $P_{LlacO-1}$:*ilvIHCD* (EC)	4
pSA55	ColE1 ori; Amp^r; $P_{LlacO-1}$:*kivd* (LL)-*ADH2* (SC)	4
pSA69	p15A ori; Kan^r; $P_{LlacO-1}$:*alsS* (BS)-*ilvCD* (EC)	4
pIAA1	pSC101 ori; Kan^r; $P_{LlacO-1}$:*ilvIHCD* (EC)	This study
pIAA2	p15A ori; Cm^r; $P_{LlacO-1}$:*leuABCD* (EC)	This study
pIAA11	pSC101 ori; Kan^r; $P_{LlacO-1}$:*alsS* (BS)-*ilvCD* (EC)	This study
pIAA12	ColE1 ori; Amp^r; $P_{LlacO-1}$:*kivd* (LL)-*ADH2* (SC); P_{Amp}:*bla-lacI* (EC)	This study
pIAA13	p15A ori; Amp^r; $P_{LlacO-1}$:*kivd* (LL)-*ADH2* (SC)	This study
pIAA15	ColE1 ori; Cm^r; $P_{LlacO-1}$:*leuABCD* (EC); P_{Cm}:*cat-lacI* (EC)	This study
pIAA16	ColE1 ori; Cm^r; $P_{LlacO-1}$:*leuA*(syn RBS)-*leuBCD* (EC); P_{Cm}:*cat-lacI* (EC)	This study
pIAA17	ColE1 ori; Cm^r; $P_{LlacO-1}$:*leuA* (*G462D*)-*leuBCD* (EC); P_{Cm}:*cat-lacI* (EC)	This study

[a] EC, *E. coli*; SC, *S. cerevisiae*; BS, *Bacillus subtilis*; LL, *Lactococcus lactis*.

TABLE 2. Primers used in this work

Name	Sequence (5′→3′)
KanLeuO1f	TTCCATCCACTGATGGCCTTTATCGCGCC CTGTAGGCTGGAGCTGCTTCG
KanLeuO2f	TTAGCGTTTGCAAATTGAGACTAATTGC TCTTCCATCCACTGATGGCCTTTA
KanlacO1SOEr	GGCCTCGTGATACGCCTATTATTCCGGG GATCCGTCGACC
lacO1KanSOEf	GGTCGACGGATCCCCGGAATAATAGGC GTATCACGAGGCCCT
lacO1LeuA1r	GCTGGCTCATGGTTTGGGTCCTTGTCTCT TGGTCAGTGCGTCCTGCTGATGT
lacO1LeuA2r	CGCGCAATGTGGTATCGAAAATAATGAC TTGCTGGCTCATGGTTTGGGTCCT
leuKOv1	GCAATGTGGTATCGAAAATAATGAC
leuKOv2	CCAGACATTCATGTCTGACCTATTC
laclSaclf	CTAGAGCTCGAAGGAGATATACCATGAA ACCAGTAACGTTATACGATG
laclSaclr	CTAGAGCTCTCACTGCCCGCTTTCCAGTC

expressed along with the ampicillin resistance gene, *bla*, creating plasmid pIAA12.

In order to overexpress the *leuABCD* operon in JCL16 from the chromosome, the native promoter and leader sequence was replaced with the $P_{LlacO-1}$ promoter (17). The $P_{LlacO-1}$ promoter was amplified from pZE12-luc (17) with KOD polymerase using the primers lacO1KanSOEf and lacO1LeuA1r. The gene encoding resistance to kanamycin, *aph*, was amplified from pKD13 (9) using the primers KanLeuO1f and KanlacO1SOEr. One microliter of product from each reaction was added as a template, along with primers KanLeuO2f and lacO1LeuA2r, and was amplified with KOD polymerase using SOE. The PCR product was electroporated and integrated into the chromosome using the phage λ Red recombinase as described previously (9). The new construct was amplified from the genomic DNA of kanamycin-resistant clones using the primers leuKOv1 and leuKOv2 and sent out for sequence verification to confirm the accuracy of cloning.

