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Research Report

Development of Genetic Switch to Control Metabolic Flow in *Saccharomyces cerevisiae*

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Report received on Apr. 2, 2012

ABSTRACT The control of metabolic flow is a prerequisite for efficient chemical production in transgenic microorganisms. Exogenous genes required for the biosynthesis of target chemicals are expressed under strong promoters, while the endogenous genes of the original metabolic pathway are repressed by disruption or mutation. These genetic manipulations occasionally cause harmful effects to the host. In the lactate-producing yeast *Saccharomyces cerevisiae*, where endogenous pyruvate decarboxylase (*PDC*) is disrupted and exogenous lactate dehydrogenase (*LDH*) is introduced. *PDC* deletion is extremely detrimental to cell growth but is required for efficient production of lactate. A suitable means to dynamically control the metabolic flow from ethanol fermentation during the growth phase to lactate fermentation during the production phase is needed. We developed a Cre-*lox* genetic switch to dynamically control the exclusive expression of two genes. With this genetic switch, we demonstrated a "metabolic shift" concept that controls the flow by the exclusive expression of *PDC* and *LDH*.

KEYWORDS Metabolic Shift, Genetic Switch, Cre-*lox* System, *Saccharomyces cerevisiae*, Synthetic Biology

1. Introduction

In recent decades, many transgenic microorganisms have been developed to produce chemicals for use in bio-plastics and bio-fuels.[1-4] In these transgenic microorganisms, one or more genes of the enzymes that convert intermediates into a target product or its precursors are introduced and strongly expressed to induce shifts from innate metabolic flow to the desired flow. As the product of the original metabolic pathway then becomes an undesirable by-product, it needs to be suppressed for efficient production of the target end-product. This suppression often results in retardation of cell growth. In this case, compatible metabolic flows are necessary: the original flow during the growth phase and the engineered flow during the production phase. However, the conversion of the original metabolic pathway to the artificial pathway is often difficult to control with traditional fermentation technology, and approaches based on synthetic biology should shed light on this problem. Here, we reviewed three of our recent works to achieve this goal.

Genetic switches are a fundamental synthetic biological tool[5] that control the expression of transgenes. As a means of controlling metabolic flow, we developed a strict Cre-*lox* genetic switch at the genomic level in *Saccharomyces cerevisiae* (**Fig. 1**). This switch comprised two parts: the improved galactose induction system[6] and the temporal Cre activation system. To achieve efficient phenotype conversion, we also confirmed that the *TPS1* terminator (*TPS1t*) confers strong gene expression.[7]

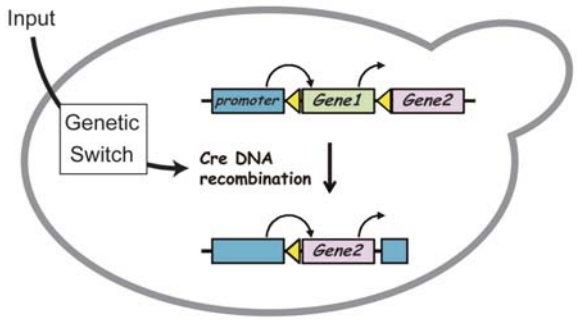

Fig. 1 Conceptual scheme for a genetic switch to control exclusive gene expression. The promoter drives expression of gene 1 in the initial state. After the genetic switch is activated by physical inputs such as chemicals or heat, Cre DNA recombinase alters the genomic structure, allowing the promoter to drive gene 2 expression. As gene 1 is excised from the genome, its expression is then arrested.

For investigation of switch performance, green and red fluorescent proteins (GFP and mKO2) were used as reporters to analyze the phenotypic changes within a few hours. After approval, this novel tool was applied to metabolically engineered yeast for lactate production, in which the central carbon flow was shifted from ethanol to lactic acid.[8] To accomplish this "metabolic shift", we used dozens of genes that are briefly summarized in **Table 1**.

