Redesigning *Escherichia coli* Metabolism for Anaerobic Production of Isobutanol

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Fermentation enables the production of reduced metabolites, such as the biofuels ethanol and butanol, from fermentable sugars. This work demonstrates a general approach for designing and constructing a production host that uses a heterologous pathway as an obligately fermentative pathway to produce reduced metabolites, specifically, the biofuel isobutanol. Elementary mode analysis was applied to design an *Escherichia coli* strain optimized for isobutanol production under strictly anaerobic conditions. The central metabolism of *E. coli* was decomposed into 38,219 functional, unique, and elementary modes (EMs). The model predictions revealed that during anaerobic growth *E. coli* cannot produce isobutanol as the sole fermentative product. By deleting 7 chromosomal genes, the total 38,219 EMs were constrained to 12 EMs, 6 of which can produce high yields of isobutanol in a range from 0.29 to 0.41 g isobutanol/g glucose under anaerobic conditions. The remaining 6 EMs rely primarily on the pyruvate dehydrogenase enzyme complex (PDHC) and are typically inhibited under anaerobic conditions. The redesigned *E. coli* strain was constrained to employ the anaerobic isobutanol pathways through deletion of 7 chromosomal genes, addition of 2 heterologous genes, and overexpression of 5 genes. Here we present the design, construction, and characterization of an isobutanol-producing *E. coli* strain to illustrate the approach. The model predictions are evaluated in relation to experimental data and strategies proposed to improve anaerobic isobutanol production. We also show that the endogenous alcohol/aldhyde dehydrogenase AdhE is the key enzyme responsible for the production of isobutanol and ethanol under anaerobic conditions. The glycolytic flux can be controlled to regulate the ratio of isobutanol to ethanol production.

Society currently relies on the use of fossil fuels to meet its growing energy demands (16). Oil, natural gas, and coal contribute about 84% of the world’s energy consumption (International Energy Outlook 2010 [39]). There is thus a pressing need to develop alternative technologies to reduce reliance on fossil fuels (26). One such alternative is the biological conversion of lignocellulosic biomass into biofuels, which has emerged as a promising route to sustainable energy (1, 9, 24).

Recent efforts have focused on engineering *Saccharomyces cerevisiae* to ferment sugars derived from lignocellulosic biomass hydrolysates to produce ethanol as a biofuel (17). Other microorganisms, such as *Zymomonas mobilis* (40) and *Escherichia coli* (20), have also been engineered to produce ethanol. However, ethanol is not an ideal biofuel, due to its miscibility in water and low energy content relative to gasoline. Recently, metabolic engineering and synthetic biology have been applied to engineer recombinant microorganisms to produce biofuels, such as butanol, isobutanol, isopentanol, fatty alcohols, biodiesel, and isoprenoid-derived biofuels, that have similar properties to gasoline or jet fuel (3, 10, 18, 25, 31). However, most of these biofuels are typically produced at low titers and with poor yields, in part because the producing organism must be cultivated under aerobic or oxygen-limited conditions to address a redox imbalance inherent in the production pathways employed.

Fermentation is the anaerobic metabolic conversion of sugars to products, involves endogeneous electron acceptors, and produces reducing equivalents, such as NADH. Fermentation is the most efficient route to synthesize biofuels that are reduced relative to the sugar source. Relevant examples are ethanol production from *S. cerevisiae* and butanol production from *Clostridium acetobutylicum* (17, 30). Under anaerobic conditions, the NADH generated from sugar metabolism is recycled to NAD⁺ by the formation of reduced metabolites, such as alcohols, thus helping to maintain the appropriate redox balance within the cell. However, most pathways that synthesize advanced biofuels are not obligately anaerobic and are active under aerobic conditions, even when the host is a facultative anaerobe. For example, *E. coli* is able to produce biodiesel and certain hydrocarbons by employing fatty acid biosynthesis pathways only under aerobic conditions (25, 31).

Under aerobic conditions, reducing equivalents in the form of NADH are most efficiently regenerated by oxidation, directly generating ATP for cell synthesis and maintenance, rather than by the production of reduced metabolites such as biofuels (37). To direct cellular metabolism toward the formation of reduced products, cell cultivation is often carried out under oxygen-limited conditions (3). Precise control of the
dissolved oxygen concentration is required to partition carbon flow between the synthesis of biomass and reduced metabolites. Therefore, converting biofuel-producing pathways to obligate anaerobic pathways would result in higher yields of reduced metabolites and be advantageous for large-scale production, because oxygen supply and control would not be required.

Isobutanol formation provides an example of a nonfermentative biofuel-producing pathway. Isobutanol is produced via retrofitted anaerobic pathways that function as obligate anaerobic pathways. This approach deconstructs the complex metabolic network of E. coli into a complete set of EMs. Each EM contains a minimum and unique set of enzymes that cells can use to function under quasi-steady-state conditions (23, 27–29). Knowledge of the complete set of EMs allows the selection of routes that enable cell growth and synthesis of isobutanol at high yields under anaerobic conditions (32, 33, 35, 36, 38).

In the present study, we demonstrate as a proof of concept that it is possible to “retrofit” E. coli to produce isobutanol through a nonnative obligate anaerobic pathway. Elementary mode (EM) analysis was employed to redesign E. coli metabolism. This approach deconstructs the complex metabolic network of E. coli into a complete set of EMs. Each EM contains a minimum and unique set of enzymes that cells can use to function under quasi-steady-state conditions (23, 27–29).

Materials and Methods

Organisms and plasmids. Table 1 shows a list of strains and plasmids used in the study.

Strain construction. All mutants with single deleted genes were obtained from the single-gene knockout library of the Keio Collection (6). These mutants were derived from BW25113, a derivative of E. coli MG1655, and constructed using the technique of one-step disruption of chromosomal genes (12). Mutants with multiple deleted genes were created by multiple steps of P1 transduction from strains with a single deleted gene (33). At each step, the recipient strain that contained one or more deleted genes had the kanamycin cassette removed by donor strains used to prepare P1 lysate had a single deleted gene with an intact kanamycin cassette. The PCR test was designed to detect complete gene disruption by using primers located outside the undeleted portion of the structural gene (see Table S1 in the supplemental material). To construct a mutant with the T7 polymerase gene, the technique of one-step disruption of chromosomal genes (12). Mutants with multiple deleted genes were created by multiple steps of P1 transduction from strains with a single deleted gene (33). At each step, the recipient strain that contained one or more deleted genes had the kanamycin cassette removed by donor strains used to prepare P1 lysate had a single deleted gene with an intact kanamycin cassette. The PCR test was designed to detect complete gene disruption by using primers located outside the undeleted portion of the structural gene (see Table S1 in the supplemental material). To construct a mutant with the T7 polymerase gene, the
prophage ADE3 (Novagen ADE3 dsygoxenization kit, catalog number 69734-3) was used to insert the gene into the specific site of the mutant chromosome.

