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Metabolic Engineering of *Clostridium cellulolyticum* for Production of Isobutanol from Cellulose[▽]

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Producing biofuels directly from cellulose, known as consolidated bioprocessing, is believed to reduce costs substantially compared to a process in which cellulose degradation and fermentation to fuel are accomplished in separate steps. Here we present a metabolic engineering example for the development of a *Clostridium cellulolyticum* strain for isobutanol synthesis directly from cellulose. This strategy exploits the host's natural cellulolytic activity and the amino acid biosynthesis pathway and diverts its 2-keto acid intermediates toward alcohol synthesis. Specifically, we have demonstrated the first production of isobutanol to approximately 660 mg/liter from crystalline cellulose by using this microorganism.

Relative to biofuels produced from corn (starch rich) and sugar cane (sucrose rich), biofuels obtained from cellulosic materials could result in lower fuel costs (16), greater petroleum displacement (13), and lower greenhouse gas emissions (16). To meet its potential, technological advances to improve the conversion efficiency of the recalcitrant lignocellulose to fermentable sugars are needed (29). So far, improvements in cellulase production (17, 20) and pretreatment techniques (1) have aided in increasing cellulose degradation efficiency in a cost-effective manner.

Another approach which has generated much interest is consolidated bioprocessing (CBP). This process utilizes microorganisms to perform biomass hydrolysis and the fermentation of the sugars into biofuel within a single process (16). Research in this area has taken one of two approaches. In one approach, referred to as the "recombinant cellulolytic strategy" (14), microorganisms that have previously demonstrated high biofuel yields are engineered to utilize cellulose and/or the sugars resulting from cellulose degradation. These organisms have been genetically engineered to expand their substrate range to include cellulose or the sugars freed from cellulose or hemicellulose degradation, as in the case of ethanologenic organisms such as *Escherichia coli* (23, 33), *Zymomonas mobilis* (4, 18), and *Saccharomyces cerevisiae* (14, 27). Research efforts continue to improve the strains' cellulolytic abilities to industrially relevant levels. For the "native cellulolytic strategy" (14), research has focused mainly on microorganisms that possess cellulosomes, which are extracellular multienzyme complexes that aid in the digestion of cellulose. While these microorganisms are capable of efficiently hydrolyzing cellulose, their biofuel productivities are significantly lower than those of existing industrial strains. In addition to improving biofuel productivity (22), research efforts are also focused on increasing ethanol yields (31), eliminating competing pathways (26), and improving ethanol tolerance (30).

Most studies employing the native cellulolytic strategy have been conducted with the thermophilic, cellulolytic *Clostridium thermocellum*. This strain is particularly attractive because it is able to thrive in high-temperature fermentations, which are conducive to high-level substrate conversion, low contamination risk, and high-level product recovery (15). Although *C. thermocellum* has potential to be a CBP organism, issues such as low transformation efficiency (28) and the lack of publications demonstrating successful overexpression of foreign proteins in *C. thermocellum* significantly impede the engineering progress of this organism to produce synthetic biofuels, such as isobutanol. One way to hasten this progress is to first establish and optimize the desired metabolic pathways in a closely related, more amenable organism. Once the specifics, such as identifying which genes to overexpress, mutate, and/or delete, have been determined, the same strategy can then be adapted to *C. thermocellum*. *Clostridium cellulolyticum*, which was originally isolated from decayed grass (21), is a useful candidate for this initial metabolic engineering work because, like *C. thermocellum*, it belongs to *Clostridium* group III, based on 16S rRNA phylogenetic analysis (7), and because it is a mesophile, many problems that are associated with the heterologous expression of proteins in thermophiles are circumvented. In addition, *C. cellulolyticum* has a sequenced genome (GenBank accession NC_011898.1) and there exist well-established DNA transfer techniques (24) and gene overexpression methods (10) for it. As a potential CBP organism in its own right, *C. cellulolyticum* can not only utilize cellulose similar to *C. thermocellum* but also utilize additional sugars freed from hemicellulose degradation, including xylose, arabinose, fructose, galactose, mannose, and ribose (9).

Previously, *C. cellulolyticum* has been genetically engineered for improved ethanol production (10). Similarly, most of the

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TABLE 1. List of plasmids and strains used in this study

