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Metabolic engineering of *Escherichia coli* for 1-butanol production

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Abstract

Compared to ethanol, butanol offers many advantages as a substitute for gasoline because of higher energy content and higher hydrophobicity. Typically, 1-butanol is produced by *Clostridium* in a mixed-product fermentation. To facilitate strain improvement for specificity and productivity, we engineered a synthetic pathway in *Escherichia coli* and demonstrated the production of 1-butanol from this non-native user-friendly host. Alternative genes and competing pathway deletions were evaluated for 1-butanol production. Results show promise for using *E. coli* for 1-butanol production.

Keywords: Biofuel; Butanol; *E. coli*

1. Introduction

Biomass as a renewable energy source has gained increased attention because of energy and environmental concerns. Typically, biomass is converted to ethanol as a transportation fuel. According to the US International Trade Commission (USITC, 2007), 4 billion gallons of bioethanol was produced in the United States in 2005, and both the production capacity and the demand for bioethanol are increasing rapidly. However, ethanol is not an ideal replacement for gasoline because of its high water content and low energy density relative to gasoline. On the other hand, 1-butanol is hydrophobic and its energy content (27 MJ/L) is similar to that of gasoline (32 MJ/L). It can completely replace gasoline or mix with gasoline at any ratio. It can also be stored and transported using existing infrastructure. Furthermore, the vapor pressure of 1-butanol (4 mmHg at 20 °C) is approximately 11 times less than that of ethanol (45 mmHg at 20 °C). As such, 1-butanol has been proposed as a substitute and supplement of gasoline as a transportation fuel.

The microbial production of 1-butanol utilizes various species of *Clostridium*, particularly *acetobutylicum* (Lin and Blaschek, 1983). *Clostridium acetobutylicum* is a Gram-positive anaerobe which also produces byproducts such as butyrate, acetone, and ethanol (Jones and Woods, 1986). As a result, its 1-butanol yield is difficult to control. Its relatively slow growth rate and spore-forming life cycle create additional problems for industrial fermentation. Furthermore, the relatively unknown genetic system and complex physiology of the microorganism present difficulties in engineering its metabolism for optimal production of 1-butanol. Therefore, there is strong incentive to produce 1-butanol from a user-friendly organism. In this regard, *Escherichia coli* is a well-characterized microorganism with a set of readily available tools for genetic manipulation and its physiological regulation is well-studied. *E. coli* has previously been shown to be a suitable host for the production of valuable metabolites (Farmer and Liao, 2000; Martin et al., 2003; Causey et al., 2004; Kim et al., 2007). However, it does not produce 1-butanol as a fermentation product. We thus seek to engineer a synthetic pathway (Fig. 1) in *E. coli* to produce 1-butanol.

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2. Materials and methods

2.1. Bacteria strains, media, and growth conditions

BW25113 ($rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) was used as wild-type (WT) (Datsenko and Wanner, 2000). XL-1 Blue (Stratagene, La Jolla, CA) was used to propogate all plasmids.

For all experiments, 16 h precultures in M9 medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 1 mM $MgSO_4$, 10 mg Vitamin B_1 and 0.1 mM $CaCl_2$ per liter water) containing 2% glucose, 0.1 M MOPS and 1000 × Trace Metal Mix (27 g $FeCl_3 \cdot 6H_2O$, 2 g $ZnCl_2 \cdot 4H_2O$, 2 g $CaCl_2 \cdot 2H_2O$, 2 g $Na_2MoO_4 \cdot 2H_2O$, 1.9 g $CuSO_4 \cdot 5H_2O$, 0.5 g H_3BO_3, 100 mL HCl per liter water) were inoculated 1% from an overnight culture in LB and grown at 37 °C in a rotary shaker (250 rpm). For the knockout strain comparisons, 0.1% casamino acids were added to the media. For the media comparison, cultures were grown semi-aerobically in M9 medium as described previously and Terrific Broth (TB) (12 g tryptone, 24 g yeast extract, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4) supplemented with 2% glucose, 2% glycerol, or no additional carbon source. Antibiotics were added appropriately (ampicillin 100 μg/mL, chloroamphenicol 40 μg/mL, spectinomycin 20 μg/mL, kanamycin 30 μg/mL).