To overexpress the *leuABCD* operon from a plasmid, the p15A ori from pSA54 was removed with SacI and AvrII and ligated into the corresponding sites of pCS22 (ColE1, Cm^r, $P_{LlacO-1}$: *leuABCD*) to create plasmid pIAA2. In order for tighter expression, *lacI* was amplified and ligated, as described previously for pIAA12, into pCS22 to be expressed along with the chloroamphenicol resistance gene, *cat*, creating plasmid pIAA15. Plasmid pIAA16, containing $leuA^{FBR}$ [*leuA*(*G1385A*)] encoding IPMS(G462D), was created by ligating the 5.5-kb fragment of pIAA15 digested with XhoI and NdeI and ligating it with the 2.3-kb fragment of pZE12-*leuABCD* [ColE1, Amp^r, $P_{LlacO-1}$: *leuA*(*G1385A*)*BCD*] cut with the same restriction enzymes. To control for the expression level, the ribosome binding site (RBS) was replaced in pIAA15 to match that of pIAA16. To do this, the 5.6-kb fragment of pIAA16 from digestion with HindIII and NdeI was ligated with the 2.2-kb fragment of pIAA15 digested with the same enzymes to create pIAA17.

Detection of metabolites. The produced alcohol compounds were quantified by a gas chromatograph (GC) equipped with a flame ionization detector. The system consisted of model 5890A GC (Hewlett-Packard, Avondale, PA) and a model 7673A automatic injector, sampler, and controller (Hewlett-Packard). The separation of alcohol compounds was carried out using a DB-WAX capillary column (30 m, 0.32 mm-inside diameter, 0.50-μm film thickness) purchased from Agilent Technologies (Santa Clara, CA). The GC oven temperature was initially held at 40°C for 5 min and raised with a gradient of 15°C/min until reaching 120°C. It was then raised with a gradient of 50°C/min until 230°C and held for 4 min. Helium was used as the carrier gas, with 9.3-lb/in^2 inlet pressure. The injector and detector were maintained at 225°C. Supernatant of culture broth (0.5 μl) was injected in split injection mode with a 1:15 split ratio. Methanol or butanol was used as the internal standard.

For other secreted metabolites, filtered supernatant was applied (20 μl) to an Agilent 1100 high-performance liquid chromatography system equipped with an auto-sampler and a Bio-Rad (Hercules, CA) Aminex HPX87 column (5 mM H_2SO_4, 0.6 ml/min; column temperature at 65°C). Glucose was detected with a refractive index detector, while organic acids were detected using a photodiode array detector at 210 nm. Concentrations were determined by extrapolation from standard curves.

RESULTS

Expression of valine and leucine biosynthesis pathway genes leads to 3-methyl-1-butanol production. To produce 3-methyl-1-butanol in *E. coli*, the entire pathway from pyruvate to 3-methyl-1-butanol was overexpressed. *ilvIHCD* (*E. coli*), *kivd* (*L. lactis*), and *ADH2* (*S. cerevisiae*) were all expressed from plasmids (pIAA1 and pIAA12) under the control of the $P_{LlacO-1}$ promoter (17). The *leuABCD* operon was overexpressed by replacing the upstream noncoding region of *leuA* with the $P_{LlacO-1}$ promoter in JCL16 (WT) (See Materials and Methods). This strain was able to produce 56 mg/liter of 3-methyl-1-butanol after 18 h of induction with IPTG (Fig. 2A). In order to increase production of 3-methyl-1-butanol,

