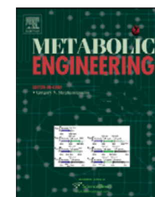
Metabolic Engineering 11 (2009) 192–198

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben



Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering

Wenjuan Zha [a], Sheryl B. Rubin-Pitel [a], Zengyi Shao [a], Huimin Zhao [a,b,*]

[a] *Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*
[b] *Departments of Chemistry, Biochemistry, and Bioengineering, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*

ARTICLE INFO

Article history:
Received 16 November 2008
Received in revised form
28 January 2009
Accepted 28 January 2009
Available online 5 February 2009

Keywords:
Malonyl-CoA
Metabolic engineering
Polyketides
Flavonoids
Type III polyketide synthases

ABSTRACT

Escherichia coli only maintains a small amount of cellular malonyl-CoA, impeding its utility for overproducing natural products such as polyketides and flavonoids. Here, we report the use of various metabolic engineering strategies to redirect the carbon flux inside *E. coli* to pathways responsible for the generation of malonyl-CoA. Overexpression of acetyl-CoA carboxylase (Acc) resulted in 3-fold increase in cellular malonyl-CoA concentration. More importantly, overexpression of Acc showed a synergistic effect with increased acetyl-CoA availability, which was achieved by deletion of competing pathways leading to the byproducts acetate and ethanol as well as overexpression of an acetate assimilation enzyme. These engineering efforts led to the creation of an *E. coli* strain with 15-fold elevated cellular malonyl-CoA level. To demonstrate its utility, this engineered *E. coli* strain was used to produce an important polyketide, phloroglucinol, and showed near 4-fold higher titer compared with wild-type *E. coli*, despite the toxicity of phloroglucinol to cell growth. This engineered *E. coli* strain with elevated cellular malonyl-CoA level should be highly useful for improved production of important natural products where the cellular malonyl-CoA level is rate-limiting.

© 2009 Elsevier Inc. All rights reserved.

1. Introduction

Malonyl-CoA is the major building block for natural products such as fatty acids, polyketides, and flavonoids, which have significant applications in medicine (antibacterials, antifungals, anticancers, and immunosuppressants), veterinary medicine (antihelmintics), and agriculture (insecticides), and potential as a source of alternative energy (microdiesel) (Dixon and Steele, 1999; Edwards et al., 1990; Forkmann and Martens, 2001; Hopwood, 1997; Jang, 1997; Kalscheuer et al., 2006; Katz and Donadia, 1993; Keating and Walsh, 1999; Khosla et al., 1999). The native producers of many natural products are of limited utility for large-scale production, because they often grow slowly and are difficult to manipulate genetically. On the other hand, *Escherichia coli* has become an attractive host for natural product manufacture, owing to its genetic tractability and favorable fermentation properties. In fact, *E. coli* has been engineered to produce the fungal polyketide 6-methylsalicylic acid (6-MSA) (Kealey et al., 1998) and the complex erythromycin precursor polyketide, 6-deoxyerythronolide B (6dEB) (Pfeifer et al., 2001). However, it has been shown that *E. coli* possesses a very low level of cellular malonyl-CoA (Takamura and Nomura, 1988), which can be a potential barrier to the wide utilization of this host for large-scale manufacturing of important polyketides. Therefore, it is desirable to create an *E. coli* strain with improved cellular malonyl-CoA level.

In the central metabolism of *E. coli* (Fig. 1), a carbon source, such as glucose, is consumed through a series of enzymatic reactions to form pyruvate. Subsequently, pyruvate is oxidatively decarboxylated by the pyruvate dehydrogenase complex to produce CO_2 and acetyl-CoA. While the majority of acetyl-CoA enters the tricarboxylic acid (TCA) cycle, a small portion participates in fatty acid biosynthesis, where acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (Acc). In *E. coli*, intracellular malonyl-CoA concentration is tightly regulated to be very low, so as to coordinate the rate of fatty acid biosynthesis with phospholipid production, macromolecule synthesis, and cell growth (for review, see Magnuson et al., 1993).

Metabolic engineering has emerged as a powerful tool to improve productivity by genetic manipulation of multistep catalytic systems involved in cell metabolism. In recent years, application of metabolic engineering has blossomed in both academia and industry (Aldor and Keasling, 2003; Stephanopoulos, 1999). Most metabolic engineering studies have focused on manipulating enzyme levels through the amplification, addition, or deletion of particular pathways. For example, these strategies were successfully applied to increase the conversion of glucose to

* Corresponding author at: Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.
Fax: +1 217 333 5052.
E-mail address: zhao5@illinois.edu (H. Zhao).

1096-7176/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2009.01.005

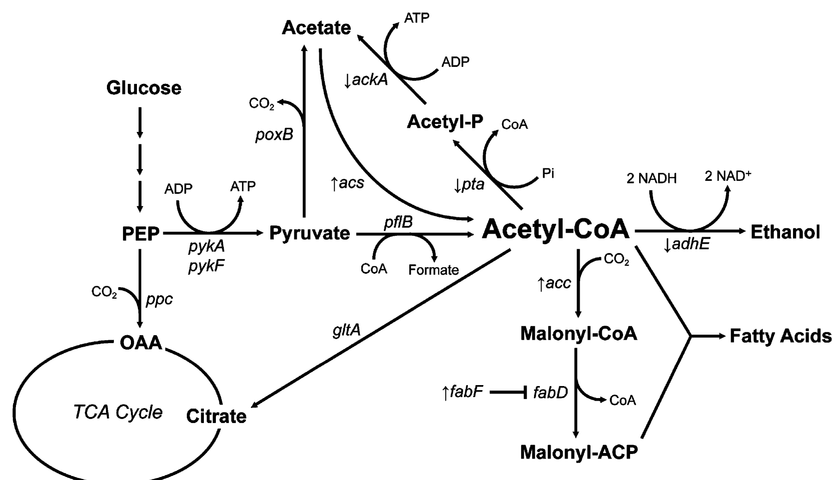


Fig. 1. Scheme of central metabolism in *E. coli*. Genes knocked out (↓) and overexpressed (↑) are noted.