For anaerobic growth, precultures were adjusted to OD_{600} 0.4 with 12 mL of fresh medium with appropriate antibiotics and induced with 0.1 mM IPTG. The culture was transferred to a sealed 12 mL glass tube (BD Biosciences, San Jose, CA) and the headspace was evacuated. Cultures were shaken (250 rpm) at 37 °C for 8–40 h. Semi-aerobic cultures were grown similarly, except that 5 mL of fresh medium was added and transferred to the sealed glass tubes without evacuation of the headspace. Aerobic cultures were diluted with 3 mL of fresh media and grown in unsealed capped test tubes.

2.2. 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).

2.3. DNA techniques

E. coli genes *adhE*, *ldhA*, *frdBC*, *fnr*, *pflB* were deleted as described (Datsenko and Wanner, 2000). Phosphate acetyltransferase, encoded by *pta*, was inactivated by P1 transduction with JW2294 (Baba et al., 2006) as the donor. F′ was transferred from XL-1 blue (Stratagene) to supply $lacI^q$. All plasmids listed in Table 1 were sequenced to verify the accuracy of the cloning. All oligonucleotides are listed in Table 2.

To clone *crt*, *bcd*, *etfBA* and *hbd*, genomic DNA of *C. acetobutylicum* ATCC824 (ATCC) was used as a PCR template and amplified using crtXmaIf and hbdSacIr (see Table 2). PCR products were digested with *Xma*I and *Sac*I and cloned into pJRB1-rc (pACYC184 derivative, Spec®, P_{BAD}) cut with the same enzymes, creating pJCL2. To replace P_{BAD} with $P_L$lacO1, pZE12-luc (Lutz and Bujard, 1997) was used as PCR template with primers A46 and A47. PCR products were digested with *Nco*I and *Xma*I and ligated into the matching sites of pJCL2 to create pJCL60.

The *atoB* gene was amplified from *E. coli* MG1655 genomic DNA using primers atoBAcc65If and atoBSphIr. PCR products were digested with *Acc*65I and *Sph*I and cloned into the corresponding sites of pZE12-luc, creating pJCL16. *adhE2* was amplified from the pSOL1 megaplasmid in a total DNA extract of *C. acetobutylicum* DNA using adhE2SphIf and adhE2XbaIr. The PCR product was digested with *Sph*I and *Xba*I and ligated into the same sites of pJCL16 to create pJCL17.

The *C. acetobutylicum* ATCC824 *thl* was amplified from genomic DNA using primers thlAcc65I and thlSphIr. The product was digested with *Acc*65I and *Sph*I and ligated into the *Acc*65I and *Sph*I sites of pZE12-luc to create pJCL43. pJCL43 was then digested with *Spe*I and *Sph*I, and the larger fragment was purified and cloned into the larger fragment created by digestion with *Spe*I and *Sph*I of pJCL17, creating pJCL50. To replace P_{LtetO1} of pZE21-MCS1 (Lutz and Bujard, 1997) with P_{LlacO1}, pZE12-luc was digested with *Aat*II and *Acc*65I. The shorter fragment was purified and cloned into the corresponding sites of pZE21-MCS1 to create pSA40. *crt* was amplified from *C. acetobutylicum* ATCC824 genomic DNA using primers A85 and A86. The PCR product was digested with *Acc*65I and *Sal*I and cloned into pSA40 cut with the same enzymes, creating pJCL33. pJCL35 was created by amplifying the *hbd* gene fragment from *C. acetobutylicum* genomic DNA with primers A89 and A90, digesting the PCR fragment with *Xma*I and *Mlu*I, and ligating the product into the corresponding sites of pJCL33. The ColE1 origin was replaced with p15A by digesting pZA31-luc (Lutz and Bujard, 1997) with *Aat*II and *Avr*II. The smaller fragment was purified and cloned into pJCL35 digested with the same enzymes, creating pJCL37. To eliminate a point mutation in the *crt* gene of pJCL37, *crt* was amplified and digested as described previously and ligated into the corresponding sites of pJCL37 to create pJCL66. The *Streptomyces coelicolor ccr* gene was amplified from genomic DNA using primers A87 and A88. The product was digested with *Sal*I and *Xma*I, and cloned into the same sites of pJCL66 to create pJCL63. *Megasphaera elsdenii bcd* and *etfBA* was amplified from a synthesized template (Epoch Biolabs, Sugar Land, TX) using primers MegBcd-op-fwd and MegBcd-op-rev. The PCR product was digested with *Xho*I and *Xma*I and ligated into the *Sal*I and *Xma*I sites of pJCL66 to create pJCL74.