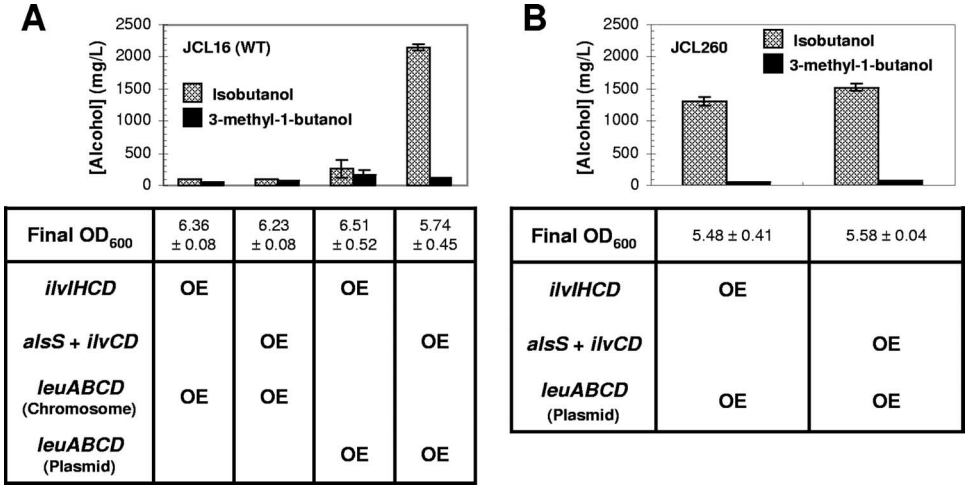


FIG. 2. Initial production of 3-methyl-1-butanol. Checkered columns indicate isobutanol; solid columns are for 3-methyl-1-butanol. "OE" indicates overexpression. (A) 3-Methyl-1-butanol production in JCL16. Strains carrying either *ilvIH* (pIAA1) or *alsS* (pIAA11) along with *kivd* and *ADH2* (pIAA12) with chromosomal or plasmid-based expression of *leuABCD* (IAA10 and pIAA2, respectively) were assayed for alcohol production. (B) 3-Methyl-1-butanol production in JCL260. Strains carrying either *ilvIH* (JCL260/pIAA1/pIAA2/pIAA12) or *alsS* (JCL260/pIAA2/pIAA11/pIAA12) were tested for alcohol production.

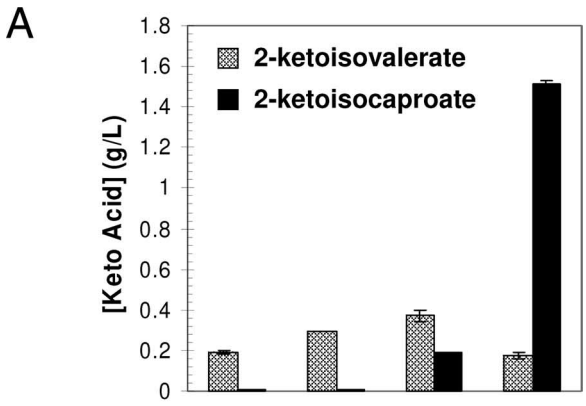
ilvIH was replaced with *alsS* (pIAA11) from *B. subtilis*. Acetolactate synthase from *B. subtilis*, encoded by *alsS*, has previously been shown to increase isobutanol production (4), which uses the valine synthesis pathway. The replacement of *ilvIH* with *alsS* showed a minimal increase in 3-methyl-1-butanol production (67 mg/liter) (Fig. 2A). To increase the production of 3-methyl-1-butanol, *leuABCD* was cloned into a p15A-derived plasmid and expressed under the control of the $P_{LlacO-1}$ promoter. Plasmid-based expression of the leucine biosynthesis genes increased 3-methyl-1-butanol production for strains containing either *ilvIH* or *alsS* (Fig. 2A), although overexpression of *alsS* lead to a dramatic increase in isobutanol production.

Deletion of native competing pathways to increase 3-methyl-1-butanol production. Similar to strategies employed previously for fuel production (3, 4), host pathways competing for carbon and reducing power were deleted. Our laboratory has shown previously that deletion of *adhE*, *frdBC*, *ldhA*, *pta*, *fnr*, and *pflB* significantly increased production of isobutanol in *E. coli* relative to a WT background (4). When the 3-methyl-1-butanol pathway was transformed into this strain, the final titer of 3-methyl-1-butanol was 76 mg/liter for the strain expressing *alsS* (Fig. 2B). Since the isobutanol titer was greater than 10 times that of the target product, the process and metabolic pathway were examined to improve the production of 3-methyl-1-butanol.