pyruvate in *E. coli* (Causey et al., 2004). The concentration of acetyl-CoA inside *E. coli* was increased by engineering pathways that consume or produce acetyl-CoA (Lin et al., 2004; Vadali et al., 2004a, b), and this strategy has also been applied to *Saccharomyces cerevisiae* (Shiba et al., 2007). In particular, the *E. coli* intracellular malonyl-CoA concentration was enhanced by overexpression of a key enzyme, acetyl-CoA carboxylase, leading to improved production of the important polyketides (2S)-flavanones (Miyahisa et al., 2005) and flavonoid (Leonard et al., 2007) as well as fatty acids (Lu et al., 2008). Similarly, overexpression of the endogenous acetyl-CoA carboxylase in *S. cerevisiae* enhanced production of 6-MSA (Wattanachaisaereekul et al., 2008). Nevertheless, there has been no effort to evaluate the combination of various distinct metabolic engineering strategies to achieve greater improvement in cellular malonyl-CoA level.

In this study, we applied various rational modification strategies to cellular pathways to redirect carbon flux toward malonyl-CoA, and investigated their synergism on improving malonyl-CoA level in *E. coli*. These strategies include overexpression of Acc, inactivation of malonyl-CoA-consuming fatty acid synthesis, overexpression of acetyl-CoA synthase (Acs), an enzyme involved in the acetate assimilation pathway, and knockout of the competing pathways for acetate and ethanol synthesis. Finally, we applied some of our engineered *E. coli* strains for the production of an important polyketide, phloroglucinol, which is a core precursor to the synthesis of various high-value bioactive compounds and energetic compounds. Previously, we have shown that phloroglucinol is produced by the type III polyketide synthase PhlD from *Pseudomonas fluorescens* via condensation of three molecules of malonyl-CoA (Achkar et al., 2005b; Zha et al., 2006). Here, we overexpressed PhlD in selected metabolically engineered *E. coli* strains and observed an increase in phloroglucinol production.

2. Materials and methods

2.1. Materials

Luria Broth (LB) supplemented with 2% glucose was used throughout as the medium for malonyl-CoA overproduction. Various combinations of ampicillin, kanamycin, and chloramphenicol were added in cultures of plasmid-bearing *E. coli*. The pACYCDuet-1 and pRSFDuet-1 expression vectors and *E. coli* BL21(DE3) strain were obtained from Novagen (Madison, WI, USA). *Phusion* DNA polymerase, T4 DNA ligase, and restriction endonucleases were purchased from New England Biolabs (Beverly, MA, USA). QIAprep Spin Plasmid Miniprep Kit, QIAquick Gel Extraction Kit, and QIAquick PCR Purification Kit were obtained from Qiagen (Valencia, CA, USA). Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). CoASH, acetyl-, butyryl-, and malonyl-CoAs were purchased from Sigma (St. Louis, MO, USA). All the other reagents unless specified were obtained from Sigma–Aldrich.

2.2. Construction of plasmids

The plasmids constructed and used in this study are listed in Table 1. The *fabF* gene was PCR-amplified from the *E. coli* K-12 genome, and cloned into pACYCDuet-1 between the EcoRV and KpnI sites, creating plasmid pACYC-FabF. Likewise, the *E. coli acs* gene was PCR-amplified and cloned into pACYCDuet-1 and pACYC-FabF between the NcoI and HindIII sites, resulting in plasmids pACYC-Acs and pACYC-FabF/Acs, respectively. The *P. fluorescens* Pf-5 *phlD* gene was cloned into pACYC between NcoI and KpnI to create plasmid pACYC-PhlD. The *phlD* gene was also cloned into pACYC-Acs between the NdeI and KpnI sites, creating plasmid pACYC-Acs/PhlD. Plasmid pRSF-Acc was kindly provided by Sueharu Horinouchi (Department of Biotechnology, University of Tokyo, Japan).

2.3. Construction of knockout strains

Strains used in this work are listed in Table 1. Deletion of *E. coli* chromosomal genes, *ackA-pta* or *adhE*, was carried out by the Red recombinase method (Datsenko and Wanner, 2000). Briefly, the *ackA-pta* or *adhE* gene was PCR-amplified from the *E. coli* genome and cloned into pKD46. Integration of linear DNA was facilitated by an arabinose-inducible Red recombinase carried in pKD46 (temperature conditional). Integrants were selected for kanamycin resistance (10 mg/L). FRT-flanked antibiotic resistance genes used for selection were deleted by using a temperature-conditional plasmid pCP20 expressing FLP recombinase from a thermo-inducible promoter. The double knockout strain (Δ*ackA-pta*+Δ*adhE*) was created by the standard P1 transduction method (Miller, 1992). At each step, mutations were verified by analyses of PCR products.

2.4. Culture conditions

For intracellular CoA-ester pool analysis, inoculants of wild type or engineered *E. coli* strains were grown in LB at 37 °C

Table 1
Plasmids and strains used in this study.