Strain or plasmid	Phenotype, genotype, or construct description[a]	Source or reference
Strains		
E. coli XL10-Gold	Tet^r Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r) Amy Cam^r]	Stratagene
C. cellulolyticum H10	ATCC 35319	ATCC
Plasmids		
pAT187	Km^r; broad-host-range plasmid	24
pWH159	Km^r; 5.5-kb EcoRI fragment of pAT187 was ligated with the EcoRI fragment of the PCR product of pAT187; For oligo WH177, Rev oligo WH178	This study
pWH168	Em^r Km^r; *ermC* was cloned into pWH159 by ligating the AatII-PstI fragment of PCR product; For oligo WH248, Rev oligo WH249, with pECN2 (11) as the template	This study
pWH199	Em^r Km^r; the ferredoxin promoter and multiple-cloning site (see Fig. S1 in the supplemental material) were cloned into pWH168 with the BssHII-AgeI fragment of PCR product; For oligo WH194, Rev oligo WH195; the template was synthesized by PCR assembly, using 18 primers (FD1 to FD18)	This study
pWH203 (**alsS-ilvC-ilvD*)	Em^r Km^r; 0.8-kb BamHI-XbaI fragment of pWH315 was cloned into the same sites of pWH277	This study
pWH277 (*alsS-ilvC-ilvD*)	Em^r Km^r; *alsS* from *B. subtilis* and *ilvCD* from *E. coli* were amplified from pSA69 (2); For oligo WH301, Rev oligo WH302; BamHI-NotI fragment of PCR product ligated into the BamHI and NotI sites of pWH199	This study
pWH278 (*alsS*)	Em^r Km^r; *alsS* from *B. subtilis* was amplified from pSA69 (2); For oligo WH261, Rev oligo WH262; BamHI-NotI fragment of PCR product ligated into the same restriction sites as pWH199	This study
pWH314 (*alsS-ilvCD-kivd-adhA*)	Em^r Km^r; *kivd* from *L. lactis* and *adhA* from *E. coli* were amplified by PCR amplification using pSA65 (2) as a template; For oligo WH886, Rev oligo WH885; NotI-BamHI fragment of PCR product ligated into NotI and BglII sites of pWH203	This study
pWH315 (**alsS-ilvC-ilvD-klvD-adhA*)	Em^r Km^r; spontaneous mutation in *alsS*	This study
pWH318 (*kivd-yqhD*)	Em^r Km^r; *kivd* from *L. lactis* and *yqhD* from *E. coli* were amplified by PCR from pCS97 (C. Shen, unpublished); For oligo WH888, Rev oligo WH887; Acc65I-BamHI fragment of PCR product ligated into the Acc65I and BamHI sites of pWH199	This study
pWH320 (*kivd-yqhD-alsS-ilvC-ilvD*)	Em^r Km^r; *alsS* from *B. subtilis* and *ilvCD* from *E. coli* were amplified from pSA69 (2); For oligo WH900, Rev oligo WH901; SpeI-NotI fragment of PCR product ligated into the XbaI and NotI sites of pWH318	This study

[a] Abbreviations: Amy, amylase; Cam^r, chloramphenicol resistance; For, forward primer; Rev, reverse primer; oligo, oligonucleotide.

research concerning the construction of a CBP organism has focused on ethanol production. Despite this, it has been asserted that higher alcohols (i.e., alcohols with more than two carbons), such as isobutanol, are better candidates for gasoline replacement because they have energy density, octane value, and Reid vapor pressure that are more similar to those of gasoline (5). Unlike ethanol, isobutanol can also be blended at any ratio with gasoline or used directly in current engines without modification (8). In this study, we have metabolically engineered *C. cellulolyticum* to produce isobutanol. By expressing enzymes that direct the conversion of pyruvate to isobutanol by using an engineered valine biosynthesis pathway, we were able to produce up to 660 mg/liter of isobutanol by using *C. cellulolyticum* growing on crystalline cellulose.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. Restriction enzymes, phosphatase (New England BioLabs, Ipswich, MA), ligase (rapid DNA ligation kit; Roche, Mannheim, Germany), and KOD DNA polymerase (EMD Chemicals, San Diego, CA) were used for cloning. Oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL).

Chemicals. Unless indicated otherwise, commercial reagents, enzymes, and coenzymes were supplied by Sigma Chemical Company (St. Louis, MO).

Media and cultivation. *C. cellulolyticum* was grown at 34°C in VM medium (11) that had been modified to reduce precipitation. The medium contained the following components: KH_2PO_4 (1.0 g/liter), K_2HPO_4 (3.4 g/liter), urea (2.14 g/liter), $MgCl_2 \cdot 6H_2O$ (1.0 g/liter), $CaCl_2 \cdot 2H_2O$ (0.15 g/liter), $FeSO_4 \cdot 6H_2O$ (1.25 mg/liter), 3-(*N*-morpholino)propanesulfonic acid (MOPS; 10.0 g/liter), resazurin (2.0 mg/liter), vitamin solution (10 ml/liter), yeast extract (2.0 g/liter), oligoelement solution (1 ml/liter), cysteine-HCl (1.0 g/liter), and cellobiose (5.1345 g/liter). The vitamin solution (100×) contained biotin (0.08 μM), pyridoxamine (0.02 μM), cyanocobalamin (0.001 μM), ρ-aminobenzoic acid (0.15 μM), thiamine (0.9 μM), and L-alanine (0.22 μM). The 1,000× oligoelement solution contained $FeSO_4 \cdot 7H_2O$ (5.0 g liter^{-1}), $ZnSO_4 \cdot 7H_2O$ (1.44 g liter^{-1}), $MnSO_4 \cdot 7H_2O$ (1.12 g liter^{-1}), $CuSO_4 \cdot 5H_2O$ (0.25 g liter^{-1}), $Na_2B_4O_7$ (0.20 g liter^{-1}), $(Mo)_7(NH_4)_6O_{24} \cdot 4H_2O$ (1.00 g liter^{-1}), $NiCl_2$ (0.04 g liter^{-1}), $CoCl_2$ (0.02 g liter^{-1}), HBO_3 (0.03 g liter^{-1}), Na_2SeO_3 (0.02 g liter^{-1}), and HCl (50 ml of a 10 M concentration).

