Journal of Bioscience and Bioengineering
VOL. 123 No. 5, 625–633, 2017

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Synthetic metabolic bypass for a metabolic toggle switch enhances acetyl-CoA supply for isopropanol production by *Escherichia coli*

Yuki Soma,[1,3] Taiki Yamaji,[1] Fumio Matsuda,[2] and Taizo Hanai[1,*]

Laboratory for Bioinformatics, Graduate School of Systems Lifesciences, Kyushu University, 804 Westwing, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan,[1] Department of Bioinformatic Engineering, Graduate School of Information Science and Technology, Osaka University, 1-5 Yamadaoka, Suita 565-0871, Osaka, Japan,[2] and Research Center for Transomics Medicine, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan[3]

Received 11 November 2016; accepted 15 December 2016
Available online 14 February 2017

Almost all synthetic pathways for biofuel production are designed to require endogenous metabolites in glycolysis, such as phosphoenolpyruvate, pyruvate, and acetyl-CoA. However, such metabolites are also required for bacterial cell growth. To reduce the metabolic imbalance between cell growth and target chemical production, we previously constructed a metabolic toggle switch (MTS) as a conditional flux redirection tool controlling metabolic flux of TCA cycle toward isopropanol production. This approach succeeded to improve the isopropanol production titer and yield while ensuring sufficient cell growth. However, excess accumulation of pyruvate, the precursor for acetyl-CoA synthesis, was also observed. In this study, for efficient conversation of pyruvate to acetyl-CoA (pyruvate oxidation), we designed a synthetic metabolic bypass composed of *poxB* and *acs* with the MTS for acetyl-CoA supply from the excess pyruvate. When this designed bypass was expressed at the appropriate expression level associated with the conditional metabolic flux redirection, pyruvate accumulation was prevented, and the isopropanol production titer and yield were improved. Final isopropanol production titer of strain harboring MTS with the synthetic metabolic bypass improved 4.4-fold compared with strain without metabolic flux regulation, and it was 1.3-fold higher than that of strain harboring the conventional MTS alone. Additionally, glucose consumption was also improved 1.7-fold compared with strain without metabolic flux regulation. On the other hand, introduction of the synthetic metabolic bypass alone showed no improvement in isopropanol production and glucose consumption. These results showed that the improvement in bioproduction process caused by synergy between the MTS and the synthetic metabolic bypass.

[**Key words:** Synthetic metabolic bypass; Synthetic genetic circuit; Metabolic toggle switch; Biosynthetic pathway; Acetyl-CoA supply]

Microbial chemical production from renewable resources has attracted increasing attention as a potential alternative to petrochemical production because of the limited amounts of fossil oils and global concerns regarding sustainability and the environment (1–3). To expand the target chemicals that microorganisms can produce, many types of biosynthetic pathways have been constructed by combining heterogeneous metabolic pathways (4). Most synthetic pathways used for production of bulk chemical, such as bio-alcohols, biodiesels, and bioplastics, have been constructed based on endogenous metabolites in the glycolysis pathway, including phosphoenolpyruvate, pyruvate, and acetyl-CoA (5–8). These metabolites are also consumed in endogenous pathways that are responsible for bacterial cell growth and physiological activity (e.g., amino acid synthesis, fatty acid synthesis, the TCA cycle). Therefore, there is a conflicting relationship between endogenous metabolism and the biosynthetic pathway, termed metabolic imbalance, and this often limits microbial chemical product titers and yields (2,9,10). To achieve economic viability of microbial chemical production, it is necessary to overcome these tradeoff relationships in order to ensure both efficient cell growth and target chemical productivity.

In recent years, synthetic biological tools have been developed for metabolic flux regulation in order to facilitate reduction of the metabolic imbalance and improve productivity, titers, and yields in the production of several microbial chemicals (11). The first demonstration of regulation of the synthetic pathway was implemented in lycopene biosynthesis in *Escherichia coli* using the *glnA*P2 promoter as a dynamic controller that responds to accumulation of acetyl-phosphate as an indicator of excess glycolytic flux (12). A dynamic sensor-regulator system has been also developed for sensing the intracellular concentration of key intermediates for fatty acid ethyl ester production (13). These systems allow microbes to avoid the metabolic imbalance by inducing heterogeneous enzymes optimally in response to perturbation of intracellular metabolism, thereby significantly improving productivity, titers, and yields.

