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Authors

Ohtake, Toshiyuki
Pontrelli, Sammy
Laviña, Walter A
et al.

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Metabolomics-driven approach to solving a CoA imbalance for improved 1-butanol production in *Escherichia coli*

Toshiyuki Ohtake[a,1], Sammy Pontrelli[b,1], Walter A. Laviña[a], James C. Liao[b], Sastia P. Putri[a,*], Eiichiro Fukusaki[a,*]

[a] Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, 5531 Boelter Hall, 420 Westwood Plaza, Los Angeles, CA 90095, USA

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ABSTRACT

High titer 1-butanol production in *Escherichia coli* has previously been achieved by overexpression of a modified clostridial 1-butanol production pathway and subsequent deletion of native fermentation pathways. This strategy couples growth with production as 1-butanol pathway offers the only available terminal electron acceptors required for growth in anaerobic conditions. With further inclusion of other well-established metabolic engineering principles, a titer of 15 g/L has been obtained. In achieving this titer, many currently existing strategies have been exhausted, and 1-butanol toxicity level has been surpassed. Therefore, continued engineering of the host strain for increased production requires implementation of alternative strategies that seek to identify non-obvious targets for improvement. In this study, a metabolomics-driven approach was used to reveal a CoA imbalance resulting from a *pta* deletion that caused undesirable accumulation of pyruvate, butanoate, and other CoA-derived compounds. Using metabolomics, the reduction of butanoyl-CoA to butanal catalyzed by alcohol dehydrogenase AdhE2 was determined as a rate-limiting step. Fine-tuning of this activity and subsequent release of free CoA restored the CoA balance that resulted in a titer of 18.3 g/L upon improvement of total free CoA levels using cysteine supplementation. By enhancing AdhE2 activity, carbon flux was directed towards 1-butanol production and undesirable accumulation of pyruvate and butanoate was diminished. This study represents the initial report describing the improvement of 1-butanol production in *E. coli* by resolving CoA imbalance, which was based on metabolome analysis and rational metabolic engineering strategies.

1. Introduction

As an important bulk chemical and potential biofuel, 1-butanol biosynthesis from microorganisms has attracted much attention. The clostridial CoA-dependent 1-butanol pathway has been successfully transferred to well-characterized and genetically tractable organisms such as *Escherichia coli* for further engineering and improvement of 1-butanol production (Atsumi et al., 2008; Bond-watts et al., 2011; Inui et al., 2008; Lan and Liao, 2012; Lim et al., 2013; Shen et al., 2011). Previously, 15 g/L of 1-butanol was produced within engineered *E.coli* by deletion of fermentative pathways, thereby requiring the use of the 1-butanol pathway as the sole NADH sink under anaerobic conditions (Shen et al., 2011). While this strategy yielded 1-butanol that is regarded as one of the highest biofuel production to date, (Baez et al., 2011; Friedlander et al., 2016; Inokuma et al., 2010; Pais et al., 2013; Wernick et al., 2016; York and Ingram, 1996) improved titers are desired. In addition to the deletion of NADH consuming pathways, many other metabolic engineering strategies have been applied in order to achieve such high titers. Furthermore, current titers extend beyond toxicity levels. Therefore, more innovative strategies need to be employed in order to further identify non-obvious targets that may be improved to increase 1-butanol production.

Metabolomics stands as a tool with great potential for strain engineering to obtain a desired phenotype. Metabolomics, the comprehensive analysis of a wide range of metabolites, has the ability to provide a dynamic and holistic understanding of how the implementation of a metabolic pathway affects the cellular metabolism in a broader sense. Metabolomics therefore may be used to complement current metabolic engineering strategies for optimizing biological production of chemicals within microorganisms (Oldiges et al., 2007; Putri et al., 2013). Through the detection of relevant metabolic perturbations, metabolomics can identify specific targets for strain improvement that

* Corresponding authors.
E-mail addresses: sastia_putri@bio.eng.osaka-u.ac.jp (S.P. Putri), fukusaki@bio.eng.osaka-u.ac.jp (E. Fukusaki).
[1] Both authors contributed equally to this work.

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may include detection of pathway bottlenecks, metabolite or product toxicity, imbalanced cofactor supply, or draining of metabolites by alternative pathways (Gold et al., 2015; Hasunuma et al., 2011; Korneli et al., 2012; Noguchi et al., 2016; Ohta et al., 2015; Teoh et al., 2015; Xu et al., 2016). Furthermore, metabolomics provides a comprehensive analysis of the metabolome that allows a deeper understanding of cellular metabolism and how gene modifications can be used for metabolic engineering. Therefore, a metabolomics-driven approach can be a powerful tool in generating strategies to enhance desired phenotypes.

A previously engineered 1-butanol producing *E. coli* strain with deleted phosphate acetyltransferase (*pta*), JCL299F, resulted in the highest improvement of 1-butanol titer by blocking acetate by-product formation and increasing flux through the 1-butanol pathway (Shen et al., 2011). While exhibiting high production titers, this strain has presented a number of metabolic perturbations that remain as targets for further engineering. In this study, we demonstrate the utility of a metabolomics-driven approach to optimize further the engineered 1-butanol producing *E. coli* strain, JCL299F, by revealing unexpected metabolic changes and identifying rate-limiting reactions within the strain. Consequently, these insights served as the basis for succeeding metabolic engineering strategies. Ultimately, successful engineering of the 1-butanol pathway to overcome this imbalance improved the production and resulted in a titer of 18.3 g/L.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli strains and plasmids used in this study are summarized in Table 1. Primers were purchased through Integrated DNA Technologies (Coralville, IA, USA). All PCR reactions were performed with KOD Hot-Start DNA polymerase or KOD Xtreme Hot-Start Polymerase (Millipore, MA, USA). Gel purification, DpnI digestion, and PCR Clean (Zymo Research, CA, USA) were used to isolate and purify the correct amplicons. Assembly of fragments was performed using T4 DNA polymerase (New England Biolabs, UK) using the following protocol: Gel purified PCR fragments with 20 bp overlaps were mixed in equimolar amounts. Approximately 300 ng of the fragment mixture was combined with NEB buffer #2 and 0.3 µL T4 polymerase to a final volume of 10 µL. The reaction was incubated at room temperature for 5 min, transformed into *E. coli* XL1Blue (Agilent Technologies, Waldbronn, Germany), and selected on LB agar plates containing appropriate antibiotics.