For agar plates, 0.8% (wt/vol) agar (Difco Laboratories, Detroit, MI) was added to the media. To make competent cells, to prepare cell lysates for enzyme assays and daily maintenance, and to determine isobutanol production on cel-

lobiose, the strains were grown in modified VM medium. To examine isobutanol, lactate, acetate, and ethanol production on cellulose, the strains were grown in modified VM medium, in which cellobiose and yeast extract were replaced with 10 g/liter of crystalline cellulose (50 μm; Sigma type 50).

Stock cultures of *C. cellulolyticum* were maintained at −80°C in 15% (vol/vol) glycerol and were grown for one transfer in cellobiose medium before the initiation of growth experiments.

Transformation. Cell transformation was conducted as described previously (11) with some modifications. Cells were grown for 17 to 24 h in 10-ml cultures of modified VM medium to late exponential phase (optical density at 600 nm [OD_{600}], 0.5 to 1.0; 5×10^6 CFU/ml). The following steps were all performed with anoxic solutions under anaerobic conditions at 4°C. The cells were washed twice with cold electroporation buffer (270 mM sucrose, 1 mM $MgCl_2$, 5 mM sodium phosphate buffer, pH 7.4). The cells were resuspended in 600 μl of electroporation buffer. For each transformation, 200 μl of the cells was mixed with 2 μg of MspI-methylated plasmid DNA. The DNA was methylated overnight with 5 units of MspI methyltransferase (New England BioLabs, Ipswich, MA) and then purified with the DNA Clean and Concentrator kit (Zymo Research Inc., Orange, CA). In 2-mm-gap electroporation cuvettes (Molecular BioProducts, San Diego, CA), the cells and plasmid DNA were electroporated (1.5 kV, 25 μF, and 48 Ω) with a Bio-Rad gene pulser apparatus (Bio-Rad Laboratories, Richmond, CA). The electroporated cells were transferred to 10 ml of fresh modified VM medium. The cells were recovered for 24 h at 34°C, then the cells were collected by centrifugation, and cell pellet was spread on modified VM cellobiose agar plates supplemented with 10 μg/ml erythromycin. The plates were incubated at 34°C anaerobically for 5 to 7 days. Single colonies were transferred to 10 ml VM cellobiose medium supplemented with 10 μg/ml erythromycin.

Analytical procedures. Bacterial growth was measured spectrophotometrically at 600 nm. For cultures containing cellulose, the cellulose was allowed to settle for at least 2 h before samples were taken for measurement.

The produced alcohol compounds were quantified by a gas chromatograph (GC) with a flame ionization detector. The system consisted of a 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 it reached 120°C. It was then raised with a gradient of 50°C/min until it reached 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. Pentanol was used as the internal standard.

Enzyme assays. The cells were grown for 17 to 24 h in 50-ml cultures of modified VM medium to late exponential phase (OD_{600}, 0.5 to 1.0; 5×10^6 CFU/ml). The cells were harvested, washed in 50 mM potassium phosphate buffer, pH 7.5, and resuspended in 0.5 ml of the same buffer. Crude extract was prepared under aerobic conditions with 0.1-mm glass beads and a Mini Bead Beater 8 (BioSpec Products, Inc., Bartlesville, OK). Total protein measurements were made with the Bradford protein assay kit from Bio-Rad (Hercules, CA).

The AlsS assay was performed as described previously (32), with the exception that the reaction mixture contained 20 mM sodium pyruvate, 100 mM MOPS buffer, pH 7.0, 1 mM $MgCl_2$, and 100 μM cocarboxylase. The concentration of acetoin produced was determined by a standard curve created using pure acetoin. One specific unit of AlsS activity corresponds to the formation of 1 nmol of acetoin per min per mg of soluble protein at 34°C.

To measure the reduction of 2-acetolactate to 2,3-dihydroxy-isovalerate, the oxidation of NADPH was monitored by a decrease in absorbance at 340 nm. The substrate, 2-acetolactate, was first produced in a separate reaction as described for the Als assay using purified, heterogeneously expressed *Bacillus subtilis* AlsS in *E. coli* strain BL21. From this reaction, 180 μl was added to 200 mM potassium phosphate buffer, pH 7.5, 4 mM $MgCl_2$, and 0.1 mM NADPH. The samples were incubated at 34°C for 5 min, and then the reaction was initiated with the addition of cell extracts. The consumption of NADPH was monitored at 340 nm (extinction coefficient, 6.22 mM^{-1} cm^{-1}). IlvC activity is expressed as nmol of NADPH oxidized per min per mg of soluble protein at 34°C.