**Plasmid construction.** To construct pCT01, the orthologous slsZ/sdhB, catalase E. coli strain MG1655 by using the primers kivd_adhE_1f_2 (5′-AAA AAA CAT ATG TAG ATA CAT ACG GAT GTT ATG TGA AAA ACG TAC CTC CTT GGT TTG TG-3′ and kivd_adhE_1r_1 (5′-AAA AAA GCG GCC GCT TAA CCC CCC AGT TTC GAT TTA TCG CCC ACC-3′). The gene amplification was carried out by PCR with a high-fidelity Pfu polymerase (Agilent Technologies catalog number 609674). The PCR product was double digested with BamHI and NotI and ligated into the backbone vector pCOLAduet (Novagen).

To construct pCT02, the orthologous kivd_adhE_1f_1 (5′-AAA AAA CAT ATG TAG ATA CAT ACG GAT GTT ATG TGA AAA ACG TAC CTC CTT GGT TTG TG-3′) and kivd_adhE_1r_2 (5′-AAA AAA GCG GCC GCT TAA CCC CCC AGT TTC GAT TTA TCG CCC ACC-3′) were amplified from the genomic DNA of E. coli MG1655 by using the primers kivd_adhE_1f_1 (5′-TTT GCT GGA CAA GAA ATA TAA AAG GAG ATA TAA TGG CTG TTA CATG ATG TCG-3′) and kivd_adhE_1r_1 (5′-AAA AAA CAT ATG GCT GTT ACT AAT GTC GCT-3′). The PCR product was double digested with NdeI and XhoI and ligated into the backbone vector pCOLAduet. To construct pCT03, the orthologous slsZ/sdhB was doubly digested with BamHI and NotI and ligated into the vector pCT01.

The orthologous kivd_adhE was constructed using the overlapping PCR extension technique. The gene kivd was amplified from the genomic DNA of E. coli strain MG1655 by using the primers kivd_adhE_1f_1 (5′-AAA AAA CAT ATG TAG ATA CAT ACG GAT GTT ATG TGA AAA ACG TAC CTC CTT GGT TTG TG-3′) and kivd_adhE_1r_1 (5′-TTT GCT GGA CAA GAA ATA TAA AAG GAG ATA TAA TGG CTG TTA CATG ATG TCG-3′) and ligated into the vector pCT01.

To construct the plasmid pA1103, the operon kivd_adhE_1f_1 (5′-AAA AAA CAT ATG TAG ATA CAT ACG GAT GTT ATG TGA AAA ACG TAC CTC CTT GGT TTG TG-3′) and kivd_adhE_1r_2 (5′-AAA AAA GCG GCC GCT TAA CCC CCC AGT TTC GAT TTA TCG CCC ACC-3′) were amplified from the plasmid pA1103 by using the primers kivd_adhE_1f_2 (5′-AAA AAA CAT ATG TAG ATA CAT ACG GAT GTT ATG TGA AAA ACG TAC CTC CTT GGT TTG TG-3′) and kivd_adhE_1r_1 (5′-AAA AAA GCG GCC GCT TAA CCC CCC AGT TTC GAT TTA TCG CCC ACC-3′). The gene kivd_adhE was amplified from the genomic DNA of E. coli MG1655 by using the primers kivd_adhE_1f_2 (5′-TTT GCT GGA CAA GAA ATA TAA AAG GAG ATA TAA TGG CTG TTA CATG ATG TCG-3′) and kivd_adhE_1r_2 (5′-AAA AAA GCG GCC GCT TAA CCC CCC AGT TTC GAT TTA TCG CCC ACC-3′). The operon kivd_adhE was constructed by amplying both of the amplified genes kivd and adhE with the primers kivd_adhE_1f_1 and kivd_adhE_1r_2. The operon kivd_adhE was digested with Ndel and Xhol and ligated into the backbone vector pCOLAduet to create the vector pCT13. To construct the vector pCT13, the operon adhE was doubly digested with BamHI and NotI and ligated into the vector pCT13A. All constructed vectors were confirmed by gel electrophoresis after being digested with either BamHI/NotI or Ndel/Xhol and by sequencing the inserts.

To construct the plasmid pET24b with the adhE gene, the gene adhE was amplified from the genomic DNA of E. coli strain MG1655 by using the primers adhE_1f_1 (5′-AAA AAA CAT ATG TAG ATA CAT ACG GAT GTT ATG TGA AAA ACG TAC CTC CTT GGT TTG TG-3′) and adhE_1r_2 (5′-AAA AAA GCG GCC GCT TAA CCC CCC AGT TTC GAT TTA TCG CCC ACC-3′). The PCR product was doubly digested with Ndel and Xhol and ligated into the vector pET24b to create the vector pET24b adhE. This gene also contained an additional 18-nucleotide sequence from pET24b that encoded the His-tag sequence at the C terminus of the enzyme AdhE.

**Cell cultivation.** (i) Medium. Strains were characterized in a medium containing 6.8 g/liter Na2HPO4, 3.0 g/liter KH2PO4, 1.0 g/liter NH4Cl, 2 ml/liter 1 M MgSO4, 100 μg/ml kanamycin, and 20 μg/ml kanamycin (unless otherwise specified) and 37°C. Anaerobic conditions were maintained by using a French press cell operated at a constant pressure of 21,300 lb/in2.

(ii) Fermentation. Batch fermentations were conducted in 3-liter bioreactors (Bioengineering AG, Switzerland) with a working volume of 2 liters, under anaerobic growth conditions. The operating conditions were 200 rpm, pH 7 (unless otherwise specified), and 37°C. Anaerobic conditions were maintained by continuously sparging the bioreactor with N2 at a volumetric flow rate of 100 ml/min throughout the fermentation. Dissolved oxygen was measured by using a polarographic dissolved oxygen probe, which was found to be 0%, confirming that the bioreactor was anaerobic. Exponentially grown cell cultures (OD600 0.5 to ~1) were used for inoculation, and the OD600 of initial cultures in the bioreactors following inoculation was about 0.05. Optical density was measured at 600 nm (OD600) of the cell culture reached about 0.5. Antifoam 204 (Sigma-Aldrich catalog number A6426) was added to the medium at 100 μl/liter before cell inoculation.