Plasmid or strain	Relevant properties	Reference(s)
Plasmids		
pACYCDuet-1	P15A(pACYC184), Cm^r	Novagen
pRSFDuet-1	RSF1030, Kan^r	Novagen
pACYC-FabF	pACYCDuet-1 carrying *fabF* from *E. coli* K-12	This study
pACYC-Acs	pACYCDuet-1 carrying *acs* from *E. coli* K-12	This study
pACYC-FabF/Acs	pACYCDuet-1 carrying *fabF* and *acs* from *E. coli* K-12	This study
pACYC-PhlD	pACYCDuet-1 carrying *phlD* from *P. fluorescens*	
pACYC-PhlD/Acs	pACYCDuet-1 carrying *phlD* from *P. fluorescens* and *acs* from *E. coli* K-12	This study
pRSF-Acc	pRSFDuet-1 carrying *accAB* from *C. glutamicum*	Miyahisa et al., 2005
Strains		
E. coli BL21(DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen
E. coli K-12	Wild-type strain	ATCC
BWZ1	*ackA-pta* deletion mutant of BL21(DE3)	This study
BWZ2	*adhE* deletion mutant of BL21(DE3)	This study
BWZ3	*ackA-pta* and *adhE* deletion mutant of BL21(DE3)	This study
B-Acc	BL21(DE3) carrying pRSF-Acc6	This study
B-FabF	BL21(DE3) carrying pACYC-FabF	This study
B-Acc/FabF	BL21(DE3) carrying pRSF-Acc and pACYC-FabF	This study
B-Acs/Acc	BL21(DE3) carrying pACYC-Acs and pRSF-Acc	This study
B-Acs/FabF	BL21(DE3) carrying pACYC-FabF/Acs	This study
BWZ1-Acc	BL21(DE3, Δ*ackA-pta*) carrying pRSF-Acc	This study
BWZ1-FabF	BL21(DE3, Δ*ackA-pta*) carrying pACYC-FabF	This study
BWZ1-Acs/Acc	BL21(DE3, Δ*ackA-pta*) carrying pACYC-Acs and pRSF-Acc	This study
BWZ1-Acs/FabF	BL21(DE3, Δ*ackA-pta*) carrying pACYC-FabF/Acs	This study
BWZ3-Acs/Acc	BL21(DE3, Δ*ackA-pta*, Δ*adhE*) carrying pACYC-Acs and pRSF-Acc	This study
BWZ3-Acs/FabF	BL21(DE3, Δ*ackA-pta*, Δ*adhE*) carrying pACYC-FabF/Acs	This study

overnight. A small portion of this culture was then diluted 1:100 in fresh LB medium containing 2% glucose, and continued growing at 37 °C until $OD_{600} = 1.0$–1.2. Then, IPTG was added to a final concentration of 1 mM, and protein expression was induced at 25 °C for 5 h. At the end of induction, aliquots of the culture were taken, and subjected to further intracellular CoA-esters analysis. The production of phloroglucinol in wild-type or engineered *E. coli* strains was carried out as described elsewhere with some modifications (Achkar et al., 2005). Specifically, cultures were grown to an $OD_{600} = 0.8$ and induced in a final volume of 2 mL. Samples were removed for analysis 24 and 48 h after induction. Phloroglucinol was quantified in the cell-free supernatant by colorimetric assay (Zha et al., 2008) and normalized against the optical density measured at 600 nm.

2.5. Extraction of intracellular CoA-esters

An aliquot of 0.5 mL cell culture was removed, chilled immediately on ice, and centrifuged at 5000 rpm, 4 °C for 10 min. The cell pellet was resuspended in 1 mL of 6% perchloric acid to facilitate cell lysis. Then, 0.3 mL of 3 M potassium carbonate was added while vortexing to neutralize the acid. The solution was centrifuged to pellet the cell debris. The supernatant was collected and stored chilled until analysis of CoA compounds using LC–ESI–MS/MS (described below). To determine the dry cell weight, 2 mL of the same culture was filtered through a 0.45 μm cellulose membrane (Millipore, Billerica, MA, USA), followed by washing with distilled water. The membranes with cell residue were dried in a conventional oven. The weight difference between empty membranes and those with cell residues represented dry cell weight.

2.6. LC–ESI–MS/MS analysis of CoA-esters

The analysis of intracellular CoA-esters extracted from cultures of *E. coli* strains was performed using an Agilent Quattro *micro* MS interface consisting of a separation module connected directly to a Micromass Quattro *micro* MS. A $150 \times 4.6\,mm^2$ C18 reverse-phase HPLC column (Agilent Technologies, Santa Clara, CA, USA) was used to perform HPLC separation. The analytes were eluted at a flow rate of 300 μL/min with a gradient of 15 mM ammonium formate (A) and 10% 10 mM ammonium acetate in methanol (v/v) (B) at 25% B for the first 5 min, to 100% B in 10 min. The column effluent was directed to an MS instrument, equipped with an ESI ion source, which was operated in the negative ion mode. The instrument was tuned by the direct infusion of a solution of CoA (100 μM) in the ion source to optimize the parameters of the ESI–MS/MS system, first based on the maximum generation of protonated molecular ions (parents) and then on those of the corresponding fragment ions (daughters) for the CoA-esters of interest. Quantification was done in the multiple reaction monitoring (MRM) mode in MS/MS using the mass ions set to detect transitions of the parent ion to the daughter ion specific to the selected analytes. The transitions (m/z parent → m/z daughter) for the four CoA-esters of our interest were as follows: (1) malonyl-CoA, 852.5 → 808.5; (2) CoA, 766.5 → 419.2; (3) acetyl-CoA, 808.6 → 461.1; and (4) butyryl-CoA, 836.5 → 489.2.