The IlvD assay was performed as described previously (12). The 500-μl reaction mixture contained 5 mM $MgSO_4$, 50 mM Tris-Cl, pH 8.0, cell extract, and 10 mM 2,3-dihydroxy-isovalerate. The substrate, 2,3-dihydroxy-isovalerate, was synthesized as described previously (6). After the reaction mixture was preincubated for 5 min at 34°C, the substrate was added to initiate the reaction. The samples were incubated for 15 min at 34°C. The reaction was terminated by the addition of 125 μl of 10% (wt/vol) trichloroacetic acid, and then 250 μl of saturated 2,4-dinitrophenylhydrazine in 2 N HCl was added to the samples. After 20 min at room temperature, 875 μl of 2.5 N NaOH was added and then the samples were incubated for another 30 min at room temperature. The samples were then spun down for 1 min to remove coagulated protein. Sample absorbances were measured at 550 nm. Standard curves based on known amounts of 2-ketoisovalerate were generated. The specific activities were calculated as 1 nmol of 2-ketoisovalerate synthesized per min per mg of soluble protein at 34°C.

The decarboxylation activity of Kivd was assayed as described previously (34), with some modifications. Kivd activity was measured at 34°C by using a coupled enzymatic assay method. ADH6 was isolated as previously described (34). Excess ADH6 was used to reduce aldehyde into alcohol, and concomitantly, cofactor NADPH was oxidized to $NADP^+$. The assay mixture contained 0.2 mM NADPH, 0.1 μM ADH6, and 20 mM 2-ketoisovalerate in assay buffer (50 mM potassium phosphate buffer, pH 7.0, 1 mM $MgSO_4$, 0.5 mM thiamine diphosphate [ThDP]) with a total volume of 0.2 ml. The reactions were started by the addition of the 2-ketoisovalerate. The consumption of NADPH was monitored at 340 nm (extinction coefficient, 6.22 mM^{-1} cm^{-1}). One specific unit of Kivd activity corresponds to the oxidation of 1 nmol of NADPH per min per mg of soluble protein at 34°C.

To measure the alcohol dehydrogenase (ADH) activities of YqhD and AdhA, the oxidation of NADPH was monitored by a decrease in absorbance at 340 nm. The assay mixture contained 50 mM MOPS, pH 7.0, 25 mM isobutyraldehyde, and 0.2 mM NAD(P)H. The samples were incubated at 34°C for 5 min, and then the reaction was initiated with the addition of cell extracts. The consumption of NAD(P)H was monitored at 340 nm (extinction coefficient, 6.22 mM^{-1} cm^{-1}). One specific unit of ADH activity corresponds to the oxidation of 1 nmol of NAD(P)H per min per mg of soluble protein at 34°C.

RESULTS

A single base pair insertion in *alsS* enables expression in *C. cellulolyticum*. In order to achieve direct isobutanol production from pyruvate, the genes encoding *B. subtilis* α-acetolactate synthase, *E. coli* acetohydroxyacid isomeroreductase, *E. coli* dihydroxy acid dehydratase, *Lactococcus lactis* ketoacid decarboxylase, and *E. coli* and *L. lactis* alcohol dehydrogenases (Fig. 1A) were cloned into a pAT187 derivative plasmid (25). These specific genes were chosen because they were the same genes utilized for isobutanol production in *E. coli* (2) and *Synechococcus elongatus* (3). The different combinations of the genes (Fig. 1B) were cloned as single synthetic operons driven by the constitutive ferredoxin (Fd) promoter from *Clostridium pasteurianum*.

The activities of the first three enzymes in the isobutanol pathway were examined by transforming plasmids expressing *alsS* or *alsS ilvCD* into *C. cellulolyticum*. While *C. cellulolyticum* was successfully transformed with the empty vector, no *C. cellulolyticum alsS* or *alsS ilvCD* transformants were obtained. The same results were observed after repeated transformation efforts. Due to the fact that *alsS* and *alsS ilvCD* transformants could not be obtained, the complete isobutanol pathway was then examined. *C. cellulolyticum* was transformed with a plasmid expressing *alsS ilvCD kivd adhA*. While transformants were obtained, sequence confirmation of the plasmid revealed that a single adenine insertion, which is not found in the wild-type *alsS* sequence, was present 54 bp downstream of the start ATG. This single insertion, by shifting the reading frame, results in a downstream premature stop codon (TGA) and, subsequently, a truncated 37-amino-acid protein (Fig. 2). This spontaneous mutation in *alsS* (**alsS*) was found to have originated in the *E. coli* strain used for cloning.