Table 1
Strains and plasmids used

Name	Relevant genotype	Reference
Strains		
BW25113	*rrnB*$_{T14}$ Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner (2000)
XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15 Tn10* (Tet^R)]	Stratagene
JCL16	BW25113/F′ [*traD36*, *proAB*+, *lacIq ZΔM15*]	This study
JCL88	As JCL16, but Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*fnr*, Δ*pta*	This study
JCL166	As JCL16, but Δ*adhE*, Δ*ldhA*, Δ*frdBC*	This study
JCL167	As JCL16, but Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*fnr*	This study
JCL168	As JCL16, but Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*fnr*, Δ*pflB*	This study
JCL170	As JCL16, but Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*fnr*, Δ*pta*, Δ*pntA*	This study
JCL171	As JCL16, but Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*pta*, Δ*pflB*	This study
JCL184	JCL166/pJCL17/pJCL60	This study
JCL185	JCL167/pJCL17/pJCL60	This study
JCL186	JCL168/pJCL17/pJCL60	This study
JCL187	JCL88/pJCL17/pJCL60	This study
JCL190	JCL171/pJCL17/pJCL60	This study
JCL191	JCL16/pJCL17/pJCL60	This study
JCL198	JCL16/pJCL50/pJCL60	This study
JCL230	JCL88/pJCL17/pJCL63	This study
JCL235	JCL88/pJCL17/pJCL74	This study
JCL260	As JCL16, but Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*fnr*, Δ*pta*, Δ*pflB*	This study
JCL262	JCL260/pJCL17/pJCL60	This study
JCL274	As JCL16, but Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*pta*	This study
JCL275	JCL274/pJCL17/pJCL60	This study
Plasmids		
pZE12-luc	ColE1 ori; Amp^R; P$_L$lacO$_1$::*luc*(VF)	Lutz and Bujard (1997)
pZE21-MCS1	ColE1 ori; Kan^R; P$_{Ltet}$O$_1$::MCS1	Lutz and Bujard (1997)
pACYC184	p15A ori; Cm^R; Tet^R	New England Biolabs
pJCL17	From pZE12, P$_L$lacO$_1$::*atoB*(EC)-*adhE2*(CA)	This study
pJCL50	From pZE12, P$_L$lacO$_1$::*thl*(CA)-*adhE2*(CA)	This study
pJCL60	p15A ori; Spec^R; P$_L$lacO$_1$::*crt*(CA)-*bcd*(CA)-*etfAB*(CA)-*hbd*(CA)	This study
pJCL63	p15A ori; Cm^R; P$_L$lacO$_1$::*crt-bcd*(ME)-*ccr*(SC)-*hbd*(CA)	This study
pJCL74	p15A ori; Cm^R; P$_L$lacO$_1$::*crt-bcd*(ME)-*etfAB*(ME)-*hbd*(CA)	This study