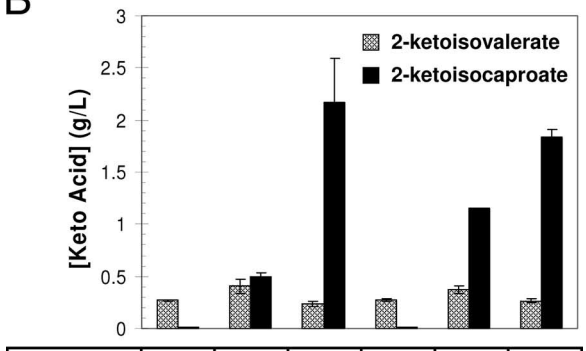
Production of 2-keto acid precursors to determine pathway bottleneck. We hypothesized that isobutanol production was much higher than 3-methyl-1-butanol production due to competition of the substrate 2-ketoisovalerate (KIV) between the gene products of *kivd* and *leuA*. In order to investigate this hypothesis, the production of the 2-keto acid precursors was examined. To achieve this, *leuABCD* was expressed on a ColE1-derived plasmid along with *alsS-ilvCD* under the control of $P_{LlacO-1}$. When this strain was tested, the isobutanol precursor and *leuA* substrate, KIV, was the main product (Fig. 3A). KIV was produced to a final concentration of 0.29 g/liter, while the 3-methyl-1-butanol precursor, KIC, was not detected (<5 mg/liter). In hopes of increasing the KIC pool, the RBS of the *leuA* gene was changed from its native sequence to more of a consensus sequence (see Materials and Methods). The use of the synthetic RBS increased the KIC concentration to 0.19 g/liter, although KIV was still the main product (0.37 g/liter) (Fig. 3A). This result suggests that the decreased 3-methyl-1-butanol production was not due completely to competition for KIV but rather was related to the low activity of the *leuA* gene product (2-isopropylmalate synthase [IPMS]).

IPMS catalyzes the condensation of KIV with acetyl-coenzyme A. The accumulation of KIV could be due to feedback inhibition of IPMS by free leucine synthesized from KIC. To relieve the feedback inhibition of IPMS, two strategies were investigated. First, we employed a feedback-insensitive mutant of IPMS [IPMS(G462D)] (13), encoded by $leuA^{FBR}$. Second, the final step in the leucine synthesis pathway was inactivated by deleting *ilvE* (branched-chain-amino-acid transferase) and *tyrB* (tyrosine aminotransferase), two isozymes responsible for converting KIC into leucine.

When $leuA^{FBR}$ was expressed, the product distribution dramatically shifted toward KIC, with a final concentration of 1.61 g/liter, while KIV accumulation decreased to 0.17 g/liter (Fig. 3A). Inactivation of *ilvE* increased production of KIC in the



Final OD_{600}	5.51 ± 0.65	6.51 ± 0.82	6.32 ± 0.19	6.04 ± 0.20
alsS + ilvCD	OE	OE	OE	OE
WT *leuABCD*		OE	OE	
$leuA^{FBR}BCD$				OE
Natural RBS		√		
Synthetic RBS			√	√



Final OD_{600}	5.24 ± 0.13	6.32 ± 0.42	6.00 ± 0.30	6.37 ± 0.59	6.11 ± 0.11	6.29 ± 0.83
WT *leuABCD*	OE	OE		OE	OE	
$leuA^{FBR}BCD$			OE			OE
Natural RBS	√			√		
Synthetic RBS		√	√		√	√
ilvE	Δ	Δ	Δ	Δ	Δ	Δ
tyrB				Δ	Δ	Δ

FIG. 3. Investigation of 2-keto acid production. Checkered columns indicate KIV; solid columns are for KIC. "OE" indicates overexpression. (A) Production of 2-keto acids in JCL260 background. Strains were tested for production of KIV (isobutanol) and KIC (3-methyl-1-butanol) using *alsS-ilvCD* (pIAA11) and WT *leuABCD* with a natural (pIAA15) or synthetic (pIAA17) RBS or $leuA^{FBR}BCD$ (pIAA16). The RBS change is for the *leuA* gene only. OD_{600}, optical density at 600 nm. (B) Production of 2-keto acids in leucine synthesis knockout backgrounds. The "Δ" symbol indicates deletion. *ilvE* encodes branched-chain-amino-acid aminotransferase, and *tyrB* encodes tyrosine aminotransferase.