The frameshift mutation in the *alsS* sequence was a cause for great concern because of the effect it could have on AlsS

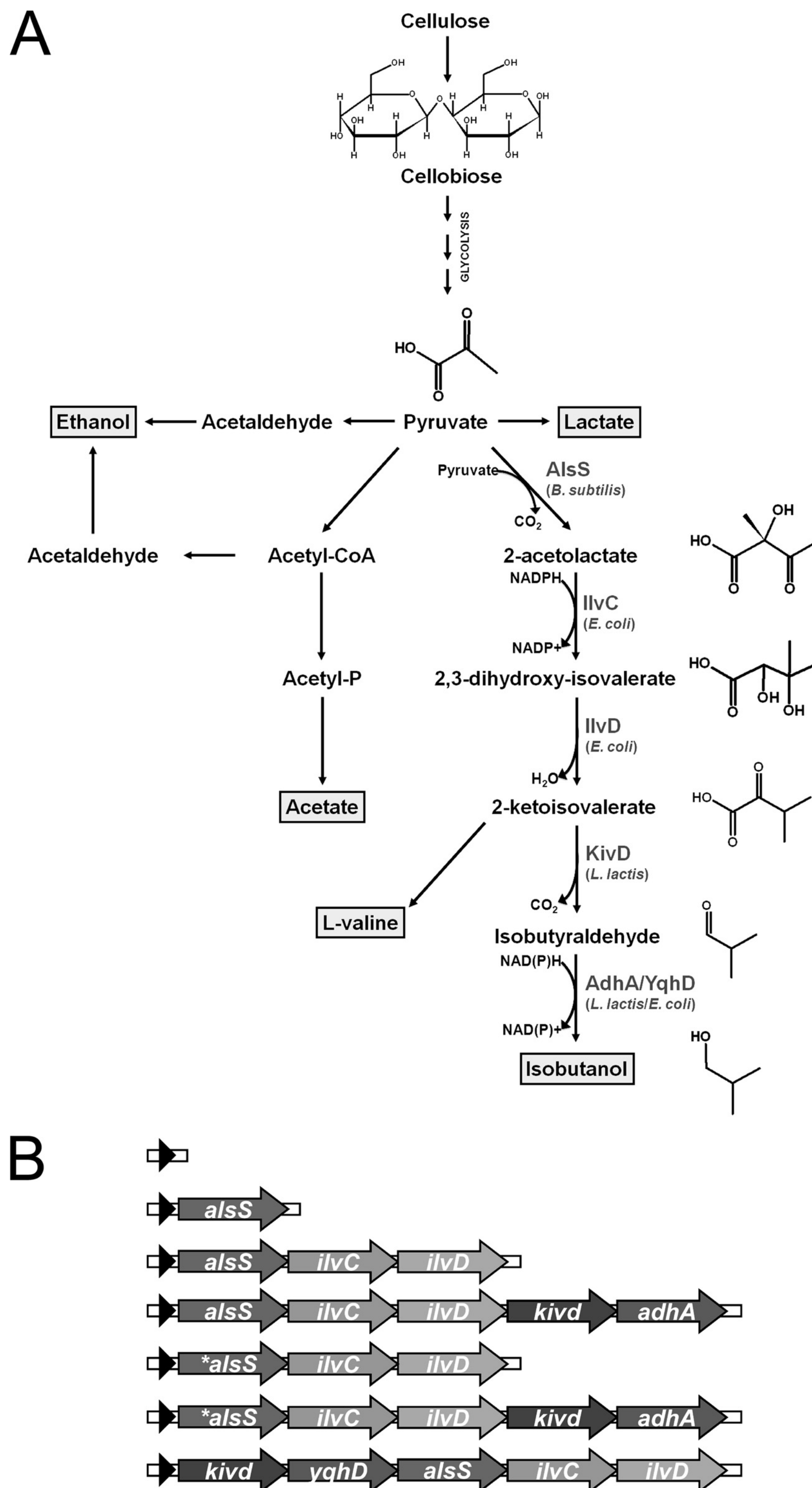

FIG. 1. The pathway for isobutanol production in *C. cellulolyticum* (A) and the ferredoxin promoter (black arrow)-driven operons used in this study (B). The asterisk indicates the adenine insertion in the *alsS* gene sequence.

activity. Thus, to determine the activities of AlsS and the other enzymes expressed from the synthetic operon, enzymatic assays were performed on lysates of the *C. cellulolyticum* strain expressing **alsS ilvCD kivd adhA* (Fig. 3A). Surprisingly, for the AlsS assay, the **alsS ilvCD kivd adhA* lysates were found to demonstrate an activity of 282 nmol min^{-1} mg^{-1}, which was significantly higher than the 11 nmol min^{-1} mg^{-1} demonstrated by the strain transformed with the vector (Fig. 3A).

```
 1 ATG ACC ATG ATT ACG AAT TCG AGC TCG GTA
    M   T   M   I   T   N   S   S   S   V

31 CCC GGG GAT CCA TGG TTG ACA AAA GCA AAC
    P   G   D   P   W   L   T   K   A   N

61 AAA AGA ACA AAA ATC CCT TGT GAA AAA CAG
    K   R   T   K   I   P   C   E   K   Q

91 AGG GGC GGA GCT TGT TGT TGA TTG CTT AGT
    R   G   G   A   C   C   *   L   L   S
```

FIG. 2. The first 120 bp of the *alsS* sequence with the adenine insertion mutation. The adenine insertion (solid box), the putative start GTG, which restores the *alsS* reading frame (underline), the premature stop codon (*), and the putative Shine-Dalgarno sequence (dashed box) are indicated.