All constructed plasmids were sequenced using Laragen (Culver City, CA, USA). pALQ97 was constructed using pCS138 as a template with insertion of *Salmonella enterica pduP*. pALQ96 was constructed using pIM8 as a template with insertion of *panK* from *E. coli*.

2.2. Construction of RBS library

RBS sequences were generated using the RBS Calculator v2.0 (Espah Borujeni et al., 2014; Salis et al., 2009). The RBS library generator function was applied to pEL11 to create variable RBS sequences between the *atoB* stop codon and *adhE2* start codon. Eight degenerate sequences were generated with predicted translation initiation rates ranging from 9344 au to 715,663 au. Degenerate primers were synthesized (Table 1) and used to amplify pEL11. The resulting fragment was assembled and transformed as previously described. Colonies formed were pooled into a single culture of LB medium, grown overnight, and plasmids were purified to yield the completed plasmid library.

2.3. Media and growth conditions

The culture media and conditions used were based on Shen et al. (2011) with minor modifications. 1-Butanol production was performed in terrific broth (TB) (12g tryptone, 24g yeast extract, 2.31g KH_2PO_4, 12.54g K_2HPO_4, 4 mL glycerol per liter of water) supplemented with 2% glucose, unless otherwise noted. Pre-cultures were grown aerobically in LB containing appropriate antibiotics (ampicillin 100 µg/mL, kanamycin 50 µg/mL, chloramphenicol 50 µg/mL, and tetracycline 12 µg/mL) at 37 °C overnight. Pre-cultures were transferred to 20 mL of fresh TB-2% glucose medium at an initial optical density of 0.04 at 600 nm (OD_{600}). Cells were grown in 250 mL screw cap flasks at 37 °C to an OD_{600} of 0.4–0.6 before induction with 0.1 mM IPTG. After 2 h of incubation, anaerobic switch was performed. Briefly, oxygen in the headspace was purged by repeated vacuum treatment and refilling with nitrogen in an anaerobic chamber. The cap was closed tightly and wrapped with parafilm. Cultures were then incubated at 37 °C at 200 rpm. For time course experiments, samples were taken inside the anaerobic chamber to maintain anaerobicity. Glucose was fed to the cultures using 30% glucose in TB and the culture pH was adjusted to around 7 using 10 M NaOH.

For set-ups using test tubes, 3 mL cell cultures were grown in 10 mL

Table 1
Bacterial strains and plasmids used in this study.

Name	Relevant characteristics	Source
***E.coli* strain**		
BW25113	$rrnB_{T14}$ $\Delta lacZ_{WJ16}$ $\Delta hsdR514$ $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$	(Datsenko and Wanner, 2000)
JCL16	BW25113/F′ [$\Delta traD36$ $proAB^+$ $\Delta lacI^qZ\Delta M15$ (Tet^r)]	(Atsumi et al., 2008)
JCL166	JCL16 $\Delta ldhA$ $\Delta adhE$ $\Delta frdBC$	(Atsumi et al., 2008)
JCL299	JCL16 $\Delta ldhA$ $\Delta adhE$ $\Delta frdBC$ Δpta	(Atsumi et al., 2008)
JCL166F	JCL166/pCS138, pEL11, pIM8	(Shen et al., 2011)
JCL299F	JCL299/pCS138, pEL11, pIM8	(Shen et al., 2011)
JCL299FT	JCL299/pCS138, pTO1, pIM8	This study
Plasmid		
pCS138	P_LlacO_1:: fdh_{CB} pSC101 *ori* Cm^r	(Shen et al., 2011)
pEL11	P_LlacO_1:: $atoB_{EC}$-$adhE2_{CA}$-crt_{CA}-hbd_{CA} ColE1 *ori* Amp^r	(Shen et al., 2011)
pIM8	P_LlacO_1:: ter_{TD} Cola *ori* Kan^r	(Shen et al., 2011)
pALQ96	P_LlacO_1:: ter_{TD}-$panK_{EC}$ Cola *ori* Kan^r	This study
pALQ97	P_LlacO_1:: fdh_{CB}-$pduP_{SE}$ pSC101 *ori* Cm^r	This study
pTO1	P_LlacO_1:: $atoB_{EC}$-$adhE2_{CA}$-crt_{CA}-hbd_{CA} ColE1 *ori* Amp^r (Improved RBS strength)	This study
Primer		
RBS lib F	ATGAAAGTTACAAATCAAAAAGAACTAAAACAAA	This study
RBS lib R	TTTTGATTTGTAACTTTCATTGSTACCTCCTCTTTTCT CGGMTACGSGCTCATTTAATTCAACCGTTCAATCACCATCG	This study

In the plasmid description, subscripts indicate the source of the gene as follows: CA, *Clostridium acetobutylicum*; CB, *Candida boidinii*; EC, *Escherichia coli*; SE, *Salmonella enterica*; TD, *Treponema denticola*. Primer sequences are shown from 5′ to 3′.