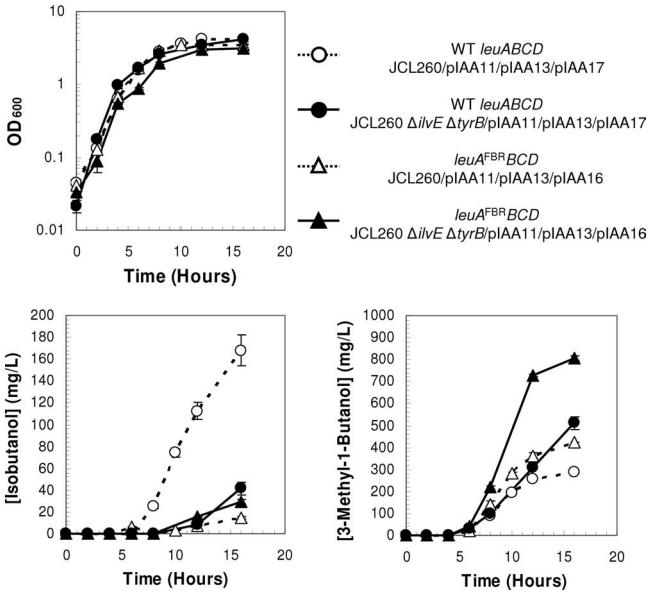


FIG. 4. 3-Methyl-1-butanol production with removal of feedback inhibition. Growth and alcohol production were compared for strains overexpressing WT *leuA* and *leuA*^{FBR} in JCL260 and JCL260 Δ*ilvE* Δ*tyrB* backgrounds after 12 h of induction at 30°C. OD_{600}, optical density at 600 nm.

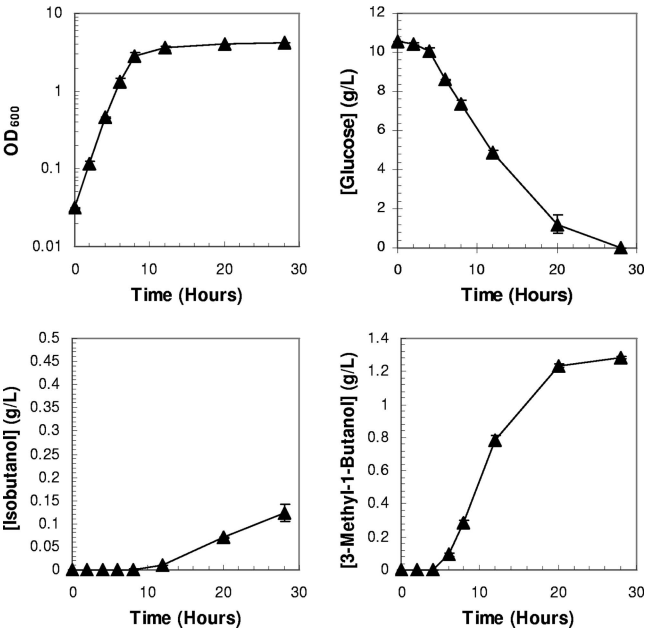


FIG. 5. Increased production of 3-methyl-1-butanol. Growth and alcohol production were quantified for JCL260 Δ*ilvE* Δ*tyrB*/pIAA11/pIAA13/pIAA16 after 24 h of induction at 30°C. OD_{600}, optical density at 600 nm.

strain expressing WT *leuA* with the synthetic RBS, while deletion of *ilvE* and *tyrB* further increased accumulation of KIC to 1.23 g/liter (Fig. 3B). The production of KIV in the JCL260 Δ*ilvE* and JCL260 Δ*ilvE* Δ*tyrB* backgrounds remained similar to that of the JCL260 *ilvE*$^+$ *tyrB*$^+$ strain, with final concentrations of 0.40 g/liter and 0.37 g/liter, respectively. When the Δ*ilvE* and Δ*ilvE* Δ*tyrB* host strains were combined with the expression of *leuA*^{FBR}, KIC increased to 2.31 g/liter and 1.95 g/liter, respectively (Fig. 3B).

