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Engineered *Escherichia coli* for simultaneous utilization of galactose and glucose

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HIGHLIGHTS

- Galactose metabolism in *Escherichia coli* was re-engineered to co-utilize glucose and galactose.
- The engineered strain showed a 44.8% increase in growth rate in galactose medium.
- This strain could simultaneously utilize glucose and galactose with similar rates.
- This system has enormous potential for broad biorefinery applications.

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ABSTRACT

In this study, the Leloir pathway of galactose metabolism was rebuilt in *Escherichia coli* to remove CCR and amplify galactose utilization rate. All genes encoding pathway enzymes were expressed under the control of a synthetic module that included promoters, 5′-untranslated regions, and terminators as a re-organized single operon in the chromosome. The engineered strain showed both an enhanced galactose utilization rate and the capacity to simultaneously assimilate galactose and glucose. This work demonstrates the feasibility of using synthetic biology tools to re-build biological systems for engineering purpose.

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1. Introduction

Microbial production of chemicals and fuels from renewable resources such as land- and marine-derived biomass has attracted considerable attention in recent decades (John et al., 2011). One of the challenges in realizing the potential of this emerging technology is designing microbial biocatalysts that can utilize multiple carbohydrate substrates (e.g., xylose, arabinose, galactose, mannose, sucrose) from these various renewable resources to facilitate the production of chemicals and fuels (Bothast et al., 1999; Chan et al., 2012). However, these alternative carbohydrates are less preferred by industrial microorganisms compared to glucose; moreover, carbon catabolite repression (CCR) limits simultaneous utilization of multiple carbohydrates (Vinuselvi et al., 2012).

One promising alternative carbohydrate is galactose, a major sugar of red seaweeds, which have been touted for their high biomass productivity, high carbon fixation rate, and easily degradable structures compared to other lignocellulosic biomass sources (Yanagisawa et al., 2011). However, galactose metabolism requires more enzymatic steps (Leloir pathway) to enter the glycolytic pathway compared to simple glucose metabolism (Horváth et al., 2010). This might reduce both uptake and assimilation rates of galactose. Additionally, CCR regulates the expression of genes encoding enzymes involved in the catabolism of galactose, including those that transport and utilize galactose in the presence of glucose (Horváth et al., 2010). Therefore, rebuilding galactose metabolism is required to enhance galactose utilization rates while allowing simultaneous utilization of galactose and glucose.

In this study, the Leloir pathway of galactose metabolism in *Escherichia coli* was refactored using a bottom-up approach to remove native regulation and amplify pathway fluxes. All genes encoding pathway enzymes were re-organized as a series of the expression units with the synthetic components, including promoters, 5′-untranslated region (5′-UTR), and terminators for each individual gene in the chromosome. Synthetic promoters and 5′-UTRs were designed to deregulate the control system of glucose and galactose consumption and to maximize the expression levels of genes involved in galactose uptake pathways. The engineered strain showed enhanced galactose utilization rate as well as the capacity to simultaneously assimilate galactose and glucose. Further implementation of downstream pathways for the production of chemicals and fuels will enable this strain to be used as an

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efficient biocatalyst. This work also demonstrates that, by applying engineering strategies, synthetic biology tools can facilitate the rebuilding of biological systems.

2. Methods

2.1. Bacterial strains, plasmids, and primers

The *E. coli* bacterial strains and plasmids used in this study are listed in Table S1. Phusion polymerase and restriction endonuclease were purchased from New England Biolabs (Beverly, MA, USA). Oligonucleotides used in this study were synthesized by Genotech (Daejeon, Korea) and Bioneer (Daejeon, Korea) and are listed in Table S2. Other reagents were obtained from Sigma (St. Louis, MO, USA).

To construct pACYCgalO, an FRT-Kan^R-FRT fragment amplified with V-KanR-F and V-KanR-R primers was first inserted in the *KpnI* and *SacI* restriction sites of a modified pACYC vector. A galE-galT fragment was assembled by overlap polymerase chain reaction (PCR) using V-galE-F/V-galE-R and V-galT-F/V-galT-R primer pairs and inserted into *Not*I and *Bam*HI restriction sites. After that, the galK-galM fragment, assembled by overlap PCR using V-galK-F/V-galK-R and V-galM-F/V-galM-R primer pairs, was inserted into *Nde*I and *Xho*I restriction sites.