BD (San Jose, CA, USA) Vacutainer sealed tubes. A needle (20 G by 1 1/2 in.; BD) was inserted through the rubber cap of the glass tube with the other end attached to a Millipore filter (0.22 μm). The needle was then removed from the cap inside the anaerobic chamber after oxygen was evacuated. The sealed tubes were then taken out, wrapped with parafilm and taped to prevent bursting of the caps due to gas build up that occurs during fermentation. Then, the tubes were incubated at 37 °C for 12 h at 200 rpm (unless otherwise noted). Cultures were fed using 30% glucose in TB and pH was adjusted to around 7 using 10 M NaOH.

2.4. Sampling and extraction

For metabolome analysis, five OD_{600} units of cells were collected 12 or 24 h after anaerobic switch by fast filtration using a 0.45 μm pore size, 47 mm diameter nylon membrane (Millipore). The cells were immersed in liquid nitrogen immediately for quenching and stored at −80 °C until extraction.

For extraction, 1.8 mL of extraction solvent (methanol/water/chloroform = 5:2:2 v/v/v%, with 20 μg/L of (+)-10 camphorsulfonic acid as an internal standard) was added to each 2-mL sampling tube with filtered sample and incubated at −30 °C for 1 h. After incubation, 700 μL of solution was transferred to a new tube containing 350 μL of water. The mixture was mixed using a vortex and centrifuged at 16,000*g* for 3 min at 4 °C to separate polar and non-polar phases. 700 μL of the upper polar phase was transferred to a new tube via syringe filtration (0.2 μm PTFE hydrophilic membrane, Millipore). The sample was centrifugally concentrated for 2 h and freeze-dried overnight. After reconstituting in 100 μL ultra-pure water, the sample was centrifuged at 16,000*g* for 3 min at 4 °C and transferred to a glass vial.

2.5. Ion-pair LC/MS/MS analysis

Ion-pair LC/MS/MS analysis was performed using a Nexera UHPLC system (Shimadzu, Kyoto, JAPAN) coupled with LCMS 8030 Plus (Shimadzu) as previously reported (Mitsunaga et al., 2015). The column used was a CERI (Chemicals Evaluation and Research Institute, Tokyo, JAPAN) L-column 2 ODS (150 mm × 2.1 mm, particle size 3 μm). The mobile phase (A) was 10 mM tributylamine and 15 mM acetate in ultra-pure water, while the mobile phase (B) was methanol. The flow rate was 0.2 mL/min and column oven temperature was 45 °C. The concentration of mobile phase (B) was increased from 0% to 15%, 50%, and 100% from 1.0 to 1.5 min, 3.0 to 8.0 min, and 8.0 to 10.0 min, respectively; held until 11.5 min, decreased to 0% from 11.5 min, and held at 0% until 20 min. The analysis mode was negative ion mode. The injection volume was 3 μL, probe position was +1.5 mm, desolvation line temperature was 250 °C, heat block temperature was 400 °C, nebulizer gas flow was 2 L/min and drying gas flow was 15 L/min. The optimized multiple reaction monitoring (MRM) parameters for each metabolite are listed in Supplementary material Table S1.

The acquired raw data was converted to an analysis base file format using a freely available file format converter (Reifycs Inc., Tokyo, JAPAN). After file conversion, MRMPROBS (Tsugawa et al., 2013) was used for automatic peak picking and calculation of the peak area. The detected peaks were also confirmed manually.

2.6. Multivariate analysis

Principal component analysis (PCA) was performed using SIMCA-P + version 13 (Umetrics, Umeå, Sweden). The metabolome data was normalized by an internal standard, mean centered and scaled to unit variance. T-test was performed using MS Excel to determine statistically significant differences.

2.7. Quantification of extracellular metabolites

The supernatant of the culture media was collected by centrifugation at 16,000*g* for 5 min at 4 °C before filtration using 0.2 μm pore size filter (Millipore). Alcohols were quantified using a GC-2010 system (Shimadzu) with a GL Science (Tokyo, JAPAN) InertCap Pure-WAX capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) equipped with a flame ionization detector and an AOC-20i/s autoinjector (Shimadzu). A half microliter (0.5 μL) supernatant of culture media was injected in split mode with a split ratio of 1:15. The injector was maintained at 225 °C. The column temperature was initially held at 40 °C for 1 min and raised with a gradient of 15 °C/min until 120 °C and held for 1 min. It was then raised with a gradient of 50 °C/min until 250 °C and held for 5 min. Nitrogen was used as the carrier gas with a column flow rate of 1.90 mL/min (linear velocity 40.0 cm/s). Isobutanol was used as an internal standard.

Organic acids were determined using a Prominence UFLC system (Shimadzu) equipped with a photo diode array detector SPD-M20A (Shimadzu) and a Bio-Rad (Hercules, CA, U.S.A.) Aminex HPX-87H column (300 mm × 7.8 mm, particle size 9 μm). The column temperature was set to 50 °C, and 10 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.3 mL/min. Organic acids were detected at 210 nm. Concentrations were determined by extrapolation from standard curves. Each component in TB was subtracted out individually.

Glucose was measured using a F-kit D-glucose (Roche Diagnostics, Manheim, Germany) following the manufacturer's instructions.

2.8. Cell extract preparation

JCL299 harboring respective plasmids were grown and induced in the same media and conditions as described above for anaerobic production of 1-butanol. After 16 h, cells were harvested by centrifugation under anaerobic conditions. Cells were lysed by adding BugBuster (1:20 bugbuster culture volume) and allowed to sit anaerobically for 20 min. The resulting lysate was used for crude assays without further purification.