Thus, despite the insertion mutation, the mutant retained a significant level of activity. However, unlike the case for AlsS, we were not able to detect enzymatic activity for IlvC (Fig. 3B), IlvD (Fig. 3C), Kivd (Fig. 3D), or AdhA (Fig. 3E). There were no statistically significant differences between the activities of these enzymes in the lysates of the **alsS ilvCD kivd adhA* strain and the vector control strain.

The presence of AlsS activity, despite the stop codon introduced by the frameshift mutation, suggests that the 37-amino-acid truncated protein is not the only translation product. It is likely that an alternate Shine-Dalgarno (SD) sequence and start site are present downstream of the insertion. After examining the sequence, we identified likely candidates for the alternative SD sequence and start site (Fig. 2), which are approximately 8 and 23 bp, respectively, downstream from the adenine insertion. This would result in an AlsS that is 25 amino acids shorter than the wild-type AlsS and explain the activity in the transformants.

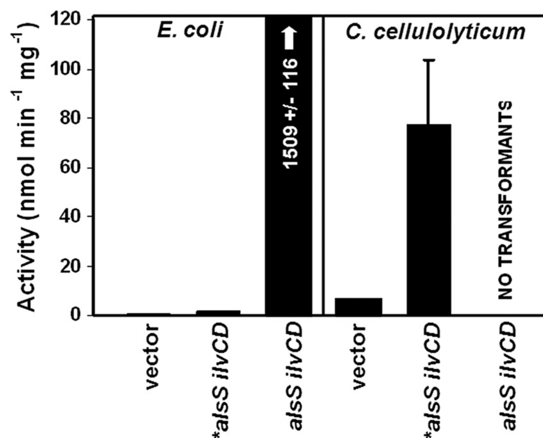

FIG. 4. AlsS activity of *E. coli* and *C. cellulolyticum* expressing the vector, the **alsS ilvCD* construct, or the *alsS ilvCD* construct. One specific unit of AlsS activity corresponds to the formation of 1 nmol of acetoin per min per mg of soluble protein at 34°C.

To further analyze the effect of the *alsS* mutation, we compared the AlsS and *AlsS activities in *E. coli* because we were unable to obtain a *C. cellulolyticum* transformant expressing the wild-type *alsS*. Figure 4 compares the AlsS activities of *E. coli* strains expressing the **alsS ilvCD* and *alsS ilvCD* constructs. While the **alsS* mutant presented no significant activity in *E. coli*, the wild-type *alsS* variant demonstrated activity that was approximately 1,000-fold greater than that of the empty vector. This result strongly suggests that the mutation significantly reduces the activity of AlsS. This difference in activity may explain why *C. cellulolyticum* cannot be transformed with constructs that contain *alsS* as the first gene in the operon, which was the case for the *alsS*, *alsS ilvCD*, and *alsS ilvC ilvD kivd adhA* strains (Fig. 1B).