3. Results and discussion

3.1. Quantification of intracellular CoA-ester concentration in E. coli

Numerous methods have been reported in the literature to determine the concentrations of intracellular CoA-esters in biological systems, including plants, animal tissues, and a variety of microbes. These methods relied on either the enzymatic assays of CoA moiety after hydrolysis (Knudsen et al., 1994) or the separation and detection of CoA-esters by HPLC equipped with an ultraviolet detector (Pogolotti and Santi, 1982). However, they generally suffer from poor selectivity or low sensitivity. Such drawbacks hinder their application in quantification of CoA-esters from microbes, where CoA-esters are present at low

concentrations in an extremely intricate sample matrix. Recently, a combined usage of LC with electrospray ionization tandem mass spectrometry (ESI–MS/MS) was reported for analysis of intracellular short organic CoA-esters from *Streptomyces venezuelae* (Park et al., 2007). Using HPLC to separate analytes and further relying on tandem MS/MS data to distinguish analytes based on their molecular weight as well as the fragmented mass profiles that are characteristic or typical of each analyte, the LC–ESI–MS/MS method provides easy separation and detection of individual CoA-ester from the complex matrix of cellular components. In addition, the amount of each CoA-ester can be readily quantified according to the peak area of their characteristic daughter ion peak on MS/MS spectra. Herein, we applied this method to quantify the three intracellular CoA-esters of interest (CoASH, acetyl-CoA, and malonyl-CoA) in our genetically engineered *E. coli* strains.

Direct injection analysis of pure CoA-esters by ESI revealed that negative ion mode gave higher signal intensity than positive ion mode, and thus, the negative ion mass spectrum $[M-H]^-$ was acquired for CoA-ester analysis throughout this study. Direct injection of pure compounds onto LC–MS/MS showed that malonyl-CoA, CoASH, acetyl-CoA, and butyryl-CoA (used as internal standard for quantification) were eluted at 4.3, 6.1, 7.9, and 11.8 min, respectively. Since the daughter fragment ions generated from a specific CoA-ester in tandem MS/MS vary depending on the structure of its acyl-side chain, for malonyl-CoA, acetyl-CoA, butyryl-CoA, and CoASH, each had a unique fragmentation pattern, and could be unambiguously identified in the sample matrix (data not shown). For quantification, the characteristic daughter ions specific to the four individual CoA-esters were analyzed with regard to their peak area in MRM mode (Fig. 2). Calibration curves were established for each CoA-ester, and there was a good linear correlation ($R^2 > 0.98$) between the concentration of CoA-ester and the peak area. The limit of quantification (LOQ), based on a signal-to-noise ratio greater than 5, was determined to be at nM scale for each of the CoA-ester standards in the MRM mode. These data demonstrate that the LC–ESI–MS/MS method developed in this work is selective and sensitive enough for analysis of CoA-esters derived from *E. coli* cells.

In order to determine the intracellular CoA-ester concentrations in real time, it is critical to rapidly quench cellular metabolism during sample preparation, since the turnover rates of most metabolic reactions are in the order of sub-seconds. To process *E. coli* samples, we used a modified protocol from Boynton et al. (1994), where perchloric acid was used to facilitate the disruption of cell membrane and simultaneously inactivate the cellular enzymes.

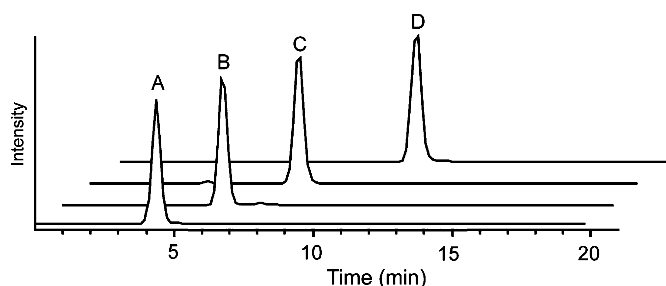


Fig. 2. MRM mass chromatograms obtained for standard CoA-esters: (A) Malonyl-CoA (852.5→808.5), (B) CoASH (766.5→419.2), (C) Acetyl-CoA (808.6→461.1), and (D) Butyryl-CoA (836.5→489.0).

3.2. Overexpression of acetyl-CoA carboxylase

Using the LC–ESI–MS/MS analytical method described above, intracellular malonyl-CoA concentration of the wild-type *E. coli* was determined to be around 0.07 nmol/mg dry cell weight, consistent with the reported value that was determined by an enzymatic method (0.01–0.23 nmol/mg dry cell weight) (Takamura and Nomura, 1988). Our previous effort to produce an important polyketide, phloroglucinol, in *E. coli* by heterologous expression of its synthase, PhlD from *P. fluorescens*, led to low yield of only 0.7 g/L (Achkar et al., 2005a). Since PhlD uses malonyl-CoA as the sole substrate to synthesize phloroglucinol, we hypothesized that the low concentration of cellular malonyl-CoA might limit phloroglucinol production in *E. coli*. It was suggested in literature that acetyl-CoA carboxylase, which catalyzes the conversion of acetyl-CoA to malonyl-CoA, is the rate-controlling enzyme in fatty acid synthesis (Davis et al., 2000). Therefore, an obvious strategy to improve cellular malonyl-CoA concentration would be to increase Acc enzyme level through episomal overexpression (Fig. 1). The most intensively studied *E. coli* Acc enzyme consists of four subunits (Davis et al., 2000) and is known to be feedback inhibited by acyl-acyl carrier proteins (ACPs) from the *E. coli* fatty acid biosynthetic pathway (Davis and Cronan, 2001). Although studies have shown that overexpression of the *E. coli* Acc enzyme led to increased cellular level of malonyl-CoA (Davis et al., 2000), we found that overexpression of this native Acc affected cell viability, and the phloroglucinol production was not improved at all (data not shown). On the other hand, Acc from *Corynebacterium glutamicum* consists of only two subunits, and it has been reported that overexpression of this Acc enzyme in *E. coli* led to increased heterologous production of flavanone, one of whose precursors is also malonyl-CoA (Miyahisa et al., 2005). We chose to introduce the same *C. glutamicum* Acc into *E. coli* for this metabolic engineering work. We found that functional expression of *C. glutamicum* Acc from the strong T7 promoter required low induction temperature, for induction at 37 °C gave no change to cellular malonyl-CoA concentration (data not shown), whereas induction at 25 °C resulted in significant increase in malonyl-CoA concentration. Therefore, 25 °C was used as the induction temperature throughout our studies. An *E. coli* BL21 (DE3) strain overexpressing *C. glutamicum* Acc and the wild-type strain were grown under the same conditions, and their intracellular CoA pool was analyzed by LC–ESI–MS/MS as described in Section 2. Overexpression of Acc alone increased the cellular malonyl-CoA concentration by 3-fold, while acetyl-CoA concentration remained almost unchanged (Table 2).