Data analysis. Product yields were calculated, and a carbon balance was determined as previously described (34, 36). Statistical analysis was performed using SigmaPlot (Systat Software, Inc.).

Protein expression and purification. From the petri dish, a single colony of BL21(DE3) pET24b adhE was picked and grown overnight in a shake tube containing Luria-Bertani (LB) medium plus 10 μl/glucose and 50 μg/ml kanamycin. The overnight culture was transferred into a 1-liter shake flask containing 500 ml of the identical medium at an inoculum ratio of 1:100. After the OD600 reached 0.5, IPTG was added to a final concentration of 0.5 mM to induce expression of the His-tagged AdhE. The cell pellet was collected after 4 h of incubation at 5,000 × g for 10 min and at 4°C. Cells were lysed by using a French press cell operated at a constant pressure of 21,300 lb/in2. The lysed cells were used to purify the His-tagged AdhE by using Ni-nitrilotriacetic acid (NTA) agarose beads. The purification procedure followed protocols 9 and 12 in the QIA expressionist manual (Qiagen Inc.). The protein concentration was measured by using the Bradford method (catalog number 500-0006; Bio-Rad, Hercules, CA).

Enzyme assay. The enzyme activity of AdhE was measured based on the oxidation of NADH by different aldehydes, including acetaldehyde, isobutanaldehyde, and butyraldehyde, at the wavelength of 340 nm. A 1-ml reaction mixture in morpholinepropanesulfonic acid buffer (pH 7.0) containing 10 μg/ml of the purified protein AdhE, 5 mM aldehyde, and various concentrations of NADH (40 to 150 μM) at 37°C. The reaction was initiated by adding the aldehyde last, and the OD600 was measured and recorded every 10 s during the course of 180 to 360 s.

Analytical techniques. Metabolites such as glucose, isobutanol, succinate, lactate, acetate, and formate were measured using the high-performance liquid chromatography Prominence system (10A; Shimadzu, Columbia, MD) as described previously (34).

**Metabolic network reconstruction and analysis.** The metabolic network of E. coli central metabolism was constructed as previously reported (36). In this work, the isobutanol-producing pathway consisting of 5 enzymatic reactions that convert pyruvate into isobutanol (Fig. 1) was also added. These reactions were as follows: IBUT1, AlsS (acetolactate synthase), 2 pyruvate + acetaldehyde + CO2 → IBUT2, InC (2,3-dihydroxy isovalerate oxidoreductase) + IVd (IVd), 2,3-dihydroxy isovalerate dehydrogenase; Kivd2, lactis (kivd2, lactis), aldehyde dehydrogenase from S. cerevisiae. AdhE, alcohol/aldehyde dehydrogenase from E. coli.

![FIG. 1. Simplified metabolic network of the designed isobutanol-producing cells, optimized for anaerobic isobutanol synthesis. PB (encoded by the gene pbPB), pyruvate formate lyase; PDHC (pdh), PDHC enzyme; AlsS_B. subtilis (alsS_B. subtilis), acetolactate synthase from B. subtilis. It should be noted that E. coli has the endogenous acetolactate synthase enzyme encoded by ivlHI, ivlCM, or ivlNB (http://www.ecocyc.org). Here, AlsS_B. subtilis is used to bypass the allosteric inhibition by valine (2, 21). IvC (ivC), 2,3-dihydroxy isovalerate oxidoreductase; IVd (IVd), 2,3-dihydroxy isovalerate dehydrogenase; Kivd2, lactis (kivd2, lactis), α-ketoacid decarboxylase from L. lactis; Adh2, corynebacterium (A. corynebacterium). (A) The endogenous acetolactate synthase enzyme encoded by ivlHI, ivlCM, or ivlNB. (B) The acetolactate synthase enzyme encoded by alsS_B. subtilis. (C) The α-ketoacid decarboxylase from L. lactis. (D) The alcohol/ aldehyde dehydrogenase from S. cerevisiae. (E) The alcohol/ aldehyde dehydrogenase from E. coli.](http://aem.asm.org/Downloaded from)
structure (27) and to rationally design optimal cells with efficient minimized metabolic functionalities (32). EMs of the metabolic network were calculated using the publicly available software program METATOOL 5.0.

RESULTS

Metabolic network analysis. (i) Metabolic network structure. EM analysis identified a total of 38,219 EMs that used glucose as a carbon source, 5,621 of which operated under anaerobic conditions. These anaerobic EMs included 1,899 isobutanol-producing EMs, only 1,458 of which could coproduce isobutanol and cell biomass (Fig. 2A). The solution space of all admissible EMs was used to study anaerobic isobutanol metabolism in E. coli and to identify the most efficient pathways for anaerobic isobutanol production.

(ii) Infeasibility of E. coli fermentation for isobutanol production as the sole product. To examine whether fermentation for isobutanol production as the sole product is feasible in E. coli, we searched for EMs that support both cell biomass and isobutanol production under anaerobic growth conditions. By eliminating all fermentative pathways that produced succinate, lactate, acetate, and ethanol (Δfrd, ΔldhA, Δpta, ΔpoxB, and ΔadhE), we found no existing EMs that could coproduce cell biomass and isobutanol under anaerobic growth conditions (Table 2). All 14 remaining EMs produced isobutanol as the sole product at a theoretical yield of 0.41 g isobutanol/g glucose. A similar result was obtained if only the anaerobic pathways that produced succinate and ethanol, but neither lactate nor acetate, were eliminated. However, if the isobutanol-producing pathway was forced to couple with one of the anaerobic pathways that could oxidize NADH, there existed EMs that coproduced both cell biomass and isobutanol. For instance, if the isobutanol-producing pathway was coupled with the ethanol-producing pathway, then 814 out of 1150 EMs could produce isobutanol. Among these isobutanol-producing pathways, 282 EMs could coproduce cell biomass and isobutanol (Table 2). This result indicates that anaerobic production of isobutanol as the sole product is infeasible due to an imbalance of the reducing equivalents. Isobutanol must be coproduced with...
other reduced metabolites, such as succinate and ethanol, to balance the reducing equivalents by recycling NADH. Our strategy was thus directed toward production of biofuels by coupling the biosynthesis of isobutanol and ethanol.