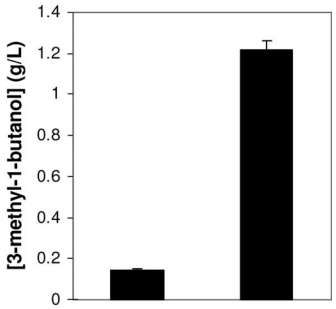
Production of 3-methyl-1-butanol. With an increased production of KIC, the entire pathway for 3-methyl-1-butanol production from pyruvate was transformed using either WT *leuA* or *leuA*^{FBR}. Similar to the results seen for keto-acid production, the strain expressing WT *leuA* still produced a significant amount of isobutanol (168 mg/liter) in a JCL260 *ilvE*$^+$ *tyrB*$^+$ background, although 3-methyl-1-butanol was the main product (286 mg/liter) (Fig. 4). As expected, when *leuA*^{FBR} was expressed in the JCL260 *ilvE*$^+$ *tyrB*$^+$ background, 3-methyl-1-butanol was the main product, with a final titer of 425 mg/liter, with isobutanol accumulating to only 15 mg/liter (Fig. 4). The removal of feedback inhibition of IPMS by mutation changed the product distribution from 1.7:1 (3-methyl-1-butanol:isobutanol) using WT *leuA* to greater than 28:1. Accumulation of other common metabolic by-products including acetate and succinate is shown in Table 3.

When the JCL260 Δ*ilvE* Δ*tyrB* strain expressing the WT *leuA* gene product was examined for 3-methyl-1-butanol production, the results mimicked those of the strain containing the mutant IPMS. 3-Methyl-1-butanol accumulated to a final concentration of 512 mg/liter, while isobutanol was present at only 42 mg/liter (Fig. 4). This corresponds to a product distribution ratio of 3-methyl-1-butanol to isobutanol of greater than 12:1. When the *leuA*^{FBR} gene was expressed in place of WT *leuA*, 3-methyl-1-butanol increased further, to 806 mg/liter, while isobutanol production was minimal (Fig. 4). The yield for this experiment was estimated to be 0.13 g/g from glucose by subtracting the 3-methyl-1-butanol produced from yeast extract alone (see Fig. 6). Increasing the glucose concentration (10 g/liter) and fermentation time (28 h) in this strain led to a final titer of 1.28 g/liter for 3-methyl-1-butanol (Fig. 5 and 6), the

TABLE 3. Metabolic by-products of 3-methyl-1-butanol-producing strains

Host strain	Plasmids	Fermentation time (h)	Yield[a] (g/g)	Final metabolite concn (g/liter)[b]		
				Glucose[c]	Acetate	Succinate
JCL260	pIAA11, pIAA13, pIAA17	16	0.03	5.49	0.30	0.14
JCL260	pIAA11, pIAA13, pIAA16	16	0.05	5.48	0.22	0.13
JCL260 Δ*ilvE* Δ*tyrB*	pIAA11, pIAA13, pIAA17	16	0.06	5.62	0.65	0.16
JCL260 Δ*ilvE* Δ*tyrB*	pIAA11, pIAA13, pIAA16	16	0.13	5.06	0.13	0.05
JCL260 Δ*ilvE* Δ*tyrB*	pIAA11, pIAA13, pIAA16	28	0.11	10.57	0.24	0.34

[a] Estimated by subtracting 3-methyl-1-butanol produced solely from yeast extract from the total and dividing by the total glucose consumed.
[b] Pyruvate, formate, lactate, and fumarate were not detected.
[c] Glucose consumed.



Final OD_{600}	2.03 ± 0.45	4.47 ± 0.59
+ 5g/L Yeast Extract	√	√
+ 10g/L Glucose		√

FIG. 6. Production of 3-methyl-1-butanol from yeast extract. Growth and alcohol production were quantified for JCL260 Δ*ilvE* Δ*tyrB*/pIAA11/pIAA13/pIAA16 after 24 h of induction at 30°C with and without glucose added to the medium. OD_{600}, optical density at 600 nm.

highest production of 3-methyl-1-butanol reported to our knowledge. The estimated yield was calculated to be 0.11 g/g. Isobutanol accumulated to less than 0.2 g/liter.