2.9. AdhE2 assay

The relative specific activity of AdhE2 within cell lysate was measured by monitoring the decrease in absorbance at 340 nm corresponding to the consumption of supplied NADH. The reaction mixture contained 100 mM Tris-HCl (pH 7.0), 400 μM NADH, and 2 mM butanoyl-CoA. The reactions were initiated by the addition of the cell extract.

2.10. Cysteine and pantothenate supplementation

Effects of nutrient supplementation on 1-butanol production were observed by the addition of 2 mM cysteine or 2 mM pantothenate. Precultures were grown aerobically in LB containing appropriate antibiotics at 37 °C overnight. These were used to inoculate fresh TB-2% glucose medium containing the respective nutrients to a final OD600 of 0.04. 1-Butanol production followed as described above.

3. Results and discussion

3.1. Metabolome analysis reveals a CoA imbalance

Previously, Shen et al. (2011) successfully engineered *E. coli* to produce 1-butanol with a titer of 15 g/L through deletion of NADH consuming pathways. This effectively created an NADH driving force that greatly enhanced production of 1-butanol in anaerobic conditions. Thus, the engineered *E. coli* strain (Δ*ldhA*, Δ*adhE*, Δ*frdBC*, Δ*pta*) expressing the modified heterologous clostridial 1-butanol pathway (*fdh*, *atoB*, *hbd*, *crt*, *ter* and *adhE*2) hereinafter called JCL299F was used

in this study as a platform for optimization of 1-butanol production (Fig. 1). Within JCL299F, *pta* deletion had the most significant impact on the improvement of 1-butanol titers. The *pta* gene, encoding a phosphate acetyltransferase, was deleted in an attempt to reduce the amount of acetate produced while simultaneously increasing acetyl-CoA pools. Increased acetyl-CoA availability is essential for driving the thermodynamically unfavorable condensation of two acetyl-CoA molecules into one acetoacetyl-molecule using the first enzyme in the 1-butanol pathway, acetyl-CoA acetyltransferase, AtoB (Fig. 1). Deletion of *pta* has previously been utilized for similar purposes (Shams Yazdani and Gonzalez, 2008; Shen et al., 2011; Vadali et al., 2004a, 2004b, 2004c).

Cross mark indicates that the pathway was blocked via gene deletion. The deleted genes encode for fumarate reductase (*frdBC*), lactate dehydrogenase (*ldhA*), alcohol dehydrogenase (*adhE*) and phosphate acetyltransferase (*pta*). PEP, phosphoenolpyruvate; *fdh*, formate dehydrogenase from *Candida boidinii*; *atoB*, acetyl-CoA acetyltransferase from *E. coli*; *hbd*, 3-hydroxybutanoyl-CoA dehydrogenase; *crt*, *crotonase*; *adhE2*, aldehyde/alcohol dehydrogenase from *Clostridium acetobutylicum, ter*, trans-enoyl-CoA reductase from *Treponema denticola*.

To gain insights into the resulting changes caused by *pta* deletion, metabolome analysis using ion pair LC/MS/MS was performed to determine and compare the intracellular metabolome profiles of JCL166F and JCL299F (JCL166F Δ*pta*) including central metabolites, amino acids, nucleotides and cofactors. A total of 78 metabolites were detected (Table S2) and the dataset was subjected to principle component analysis (PCA), an unsupervised multivariate analysis method. The resulting PCA score plot (Fig. 2A) showed that PC1, accounting for 50% of the total variance, separated between different genotypes, while PC2, accounting for 20% of the total variance, separated between replicates. Then, compounds contributing to the separation of samples were examined in the loading plot (Fig. 2B). PCA results revealed that within JCL299F, there was an accumulation of CoA-containing intermediates of the 1-butanol pathway such as acetyl-CoA, 3-hydroxybutanoyl-CoA and butanoyl-CoA. An overall decrease in free CoA was also observed in JCL299F (Fig. 2C). Additionally, extracellular metabolome analysis revealed a significant accumulation of pyruvate and butanoate as major by-products (2.0 g/L pyruvate and 1.8 g/L butanoate after 24 h of anaerobic fermentation) from JCL299F (Fig. 2D). This phenomenon was also observed when cultivating the strains in other media, indicating that pyruvate and butanoate accumulation is a major problem in JCL299F regardless of the media used in fermentation (Fig. S1).

Metabolome analysis points to several metabolic perturbations caused by *pta* deletion that may be a result of a CoA imbalance or insufficient CoA recycling. Elevated levels of intracellular CoA compounds and pathway intermediates support the notion that deletion of *pta* gene in the production strain prevents the release of a free CoA moiety that would otherwise be released during the production of acetate. Furthermore, the entrapment of CoA within the pathway may be the cause of pyruvate and butanoate accumulation throughout production. Pyruvate formate-lyase (Pfl) catalyzes the conversion of pyruvate and CoA into formate and acetyl-CoA. The lack of CoA recycling may be the reason for the insufficient rate of this reaction, as it is required for the formation of additional CoA containing compounds.

This is parallel to previous reports that carbon flux through the pyruvate node is greatly affected by redox and cofactor supply (Chang et al., 1999; Lim et al., 2013; Shen et al., 2011; Vadali et al., 2004a, 2004b, 2004c; Yang et al., 2001). Butanoate by-product formation, similar to acetate by-product formation, provides the release of a CoA molecule from the 1-butanol production pathway. This butanoate formation may be compensating for the lack of Pta function in releasing a CoA molecule thus, butanoate is one effect of inefficient CoA recycling. A similar CoA imbalance has been recently observed in cyanobacteria (Noguchi et al., 2016). Furthermore, elevated intracellular levels of butanoyl-CoA may also result in butanoate formation. Taken together, it is evident that a CoA imbalance may provide a target for further engineering to improve flux through the 1-butanol pathway.