**Rational design of the most efficient isobutanol-producing E. coli strain.** (i) **Strain design strategy.** The optimal pathways for supporting cell growth and converting glucose into isobutanol can be selected and strictly enforced by removing the inefficient pathways from the metabolic network. The search algorithm to remove the inefficient pathways has been developed and reported in previous publications (15, 32, 33, 35, 36, 38). Briefly, the algorithm identifies the minimal set of reactions that should be deleted from the metabolic network in order to (i) remove as many inefficient pathways for isobutanol production as possible, (ii) retain a small subset of efficient isobutanol-producing pathways that can achieve maximal isobutanol yield, and (iii) tightly couple isobutanol and biomass synthesis during cell growth.

(ii) **Strain design implementation.** In order to achieve maximum efficiency of isobutanol production, cellular metabolism must be redirected to use only the optimal anaerobic isobutanol-producing pathways. By deleting 6 reactions (ΔPDP1, ΔTCA9r, ΔTRA5, ΔOPM4r, ΔTRA2, and ΔTRA4), corresponding to knockout of 7 genes (Δzwf, Δmdh, ΔfrdA, Δndh, ΔaceF, ΔpoxB, and ΔadhE), the total numbers of EMs, biomass-producing EMs, isobutanol-producing EMs, and biomass- and isobutanol-coproducing EMs were reduced from 5,621, 4,500, 1,899, and 1,458 to 12, 4, 6, and 2, respectively (Fig. 2A). After removing the 6 reactions, half of the total 12 EMs produced solely isobutanol while the other half made only ethanol. Interestingly, all 6 EMs that produced solely ethanol used the pyruvate dehydrogenase complex (PDHC) enzyme (the reaction GG13) to convert pyruvate into acetyl coenzyme A (CoA). This enzyme is known to be downregulated by a high intracellular redox state (NADH/NAD⁺ ratio) under anaerobic conditions (15). In principle, these 6 EMs should not be functional under anaerobic conditions.

If the additional reaction GG13 was deleted by knocking out the gene aceF, the total number of EMs, biomass-producing EMs, isobutanol-producing EMs, and biomass- and isobutanol-coproducing EMs could be reduced from 12, 4, 6, and 2 to 6, 2, 6, and 2, respectively (Fig. 2A). With the deletion of the complete set of seven reactions, the total number of EMs was equal to that of the isobutanol-producing EMs, which empha-

**TABLE 2. Effects of deleting anaerobic pathways on feasibility of producing isobutanol as the sole product**

<table>
<thead>
<tr>
<th>Deletions</th>
<th>No. of EMs based on:</th>
<th>Range of iBuOH yield on glucose (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Biomass</td>
</tr>
<tr>
<td>Δfrd ΔldhA Δpta ΔpoxB ΔadhE</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Δfrd ΔldhA Δpta ΔpoxB</td>
<td>1,150</td>
<td>900</td>
</tr>
<tr>
<td>Δfrd ΔldhA ΔadhE</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Δfrd Δpta ΔpoxB ΔadhE</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>ΔldhA Δpta ΔpoxB ΔadhE</td>
<td>427</td>
<td>251</td>
</tr>
<tr>
<td>Δfrd ΔadhE</td>
<td>28</td>
<td>0</td>
</tr>
</tbody>
</table>


b iBuOH, isobutanol.

sizes that the cell was designed to obligately utilize the isobutanol-producing pathway for redox balancing. In addition, the number of the biomass-producing EMs was equal to that of the biomass- and isobutanol-coproducing EMs, which indicated that cell growth and isobutanol production were coupled during the growth-associated phase. In this phase, the designed E. coli strain could produce isobutanol with a yield of 0.29 (g isobutanol/g glucose). In the no-growth phase, all 4 EMs could produce isobutanol at the maximum theoretical yield of 0.41 (g isobutanol/g glucose) (Fig. 2B).

From the analysis, the optimal designed E. coli strain that can efficiently convert glucose into isobutanol by retrofitting the heterologous isobutanol-producing pathway as an obligately anaerobic pathway has the following deletions: Δzwf, Δmdh, ΔfrdA, Δndh, ΔaceF*, and ΔpoxB. The asterisk for the deleted gene aceF* implies that if the negative regulation of the high intracellular redox state NADH/NAD⁺ with the PDHC enzyme is strongly exerted under anaerobic conditions, the PDHC enzyme will be completely downregulated and replaced by pyruvate formate lyase (PFL) to convert pyruvate to acetyl-CoA. Thus, the gene aceF may not need to be deleted.

**Phenotypic operating space of the designed isobutanol-producing E. coli strain.** From the stoichiometry reactions of the 12 remaining EMs of the designed isobutanol-producing strain (see Table S2 of the supplemental material), the yields of ethanol and isobutanol on glucose were calculated and mapped on the two-dimensional (2-D) phenotypic space (Fig. 3). This phenotypic space is shown by the bold trapezoid and constructed by connecting the four star symbols located at the edge of the trapezoid. These symbols represent four groups of EMs. The group G1 is comprised of EMs that can produce isobutanol at the theoretical yield of 0.41 (g/g) without biomass production, while the group G2 coproduces isobutanol and biomass with an isobutanol yield of 0.29 (g/g). It should be emphasized that both groups G1 and G2 contain the 6 optimal isobutanol-producing pathways that do not use PDHC. This group of EMs is also constrained to produce a small amount of ethanol, 0.0012 (g/g), to maintain the redox balance. The isobutanol-to-ethanol mass ratio in this group is 236:1.

The group G3 produces only ethanol, at a theoretical yield of 0.51 (g/g), whereas group G4 coproduces ethanol and biomass with a high ethanol yield of 0.36 (g/g). The EMs of both G3 and G4 use the PDHC enzyme to convert pyruvate into
ethanol, which is typically inhibited under anaerobic conditions. The designed isobutanol-producing strain can operate anywhere in the trapezoidal phenotypic space (Fig. 3). For isobutanol production, it is desirable to have the designed strain operate close to groups G1 and G2.