2. Improved Galactose Induction System

The galactose induction system in *Saccharomyces cerevisiae* has been widely used to conditionally overexpress genes because of strong induction and catabolite repression;[9,10] however, this system does not initiate rapid gene expression. To enhance galactose response without altering characteristics of glucose repression, we modulated three limiting factors of galactose signal transduction; galactose sensor (Gal1p), transcriptional activator (Gal4p) and galactose transporter (Gal2p). After transgenic yeasts harboring the appropriate constructs were prepared as described in **Table 2**, we evaluated their galactose response.

The kinetics of galactose induction during the early 4 h was quantitatively compared in the recombinant yeasts using flow cytometry (**Fig. 2**). After the cell cultures induced with 0.5% galactose medium were sampled at the indicated time and diluted ten-fold with physiological saline, the fluorescent intensity (FI) of at least 10,000 cells in each sample was measured using Cell Lab Quanta SC MPL flowcytometer (Beckman-Coulter) equipped with a filter set (510/10) and excited at 488 nm. Three independent measurements were averaged, and standard deviation was calculated. The FI of the control strain at 0 h was used as a basal expression, and the fluorescence of all the strains was comparable before induction (Fig. 2). In the strain carrying *HIS3pro-GAL1*, *HIS3pro-GAL2* and *GAL10pro-GAL1*, the expression of GFP protein was increased about 100-fold within 1 h and 1000-fold within 2 h after galactose induction. Because *GAL1* expression is repressed 1000-fold in glucose comparable to in galactose,[11] this transformant

Table 1 List of genes used in this study.

Name	Description	Derived organism
CYC1	Cytochrome c	*S. cerevisiae*
GAL1	Galactokinase	*S. cerevisiae*
GAL2	Galactose permease	*S. cerevisiae*
GAL4	DNA-binding transcription factor	*S. cerevisiae*
GAL10	UDP-glucose-4-epimerase	*S. cerevisiae*
HIS3	Imidazoleglycerol-phosphate dehydratase	*S. cerevisiae*
MFA2	Mating pheromone a-factor	*S. cerevisiae*
MIG1	Transcription factor	*S. cerevisiae*
PDC1	Pyruvate decarboxylase	*S. cerevisiae*
PDC5	Pyruvate decarboxylase	*S. cerevisiae*
PDC6	Pyruvate decarboxylase	*S. cerevisiae*
PGK1	3-Phosphoglycerate kinase	*S. cerevisiae*
TDH3	Glyceraldehyde-3-phosphate dehydrogenase	*S. cerevisiae*
TPS1	Subunit of trehalose-6-phosphate synthase	*S. cerevisiae*
TPS2	Subunit of trehalose-6-phosphate synthase	*S. cerevisiae*
TPS3	Subunit of trehalose-6-phosphate synthase	*S. cerevisiae*
TSL1	Subunit of trehalose-6-phosphate synthase	*S. cerevisiae*
GFP	Green fluorescent protein	*Aequorea victoria*
mKO2	Red fluorescent protein	*Fungia concinna*
LDH	Lactate dehydrogenase	*Bos taurus*
Cre	Cre DNA recombinase	Bacteriophage P1

Table 2 List of strains.

Strain number	Relative genotype
GSW111	*trp1-1* :: *TRP1 GAL1pro-GFP ura3-1* :: *URA3 leu2-3, 112* :: *LEU2*
GSW112	*trp1-1* :: *TRP1 GAL1pro-GFP ura3-1* :: *URA3 leu2-3, 112*:: *LEU2 GAL10pro-GAL4*
GSW131	*trp1-1* :: *TRP1 GAL1pro-GFP ura3-1* :: *URA3 HIS3pro-GAL1 leu2-3, 112*:: *LEU2 GAL10pro-GAL4*
GSW132	*trp1-1* :: *TRP1 GAL1pro-GFP ura3-1* :: *URA3 HIS3pro-GAL2 leu2-3, 112*:: *LEU2 GAL10pro-GAL4*
GSW141	*trp1-1* :: *TRP1 GAL1pro-GFP ura3-1* :: *URA3 HIS3pro-GAL1 HIS3pro-GAL2 leu2-3, 112*:: *LEU2*
GSW142	*trp1-1* :: *TRP1 GAL1pro-GFP ura3-1* :: *URA3 HIS3pro-GAL1 HIS3pro-GAL2 leu2-3, 112*:: *LEU2 GAL10pro-GAL4*

All strains are derivatives of W303-1a; only modifications to the wild-type background are indicated. GSW131 and GSW132 are used only in the Supplementary materials.