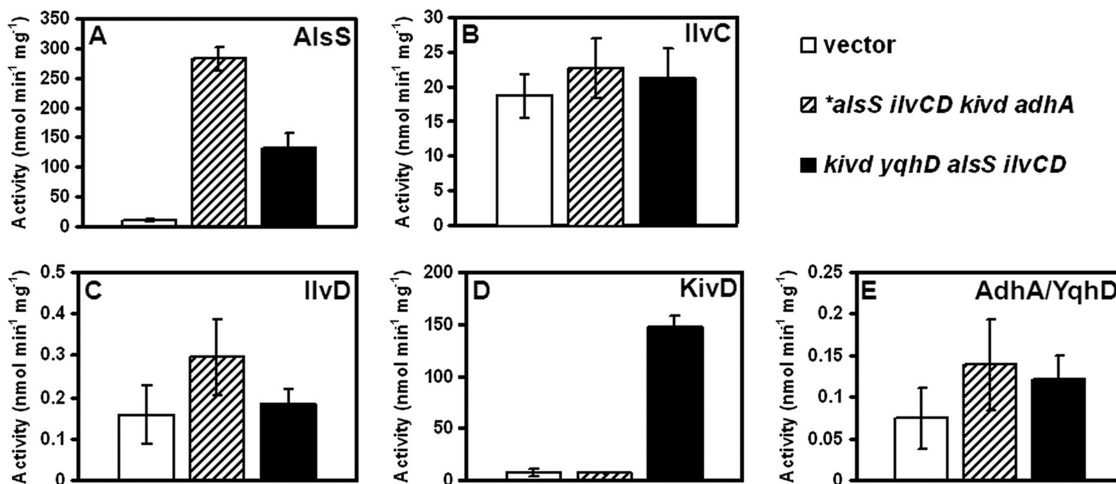

FIG. 3. Activity assays of isobutanol pathway enzymes for *C. cellulolyticum* strains expressing the empty vector, **alsS ilvCD kivd adhA*, and *kivd yqhD alsS ilvCD*, determining the activity for AlsS (one specific unit of Als activity corresponds to the formation of 1 nmol of acetoin per min per mg of soluble protein at 34°C) (A), IlvC (one specific unit of IlvC activity corresponds to the oxidation of 1 nmol of NADPH per min per mg of soluble protein at 34°C) (B), IlvD (one specific unit of IlvD activity corresponds to the formation of 1 nmol of 2-ketoisovalerate per min per mg of soluble protein at 34°C) (C), Kivd (one specific unit of Kivd activity corresponds to the oxidation of 1 nmol of NADPH per min per mg of soluble protein at 34°C) (D), and AdhA and YqhD activity [one specific unit of ADH activity corresponds to the oxidation of 1 nmol of NAD(P)H per min per mg of soluble protein at 34°C] (E).

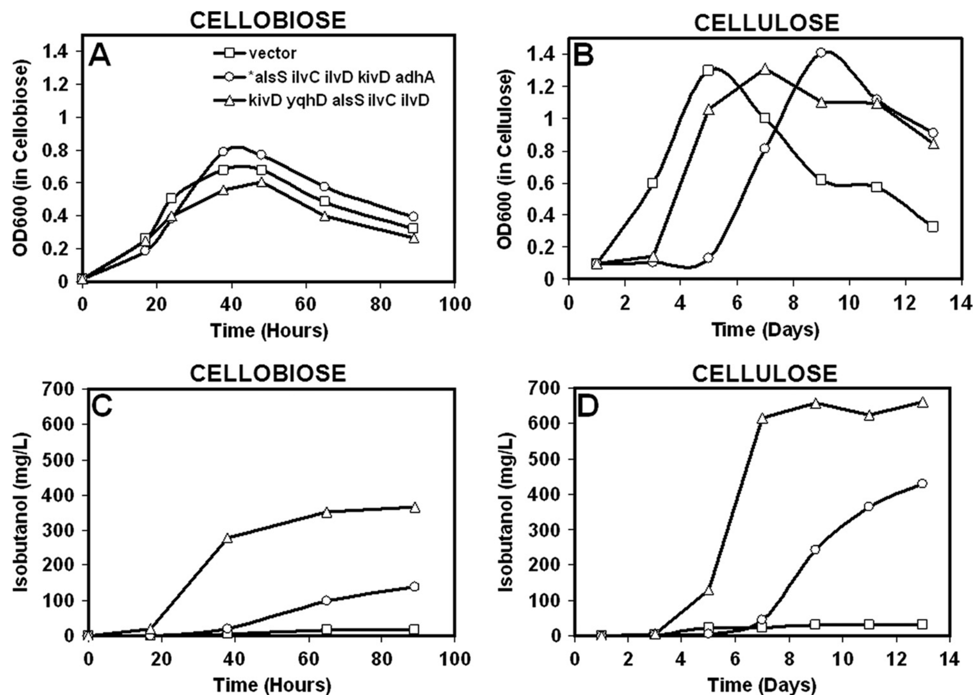

FIG. 5. Growth of *C. cellulolyticum* strains on cellobiose (A) and cellulose (B) and the isobutanol production (mg/liter) on cellobiose (C) and cellulose (D). The figure shows one data set representative of three independent experiments, with all three showing comparable results.

Production of isobutanol from cellobiose and cellulose. Despite the mutation in *alsS*, the **alsS ilvCD kivd adhA* strain was found to produce isobutanol titers of 140 mg/liter from cellobiose over a period of 90 h (Fig. 5C) and 420 mg/liter (Fig. 5D) on cellulose over a period of 13 days. These titers are significantly higher than the 17 mg/liter and 30 mg/liter of isobutanol that are produced by the strains transformed with the empty vector on cellobiose and cellulose, respectively (Fig. 5C and D).

In order to test our hypothesis of the wild-type AlsS's toxic effect on *C. cellulolyticum* growth during transformation and to obtain transformants with the wild-type AlsS, it was necessary to decrease the activity of the wild-type AlsS. To achieve this, a *kivd yqhD alsS ilvCD* strain in which *alsS* was the third gene in the operon was constructed. Previously, it has been shown that mRNA abundance decreases with increasing distance of the gene from the promoter, irrespective of gene content (19). Specifically, for the operons that were studied, the researchers found that mRNA abundance decreased by approximately 50% from one gene to the next (19). Thus, as *alsS* was the third gene in the operon, it would be expected that the *alsS* mRNA abundance would be less than that if *alsS* was the first gene in the operon. After successful transformation of the *kivd yqhD alsS ilvCD* strain, the resulting transformants were found to produce up to 364 mg/liter of isobutanol on cellobiose over a period of 90 h (Fig. 5C) and 660 mg/liter of isobutanol on cellulose within 7 to 9 days (Fig. 5D).