Strain comparisons. Other E. coli strains have been engineered to produce isobutanol, including JCL260 pSA55/pSA69 (3). The following genes are deleted from the host JCL260: \( \Delta \text{adhE}, \Delta \text{fmr}, \Delta \text{pflB}, \Delta \text{frdBC}, \Delta \text{pta}, \) and \( \Delta \text{ldhA} \). According to EM analysis, deletion of these genes disrupts the cell metabolism required to support cell growth, since no existing EMs can produce cell biomass (Table 3).

Construction of the designed E. coli strain. (i) Host strain construction. The designed host E. coli strain BFA7.001(DE3) was constructed with seven genes knocked out (\( \Delta \text{swf}, \Delta \text{ldhA}, \Delta \text{pta}, \Delta \text{poxB}, \Delta \text{ndh}, \Delta \text{frdA}, \) and \( \Delta \text{aceF}^* \)) according to the model prediction for anaerobic conversion of glucose to isobutanol. In this study, we did not delete the gene \( \text{aceF} \) to remove the PDHC reaction GG13, operating under the assumption that the PDHC enzyme is downregulated and the PFL enzyme is solely responsible for converting pyruvate to acetyl-CoA. In addition, we were interested in exploring how the level of downregulation of the PDHC enzyme affects the production of isobutanol and ethanol. The knockout genes in the designed strain were confirmed by PCR. The designed strain also had the T7 polymerase gene inserted into the chromosome (see Fig. S1 in the supplemental material).

(ii) Construction of the isobutanol-producing pathway. The isobutanol-producing pathway (Fig. 1; see also Fig. S2 in the supplemental material) was constructed by using a single plasmid, pCT03, that had a low copy number, a strong T7 promoter, and a kanamycin selection. The plasmid pCT03 derived from pCOLADuet contains two operons, including a complete set of five genes for converting pyruvate to isobutanol. The first operon, \( \text{p}_T^7::\text{alsS}::\text{iivD} \), contained 3 genes \( \text{alsS} \), \( \text{poxB} \), and \( \text{iivD} \) under the T7 promoter that were used to amplify fluxes from pyruvate to \( \alpha \)-ketoisovalerate. It should be noted that the native E. coli has these endogeneous enzymes to generate the precursor \( \alpha \)-ketoisovalerate for L-valine biosynthesis. The second operon \( \text{p}_T^7::\text{kivd}::\text{adh2} \) contained two genes \( \text{kivd} \) and \( \text{adh2} \) under the T7 promoter that were used to convert \( \alpha \)-ketoisovalerate to isobutanol. Native E. coli does not have \( \alpha \)-ketoacid deacetylase (encoded by the Kivd gene), an important enzyme for converting \( \alpha \)-ketoisovalerate to isobutyraldehyde.

Besides pCT03, we also constructed different incomplete parts of the isobutanol-producing pathway and expressed different alcohol dehydrogenases, such as AdhE (encoded by \( \text{adhE}_{\text{E. coli}} \)). Specifically, the plasmid pCT01 contained the first operon, \( \text{p}_T^7::\text{alsS}::\text{iivC}::\text{iivD} \). The plasmid pCT02 contained the second operon, \( \text{p}_T^7::\text{kivd}::\text{adh2} \). The plasmid pCT13 contained both the first operon, \( \text{p}_T^7::\text{alsS}::\text{iivC}::\text{iivD} \), and the second operon, \( \text{p}_T^7::\text{kivd}::\text{adh2} \). It should be noted that pCT03 had the alcohol dehydrogenase Adh2 from S. cerevisiae while pCT13 had the alcohol dehydrogenase AdhE from E. coli.

Characterization of the designed isobutanol-producing strains. (i) Anaerobic growth of isobutanol-producing E. coli strains. The designed isobutanol-producing E. coli strain BFA7.001(DE3) pCT03 was first tested for its ability to grow anaerobically under defined cultivation conditions (pH 7, 37°C, 200 rpm, 100 ml/min nitrogen). Consistent with the model prediction, the designed strain was able to completely ferment 20 g/liter glucose after 36 h and had a specific growth rate of 0.37 ± 0.03 h⁻¹ (mean ± standard deviation) (Fig. 4).

To confirm that the ethanol-producing pathway is required to couple with the isobutanol-producing pathway in order to support cell growth, we constructed a variant E. coli strain, BFA8.001(DE3), that was derived from BFA7.001(DE3) and had the endogenous alcohol dehydrogenase gene \( \text{adhE} \) deleted.

TABLE 3. Elementary mode analysis for the effects of multiple deleted genes applied to different designed E. coli strains, including JCL260 pSA55/pSA69 and BFA7.001(DE3) pCT03 or BFA7.001(DE3) pCT13* under anaerobic conditions

<table>
<thead>
<tr>
<th>Deleted genes or summary value</th>
<th>JCL260 pSA55/pSA69</th>
<th>BFA7.001(DE3) pCT03</th>
<th>BFA7.001(DE3) pCT13*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deleted genes</td>
<td>( \Delta \text{adhE} )</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>( \Delta \text{fmr} )</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>( \Delta \text{pflB} )</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
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<td>6</td>
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* Information on JCL260 pSA55/pSA69 is from reference 3, and that for BFA7.001(DE3) pCT03 and BFA7.001(DE3) pCT13 is from this study.
leted. Strains BFA8.001(DE3) pCT01, BFA8.001(DE3) pCT02, and BFA8.001(DE3) pCT03, which contained different portions of the isobutanol-producing pathway, were also constructed and tested for their fermentation capabilities. The results show that none of these strains was able to grow anaerobically (Fig. 5), presumably due to the reducing equivalent (NADH) imbalance. However, when the ethanol-producing pathway containing the endogenous enzyme AdhE was restored, the resulting *E. coli* strain, BFA8.001(DE3) pCT06, was able to grow even though it exhibited a long lag phase (Fig. 5). Here, the plasmid pCT06 contained two operons, pT7::alsS::ilvC::ilvD and pT7::adhE.

We also tested the fermentation capability of JCL260 pSA55 pSA69 (3). The results showed that the strain was unable to consume glucose anaerobically as a result of a genetic manipulation that led to a redox imbalance, as predicted by EM analysis. The strain was only able to grow aerobically with a supply of 100 ml/min air instead of nitrogen, with other operating conditions remaining the same (Fig. 6). In the experiment, the oxygen transfer coefficient ($k_{L}a$) was set at 1.2 h$^{-1}$ to test the growth sensitivity of JCL260 pSA55/pSA69 under oxygen-limited growth conditions. Within 3 h after inoculation, the dissolved oxygen in the bioreactor was reduced to zero, indicating that oxygen became limiting. The strain had an oxygen consumption rate of 8.16 mg/liter/h. Under the oxygen-limited condition, the growth of JCL260 pSA55/pSA69 was dramatically affected and exhibited a specific growth rate of 0.08 h$^{-1}$ (Fig. 6). This result demonstrates the challenge of precisely supplying oxygen to support cell growth and isobutanol production under oxygen-limited conditions, which becomes especially difficult for commercial production of isobutanol.

