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Engineering *Escherichia coli* for an efficient aerobic fermentation platform

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ABSTRACT

Acetate, as a major by-product, was excreted by *Escherichia coli* when aerobic fermentation runs at high growth rates. In order to reduce the acetate secretion during the fermentation fundamentally, a list of genes related to acetate accumulation in *E. coli* was selected and knocked out. Physiological characterization of each mutant demonstrated that the growth and metabolites accumulation properties of these mutations exhibited significant change upon pathway engineering. The final engineered *E. coli* QZ1110 with *ptsG*, *poxB*, *pta* and *iclR* gene mutations was confirmed to accumulate 270% more biomass with 90% less acetate secretion than that of wild type *E. coli* in LB medium supplied with 1% glucose. Polyhydroxybutyrate biosynthesis experiment showed that the acetate reduction of the engineered strain in minimal medium also reduced 90% while the PHB accumulation increased almost 100% compare to wild type *E. coli*.

1. Introduction

Escherichia coli is the most well known microorganism for biosynthetic pathway engineering in synthetic biology (Keasling, 2008). It has also been widely applied in industrial biotechnology for proteins and biochemicals production. Aerobic high cell density cultivation of *E. coli* was frequently used to arrive at high biomass yields and high metabolite/protein concentrations (De Mey et al., 2007). However, *E. coli* excretes acetate as a major by-product of its aerobic metabolism and acetate production represents a diversion of carbon that might otherwise have generated biomass or protein product (Andersen and von Meyenburg, 1980; March et al., 2002). Meanwhile, acetate was known to reduce the rate of RNA, DNA, protein and lipid synthesis (Cherrington et al., 1990), particularly those involved in the *E. coli* transcription—translation machinery, the general stress response and regulation. Additionally, acetate interferes with methionine biosynthesis, which causes the inhibitor homocysteine to accumulate (Roe et al., 2002). It is commonly observed that *E. coli* excretes 10–30% of carbon flux from glucose to acetate in glucose-containing media even when the culture is fully aerated.

Acetate is produced under an imbalance between the glycolytic and the TCA cycle fluxes (Farmer and Liao, 1997; Majewski and Domach, 1990; Wong et al., 2008). To solve this problem, many methods have been developed through controlling the glucose concentration in the fed-batch fermentation process (Kleman et al., 1991a,b; Paalme et al., 1990; Shiloach et al., 1996). Although most of these approaches have reduced acetate production, they undermine maximum growth and production capacity, or lead to undesirable pyruvate accumulation (Diaz-Ricci et al., 1991). Thus, a number of attempts with metabolic engineering to reduce carbon flow to acetate-producing pathways have been tried (Aristidou et al., 1994; Chou et al., 1994; Delgado and Liao, 1997; Farmer and Liao, 1997; Hosono et al., 1995; San et al., 1994). In *E. coli*, the two major aerobically active acetate-producing pathways are phosphotransacetylase/acetate kinase (Pta–AckA) and pyruvate oxidase (PoxB). In the former pathway, acetate is produced from acetyl coenzyme A via acetyl phosphate by the Pta–AckA; while in the later pathway, PoxB converts pyruvate, ubiquinone and H_2O to acetate, ubiquinol and CO_2 (De Mey et al., 2007). Dittrich et al. recently confirmed that the PoxB pathway is more active in *E. coli* during the late exponential and stationary phases, whereas the Pta–AckA pathway is more active in the exponential stage of the cell growth (Dittrich et al., 2005). However, most of these genetic modifications were only cloning or deleting one or two genes.

In the present study, based on the analysis of available information (March et al., 2002; Farmer and Liao, 1997; Dittrich et al., 2005; De Mey et al., 2007), a list of genes *ptsG*, *poxB*, *pta* and *iclR* in *E. coli* related to acetate secretion were selected and knocked out. The growth and physiological properties of these mutants were characterized. As an example, the production of polyhydroxybutyrate was performed.

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Table 1
Strains and plasmids used in this study.

Strains and plasmids	Genotype	Reference or source
Strains		
E. coli MG1655	*rph*$^{-1}$ *fnr*	Lab stock
E. coli DH5α	F–Φ80 *lacZ*Δ*M15 recA endA1*Δ(*lacZYA-argF*) U169 *deoR gyrA*96 *thi-I hsdR*17 *supE*44 *relAI*	Invitrogen
LR1010	MG1655(*ΔptsG*)	Li et al. (2007)
QZ1011	MG1655(*ΔptsGΔpoxB*)	This study
QZ1100	MG1655(*ΔptsGΔpoxBΔpta*)	This study
QZ1110	MG1655(*ΔptsGΔpoxBΔptaΔiclR*)	This study
QZ2000	MG1655 harboring plasmid pHBS01	This study
QZ2001	QZ1110 harboring plasmid pHBS01	This study
Plasmids		
pKD3	oriR6Kγ, Cm^R, rgnB(Ter)	CGSC (Datsenko and Wanner, 2000)
pKD4	oriR6Kγ, km^R, rgnB(Ter)	CGSC (Datsenko and Wanner, 2000)
pKD46	araBp-gam-bet-exo, bla(Ap^R), repA101(ts), oriR101	CGSC (Datsenko and Wanner, 2000)
pCP20	Ap^R,Cm^R, FLP recombinance	CGSC (Datsenko and Wanner, 2000)
pCL1920	spc^R, p_{lac}, low copy number	Lerner and Inouye (1990)
pBHR68	pBluescript SK$^-$, *phbCAB* operon from *R. eutropha*	Spiekermann et al. (1999)
pSCP	Spc^R, P_{rpoS}, low copy number	This study
pHBS01	Spc^R, P_{rpoS}, *phbCAB* operon	This study

2. Materials and methods

2.1. Genetic methods

E. coli strains, plasmids and oligonucleotides used in this study were summarized in Tables 1 and 2. Molecular cloning and manipulation of plasmids were done with *E. coli* DH5α.