In our previous study on isopropanol production by engineered *E. coli*, we demonstrated metabolic flux redirection using a synthetic genetic circuit, called a metabolic toggle switch (MTS) (14). Isopropanol is one of the simplest secondary alcohols that can be dehydrated to yield bio-propylene, which is currently derived from petroleum as a monomer for making polypropylene. Because polypropylene is currently used as a material for many industrial

* Corresponding author. Tel.: +81 92 642 6751; fax: +81 92 642 6744.
E-mail address: taizo@brs.kyushu-u.ac.jp (T. Hanai).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.12.009

products, it is expected that the world demand for propylene will grow in the future. To date, we have engineered a biosynthetic pathway and *E. coli* strains for the microbial production of isopropanol from inexpensive and renewable feedstock, such as biomass-derived saccharides (5,15,16). The biosynthetic pathway for isopropanol production uses acetyl-CoA as the precursor (Fig. 1A). Therefore, the production titer and yield of isopropanol are also decreased by the metabolic imbalance between aerobic bacterial growth depending on the TCA cycle and the biosynthetic pathway. To address this problem, we developed the MTS, which can redirect metabolic fluxes in the TCA cycle toward a biosynthetic pathway in the response to the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; Fig. 1A, B) (14). The MTS can interrupt the metabolic influx into the TCA cycle by regulating the expression of citrate synthase encoded by *gltA* (Fig. 1). Using this system, we succeeded in solving this trade-off relationship appropriately by redirecting excess flux caused by conditional interruption of the TCA cycle after ensuring that a sufficiently high cell density was obtained. The titers and yields of isopropanol production were improved by more than 3-fold (14).

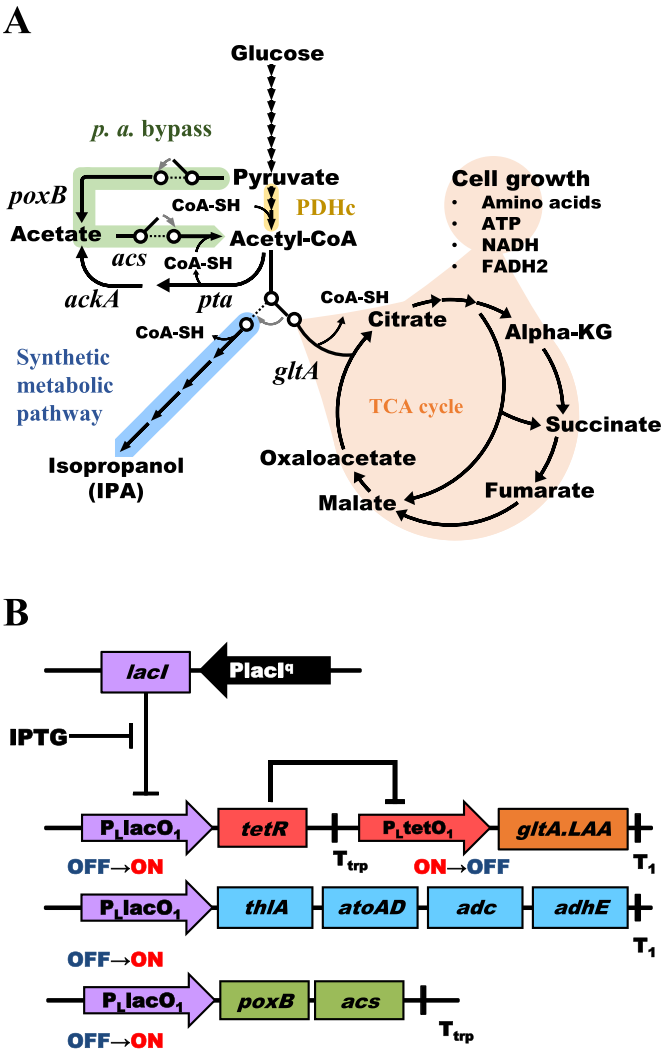

FIG. 1. Conditional boosting of acetyl-CoA supply for isopropanol production using the metabolic toggle switch (MTS) installed at the synthetic metabolic bypass. (A) Strategy to boost acetyl-CoA synthesis through metabolic flux regulation using the MTS installed at the synthetic metabolic bypass. Using the MTS, metabolic influx into the TCA cycle could be interrupted and redirected toward isopropanol production in response to the addition of IPTG, causing pyruvate accumulation. To prevent pyruvate accumulation, the synthetic metabolic bypass was induced, along with the metabolic flux redirection, which was composed of *poxB* and *acs* under control of the IPTG-inducible promoter $P_L lacO_1$. (B) Design of the MTS installed at the synthetic metabolic bypass. The MTS was divided into three parts: (i) the repressor source (pTA216: $PlacI^q$::*lacI*), (ii) the ON-OFF-module (under control of $P_L tetO_1$), and (iii) the OFF-ON-module (under control of $P_L lacO_1$). The LacI repressor from pTA216 inhibits transcription from the $P_L lacO_1$ promoter. When transcription by the $P_L lacO_1$ promoter was induced by the addition of IPTG, expression of *tetR* and genes involved in the isopropanol production pathway (e.g., *thlA*, *atoAD*, *adc*, *adhE*) and p.a. bypass (e.g., *poxB*, *acs*) were upregulated. Thus, expression of *gltA* under the $P_L tetO_1$ promoter would be inhibited by the TetR repressor, resulting in interruption of the TCA cycle. Error bars show standard deviations (n = 3).