**Metabolite production of the designed isobutanol-producing strain.** Figure 4 shows the distribution of the fermentative products that BFA7.001(DE3) pCT03 produced during fermentation. The fraction of carbon in the products that were derived from glucose was 0.83 ± 0.02. Ethanol and isobutanol were produced as the main alcohols, with a mass ratio of (0.26 ± 0.01):1. The designed strain was able to produce isobutanol during the growth-associated (7 to 24 h) and no-growth (24 h) phases and to recycle the reducing equivalent NADH. Ethanol was produced as the major product, indicating that the isobutanol-producing pathway was limiting and the ethanol-producing pathway contributed more in recycling NADH. The low mass ratio of isobutanol to ethanol implies that pyruvate dehydrogenase may be active in converting pyruvate to acetyl-CoA, as suggested by the EM analysis.

Formate was also produced as an unavoidable fermentative product (Fig. 4). From the stoichiometry of *E. coli* fermentative metabolism, for every mole of ethanol (46 g/g-mole) produced from pyruvate via the PFL enzyme, one mole of formate (46 g/g-mole) is made, together with 1 mole of acetyl-CoA, which is a precursor for cell biomass. During the first 12 h of...
ferrmentation, formate production paralleled ethanol production, indicating the major role of the PFL enzyme. After 12 h, formate production decreased as a result of either the oxidation of formate to CO2 by formate hydrogenlyase (FHL) or the dehydrogenation of pyruvate directly to CO2 and acetyl-CoA by the PDHC enzyme.

Effect of overexpression of different portions of the isobutanol-producing pathway on isobutanol production and cell physiology. Different portions of the isobutanol-producing pathways were overexpressed and characterized in the designed host strain BFA7.001(DE3) under identical anaerobic conditions. Figure 7 compares fermentations of all strains, including BFA7.001(DE3) pCT01, BFA7.001(DE3) pCT02, and BFA7.001(DE3) pCT03. Both BFA7.001(DE) pCT01 (μ = 0.45 ± 0.02 h⁻¹) and BFA7.001(DE3) pCT02 (μ = 0.49 ± 0.01 h⁻¹) grew faster than BFA7.001(DE) pCT03 (μ = 0.37 ± 0.03 h⁻¹), which probably suffered from the significant metabolic burden of carrying a larger plasmid with the complete isobutanol-producing pathway. Similarly, both BFA7.001(DE) pCT01 (4.71 ± 0.20 g glucose/g DCW/h) and BFA7.001(DE3) pCT02 (4.43 ± 0.15 g glucose/g DCW/h) had higher specific glucose uptake rates than BFA7.001(DE3) pCT03 (4.20 ± 0.39 g glucose/g DCW/h) (Fig. 7A).

The strain BFA7.001(DE3) pCT01 overexpressed the first portion of the isobutanol-producing pathway to convert pyruvate to α-ketoisovalerate. It was not expected to produce isobutanol, because the native E. coli is not known to have an α-ketoacid decarboxylase to convert α-ketoisovalerate into isobutyraldehyde. However, the strain was unexpectedly capable of producing isobutanol under anaerobic conditions (Fig. 7C). This result implies that AlsS subtilis has the dual function of not only a known acetolactate synthase, converting pyruvate into 2-acetolactate, but also an α-ketoacid decarboxylase converting α-ketoisovalerate into isobutyraldehyde. In addition, the result strongly suggests that the endogenous alcohol dehydrogenase(s) of E. coli converted isobutyraldehyde to isobutanol. These observations are consistent with the recent reports of Atsumi et al. (4, 5).

In strain BFA7.001(DE3) pCT02 the second portion of the isobutanol-producing pathway was amplified to convert α-ketoisovalerate into isobutanol. Nevertheless, BFA7.001(DE3) pCT02 could not produce isobutanol (Fig. 7C) but did secrete pyruvate, a precursor metabolite for isobutanol production that was not detected in the fermentation of either BFA7.001(DE3) pCT01 or BFA7.001(DE3) pCT03 (data not shown). This result implies that the first section of the isobutanol-producing pathway was limiting. Because BFA7.001(DE3) pCT02 could not synthesize isobutanol, it produced ethanol at a higher rate and titer than BFA7.001(DE3) pCT01 and BFA7.001(DE3) pCT03 in order to recycle the reducing equivalent of NADH (Fig. 7B).

Strain BFA7.001(DE3) pCT03 contained not only the overexpressed α-ketoacid decarboxylase from Kivd L. lactis and AlsS subtilis but also the amplified alcohol dehydrogenase from Adh2 S. cerevisiae. However, the strain produced isobutanol at almost the same titer as BFA7.001(DE3) pCT01 (Fig. 7C). This result strongly implies that Adh2 S. cerevisiae had either low or no activity for isobutyraldehyde, at least under anaerobic conditions, in sharp contrast to the report by Atsumi et al. (3), and that alcohol/aldehyde dehydrogenase was a rate-limiting step. Recently, Atsumi et al. (5) demonstrated that the endogenous alcohol/aldehyde dehydrogenase YqhD was responsible for aerobic isobutanol production, not the exogenous enzyme Adh2 S. cerevisiae as originally reported (3). Because the enzyme YqhD is NADPH dependent and the source of NADPH produced by the glucose-6-phosphate dehydrogenase (Zwf) from the oxidative pentose phosphate pathway was knocked out in the designed isobutanol-producing strain, it is unlikely that YqhD played a role in anaerobic isobutanol production.