Table 2
Primer sequences

Primer name	Sequence 5′→3′
crtXmaIf	GCGCCCGGGTTAGGAGGATTAGTCATGGAACTAA
hbdSacIr	GGCGAGCTCCCCCATTTGATAATGGGGATTCTTG
A46	AATAATCCATGGCGTATCACGAGGCCCTTTCGTCT
A47	AATAACCCGGGTCAGTGCGTCCTGCTGATGTGCT
atoBAcc65If	CGAGCGGTACCATGAAAAATTGTGTCATCGTCAGTG
atoBSphIr	CCGCATGCTTAATTCAACCGTTCAATCACCATC
adhE2SphIf	CCGCATGCAGGAGAAAGGTACCATGAAAGTTACAAATCAAAAAGAACTAAAACAA
adhE2XbaIr	GCGCATCTAGATTAAAATGATTTTATATAGATATCC
thlAcc65If	TCAGGTACCATGAAAGAAGTTGTAATAGCTAGTGCAGTA
thlSphIr	TCAGCATGCCTAGCACTTTTCTAGCAATATTGCTGTT
A85	CGAGCGGTACCATGGAACTAAACAATGTCATCCTTG
A86	ACGCAGTCGACCTATGAAAGCTGTCATTGCATCCTT
A89	AATAACCCGGGAGGAGATATACCATGAAAAAGGTATGTGTTATAGGTG
A90	CGAGCACGCGTTTATTTTGAATAATCGTAGAAACCT
A87	ACGCAGTCGACAGGAGATATACCATGACCGTGAAGGACATCCTGGACG
A88	AATAACCCGGGTCAGATGTTCCGGAAGCGGTTGATG
MegBcd-op-fwd	TAATCTCGAGTAAGGAGAGTGGAACATCATGGATT
MegBcd-op-rev	TTAACCCGGGCTTATGCAATGCCTTTCTGTTTCTT

2.4. AdhE2 enzyme assay

Cultures were grown in 50 mL SOB medium in a sealed 50 mL tube at 37 °C in a rotary shaker (250 rpm). At OD_{600} 0.8, cultures were induced with 0.1 mM IPTG and grown for 1 additional hour before 50-fold concentration in 100 mM Tris–HCl buffer (pH 7.0) and lysing with 0.1 mM glass beads. The crude extracts were then assayed according to the method described earlier (Dürre et al., 1987).

2.5. Detection of metabolites

The produced alcohol compounds were quantified by a gas chromatograph (GC) equipped with 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 by A DB-WAX capillary column (30 m, 0.32 mm i.d., 0.50 μm film thickness) purchased from Agilent Technologies (Santa Clara, CA). GC oven temperature was initially held at 40 °C for 5 min and raised with a gradient of 15 °C/min until 120 °C. And then it was raised with a gradient 50 °C/min until 230 °C and held for 4 min. Helium was used as the carrier gas with 9.3 psi inlet pressure. The injector and detector were maintained at 225 °C. The 0.5 μL supernatant of culture broth was injected in split injection mode (1:15 split ratio). Isobutanol was used as the internal standard.

For other secreted metabolites, filtered supernatant was applied (20 μl) to an Agilent 1100 HPLC equipped with an auto-sampler (Agilent Technologies) and a BioRad (Biorad Laboratories, Hercules, CA) Aminex HPX87 column (0.5 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.

3. Results

3.1. Expression of C. acetobutylicum pathway in E. coli leads to 1-butanol production

In *C. acetobutylicum*, the 1-butanol pathway branches off to produce acetone and butyrate (Jones and Woods, 1986). To produce 1-butanol in *E. coli*, we transferred only an essential set of genes for 1-butanol production (Fig. 1). These genes (*thl*, *hbd*, *crt*, *bcd*, *etfAB*, *adhE2*) were cloned and expressed in *E. coli* using two plasmids (pJCL50 and pJCL60, see Table 1) under the control of the IPTG-inducible $P_L lacO_1$ promoter. We successfully detected the activity of these gene products by enzyme assays consistent with previous reports (Wiesenborn et al., 1988; Boynton et al., 1996; Fontaine et al., 2002) except *bcd* and *etfAB* which code for butyryl-CoA dehydrogenase (Bcd) and an electron transfer flavoprotein (Etf). The activity of butyryl-CoA dehydrogenase was not conclusively demonstrated using crude extract from cells that expressed *bcd* and *etfAB*. This difficulty was also reported (Hartmanis and Gatenbeck, 1984; Boynton et al., 1996), possibly due to the instability of the enzyme.