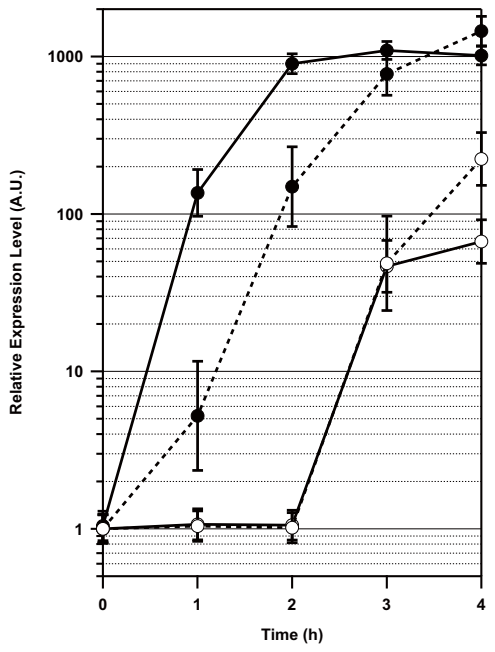

Fig. 2 Quantitative analysis on the galactose response of the recombinants. The control GSW111 strain (white circle and dotted line), the *GAL10pro-GAL4* GSW121 strain (open circle and solid line), the *HIS3pro-GAL1* and *HIS3pro-GAL2* GSW141 strain (filled circle and dotted line) and the GSW142 strain harboring *HIS3pro-GAL1*, *HIS3pro-GAL2* and *GAL10pro-GAL4* (filled circle and solid line) were investigated. The fluorescent intensity of the GSW111 strain at 0 h was used as a basal expression. The error bar means standard deviation (±SD). These data are average of three separate experiments.

seemed to achieve full induction within 2 h. The *HIS3pro-GAL1* and *HIS3pro-GAL2* strain exhibited about 5-fold FI within 1 h and 100-fold within 2 h after the induction. The GFP fluorescence of both the control and *GAL10pro-GAL4* strains was not significantly induced within 2 h but comparably enhanced about 50-fold within 3 h. At 4 h, the FI of the control strain was observed 3 times as strongly as that of the *GAL10pro-GAL4* strain. These results well coincided with those from immunoblot analysis. A galactose concentration of 5 g l^{-1} (0.5%) was empirically optimized to induce GFP expression among the concentrations to have been investigated (data not shown).

3. Identification of TPS1t as a Strong Terminator

In our genetic switch (Fig. 1), the activation of the second gene in the reporter construct is a bit problematic as indicted in the previous study.[12] The promoter upstream of the second gene is not exchangeable but the terminator downstream of it could be selectable.

In contrast to promoters, little attention has been paid to the selection of terminators for transgenes, and only a small subset of terminators, comprised of *CYC1t*, *TDH3t*, and *PGK1t*, are used routinely. Terminator is transcribed as 3'-UTR, which is involved in mRNA 3'-processing (cleavage and polyadenylation). The 3'-UTR also regulates the level of gene expression through various means, for example, by affecting mRNA stability, translation efficiency, and nuclear-to-cytoplasmic export.[13-16] In *S. cerevisiae*, the 3'-UTR

of mating pheromone a-factor (*MFA2*) has been investigated in detail; *MFA2* mRNA has a short half-life of 3.5 min.[17-19] When the 3'-UTR of *PGK1* ($t_{1/2}$ = 45 min) was replaced with the 3'-UTR of *MFA2*, the stability of the chimeric *PGK1-MFA2* transcript was about half that of the parental *PGK1* transcript.[20] Thus, substitution of the 3'-UTR potentially modulates the stability of chimeric transcripts.