Plasmid pSCP derived from pCL1920, in which *lac* promoter was replaced by a stress-induced promoter (Kang et al., 2008). After amplification and double enzyme *BamHI* and *ApaI* digestion, the purified fragments *ApaI*-spc-pSC101ori-*BamHI* and *BamHI*-SIR-*ApaI* were linked by T4 ligase and the new plasmid was named as pSCP. The *phbCAB* operon which encodes PHB synthase (PhbC), β-ketothiolase (PhbA) and acetoacetyl-CoA reductase (PhbB), respectively, was amplified from plasmid pBHR68 by PCR and subcloned to pSCP to generate plasmid pHBS01. Plasmid pHBS01 was transformed into *E. coli* MG1655 and *E. coli* QZ1110. Transformants were designated *E. coli* QZ2000 and *E. coli* QZ2001, respectively.

E. coli mutants were created using the one-step inactivation method with some modification (Datsenko and Wanner, 2000). This method includes following steps: amplification of the resistance gene with polymerase chain reaction (PCR) using pKD4 or pKD3 as template. PCR products flanked by FRT (FLP recognition target) sites and homologous sequences to the gene of interest were purified and transformed into the cells by electroporation (Bio-Rad, Gene Pulser). The knocking out of the target gene in the cells which carried with plasmid pKD46 that expresses λ Red recombinase was happened by recombining the PCR product into the chromosome. Transformants were selected with antibiotic-resistance plate. The kanamycin or chloramphenicol cassette was removed with the helper plasmid pCP20 that expresses FLP before next mutation. All other genetic operations were performed according to the protocols provided by the manufactures.

2.2. Medium and growth conditions

Cultivation of *E. coli* was performed in LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl, pH 7.2). Antibiotic, such as chloramphenicol (25 μg mL^{-1}), kanamycin (25 μg mL^{-1}), Spectinomycin (25 μg mL^{-1}) or ampicillin (100 μg mL^{-1}), was added when necessary. For characterization of the engineered *E. coli*, 1% (w/v) glucose was added. Flask cultivations were carried out in 300 mL Erlenmeyer flask supplied with 50 mL LB medium at 37 °C at an agitation of 250 rpm. pH controlled cultivation was performed in 1 L bioreactor using 2 M NaOH as neutralization agent.

M9 mineral salts medium (Sambrook et al., 1989) was used to characterize the performance of the engineered *E. coli*. Glucose as the sole carbon source was added at the indicated amount. PHB fermentation was performed in 5 L fermentor. The inlet airflow was 0.6 L min^{-1} while the dissolved oxygen was maintained above 50% saturation throughout the experiment by changing the stirrer speed.

2.3. Analytical methods

Bacterial growth was monitored by measuring the optical density (OD) at 600 nm, the culture was diluted to the linear range with 0.15N NaCl.

For analyzing the substrate and the extracellular metabolite concentrations, 1 mL of culture was centrifuged at 12,000 × *g* for 5 min

Table 2
Oligonucleotides used in this study.

Primer	Sequence
pKD-poxB F	5′-AAACTTGTTACCGTTATCACATTCAGGAGATGGAGAACCGTGTAGGCTGGAGCTGCTTC-3′
pKD-poxB R	5′-CATGGCATGTCCTTATTATGACGGGAAATGCCACCCTTTATGGGAATTAGCCATGGTCC-3′
poxB test F	5′-TCCCCCTCCGTCAGATGA-3′
poxB test R	5′-GGTATCACTGCGTAAATCAA-3′
pKD-pta F	5′-GTAACCCGCCAAATCGGCGGTAACGAAAGAGGATAAACCGTGTAGGCTGGAGCTGCTTC-3′
pKD-pta R	5′-TCAGATATCCGCAGCGCAAAGCTGCGGATGATGACGAGAATGGGAATTAGCCATGGTCC-3′
Pta test F	5′-TCAGCTGGCGGTGCTGTTT-3′
Pta test R	5′-ACCGGAAATAGTGATTATTTCCGG-3′
pKD-iclR F	5′-ATGAAAATGATTTCCACGATACAGAAAAAAGAGACTGTCGTGTAGGCTGGAGCTGCTTC-3′
pKD-iclR R	5′-TATGATGGGCAGAATATTGCCTCTGCCCGCCAGAAAAAGATGGGAATTAGCCATGGTCC-3′
iclR test F	5′-TAAAAGCGACCACCACG-3′
iclR test R	5′-GCGATTAACAGACACCCT-3′
pSCP F	5′-TTTGGATCCCGACAGTAAGACGGGTAAGCCTGTTGATGAT-3′
pSCP R	5′-ACTGGGCCCGAGCTCCTTGAACGAATTGTTAGACATTATTTG-3′
SIR F	5′-AAAGGATCCGCCTGCACAAAATTCCACCGTTGCTG-3′
SIR R	5′-TTTGGGCCCCCCCTCGAGGTCGACGGTAT-3′

Annotation: the underlined bases meant enzyme site.