Despite the inconclusive demonstration of Bcd activity, the expression of this synthetic pathway produced 13.9 mg/L of 1-butanol under anaerobic conditions (Fig. 2). In contrast to the suspected oxygen sensitivity, a slight increase in the oxygen level increased the production of 1-butanol, suggesting that the NADH produced anaerobically was insufficient to supply for 1-butanol production. In a completely aerobic condition, on the other hand, *E. coli* consumes both acetyl-CoA and NADH in TCA cycle and respiration, and thus likely contributes to the decreased 1-butanol production (Fig. 2).

3.2. Alternative enzymes from other organisms

In addition to the *C. acetobutylicum* thiolase (coded by *thl*), acetyl-CoA acetyltranserase from *E. coli* (coded by *atoB*) was overexpressed to examine its ability to catalyze the reaction of acetyl-CoA to acetoacetyl-CoA (Duncombe and Frerman, 1976). Interestingly, the production of 1-butanol increased more than three-fold (Fig. 2), possibly because of the higher activity of this native enzyme. To determine whether homologues and isoenzymes of Bcd from other organisms would be more effective in *E. coli*, we expressed *bcd* (Becker et al., 1993) and *etfAB* (O'Neill et al., 1998) from *M. elsdenii* and *ccr* from *S. coelicolor*, which encodes a crotonyl-CoA reductase (Ccr) (Wallace et al., 1995) that does not require an Etf for activity, in place of their counterparts from *C. acetobutylicum*. The activity of *S. coelicolor* Ccr, but not *M. elsdenii* Bcd, was detected conclusively by enzyme assays using crude extracts (data

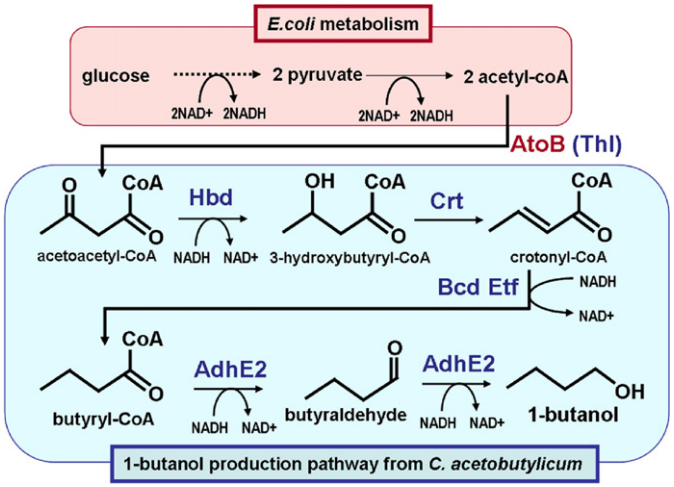

Fig. 1. Schematic representation of 1-butanol production in engineered *E. coli*. The engineered 1-butanol production pathway consists of six enzymatic steps from acetyl-CoA. *AtoB*, acetyl-CoA acetyltransferase; *Thl*, acetoacetyl-CoA thiolase; *Hbd*, 3-hydroxybutyryl-CoA dehydrogenase; *Crt*, crotonase; *Bcd*, butyryl-CoA dehydrogenase; *Etf*, electron transfer flavoprotein; *AdhE2*, aldehyde/alcohol dehydrogenase.