Role of endogenous AdhE in the isobutanol-producing pathway. BFA7.001(DE3) pCT01 produced isobutanol without the exogenous alcohol dehydrogenase Adh2 S. cerevisiae, indicating that the endogenous alcohol dehydrogenase(s) of E. coli was responsible for anaerobic isobutanol production. BFA7.001(DE3) pCT01 also produced isobutanol at the same titer as BFA7.001(DE3) pCT03, indicating that Adh2 S. cerevisiae was not important in anaerobic isobutanol production. In addition, BFA8.001(DE3), which was derived from the parent strain BFA7.001(DE3) and had the gene adhE deleted, could...
not consume glucose anaerobically when carrying the plasmids pCT01, pCT02, or pCT03 (Fig. 5). Together, these results suggest that the endogenous enzyme AdhE is involved in anaerobic isobutanol production. To confirm the role of AdhE in anaerobic isobutanol production, His-tagged AdhE was produced in BL21(DE3) pET24b adhE, purified using Ni-NTA agarose beads (see Fig. S3 in the supplemental material), and assayed for activity. The activity assay showed that AdhE was NADH dependent and had activity not only for acetaldehyde but also for isobutyraldehyde and butyraldehyde. It is interesting that AdhE had the highest enzymatic activity for butyraldehyde under the assay conditions employed (Table 4).

**Alleviating the alcohol dehydrogenase rate-limiting step.** To alleviate the alcohol dehydrogenase rate limitation, we constructed strain BFA7.001(DE3) pCT13 to overexpress the entire isobutanol-producing pathway and to amplify the endogenous alcohol dehydrogenase AdhE instead of Adh2 from *S. cerevisiae*. Even though BFA7.001(DE3) pCT13 (0.42 ± 0.1 h⁻¹) carried a larger plasmid than BFA7.001(DE3) pCT03 (see Fig. S2 in the supplemental material), it was still able to grow at a higher rate under identical anaerobic conditions (pH 7, 200 rpm, 37°C, 100 ml/min nitrogen), further supporting the unimportant role of Adh2 from *S. cerevisiae* (Fig. 8A). BFA7.001(DE3) pCT13 produced isobutanol at a higher titer than either BFA7.001(DE3) pCT01 (66% higher) or BFA7.001(DE3) pCT03 (28% higher) (Table 5).

**Effect of glycolytic flux on isobutanol production.** Our recent study of *E. coli* KO11 in both batch and continuous fermentation showed that an increase in glycolytic flux led to an increasing specific glucose uptake rate. This increased uptake rate of glucose resulted in rapid cell growth and production of lactate from pyruvate as a route to balance the redox potential (34). However, since the designed strain BFA7.001(DE3) pCT13 had the lactate dehydrogenase LdhA deleted and the endogenous alcohol/aldheyde dehydrogenase AdhE had higher specific activity toward acetaldehyde than isobutyraldehyde (Table 4), we hypothesized that the reducing equivalent NADH was more quickly and efficiently recycled for faster cell growth by using the ethanol-producing pathway. This phenotype might explain the higher production of ethanol than isobutanol, with a mass ratio of isobutanol to ethanol of (0.31 ± 0.02):1 (Table 5).

To test this hypothesis, BFA7.001(DE3) pCT13 was grown at pH 6 and 7 in order to change both the cell growth and specific glucose uptake rates that regulate the glycolytic fluxes. The results in Fig. 8A show that when the pH was reduced from 7 to 6, the specific growth rate decreased by 16%, from 4.17 ± 0.08) to 3.5 ± 0.07 (g glucose/g DCW/h). However, the isobutanol production increased by 28%, from 1.34 ± 0.05 to 1.74 ± 0.22 g/liter, and the ethanol production decreased by 12%, from 4.71 ± 0.20 to 4.17 ± 0.05 g/liter (Fig. 8B and C). The mass ratio of isobutanol to ethanol shifted from (0.31 ± 0.02):1 to (0.53 ± 0.08):1 (Table 5). It should be noted that each value represents the mean ± the standard deviation (n ≥ 6). The t test for statistical analysis in SigmaPlot gave P values of <0.05 for comparing the respective concentrations, indicating that these results are statistically significant with greater than 95% confidence.

One interesting observation during fermentation at low pH is that formate production was significantly reduced and was lower than that of ethanol (Fig. 8B). The lower production was due to the conversion of formate into CO₂ and H₂ via formate hydrogenlyase, which is known to be overexpressed in acidic environments (8, 22, 41). This phenotype is useful for the cell because the accumulation of formate is toxic to cell growth and hence benefits cell maintenance.

**Model validation.** Experimental data on the physiological states of all isobutanol-producing strains, including BFA7.001(DE3) pCT13 at pH 6 and pH 7. Kinetic profiles include glucose consumption and biomass production (A), ethanol and formate production (B), and isobutanol production (C).
EM analysis showed that deleting only fumarate reductase (frdABC) genes resulted in a redox imbalance. This was predicted by EM analysis in order to identify the optimal pathways for the strain to grow and produce isobutanol anaerobically. The designed strain was then constructed and its performance characterized under controlled anaerobic conditions.

It is noteworthy that the designed isobutanol-producing strain BFA7.001(DE3) pCT13 could operate in the predicted phenotypic space (Fig. 3). The designed strain was able to grow and produce isobutanol anaerobically. Due to the infeasibility of solely producing isobutanol, ethanol must be coproduced with isobutanol to balance the high intracellular redox state under anaerobic conditions. Thus, the designed strain BFA7.001(DE3) pCT13 produced mixed alcohols, with isobutanol and ethanol as the major fermentative products. The designed isobutanol-producing strain BFA7.001(DE3) pCT13, in which the gene adhE derived from the host BFA7.001(DE3) could not grow anaerobically unless complemented with the gene adhE.

In a previous study, JCL260 pSA55/pSA69 was reported to produce isobutanol under oxygen-limited conditions (3). The strain could not consume glucose anaerobically because the aldehyde dehydrogenase AdhE could help the cell to quickly recycle NADH and alleviate NADH inhibition of the PDHC enzyme. In addition, the lower or nonspecific activity of the isobutyraldehyde dehydrogenase Adh2S cerevisiae contributed significantly to the low isobutanol and high ethanol production. Thus, the isobutanol-producing pathway is limited at the final conversion step. The observed phenotype provides valuable insights into the metabolism of the designed isobutanol-producing strain and suggests the next step of strain optimization.

Anaerobic isobutanol production can be improved by (i) using a more specific isobutyraldehyde dehydrogenase in place of the PDHC enzyme. In addition, the low or nonspecific activity of the aldehyde dehydrogenase AdhE could help the cell to quickly recycle NADH and alleviate NADH inhibition of the PDHC enzyme. In addition, the low or nonspecific activity of the isobutyraldehyde dehydrogenase Adh2S cerevisiae contributed significantly to the low isobutanol and high ethanol production. Thus, the isobutanol-producing pathway is limited at the final conversion step. The observed phenotype provides valuable insights into the metabolism of the designed isobutanol-producing strain and suggests the next step of strain optimization.