3.3. Inhibition of fatty acid synthesis

In *E. coli*, malonyl-CoA is only consumed during biosynthesis of fatty acids. In addition, multiple components in the fatty acid synthase complex regulate the cellular concentration of malonyl-CoA to be at a very low level (Magnuson et al., 1993). Thus, inhibition of fatty acid synthesis in *E. coli* would not only abolish a competing pathway consuming malonyl-CoA, but also alleviate the inherent negative regulation. It has been shown that blocking fatty acid synthesis by the antibiotic cerulenin triggered a large accumulation of malonyl-CoA (Davis et al., 2000). This inhibitor specifically inactivates the β-ketoacyl-acyl carrier protein synthases (KAS) I and II (FabB and FabF). However, the cost of cerulenin (more than $20 mg^{-1}) makes it impractical to use in our metabolic engineering work. Subrahmanyam and coworkers reported that overexpression of β-ketoacyl-ACP synthase II (FabF) also caused cessation of fatty acid synthesis, and subsequently a temporary increase in malonyl-CoA concentration within a few

Table 2
Intracellular concentration of CoA-esters from engineered *E. coli* strains manipulating malonyl-CoA formation and/or consumption.

	Malonyl-CoA		Acetyl-CoA	
	Concentration (nmol/mg DCW)	Fold change relative to WT	Concentration (nmol/mg DCW)	Fold change relative to WT
Wild-type (WT)	0.07 ± 0.01	1	0.93 ± 0.05	1
[a]Acc expression	0.22 ± 0.01	3.1	0.78 ± 0.12	0.9
[b]FabF expression	0.28 ± 0.01	4	0.72 ± 0.06	0.8
[c]Acc and FabF coexpression	0.09 ± 0	1.3	0.91 ± 0.15	1

DCW: dry cell weight.
Engineered strains are [a]B-Acc, [b]B-FabF, and [c]B-Acc/FabF.

Table 3
Intracellular concentration of CoA-esters from engineered *E. coli* strains manipulating acetyl-CoA availability in combination with Acc overexpression.

	Malonyl-CoA		Acetyl-CoA	
	Concentration (nmol/mg DCW)	Fold change relative to WT	Concentration (nmol/mg DCW)	Fold change relative to WT
Wild-type (WT)	0.07 ± 0.01	1	0.93 ± 0.05	1
[a]Acc expression	0.22 ± 0.01	3.1	0.78 ± 0.12	0.9
[b]Acs expression	0.19 ± 0.02	2.7	0.81 ± 0.13	0.9
[c]Δ*ackA-pta*	0.45 ± 0.02	6.4	0.55 ± 0.09	0.6
[d]Acs expression and Δ*ackA-pta*	1.06 ± 0.01	15.1	0.34 ± 0.03	0.4
[e]Acs expression, Δ*ackA-pta* and Δ*adhE*	1.10 ± 0.04	15.7	0.40 ± 0.08	0.4

Strains WT and B-Acc are included as a reference for comparison.
DCW: dry cell weight.
Engineered strains are [a]B-Acc, [b]B-Acs/Acc, [c]BWZ1-Acc, [d]BWZ1-Acs/Acc, and [e]BWZ3-Acs/Acc.

minutes of inducing FabF overexpression (Subrahmanyam and Cronan, 1998). A possible model for such inhibition was proposed that malonyl-CoA:ACP transacylase (FabD), with whom the association is essential for ketosynthases' condensation activity, was exhausted by large excess expression of FabF, thus leaving little FabD for other KAS isozymes (such as FabB) to carry out the complete fatty acid synthesis. However, blocking fatty acid synthesis by any means would dreadfully affect cell viability, as demonstrated by many inhibition studies. Here, we hypothesized that if overexpression of FabF is induced right before the *E. coli* culture enters its stationary growth phase, a continuous and long-term increase in the malonyl-CoA concentration would occur without significantly compromising the cell density. To test this hypothesis, the *E. coli fabF* gene was cloned under the control of a T7 promoter for overexpression. Analysis of the intracellular CoA pool from BL21(DE3) cells overexpressing FabF showed that blocking fatty acid synthesis resulted in 4-fold higher concentration of intracellular malonyl-CoA (Table 2). However, when Acc and FabF were coexpressed in *E. coli*, we did not observe an additive effect on improving the malonyl-CoA level. This antagonism may arise due to non-obvious and complex interactions between FabF and Acc, which are involved in large metabolic and regulatory networks. Therefore, the combination of Acc and FabF was not investigated further in our metabolic engineering efforts.

3.4. *Increase of acetyl-CoA availability*

The immediate precursor to malonyl-CoA is acetyl-CoA. In *E. coli* metabolism, acetyl-CoA serves as a key metabolic intermediate to the tricarboxylic acid cycle, where the acetyl group is broken down to CO_2 and H_2O, accompanied with energy generation. Besides this major function, acetyl-CoA is also involved in several other pathways, such as those leading to the formation of acetate and ethanol (Fig. 1) (Causey et al., 2004). Acetate is a toxic byproduct that inhibits the growth of *E. coli* cells (Luli and Strohl, 1990). Two enzymes, phosphotransacetylase (Pta) and acetate kinase (AckA), are responsible for conversion of acetyl-CoA to acetate. In addition, an assimilation pathway recycles acetate back to acetyl-CoA through the action of acetyl-CoA synthetase (Acs). Both pathways have been manipulated in previous studies. Deletion of the AckA-Pta pathway was shown to give decreased extracellular acetate production while increasing the cellular level of acetyl-CoA (Yang et al., 1999), but at the expense of cell survival (Chang et al., 1999). Recently, Leonard and coworkers reported an improved heterologous production of flavonone in *E. coli* upon overexpression of Acs, which was thought to be a result of increased availability of the precursor, acetyl-CoA by Acs overexpression (Leonard et al., 2007). Ethanol is another byproduct from acetyl-CoA, and the conversion is catalyzed by the bifunctional alcohol/aldehyde dehydrogenase (AdhE) in *E. coli*. Therefore, eliminating this enzyme activity would also be expected to improve the availability of acetyl-CoA.