DISCUSSION

Previously our laboratory reported success in producing isobutanol and 1-butanol in *E. coli* by using the native amino acid biosynthesis pathways to generate precursors for alcohol production (4, 21a). Here we demonstrated the first *E. coli* strain developed to produce 3-methyl-1-butanol using the valine and leucine biosynthesis pathways. This is also the first microorganism designed for the hyperproduction of 3-methyl-1-butanol. The maximum production achieved was 1.28 g/liter after 28 h for strain JCL260 Δ*ilvE* Δ*tyrB* containing pIAA11, pIAA13, and pIAA16. The overall yield from this experiment was estimated to be 0.11 g/g, with a maximum productivity of 0.12 g/liter/h between 8 and 12 h. Also, the accumulation of undesirable metabolic by-products was minimized and kept under 0.6 g/liter total.

This work demonstrates that the main bottleneck to 3-methyl-1-butanol production in *E. coli* is due to feedback inhibition of the *leuA* gene product by free leucine. The elimination of the leucine synthesis genes *ilvE* and *tyrB* led to an increased production of KIC using WT *leuA* with a synthetic RBS. Expression of $leuA^{FBR}$ resulted in a further increase in the production of KIC and 3-methyl-1-butanol in the JCL260 Δ*ilvE* Δ*tyrB* background. Production of KIC was not detected in any strains expressing WT *leuABCD* with its natural RBS. This is most likely due to the low expression level of *leuA*, which may lead to inhibition of IPMS by low levels of free leucine contained in the yeast extract. All strains expressing WT *leuABCD* with the synthetic RBS were able to synthesize KIC, suggesting that the increased expression level of *leuA* was sufficient to overcome complete inhibition of IPMS by leucine contained in the yeast extract.

Further improvement in the production of 3-methyl-1-butanol can be achieved by increasing the glucose concentration and fermentation time to allow extended production, although 3-methyl-1-butanol production slowed after 20 h to a rate similar to that of isobutanol production. This may be due to several reasons, such as a depleted glucose concentration or precursor availability, since no acetyl-coenzyme A is required for isobutanol synthesis as in 3-methyl-1-butanol production. These late-stage production rates (6 to 7 mg/liter/h), however, are minor compared to the average 3-methyl-1-butanol production rate between 4 and 20 h (>75 mg/liter/h). Culture conditions may also need to be investigated due to an unfavorable redox balance in the absence of oxygen, since the production of 3-methyl-1-butanol from glucose creates a surplus of NADH. Isobutanol production, however, is electronically balanced and may also help to explain the increased rate of isobutanol production during the later stages of fermentation.

Our success in engineering *E. coli* to produce 3-methyl-1-butanol using conserved and native amino acid biosynthesis pathways opens the possibility of using a variety of nonnative hosts for 3-methyl-1-butanol production. Existing amino acid production technology can also be applied to increase productivity and yield (8, 19). *E. coli*, however, is still an advantageous host due to its well-known physiology and metabolism and easily manipulated genetics and has already been shown to be a suitable host for the production of many valuable metabolites (7, 12, 14, 18). Also, a variety of methods have been developed to increase tolerance to alcohol stress in various organisms (2, 6, 24), which may be applied to *E. coli* to improve its tolerance to 3-methyl-1-butanol, which will become important as production titers increase.

ACKNOWLEDGMENT

This work was supported in part by the UCLA-DOE Institute for Genomics and Proteomics.

REFERENCES

1. **Abe, F., and K. Horikoshi.** 2005. Enhanced production of isoamyl alcohol and isoamyl acetate by ubiquitination-deficient *Saccharomyces cerevisiae* mutants. Cell. Mol. Biol. Lett. **10:**383–388.
2. **Alper, H., J. Moxley, E. Nevoigt, G. R. Fink, and G. Stephanopoulos.** 2006. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science **314:**1565–1568.
3. **Atsumi, S., A. F. Cann, M. R. Connor, C. R. Shen, K. M. Smith, M. P. Brynildsen, K. J. Chou, T. Hanai, and J. C. Liao.** 2007. Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab. Eng. doi:10.1016/j.ymben.2007.08.003.
4. **Atsumi, S., T. Hanai, and J. C. Liao.** 2008. Engineering synthetic nonfermentative pathways for production of branched-chain higher alcohols as biofuels. Nature **451:**86–89.
5. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knock-out mutants—the Keio collection. Mol. Syst. Biol. **2:**2006.0008.
6. **Borden, J. R., and E. T. Papoutsakis.** 2007. Dynamics of a genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **73:**3061–3068.
7. **Causey, T. B., K. T. Shanmugam, L. P. Yomano, and L. O. Ingram.** 2004. Engineering *Escherichia coli* for efficient conversion of glucose to pyruvate. Proc. Natl. Acad. Sci. USA **101:**2235–2240.
8. **Cusyatiner, M. M., E. B. Voroshilova, Y. G. Rostova, L. V. Ivanovskaya, M. G. Lunts, and E. M. Khourges.** June 2003. Method for producing L-leucine. U.S. patent 7,220,572.
9. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
10. **Davis, S. C., and S. W. Diegel (ed.).** 2007. Transportation energy data book, 26th ed. Oak Ridge National Laboratory, Oak Ridge, TN.