An important finding of this study is that the endogenous NADH-dependent alcohol/aldehyde dehydrogenase AdhE plays a key role in anaerobic isobutanol production by converting isobutyraldehyde to isobutanol. Its unique role is supported by several lines of evidence. First, the designed strains BFA8.001(DE3) pCT01, BFA8.001(DE3) pCT02, and BFA8.001(DE3) pCT03, in which the gene adhE was deleted, were unable to grow. However, the complemented strain BFA8.001(DE3) pCT06 was able to grow when AdhE function was restored. In addition, overexpression of the gene adhE in the designed isobutanol-producing strain BFA7.001(DE3) pCT13 increased isobutanol production. Finally, the role of AdhE role was confirmed by the in vitro enzyme assay with the purified His-tagged AdhE. Interestingly, the enzyme AdhE had the highest activity on butyraldehyde, and its activity on acetaldehyde was about 3-fold higher than on isobutyraldehyde. This result explains why overexpression of adhE in the designed strain did not significantly improve isobutanol production.

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<th>Strain</th>
<th>pH</th>
<th>Specific growth rate (h⁻¹)</th>
<th>Isobutanol (g/liter)</th>
<th>IBUT:ETOH ratio (g/g)</th>
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<td>BFA7.001(DE3) pCT13</td>
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<td>0.35 ± 0.01</td>
<td>1.72 ± 0.22</td>
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DISCUSSION

In this proof-of-concept study, the central metabolism of E. coli was rewired to convert the heterologous isobutanol-producing pathway to an obligately anaerobic pathway. The engineered E. coli strain was rationally designed based on EM analysis in order to identify the optimal pathways for the strain to grow and produce isobutanol anaerobically. The designed strain was then constructed and its performance characterized under controlled anaerobic conditions.

It is noteworthy that the designed isobutanol-producing strain BFA7.001(DE3) pCT13 could operate in the predicted phenotypic space (Fig. 3). The designed strain was able to grow and produce isobutanol anaerobically. Due to the infeasibility of solely producing isobutanol, ethanol must be coproduced with isobutanol to balance the high intracellular redox state under anaerobic conditions. Thus, the designed strain BFA7.001(DE3) pCT13 produced mixed alcohols, with isobutanol and ethanol as the major fermentative products. The adhE-deficient strain derived from the host BFA7.001(DE3) could not grow anaerobically unless complemented with the gene adhE.

In a previous study, JCL260 pSA55/pSA69 was reported to produce isobutanol under oxygen-limited conditions (3). The strain could not consume glucose anaerobically because multiple genes were inactivated to remove competitive pathways, resulting in a redox imbalance. This was predicted by EM analysis and experimentally verified in the controlled bioreactor experiments performed in the present study. In particular, EM analysis showed that deleting only fumarate reductase (frdABC) and alcohol/aldehyde dehydrogenase (adhE) was sufficient to inactivate anaerobic growth in JCL260 pSA55/pSA69 (Table 2). Even though JCL260 pSA55/pSA69 could convert glucose to isobutanol microaerobically, the precise control of oxygen to support cell growth and enable isobutanol production is a difficult challenge.

Both the isobutanol yield (−20% of the theoretical yield) and mass ratio of isobutanol to ethanol (0.52) were lower than the predicted values derived from the 6 most efficient isobutanol-producing pathways (Fig. 2; see also Table S2 in the supplemental material), which are located on the line connecting groups G1 and G2 in Fig. 3. The key reason is that the PDHC enzyme that was assumed to be strongly inhibited under anaerobic conditions could be unexpectedly employed for anaerobic isobutanol production. The PDHC enzyme is typically present at low levels under anaerobic conditions due to the inhibition of NADH on the E2 subunit of the enzyme, reducing the production of NADH. Its anaerobic function is replaced by the PFL enzyme (13), but this does not imply that the PDHC enzyme is not expressed and produced (19). In the designed isobutanol-producing strains, the PDHC enzyme was likely activated, because overexpression of the endogenous alcohol/aldehyde dehydrogenase AdhE could help the cell to quickly recycle NADH and alleviate NADH inhibition of the PDHC enzyme. In addition, the low or nonspecific activity of the isobutyraldehyde dehydrogenase Adh2S cerevisiae contributed significantly to the low isobutanol and high ethanol production. Thus, the isobutanol-producing pathway is limited at the final conversion step. The observed phenotype provides valuable insights into the metabolism of the designed isobutanol-producing strain and suggests the next step of strain optimization.

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Control of glycolytic flux plays a significant role in improving
isobutanol production and changing the isobutanol-to-ethanol mass ratio in the designed isobutanol-producing strain. The high glycolytic flux causes a high intracellular redox state (ratio of NADH to NAD\(^+\)) that requires the strain to recycle NADH as quickly as possible from the ethanol-producing pathway. Thus, the strain will make more ethanol and less isobutanol. However, if the glycolytic flux is adjusted, the isobutanol-to-ethanol mass ratio can be controlled. We demonstrated this by carrying out controlled batch fermentations at pH 6 and 7. Lowering the pH decreased the glycolytic flux, reduced the high intracellular redox state, and hence increased the isobutanol-to-ethanol mass ratio. Other cultivation techniques, such as chemostat operation, can also be applied to demonstrate the observed phenotype.

In summary, we have demonstrated a powerful approach to rationally design an \textit{E. coli} strain that employs the heterologous isobutanol-producing pathway as an obligately anaerobic pathway. Metabolic pathway analysis was used to dissect \textit{E. coli} cellular metabolism and identify the most efficient pathways for anaerobic isobutanol production. The designed strain functioned in accordance with these selected optimal pathways, and its metabolism closely matched the model predictions. With the anaerobic isobutanol metabolism of the designed strain characterized, strain optimization to improve the isobutanol production and control the isobutanol-to-ethanol mass ratio can be addressed. The present approach has thus proven useful in rational strain development for conversion of renewable and sustainable biomass feedstocks to biofuels and biochemicals.

\textbf{ACKNOWLEDGMENT}

We thank James C. Liao at UCLA for kindly providing the strain JCL260 and the plasmids pSA55 and pSA69.

\textbf{REFERENCES}