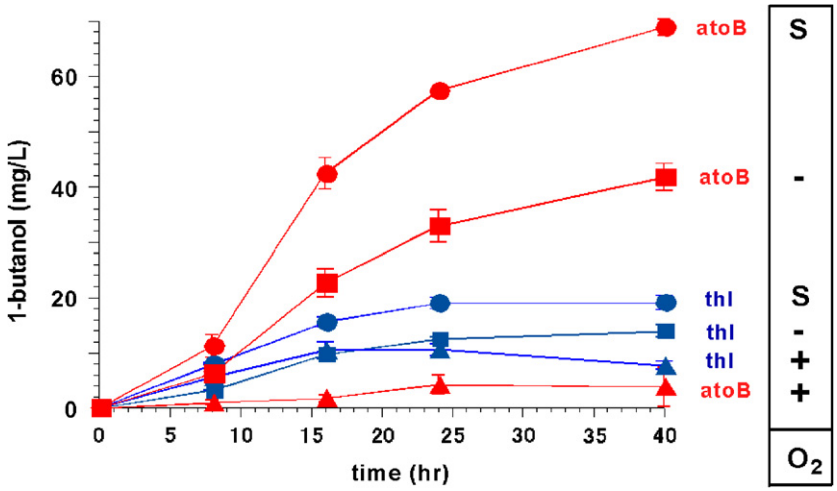

Fig. 2. 1-Butanol production from engineered *E. coli*. Investigation of growth conditions and comparison of *thl* and *atoB* on production of 1-butanol. JCL191 and JCL198 were grown in an anaerobic condition (squares, '−'), an aerobic condition (triangles, '+'), and a semi-aerobic condition (circles, 'S') at 37 °C for 8–40 h.

not shown). However, the *M. elsdenii* and *S. coelicolor* genes led to much lower production of 1-butanol in *E. coli* (Fig. 3). Nevertheless, possibilities still exist that alternative genes from other organisms might improve 1-butanol production in *E. coli*. The use of a user-friendly host facilitates such exploration.

3.3. Host gene deletion to increase 1-butanol production

To further improve 1-butanol production, we deleted the host pathways that compete with the 1-butanol pathway for acetyl-CoA and NADH. Fig. 4 shows that deletion of *ldhA, adhE*, and *frdBC* from WT, complete with the 1-butanol production pathway (JCL184), doubled the production of 1-butanol by significantly reducing the amount of lactate, ethanol, and succinate produced (Table 3), consistent with the result shown for pyruvate production (Causey et al., 2004). The decision to knock out the native *adhE* in *E. coli* and replace it with *adhE2* from *C. acetobutylicum* was based on the relative affinities of each ADH enzyme towards acetyl-CoA and butyryl-CoA (Table 4). While the activity of the *E. coli* ADH towards butyryl-CoA is not much less than the *C. acetobutylicum* ADH, its activity torwards acetyl-CoA is four times h igher than the *C. acetobutylicum* ADH for the same substrate. This ratio favors *adhE2* over *adhE* for 1-butanol production.

Although the deletions in JCL184 (Δ*ldhA*, Δ*adhE*, Δ*frdBC*) resulted in the decrease of most fermentation products, a significant amount of acetate was produced. To further increase 1-butanol production, we deleted *pta*. While acetate production was decreased considerably, JCL275 (Δ*ldhA*, Δ*adhE*, Δ*frdBC*, Δ*pta*) led to a lower production of 1-butanol.

The deletion of *pflB* nearly abolished 1-butanol production, indicating that pyruvate-formate lyase (Pfl) was the primary enzyme responsible for the production of acetyl-CoA from pyruvate under the experimental condition (Fig. 4). The use of Pfl to produce acetyl-CoA rather than the pyruvate dehydrogenase complex (PDHc) suggests that our condition does not provide enough NADH to fully reduce glucose to 1-butanol. This is supported by the data in Fig. 2 which shows that allowing a small amount of oxygen during growth, and thus elevating the activity of PDHc, increases the amount of 1-butanol produced compared to a completely anaerobic condition. This strain also produces a large amount of pyruvate due to insufficient NADH to make 1-butanol and the host's inability to produce lactate or acetate. It is therefore desirable to activate PDHc for the production of 1-butanol, since the reducing power is stored in NADH rather than formate. To achieve elevated expression of PDHc, we deleted *fnr*, an anaerobic regulator that represses the expression of PDHc genes during anaerobic growth (Salmon et al., 2003). Yet the deletion of *fnr* from the host decreased 1-butanol production. However, when both *pta* and *fnr* were deleted, production of 1-butanol improved nearly three-fold over WT levels (373 mg/L).