As is evident from the metabolome analysis, we hypothesize that insufficient butanoyl-CoA reductase activity is resulting in suboptimal 1-butanol production. Thus, in order to prevent loss of extracellular metabolites as a result of inefficient CoA recycling, and to increase production of 1-butanol, butanoyl-CoA reductase activity must be optimized in JCL299F (Fig. 3A).

3.2. Fine-tuning of butanoyl-CoA reductase activity

In our initial attempt to improve the butanoyl-CoA reductase

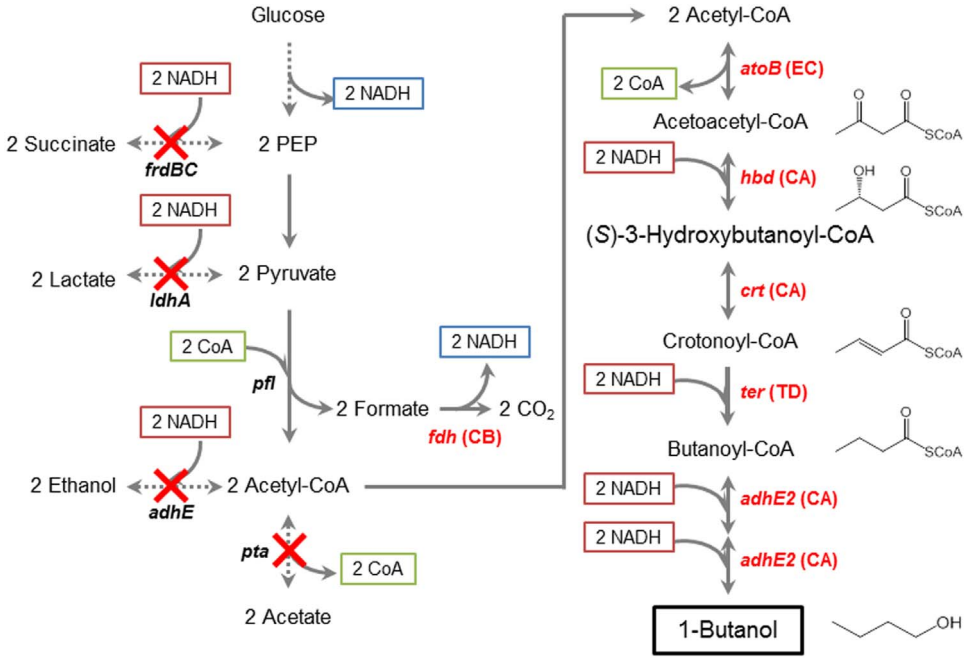

Fig. 1. Schematic of the modified clostridial CoA-dependent 1-butanol production pathway in *E. coli*.

