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

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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.

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

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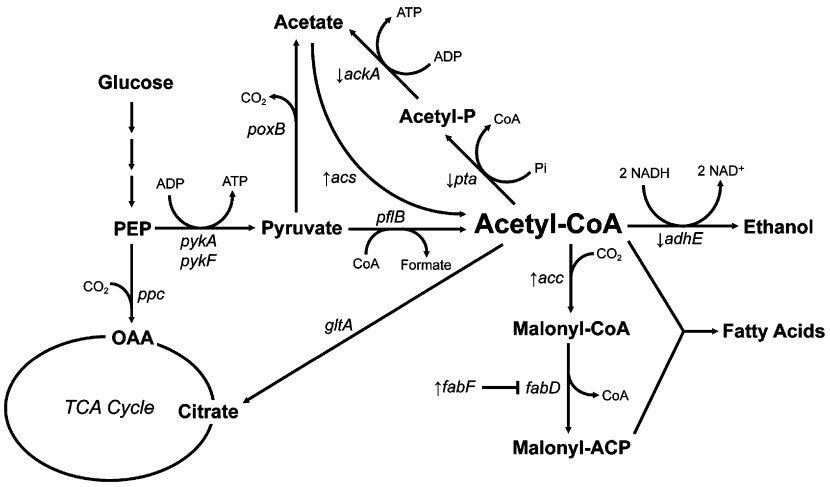

Fig. 1. Scheme of central metabolism in *E. coli*. Genes knocked out (↓) and overexpressed (↑) are noted.

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 thermoinducible 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