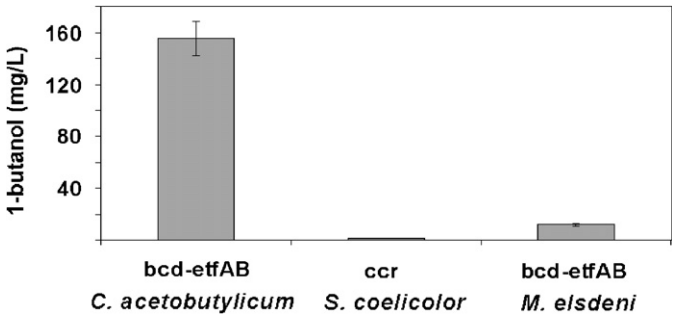

Fig. 3. Evaluation of 1-butanol production using various enzymes for the reduction of crotonyl-CoA to butyryl-CoA. JCL187, JCL230 and JCL235 contain *bcd–etfAB* from *C. acetobutylicum, ccr* from *S. coelicolor* and *bcd–etfAB* from *M. elsdenii*, respectively. Cultures were grown semi-aerobically in shake flasks at 37 °C for 24 h.

Table 3
Metabolic byproducts of 1-butanol producing strains

Knockout genes						Butanol	Product concentrations (mM)						
adh	ldh	frd	fnr	pta	pfl		Acetate	Ethanol	Formate	Pyruvate	Lactate	Succinate	Glucose[a]
						1.9	13.5	15.2	19.5	2.1	41.8	3.4	44.9
Δ	Δ	Δ				3.7	15.2	6.0	23.1	4.0	5.4	0.7	30.7
Δ	Δ	Δ	Δ			2.1	11.8	5.0	16.4	2.4	2.5	1.2	22.2
Δ	Δ	Δ		Δ		2.7	1.3	3.0	18.5	12.7	2.4	1.1	28.2
Δ	Δ	Δ	Δ	Δ		5.0	1.5	15.5	21.0	23.4	3.0	1.7	42.8
Δ	Δ	Δ	Δ		Δ	0.1	4.9	1.0	3.5	6.0	2.9	2.5	14.1
Δ	Δ	Δ		Δ	Δ	0.1	0.7	0.5	2.1	10.9	1.9	1.2	14.3
Δ	Δ	Δ	Δ	Δ	Δ	0.2	0.7	1.7	3.0	11.8	2.9	2.3	18.2

Cells were grown semi-aerobically in M9 media with the addition of 0.1% casamino acids at 37 °C for 24 h.
[a]Glucose consumed.

Table 4
Comparison of activities of Adh enzymes from *E. coli* (*Eco*) and *C. acetobutylicum* (*Cac*)

Enzyme	Butyryl-CoA	Acetyl-CoA	Ratio (B:A)
AdhE (*Eco*)	0.054	0.218	0.25
AdhE2 (*Cac*)	0.082	0.054	1.52

Enzyme activities are from crude cell extracts and given as μmol min^{-1} (mg protein)$^{-1}$.

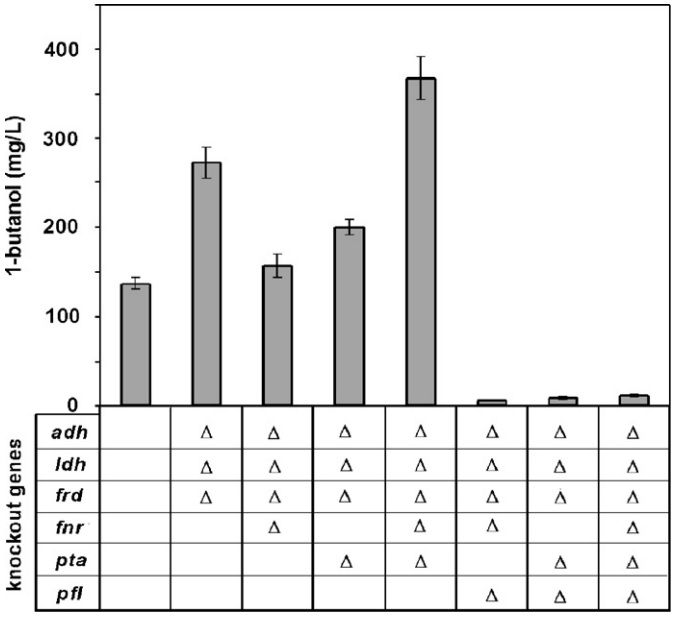

Fig. 4. Comparison of the effect of gene deletions on the production of 1-butanol in *E. coli*. Cells were grown semi-aerobically in M9 with the addition of 0.1% casamino acids in shake flasks at 37 °C for 24 h. "△" indicates gene deletion.