All chromosomal manipulations were done using the Red recombination system with pKD46 and pCP20, as described in previous studies (Datsenko and Wanner, 2000). To construct GS, GR, and GSR strains, either *galS* (GS strain), *galR* (GR strain) or both (GSR strain) were deleted by insertion of *FRT-Kan^R-FRT* fragments amplified by D-galS-F/D-galS-R and D-galR-F/D-galR-R primer pairs, respectively. To construct the GRT strain, the native promoter and 5′-UTR of *galP* of the GR strain were replaced with a BBa_J23100 promoter and optimized 5′-UTR using an *FRT-Kan^R-FRT* fragment amplified by O-galP-F, O-galP-R1, and O-galP-R2 primers. To construct the GRTOP strain, the native galactose operon was replaced by synthetic single-operon clusters amplified by O-galETKM-F and O-galETKM-R primers using pACYCgalO as a template after deleting the native operon of GRT using an FRT-Kan^R-FRT fragment amplified by the primers D-galETKM-F and D-galETKM-R. Next, the native promoter, 5′-UTR, and first 10 codons of *pgm* were replaced with synthetic components to ensure maximum expression using a PCR fragment amplified by O-pgm-F and O-pgm-R primers. It should be noted that each gene (*galP*, *pgm*, *galE*, *galT*, *galK*, and *galM*) was expressed under the control of a strong constitutive promoter (BBa_J23100 from the Registry of Standard Biological Parts, http://partsregistry.org) and contained optimized 5′-UTR sequences designed by UTR Designer (http://sbi.postech.ac.kr/utr_designer) to achieve maximum expression (Seo et al., 2012).

2.2. Cell cultures, growth measurement, and detection of carbohydrates

M9 media containing 4 g/L glucose or galactose, M9 salt solutions (Sigma, St. Louis, MO, USA), 5 mM $MgSO_4$, 0.1 mM $CaCl_2$, and appropriate antibiotics were used for cultivating each *E. coli* strain. For mixed-sugar fermentation, 2 g/L of each glucose and galactose was supplemented as a carbon source.

Cells were cultured at 37 °C with shaking (200 rpm). The density of *E. coli* was determined by measuring optical density at a wavelength of 600 nm (OD_{600}) using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan).

Seed cultures were prepared by inoculating colonies from an LB plate into 3 ml of glucose or galactose M9 minimal medium. After adaptation, this initial seed culture was used to seed 3 ml of the same medium. When these second-round seed cultures reached at an OD_{600} of 0.8 ∼ 0.9, they were washed twice and individually inoculated into 25 ml of M9 in 300 ml flask at an OD_{600} of 0.05. All cell culture experiments were conducted in duplicate.

Concentrations of glucose and galactose consumed were determined by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA) with a flow rate of 0.6 ml/min at 65 °C using 5 mM H_2SO_4 as the mobile phase. Glucose and galactose signals were detected with a Shodex RI-101 refractive index detector (Shodex, Klokkerfaldet, Denmark).

3. Results and discussion

3.1. Effect of deleting transcriptional repressors on galactose metabolism in E. coli

The growth rate of *E. coli* in galactose medium is substantially slower than that in glucose, reflecting the relatively complex enzymatic steps required for galactose to enter glycolysis (Fig. S1). Both maximum specific uptake and growth rate were decreased when *E. coli* W3110 was grown in galactose medium compared to glucose medium (Table 1), indicating that galactose metabolism should be alleviated for the enhanced galactose uptake and utilization.

Two highly homologous transcriptional repressors, *galS* and *galR*, are known to regulate galactose metabolism through binding to regions upstream of promoters of genes involved in galactose uptake and assimilation (Geanacopoulos and Adhya, 1997). In a previous study, deletion of a transcriptional repressor increased the expression level of one of the enzymes in the utilization pathway, *galE* (Mangan et al., 2006). To accelerate galactose metabolism, each transcriptional repressor was removed. The physiological parameters of GS and GR which lacked *galS* and *galR*, respectively, were similar to W3110 when media were supplemented with glucose as a sole carbon source (GS and GR strains; Table 1). When these strains were cultivated in galactose supplemented medium, each deletion had a measurable effect on the specific growth rate, as expected. Although the maximum specific galactose uptake rates of both strains were similar, the GR strain showed a higher specific growth rate compared to the GS strain (GR, 23.7% increase compared to W3110; GS, 14.1% increase compared to W3110). This result may reflect the fact that the repressor encoded by *galS* plays a minor role in regulation compared to *galR* and is only activated by a sudden drop in extracellular galactose level (Geanacopoulos and Adhya, 1997; Semsey et al., 2009). Interestingly, deletion of both repressors had the opposite effect, significantly decreasing the specific growth rate compared to *E. coli* W3110; it also decreased sugar uptake rate (GSR strain; Table 1). This decrease in growth might be caused by another function of repressors which activate expression of galactose-metabolism related genes (Roy et al., 1998). Moreover, one of intermediates is a precursor of cell-wall biosynthesis pathway (Fig. S1) and thus, loss of regulation as well as unknown additional functions of both repressors that are important for cell growth might lead reduced growth in both glucose and galactose medium. Since the GR strain showed the fastest growth rate on galactose medium, it was chosen for further modifications designed to enhance galactose utilization capacity and allow co-utilization of glucose and galactose.

3.2. Refactoring galactose metabolism in E. coli to facilitate galactose uptake and utilization

For effective co-utilization of glucose and galactose, the utilization rate of each carbohydrate should be identically fast and the CCR caused by a preferred carbohydrate (glucose) should be eliminated. To further increase the rate of galactose assimilation,

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