Here, we sought to investigate the effect of manipulating the above-mentioned pathways on cellular levels of malonyl-CoA. For this purpose, the *E. coli acs* gene was overexpressed through the regulation of a T7 promoter. The *ackA-pta* genes were deleted from *E. coli* BL21(DE3) chromosome to generate the Δ*ackA-pta* strain (BWZ1). Likewise, the *adhE* gene was deleted to generate the Δ*adhE* strain (BWZ2). To create the double knockout BL21(DE3, Δ*ackA-pta* Δ*adhE*) strain (BWZ3), P1 transduction was used to transduce the Δ*adhE* deletion to BWZ1. We then evaluated the improvement of cellular malonyl-CoA level by these strategies, which would provide more acetyl-CoA, together with overexpression of Acc or FabF. Overexpression of the acetate assimilation enzyme, Acs, that recycles acetate back into acetyl-CoA, did not significantly affect the cellular malonyl-CoA concentration in the Acc and/or FabF overexpression strains (Tables 3 and 4). However, deletion of the *ackA-pta* pathway consuming acetyl-CoA for the byproduct acetate in conjunction with Acc overexpression

Table 4
Intracellular concentration of CoA-esters from engineered *E. coli* strains manipulating acetyl-CoA availability in combination with FabF overexpression.

	Malonyl-CoA		Acetyl-CoA	
	Concentration (nmol/mg DCW)	Fold change relative to WT	Concentration (nmol/mg DCW)	Fold change relative to WT
Wild-type (WT)	0.07 ± 0.01	1	0.93 ± 0.05	1
[a]FabF expression	0.28 ± 0.01	4	0.72 ± 0.06	0.8
[b]Acs expression	0.20 ± 0.01	2.8	0.8 ± 0.13	0.9
[c]Δ*ackA-pta*	0.12 ± 0.02	1.7	0.88 ± 0.10	0.9
[d]Acs expression and Δ*ackA-pta*	0.19 ± 0.02	2.7	0.81 ± 0.07	0.9
[e]Acs expression, Δ*ackA-pta* and Δ*adhE*	0.45 ± 0.07	6.4	0.55 ± 0.04	0.6

Strains WT and B-FabF are included as a reference for comparison.
DCW: dry cell weight.
Engineered strains are [a]B-FabF, [b]B-Acs/FabF, [c]BWZ1-FabF, [d]BWZ1-Acs/FabF, and [e]BWZ3-Acs/FabF.

Table 5
Maximum concentration of phloroglucinol ($mg\,L^{-1}$) and concentration of phloroglucinol normalized against cell density ($mg\,L^{-1}\,OD_{600}^{-1}$) after 48 h culture for selected strains from this study and previously reported data.

Strain	Phloroglucinol			
	Concentration ($mg\,L^{-1}$)	Fold change relative to WT	Normalized concentration ($mg\,L^{-1}\,OD_{600}^{-1}$)	Fold change relative to WT
[a]Wild-type (WT)	720	–	–	–
[b]WT	510	1	58	1
[c]Acc expression	1110	2.2	160	2.7
[d]Acs expression, Acc expression, Δ*ackA-pta* and Δ*adhE*	1280	2.5	220	3.7

Results from this study represent the average of triplicate experiments.
Engineered strains are [a]BL21(DE3) expressing *phlD* on plasmid pJA2.042, [b]BL21(DE3) expressing *phlD* on plasmid pACYC-PhlD, [c]B-Acc expressing *phlD* on plasmid pACYC-PhlD, and [d]BWZ3-Acs/Acc expressing *phlD* on plasmid pACYC-PhlD.

resulted in a 2-fold increase in the malonyl-CoA level compared with that from Acc overexpression alone (Tables 2 and 3). More importantly, the dual manipulation of acetate production from acetyl-CoA, involving assimilation (Acs overexpression) and pathway knockout (Δ*ackA-pta*), showed synergistic effect together with Acc overexpression, giving a total of 15-fold improved cellular level of malonyl-CoA compared with that in the wild-type *E. coli* (Table 3). Moreover, deletion of a second competing pathway (Δ*adhE*) that produces byproduct ethanol from acetyl-CoA led to a slightly further increase of cellular malonyl-CoA concentration (Table 3). On the other hand, the effect of FabF overexpression on cellular malonyl-CoA level seemed to be impaired by the increased availability of acetyl-CoA (Table 4). We speculated that there might be a certain degree of inhibition between the concentration of acetyl-CoA and activity of FabF. Throughout the engineering effort, there were only small changes in the cellular acetyl-CoA concentration, with the exception of strains possessing the Δ*ackA-pta* knockout, where acetyl-CoA is converted to malonyl-CoA rather than being cycled through acetate metabolism. In particular, under the condition of Acc/Acs coexpression and Δ*ackA-pta* (or Δ*ackA-pta*+Δ*adhE*), a 15-fold improved cellular malonyl-CoA concentration was accompanied by approximately 2-fold decrease in acetyl-CoA concentration. The increased flux toward malonyl-CoA production results from increased availability of acetyl-CoA (due to loss of the acetate-acetyl-CoA recycling pathway) and simultaneous overexpression of *C. glutamicum* Acc, which is likely not effectively inhibited by *E. coli* acyl-ACP (Table 3). Finally, the cellular CoASH concentration was always below the detection limit of ESI–MS/MS, even in the wild-type *E. coli* strain. This phenomenon might be caused by extra glucose in the culture medium, as there have been reports that the CoASH pool dramatically decreased when cells were grown in sufficient glucose medium (Chohnan et al., 1997).