11. **Dickinson, J. R., M. M. Lanterman, D. J. Danner, B. M. Pearson, P. Sanz, S. J. Harrison, and M. J. Hewlins.** 1997. A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. **272:**26871–26878.
12. **Farmer, W. R., and J. C. Liao.** 2000. Improving lycopene production in *Escherichia coli* by engineering metabolic control. Nat. Biotechnol. **18:**533–537.
13. **Gusyatiner, M. M., M. G. Lunts, Y. I. Koslov, L. V. Ivanovskaya, and E. B. Voroshilova.** July 2002. DNA coding for mutant isopropylmalate synthase L-leucine producing microorganism and method for producing L-leucine. U.S. patent 6,403,342.
14. **Hanai, T., S. Atsumi, and J. C. Liao.** 2007. Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl. Environ. Microbiol. **73:** 7814–7818.
15. **Horton, C. E., K. X. Huang, G. N. Bennett, and F. B. Rudolph.** 2003. Heterologous expression of the *Saccharomyces cerevisiae* alcohol acetyltransferase genes in *Clostridium acetobutylicum* and *Escherichia coli* for the production of isoamyl acetate. J. Ind. Microbiol. Biotechnol. **30:**427–432.
16. **Lide, D. R. (ed.).** 2007. CRC handbook of chemistry and physics, Internet version 2007, 87th ed. Taylor and Francis, Boca Raton, FL. http://www.hbcpnetbase.com.
17. **Lutz, R., and H. Bujard.** 1997. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I_1-I_2 regulatory elements. Nucleic Acids Res. **25:**1203–1210.
18. **Martin, V. J., D. J. Pitera, S. T. Withers, J. D. Newman, and J. D. Keasling.** 2003. Engineering a mevalonate pathway in *Escherichia coli* for the production of terpenoids. Nat. Biotechnol. **21:**796–802.
19. **Nakano, T., M. Ikeda, K. Kino, and S. Furukawa.** June 1995. Process for producing L-leucine. U.S. patent 5,744,331.
20. **Salis, A., M. Pinna, M. Monduzzi, and V. Solinas.** 2005. Biodiesel production from triolein and short chain alcohols through biocatalysis. J. Biotechnol. **119:**291–299.
21. **Schoondermark-Stolk, S. A., M. Jansen, J. H. Veurink, A. J. Verkleij, C. T. Verrips, G. J. Euverink, J. Boonstra, and L. Dijkhuizen.** 2006. Rapid identification of target genes for 3-methyl-1-butanol production in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **70:**237–246.
21a. **Shen, C. R., and J. C. Liao.** Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab. Eng., in press.
22. **Society of Chemistry and Industry.** 12 March 2007. US bioethanol production topped 4.86 bn gallons in 2006. (Biofuel briefs.) Chem. Ind. **5:**7.
23. **Vadali, R. V., C. E. Horton, F. B. Rudolph, G. N. Bennett, and K. Y. San.** 2004. Production of isoamyl acetate in *ackA-pta* and/or *ldh* mutants of *Escherichia coli* with overexpression of yeast ATF2. Appl. Microbiol. Biotechnol. **63:**698–704.
24. **Yomano, L. P., S. W. York, and L. O. Ingram.** 1998. Isolation and characterization of ethanol tolerant mutants of *Escherichia coli KO11* for fuel ethanol production. J. Ind. Microbiol. Biotechnol. **20:**132–138.