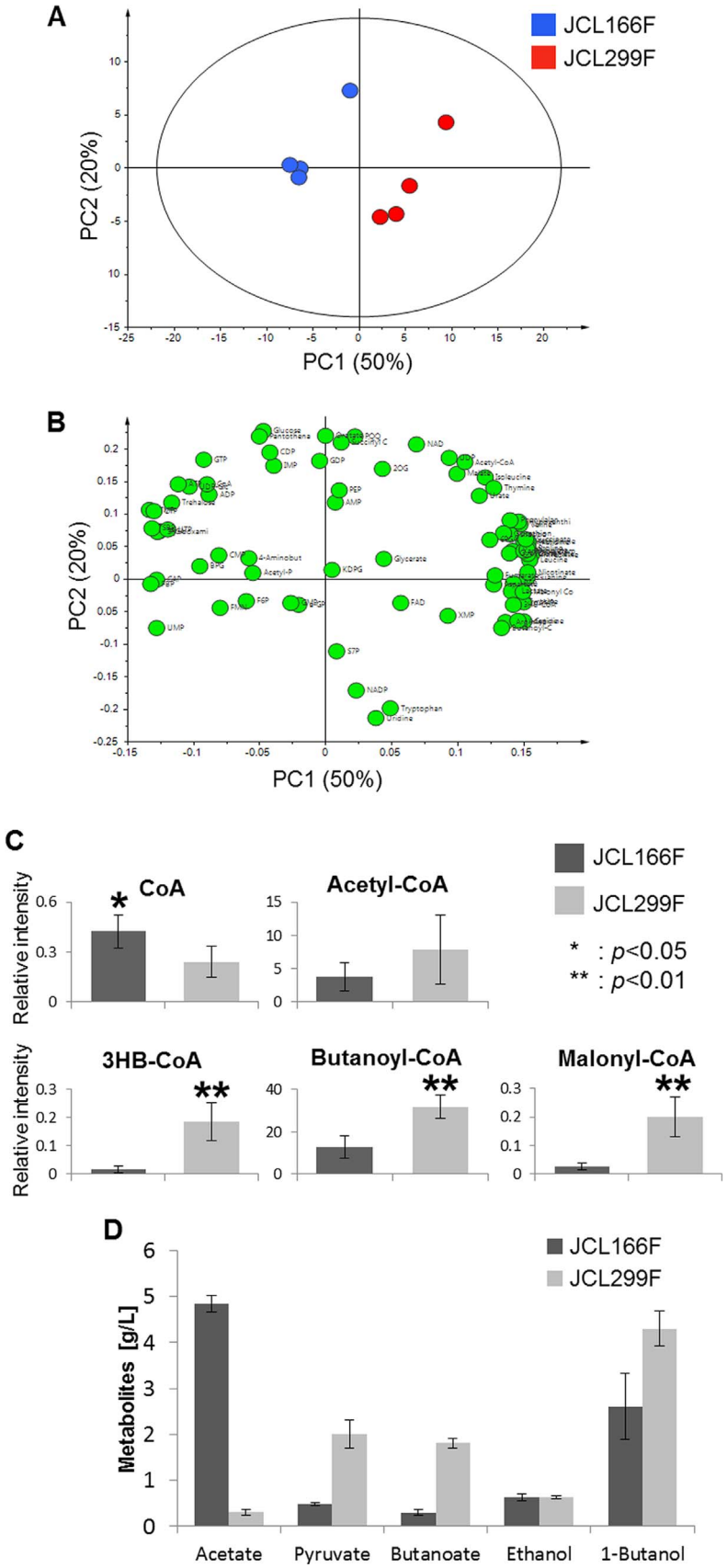

Fig. 2. (A) PCA score plot for metabolic profiling of JCL166F and JCL299F. The ellipse indicates the 95% confidence border based on Hotelling's T^2. (B) The corresponding loading plot illustrating metabolites that contributed to the separation on PC1 and PC2. (C) The result of intracellular CoA profiling. Metabolite intensities shown in the y-axis were normalized to an internal standard. Asterisks indicate significant difference between strains (*: $p < 0.05$, **: $p < 0.01$). The error bars indicate standard deviations obtained from four replications. 3HB-CoA, 3-hydroxybutanoyl-CoA. (D) Comparison of fermentation results between JCL166F and JCL299F. Samples were taken after 24 h of fermentation. The error bars indicate standard deviations obtained from four replications.

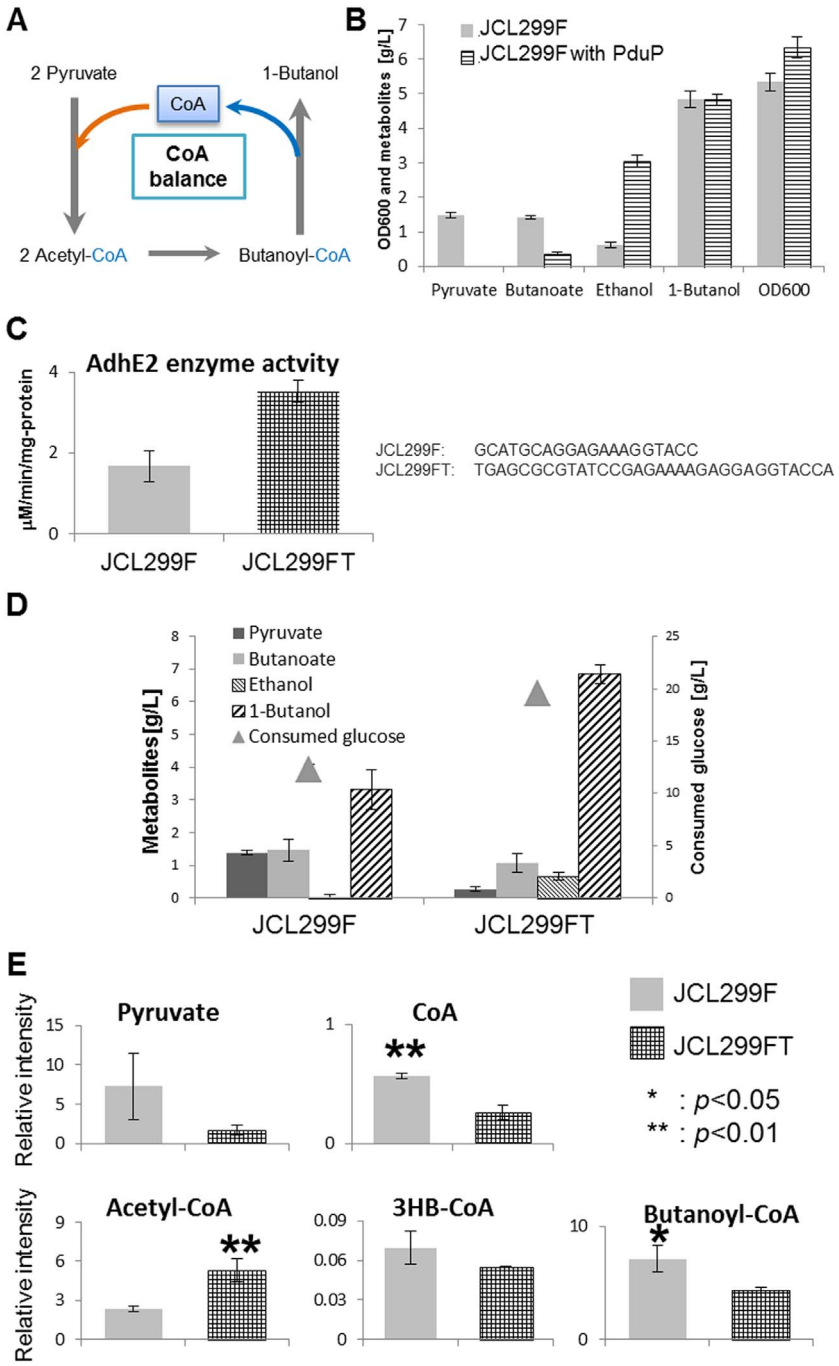

Fig. 3. (A) Strategy of CoA balancing for optimization of the 1-butanol pathway in *E. coli.* (B) Effect of PduP introduction on anaerobic fermentation. Samples were taken after 24 h of anaerobic fermentation. The error bars indicate standard deviations obtained from four replications. (C) The relative specific activity of AdhE2 within cell lysate with improved RBS sequence. (D) Comparison of fermentation results between JCL299F and JCL299FT. Samples were taken after 12 h of anaerobic fermentation in test tubes containing 3 mL of TB with 2.5% glucose medium. The error bars indicate standard deviations obtained from three replications. (E) The result of intracellular metabolites profiling. Metabolite intensities shown in the y-axis were normalized to an internal standard. Asterisks indicate significant difference between strains (*: $p < 0.05$, **: $p < 0.01$). The error bars indicate standard deviations obtained from four replications.