This improvement in 1-butanol production was accompanied by an increase of ethanol production to WT levels, as well as a further increase in the secretion of pyruvate. From these data, it appears that deletion of *fnr* did not fully activate PDHc and so this is an area for further improvement. Thus, the exact mechanism for the elevated 1-butanol production in the strain appears to be complex and requires further investigation.

Various growth media were examined to increase the titer of 1-butanol. JCL187 (Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*fnr*, Δ*pta* containing pJCL17 and pJCL60) was grown in rich media (TB) supplemented with different carbon sources as well as minimal media for comparison. Fig. 5 shows that growth in rich media increased 1-butanol production, as cultures in TB supplemented with glycerol produced five-fold more 1-butanol (552 mg/L) than cultures grown in M9 (113 mg/L).

4. Discussion

The transfer of a biosynthetic pathway from a native producer to a non-native producer may face several difficulties. Overexpression of non-native pathways may disturb the native metabolism in the hosts by competing for precursors necessary for growth or maintenance. In addition, the re-engineering of pathways often leads to imbalanced gene expression, creating a bottleneck in the biosynthetic pathway that diminishes production of the target compound. Further improvement of 1-butanol production requires balancing of each reaction step. In particular, the pathway requires four moles of NADH to produce 1 mol of 1-butanol. Balancing NADH requires active PDHc under anaerobic conditions. Therefore, activation of this enzyme under anaerobic conditions (Kim et al., 2007) would be desirable. Furthermore, the 1-butanol production pathway of *C. acetobutylicum* (Fig. 1) uses acetyl-CoA as a substrate, which, if depleted, can lead to diminished growth (Kim et al., 2007). This pathway also has four intermediate metabolites carrying coenzyme-A. If the synthetic pathway is not well balanced it could cause the depletion of the pool of free CoA.

Our success in producing 1-butanol from the engineered *E. coli* opens the possibility for using non-native, easily manipulated organisms for 1-butanol production. Since the

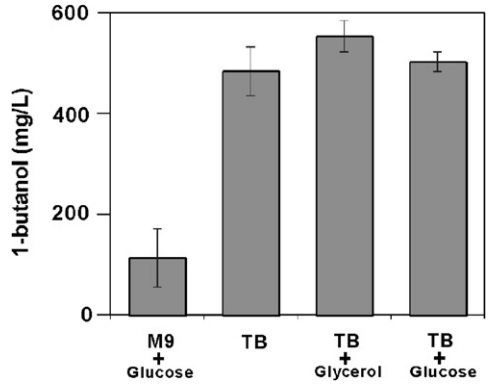

Fig. 5. Comparison of the effect of media on the production of 1-butanol in *E. coli*. Cells were grown semi-aerobically in M9 medium and TB medium supplemented with 2% glucose, 2% glycerol, or no additional carbon source at 37 °C for 24 h.

metabolism, physiology, and genetics of *E. coli* are better understood, design and construction of ideal pathways for homo-fermentative production can benefit from the vast amount of accumulated knowledge. Additionally, we found that *E. coli* can tolerate 1-butanol up to a concentration of 1.5% (data not shown), which is similar to published results found for the native producer *C. acetobutylicum* (Lin and Blaschek, 1983). As 1-butanol production in *E. coli* is optimized and product titers increase, improvement in the tolerance to 1-butanol can be achieved using similar strategies that have resulted in ethanol tolerant mutants (Yomano et al., 1998; Alper et al., 2006).

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