3.5. Production of phloroglucinol in the engineered *E. coli* strains

To demonstrate the utility of engineered *E. coli* strains with elevated malonyl-CoA concentration, we used the biosynthesis of phloroglucinol as a model system. The phloroglucinol synthetase PhlD was expressed in wild-type BL21(DE3) and two engineered *E. coli* strains, i.e. (1) B-Acc, BL21(DE3) overexpressing Acc, and (2) BWZ3-Acc/Acs, BL21(DE3) with *ackA-pta* and *adhE* double deletion, and coexpression of Acc and Acs. After inducing protein expression by IPTG, phloroglucinol production was monitored at 24 and 48 h. As shown in Table 5, at 48 h culture time the two engineered strains improved phloroglucinol concentration approximately 1.5-fold compared with previously published data, and approximately 2-fold compared with wild-type production in this study. When phloroglucinol yield is normalized against cell density to account for the slower growth rate of the engineered strains, improvements of strains B-Acc and BWZ3-Acc/Acs relative to BL21(DE3) expressing PhlD are 2.7- and 3.7-fold, respectively (Table 5). Given the fact that malonyl-CoA is the sole substrate for phloroglucinol synthesis, the 2.7-fold improved phloroglucinol production in the B-Acc strain correlates well with the 3-fold elevated malonyl-CoA concentration from Acc overexpression. However, the 3.7-fold improvement is lower than expected for phloroglucinol production in the BWZ3-Acc/Acs strain, which had more than 15-fold increased cellular malonyl-CoA concentration. This discrepancy may be explained by the toxicity of phloroglucinol to *E. coli*. When added externally into a culture grown in

minimal medium, phloroglucinol significantly inhibits cell growth at a concentration of 500 mg/L, whereas the observed phloroglucinol concentrations in the B-Acc and BWZ3-Acc/Acs strains exceeded 500 mg/L by 24 h (data not shown) and were slightly above 1000 mg/L by 48 h, shown in Table 5. It is possible that the toxicity of phloroglucinol at this concentration might have prevented further increase in phloroglucinol production. For compounds exhibiting no or less toxicity to *E. coli*, a much larger improvement in production should be reasonably expected from the BWZ3-Acc/Acs strain.

In conclusion, by applying various metabolic engineering strategies individually and in combination to redirect the carbon flux inside *E. coli* to pathways responsible for the generation of malonyl-CoA, we engineered several *E. coli* strains with highly elevated cellular malonyl-CoA concentration. The usefulness of theses strains was demonstrated by the improved heterologous production of an important polyketide, phloroglucinol. We believe that these engineered *E. coli* strain will be useful for biosynthesis of natural products where the cellular malonyl-CoA level is rate-limiting.

Acknowledgments

This research was supported by a grant from the Office of Naval Research (N00014-02-1-0725). S. B. R.-P. acknowledges support from the National Institutes of Health Cell and Molecular Biology Training Grant Program and the National Science Foundation Graduate Research Fellowship Program.

References

Achkar, J., Xian, M., Zhao, H.M., Frost, J.W., 2005. Biosynthesis of phloroglucinol. J. Am. Chem. Soc. 127, 5332–5333.

Aldor, I.S., Keasling, J.D., 2003. Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates. Curr. Opin. Biotechnol. 14, 475–483.

Boynton, Z.L., Bennett, G.N., Rudolph, F.B., 1994. Intracellular concentrations of coenzyme A and its derivatives from *Clostridium acetobutylicum* ATCC 824 and their roles in enzyme regulation. Appl. Environ. Microbiol. 60, 39–44.

Causey, T.B., Shanmugam, K.T., Yomano, L.P., Ingram, L.O., 2004. Engineering *Escherichia coli* for efficient conversion of glucose to pyruvate. Proc. Natl. Acad. Sci. USA 101, 2235–2240.

Chang, D.E., Shin, S., Rhee, J.S., Pan, J.G., 1999. Acetate metabolism in a *pta* mutant of *Escherichia coli* W3110: importance of maintaining acetyl coenzyme a flux for growth and survival. J. Bacteriol. 181, 6656–6663.

Chohnan, S., Furukawa, H., Fujio, T., Nishihara, H., Takamura, Y., 1997. Changes in the size and composition of intracellular pools of nonesterified coenzyme A and coenzyme A thioesters in aerobic and facultatively anaerobic bacteria. Appl. Environ. Microbiol. 63, 553–560.

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97, 6640–6645.

Davis, M.S., Cronan Jr., J.E., 2001. Inhibition of *Escherichia coli* acetyl coenzyme A carboxylase by acyl–acyl carrier protein. J. Bacteriol. 183, 1499–1503.

Davis, M.S., Solbiati, J., Cronan Jr., J.E., 2000. Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. J. Biol. Chem. 275, 28593–28598.

Dixon, R.A., Steele, C.L., 1999. Flavonoids and isoflavonoids—a gold mine for metabolic engineering. Trends Plant Sci. 4, 394–400.

Edwards, M.L., Stemreick, D.M., Sunkara, P.S., 1990. Chalcones: a new class of antimitotic agents. J. Med. Chem. 33, 1948–1954.

Forkmann, G., Martens, S., 2001. Metabolic engineering and applications of flavonoids. Curr. Opin. Biotechnol. 12, 155–160.

Hopwood, D.A., 1997. Genetic contributors to understanding polyketide synthases. Chem. Rev. 97, 2465–2497.