Although successful transformation suggested that AlsS activity had been successfully attenuated, enzyme assays were performed to quantify the activity of AlsS and the products of the other genes in the operon. As seen in Fig. 3A, AlsS activity for the strain expressing *kivd yqhD alsS ilvCD* resulted in a level of AlsS activity approximately 10-fold higher than that of the vector control, with activities of 133 and 11 nmol min^{-1} mg^{-1}, respectively (Fig. 3A). For Kivd activity, the *kivd yqhD alsS ilvCD* strain had 19-fold higher Kivd activity than the strain expressing the vector alone, with activities of 147.1 and 7.9 nmol min^{-1} mg^{-1}, respectively (Fig. 3D). Unlike the cases for AlsS and Kivd, no activity could be detected for IlvC (Fig. 3B), IlvD (Fig. 3C), and YqhD (Fig. 3E) when *kivd yqhD alsS ilvCD* was expressed. There were no statistically significant differences between the activities of these enzymes when comparing the *kivd yqhD alsS ilvCD* strain and the vector control strain.

DISCUSSION

Previously, we successfully showed that *E. coli* can be metabolically engineered to produce isobutanol by manipulating *E. coli*'s amino acid biosynthesis pathway by diverting the 2-keto acid intermediates toward biofuel production (2). Using the same metabolic engineering strategy, we were able to achieve an isobutanol titer of 660 mg/liter by the cellulolytic mesophile *C. cellulolyticum* by expressing *kivd yqhD alsS ilvCD*. To our knowledge, this is the first demonstration of isobutanol production directly from cellulose.

We encountered several difficulties with *C. cellulolyticum* that we did not meet with *E. coli* regarding the expression of the isobutanol pathway. One of these difficulties arose from the lack of an inducible expression system in *C. cellulolyticum*. As we were without the ability to control gene expression, the toxicity of some of the genes had a greater effect on the microorganism's growth than they would have otherwise. Specifically, the expression of the gene that encodes acetolactate synthase, *alsS*, appears to have a toxic effect in *C. cellulolyti-*

cum, an effect which is evidenced by the lack of *alsS*, *alsS ilvCD*, and *alsS ilvCD kivd adhA* transformants. Moreover, this problem with transformation is alleviated when the amount of *alsS* mRNA is decreased, as in the case for **alsS*, **alsS ilvCD*, **alsS ilvCD kivd adhA*, and *kivd yqhD alsS ilvCD* constructs. It is likely that the control conferred by an inducible system would aid in tempering the expression level of AlsS and, subsequently, its inhibitory growth effects.

Another difficulty we encountered was the lack of detectable activity for IlvC, IlvD, and the alcohol dehydrogenases (ADHs) AdhA and YqhD. From the enzyme activity assays (Fig. 3), we were unable to detect activities that were significantly greater than that found for the vector control. However, despite the results of the enzyme assays, it appears that some activity is present. For example, although no Kivd and AdhA activity in *C. cellulolyticum* transformed with **alsS ilvCD kivd adhA* was detected, it appears that there is *in vivo* activity, as the **alsS ilvCD kivd adhA* transformants were found to produce an isobutanol titer of 428 mg/liter, while **alsS ilvCD* transformants had a titer of 278 mg/liter. It is not surprising that the lack of these enzyme activities did not preclude isobutanol production, because *C. cellulolyticum* possesses native enzymes that can perform the same functions. Homologues of *ilvC* and *ilvD* are part of *C. cellulolyticum*'s valine biosynthesis pathway, and *C. cellulolyticum* possesses ADHs for ethanol fermentation. Still, additional IlvC, IlvD, and ADH activity would most likely lead to higher isobutanol titers. Differences between GC contents and codon usage frequencies of *C. cellulolyticum* and *E. coli* may explain the lack of expression of the *E. coli* genes in the host *C. cellulolyticum*. The utilization of *C. cellulolyticum ilvC*, *ilvD*, and ADH genes or the codon optimization of the *E. coli* genes may resolve this problem.

A significant amount of research has been dedicated to engineering organisms that are capable of consolidated bioprocessing. These CBP organisms are anticipated to have the ability to efficiently degrade cellulose and to convert the resulting sugars to biofuels at high productivities. Toward this goal, the production of isobutanol from cellulose has been shown to be feasible in the mesophilic *C. cellulolyticum*. Both the successes and problems encountered in establishing this pathway in *C. cellulolyticum* will aid in the adaptation of this strategy in related cellulolytic thermophiles, such as *C. thermocellum* and *Caldicellulosiruptor bescii*.

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