activity, we utilized a CoA-acylating aldehyde dehydrogenase (PduP) from *Salmonella enterica* that has previously exhibited a high catalytic activity for butanoyl-CoA (Lan et al., 2013). As anticipated, overexpression of PduP in addition to AdhE2 within JLC299F resulted in significant reduction of pyruvate and butanoate. However, as PduP also exhibits activity for acetyl-CoA, large amounts of ethanol were also produced (Fig. 3B).

We therefore attempted to optimize AdhE2 activity through the improvement of the RBS translation initiation rate. A library of 8 clones was generated using the Salis RBS Calculator v1.1 (Espah Borujeni et al., 2014; Salis et al., 2009) and transformed into JCL299F. With the Clarke Carbon formula, it is calculated that screening through 23 colonies is adequate to ensure that all variants have been screened at least once with 95.3% confidence. Out of 23 random colonies that were screened, 7 colonies produced more than 7 g/L of 1-butanol after 24 h, compared to only 3.9 g/L produced from the unmodified JCL299F strain (Fig. S2). These 7 strains were further characterized to isolate the clone that exhibited the highest level of 1-butanol production.

The plasmid contained within the new highest 1-butanol producer, JCL299FT, was sequenced and confirmed to contain a modified RBS

region (Fig. 3C). When the specific activity of the optimized butanoyl-CoA reductase was measured in the crude cell lysate of JCL299FT, it exhibited a 2.1 fold increase in AdhE2 activity compared to that of JCL299F (Fig. 3C). In addition to the increased 1-butanol production, pyruvate and butanoate formation were also decreased by 80% and 27%, respectively in JCL299FT (Fig. 3D). Metabolome analysis by ion pair LC/MS/MS also revealed that acetyl-CoA levels were subsequently increased, indicating improved flux through the pyruvate/acetyl-CoA junction (Fig. 3E). Together, these metabolic changes indicated that optimization of AdhE2 expression aided to alleviate the CoA imbalance created by *pta* deletion.

3.3. Supplementation to increase CoA supply

Although improving CoA recycling through the pathway increased 1-butanol titers, intracellular levels of free CoA were decreased in JCL299FT compared to JCL299F (Fig. 3E). Previously, strategies to increase overall free CoA supply in the cell have been shown to enhance the biochemical production of various CoA-derived compounds like succinate and isoamyl acetate (Lin et al., 2004; Vadali et al., 2004a, 2004b, 2004c). Therefore, overall CoA concentrations within the JCL299FT may be suboptimal and limiting for production of 1-butanol.

Several variables were tested in order to improve 1-butanol titers through augmentation of intracellular CoA pools (Fig S4). PanK, pantothenate kinase, was overexpressed in addition to the 1-butanol enzymes within JCL299FT. Pantothenate kinase has been identified as the rate limiting enzyme of CoA biosynthesis in *E.coli* (Jackowski and Rock, 1984; Vallari et al., 1987). Supplementation with pantothenate and L-cysteine were also tested as they are precursors to CoA (Leonardi et al., 2005). The effect of supplementation and PanK overexpression can be clearly seen after 48 h of cultivation. Cysteine supplementation enhanced 1-butanol titers by 32% after 48 h, while pantothenate supplementation and PanK overexpression did not. Since JCL299FT is able to consume almost all glucose before 24 h, the observed increase only became apparent after an additional glucose feeding and pH adjustment at 24 h. Intracellular CoA was measured within these strains (Fig S3). Pantothenate conferred the greatest increases in CoA concentrations, but it did not result in a significant increase in 1-butanol titers. Cysteine supplementation only resulted in slightly increased measurements of intracellular CoA. However, its use in aiding protein expression may also be another factor that contributed to the improved titers (Fig S3).

Production was further optimized with more frequent pH adjustments and glucose feedings. In combination with optimized AdhE2 expression and cysteine supplementation, titers of 18.3 g/L 1-butanol were reached after 78 h of fermentation (Fig. 4A). This represents 76% of the theoretical maximum.

4. Discussion

In the previous work, anaerobic production of 1-butanol was enabled by establishing an NADH driving force, which relied on the production of 1-butanol as the sole electron sink for fermentation. Key to the high titer 1-butanol production of JCL299F was a *pta* deletion, which blocked degradation of acetyl-CoA into acetate. Simultaneously, metabolome analysis found that it caused a pathway imbalance by preventing release of CoA and identified the rate-limiting AdhE2 reaction (reduction of butanoyl-CoA to butanal) as a target for strain improvement. Using an RBS library, the activity of AdhE2 was optimized for 1-butanol production while simultaneously relieving by-product formation of pyruvate and butanoate.

1-Butanol titers from JCL299FT after 78 h of anaerobic fermentation measured 18.3 g/L with the addition of cysteine. This is a 33% improvement over the base strain JCL299F. Cysteine was supplemented in an attempt to improve intracellular CoA supply. While pools were slightly increased, there may be ancillary effects due to this supplementation that contribute to the improved titers. This includes improved expression of the exogenous 1-butanol enzymes. Increases in titer are unlikely caused by utilization of cysteine as a carbon source for production as its conversion to acetyl-CoA only yields one molecule of formate. This means that even if formate is converted to CO_2 and NADH at 100% efficiency, then these reducing equivalents will only suffice to produce a maximum of 37 mg/L of 1-butanol from all of the cysteine provided.