Jang, M., 1997. Cancer chempreventive activity of resveratrol, a natural product derived from grapes. Science 275, 218–220.

Kalscheuer, S., Stolting, T., Steinbuchel, A., 2006. Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152, 2529–2536.

Katz, L., Donadia, S., 1993. Polyketide synthesis: prospects for hybrid antibiotics. Annu. Rev. Microbiol. 47, 875–912.

Kealey, J.T., Liu, L., Santi, D.V., Betlach, M.C., Barr, P.J., 1998. Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts. Proc. Natl. Acad. Sci. USA 95, 505–509.

Keating, T.A., Walsh, C.T., 1999. Initiation, elongation, and termination strageties in polyketide and polypeptide antibiotic biosynthesis. Curr. Opin. Chem. Biol. 3, 598–606.

Khosla, C., Gokhale, R.S., Jacobsen, J.R., Cane, D.E., 1999. Tolerance and specificity of polyketide synthases. Annu. Rev. Biochem. 68, 219–253.

Knudsen, J., Faergeman, N.J., Skott, H., Hummel, R., Borsting, C., Rose, T.M., Andersen, J.S., Hojrup, P., Roepstorff, P., Kristiansen, K., 1994. Yeast acyl-CoA-binding protein: acyl-CoA-binding affinity and effect on intracellular acyl-CoA pool size. Biochem. J. 302, 479–485.

Leonard, E., Lim, K.H., Saw, P.N., Koffas, M.A., 2007. Engineering central metabolic pathways for high-level flavonoid production in *Escherichia coli*. Appl. Environ. Microbiol. 73, 3877–3886.

Lin, H., Vadali, R.V., Bennett, G.N., San, K.Y., 2004. Increasing the acetyl-CoA pool in the presence of overexpressed phosphoenolpyruvate carboxylase or pyruvate carboxylase enhances succinate production in *Escherichia coli*. Biotechnol. Prog. 20, 1599–1604.

Lu, X., Vora, H., Khosla, C., 2008. Overproduction of free fatty acids in *E. coli*: implications for biodiesel production. Metab. Eng. 10, 333–339.

Luli, G.W., Strohl, W.R., 1990. Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. Appl. Environ. Microbiol. 56, 1004–1011.

Magnuson, K., Jackowski, S., Rock, C.O., Cronan Jr., J.E., 1993. Regulation of fatty acid biosynthesis in *Escherichia coli*. Microbiol. Rev. 57, 522–542.

Miller, J.H., 1992. A Short Course in Bacterial Genetics: A Laboratory Manual and Hand Book for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, Plainview, NY.

Miyahisa, I., Kaneko, M., Funa, N., Kawasaki, H., Kojima, H., Ohnishi, Y., Horinouchi, S., 2005. Efficient production of (2S)-flavanones by *Escherichia coli* containing an artificial biosynthetic gene cluster. Appl. Microbiol. Biotechnol. 68, 498–504.

Park, J.W., Jung, W.S., Park, S.R., Park, B.C., Yoon, Y.J., 2007. Analysis of intracellular short organic acid-coenzyme A esters from actinomycetes using liquid chromatography-electrospray ionization-mass spectrometry. J. Mass Spectrom. 42, 1136–1147.

Pfeifer, B.A., Admiraal, S.J., Gramajo, H., Cane, D.E., Khosla, C., 2001. Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. Science 291, 1790–1792.

Pogolotti Jr., A.L., Santi, D.V., 1982. High-pressure liquid chromatography—ultraviolet analysis of intracellular nucleotides. Anal. Biochem. 126, 335–345.

Shiba, Y., Paradise, E.M., Kirby, J., Ro, D.K., Keasling, J.D., 2007. Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. Metab. Eng. 9, 160–168.

Stephanopoulos, G., 1999. Metabolic fluxes and metabolic engineering. Metab. Eng. 1, 1–11.

Subrahmanyam, S., Cronan Jr., J.E., 1998. Overproduction of a functional fatty acid biosynthetic enzyme blocks fatty acid synthesis in *Escherichia coli*. J. Bacteriol. 180, 4596–4602.

Takamura, Y., Nomura, G., 1988. Changes in the intracellular concentration of acetyl-CoA and malonyl-CoA in relation to the carbon and energy metabolism of *Escherichia coli* K12. J. Gen. Microbiol. 134, 2249–2253.

Vadali, R.V., Bennett, G.N., San, K.Y., 2004. Applicability of CoA/acetyl-CoA manipulation system to enhance isoamyl acetate production in *Escherichia coli*. Metab. Eng. 6, 294–299.

Vadali, R.V., Bennett, G.N., San, K.Y., 2004. Cofactor engineering of intracellular CoA/acetyl-CoA and its effect on metabolic flux redistribution in *Escherichia coli*. Metab. Eng. 6, 133–139.

Wattanachaisaereekul, S., Lantz, A.E., Nielsen, M.L., Nielsen, J., 2008. Production of the polyketide 6-MSA in yeast engineered for increased malonyl-CoA supply. Metab. Eng. 10, 246–254.

Yang, Y.T., Aristidou, A.A., San, K.Y., Bennett, G.N., 1999. Metabolic flux analysis of *Escherichia coli* deficient in the acetate production pathway and expressing the *Bacillus subtilis* acetolactate synthase. Metab. Eng. 1, 26–34.

Zha, W.J., Rubin-Pitel, S.B., Zhao, H.M., 2006. Characterization of the substrate specificity of PhlD, a type III polyketide synthase from *Pseudomonas fluorescens*. J. Biol. Chem. 281, 32036–32047.

Zha, W.J., Rubin-Pitel, S.B., Zhao, H.M., 2008. Exploiting genetic diversity by directed evolution: molecular breeding of type III polyketide synthases improves productivity. Mol. Biosyst. 4, 246–248.