Fig. 1A, B) (14). The MTS can interrupt the metabolic influx into the TCA cycle by regulating the expression of citrate synthase encoded by *gltA* (Fig. 1). Using this system, we succeeded in solving this trade-off relationship appropriately by redirecting excess flux caused by conditional interruption of the TCA cycle after ensuring that a sufficiently high cell density was obtained. The titers and yields of isopropanol production were improved by more than 3-fold (14).

These designed regulatory processes for local metabolic flux have been shown to contribute to improvement of titers and yields. However, such regulation of metabolic flux may cause unexpected metabolic discord. In fact, conditional interruption of the TCA cycle by the MTS caused significant accumulation of pyruvate, a precursor of acetyl-CoA synthesis, in *E. coli* cells (14). For additional improvement of isopropanol production, the metabolic flux capacity of acetyl-CoA synthesis should be expanded to prevent the accumulation of pyruvate associated with conditional interruption of the TCA cycle. In the endogenous metabolism of *E. coli*, acetyl-CoA is mainly formed from pyruvate via pyruvate decarboxylation catalyzed by the pyruvate dehydrogenase complex (PDHc). The PDHc is a large complex composed of multiple copies of three subunits, i.e., E1 (pyruvate dehydrogenase; E.C. 1.2.4.1), E2 (dihydrolipoyl transacetylase; E.C. 2.3.1.12), and E3 (dihydrolipoyl dehydrogenase, E.C. 1.8.1.4) (17). The expression of PDHc is regulated by a complicated endogenous regulon, and its enzymatic activity is highly regulated by a variety of allosteric effectors and covalent modification (18). On the other hand, pyruvate can also be converted to acetate by pyruvate oxidase, encoded by *poxB* (E.C. 1.2.5.1), and acetate can be converted to acetyl-CoA by acetyl-CoA synthase, encoded by *acs* (E.C. 6.2.1.1). The sequential reactions of pyruvate oxidase and acetyl-CoA synthase can represent an alternative pathway for acetyl-CoA synthesis by PDHc. Thus, using *poxB* and *acs*, we designed a synthetic metabolic bypass to boost the acetyl-CoA supply independent from PDHc (Fig. 1A).

In our previous work, MTS focused only competition in carbon flux between cell growth and isopropanol production. In this study, we redesigned the MTS controlling Acetyl-CoA supply from pyruvate by using the synthetic metabolic bypass, which is potentially resulting in a bottleneck associated with the metabolic flux redirection. For construction of the synthetic metabolic bypass, the *poxB* and *acs* genes were overexpressed under control of the IPTG-inducible promoter $P_L lacO_1$. This expression module was integrated with the MTS (Fig. 1B). In this system, the addition of IPTG induced the expression of the *tetR* repressor under the $P_L lacO_1$ promoter. The induced *tetR* repressor inhibited the expression of genes under control of the $P_L tetO_1$ promoter. Using this system, the expression of *gltA*, encoding citrate synthase, could be inhibited during fermentation in response to the addition of IPTG. As a result, metabolic influx into the TCA cycle could be interrupted. At the same time, isopropanol synthesis and bypass were induced. We implemented this system in engineered *E. coli* to demonstrate isopropanol production and investigated the effects of the conditional enlargement of synthetic metabolic bypass on pyruvate accumulation and acetyl-CoA supply for isopropanol production.

MATERIALS AND METHODS

Chemicals and reagents All chemicals were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan) unless otherwise specified. Restriction enzymes and phosphatase were from New England Biolabs (Ipswich, MA, USA), ligase (rapid DNA ligation kit) was from Roche (Manheim, Germany), and KOD Plus Neo DNA polymerase was from Toyobo Co., Ltd. (Osaka, Japan). Oligonucleotides were synthesized by Life Technologies Japan Ltd. (Tokyo, Japan).

Bacterial strains and plasmid construction Table 1 shows strains and plasmids used for this study. XL10-Gold (Agilent Technologies, Santa Clara, CA, USA) and DH5alphaZ1 (Expressys, Ruelzheim, Germany) were used to construct plasmids.