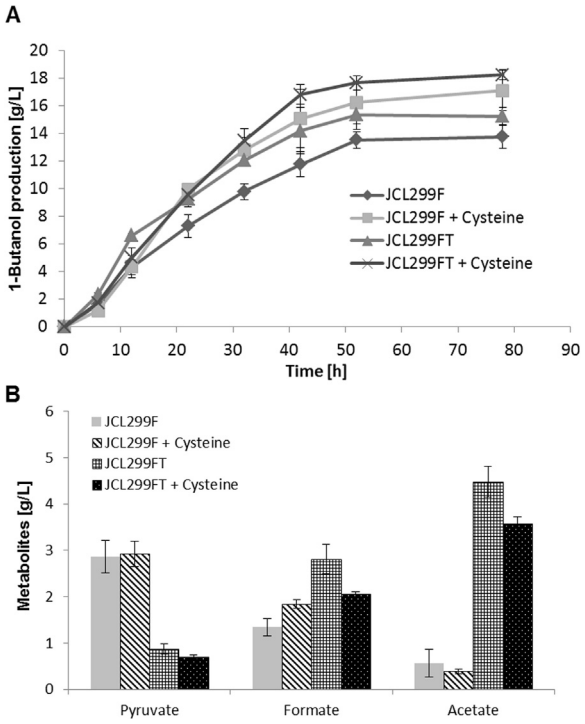

Fig. 4. (A) Time course experiment on 1-butanol production. 1% Glucose was fed to the cultures at 6, 12, 22, 32, 42 h and pH was adjusted to around 7 at 6, 12, 22, 32, 42, 52 h. The error bars indicate standard deviations obtained from three replications. "Time" indicates time since anaerobic switch. (B) Extracellular product formation at 78 h of anaerobic fermentation.

After 78 h within anaerobic fermentation conditions, pyruvate levels were not affected by cysteine supplementation in both strains. This observation suggests that the inefficient conversion of pyruvate to acetyl-CoA in JCL299F is caused by poor CoA recycling rather than simply low concentrations of CoA. Maximizing intracellular concentrations of acetyl-CoA is essential for driving the thermodynamically unfavorable ($\Delta G = 26$ kJ/mol) condensation of two acetyl-CoA molecules into one acetoacetyl-CoA molecule. Previous works have demonstrated this concept through deletion of *pta*, encoding for phosphate acetyltransferase, which prevented carbon leakage from acetyl-CoA to acetate (Shen et al., 2011). This improved 1-butanol production dramatically, and may explain why higher 1-butanol titers are observed through efficient CoA recycling. Similarly, increased acetyl-CoA pools may also be responsible for the observed acetate by-product formation. While *pta* deletion was shown to decrease acetate formation, EutD remains as an isozyme of Pta. Deletion of *eutD,* or other means of rerouting carbon flux from acetate towards 1-butanol production, serves as a potential target for further engineering. Another approach to further increase the production titer of 1-butanol may be to increase the tolerance of *E. coli* towards 1-butanol as the highest production titer exceeds the toxicity level of 10 g/L (Atsumi et al., 2010).

In this work, a titer of 18.3 g/L of 1-butanol and a yield of 76% of the theoretical maximum was reached after 78 h of fermentation (Fig. 4A). Other works have aimed at improving 1-butanol production within *E. coli* utilizing a number of other strategies. In one example, a self-regulated strain of *E. coli* was engineered to have capabilities of

adjusting the expression of fermentation pathways to maximize 1-butanol production (Wen and Shen, 2016). A separate study adjusted the intracellular redox state by targeting central carbon metabolism to push flux of carbon through the pentose phosphate pathway (Saini et al., 2016). Additionally, other strains were engineered to produce 1-butanol from butanoate (Saini et al., 2015). 1-Butanol titers from *E. coli* and *Clostridium* species are comparable. Recently reported engineered *Clostridium* species utilizing acetone-butanol-ethanol fermentation have been able to produce 19.1 g/L within 78 h (Xue et al., 2012), or 15.7 g/L using co-utilization of glucose and xylose (Yu et al., 2015). The titer reported here is much higher compared to other non-native hosts such as in *Saccharomyces cerevisiae* (835 mg/L in 96 h) (Shi et al., 2016), cyanobacterium *Synechococcus elongatus* PCC 7942 (404 mg/L in 12 days) (Lan et al., 2013) and *Lactobacillus brevis* (300 mg/L in 60 h) (Berezina et al., 2010). The efficient genetic tractability of *E. coli* allows for more complex genetic modifications than other *Clostridium* species, and therefore *E.coli* has potential to be further engineered for higher 1-butanol production.

5. Conclusions

Metabolomics, the comprehensive profiling of metabolites in the biological sample, allows the detection of complex biological changes using chemometrics. Metabolomics has been proven to strongly complement other “omics” and has essential applications in various fields such as functional genomics, medical science and food science. In metabolic engineering, metabolomics can be employed to search for rate-limiting steps and detect metabolic changes in samples such as metabolite toxicity and reaction/cofactor imbalance.

In this study, we have successfully used metabolomics in finding the bottleneck for 1-butanol production in engineered *E. coli*. This has proven to be useful in guiding metabolic engineering strategies for strain improvement and eventually increasing the 1-butanol production titer.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2017.04.003.

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