A comprehensive microarray analysis of mRNA half-life in *S. cerevisiae* suggested that, among 4687 mRNAs, the transcripts from four distinct genes (*TPS1*, *TPS2*, *TPS3*, and *TSL1*) encoding protein subunits involved in trehalose phosphate synthesis are extremely stable, with $t_{1/2} = 105 \pm 12$ min.[21] The 3'-UTRs of these genes are therefore good candidates for what we call "long-life" terminators. Here, we compared the level of gene expression obtained with these terminators to that obtained with other terminators including *CYC1t*, *TDH3t*, and *PGK1t* (the corresponding mRNA half-lives $t_{1/2}$ = 15, 45, and 45 min, respectively).[21,22]

To determine the amount of the gene products produced in the presence of the various terminators, we measured the fluorescence intensity (FI) of the mKO2 or GFP protein in each transformant. Cells were cultured in complete synthetic medium with 20 g/L glucose and 40 mg/L adenine at 30°C in test tubes shaken at 70 rpm. Overnight cultures were diluted with 6 mL fresh medium to produce an OD_{660} of about 0.1, and the diluted cultures were incubated until the OD_{660} reached between 0.8 and 1.2. After the cultures were diluted 10-fold with physiological saline, the FI of at least 5000 cells in each sample was measured using a Cell Lab Quanta SC MPL flow cytometer (Beckman-Coulter) equipped with a filter set (570/15) for mKO2 or a filter set (510/10) for GFP and a 488 nm laser. The FI of the *CYC1t* transformant was used as a standard, and the Welch's t-test was used to determine the significance of the differences in fluorescent protein output in pairwise comparisons among these transformants in three independent experiments.

The shorter mRNA half-life of the *MFA2t* transformant was reflected in an FI of 60% of the *CYC1t* control (**Fig. 3**). This result seemed consistent to the previous results.[17] The FIs of both the *TDH3t* and *PGK1t* transformants were 140% that of the *CYC1t* transformant; this significant difference reflected the differences in their mRNA half-lives. Among the eight transformants, the *TPS1t* transformant exhibited the strongest fluorescence (160% of *CYC1t*), whereas the *TPS3t* and *TSL1t* transformants were comparable to the *PGK1t* transformant, and the *TPS2t* transformant was similar to the *CYC1t* transformant.

These and the other results together indicated that the *TPS1* terminator is a more effective terminator for yielding transgene products in *S. cerevisiae* than the traditional terminators *CYC1t*, *TDH3t,* and *PGK1t*.

4. A Cre-*lox* Genetic Switch to Dynamically Control Two Exclusive Phenotypes

The Cre-*lox* system has become a key technology for investigating the development of eukaryotic cells because it allows the strict control of gene expression

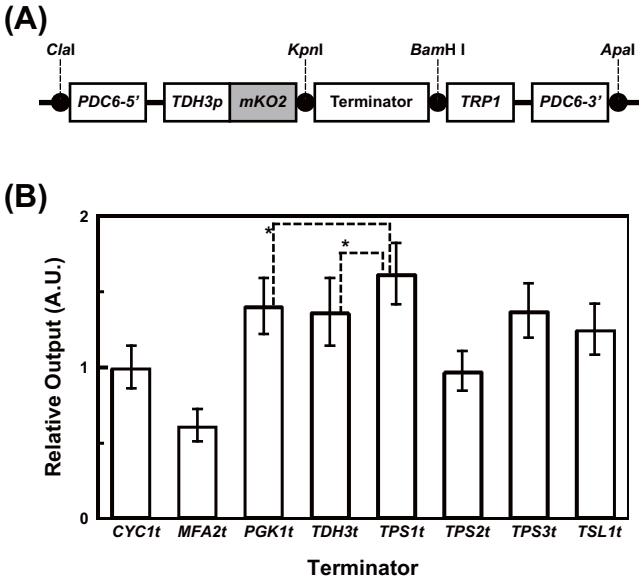

Fig. 3 Comparison of mKO2 Fluorescence Intensity among Various Terminator Transformants.
(A) Schematic diagram of gene constructs. The various terminators (*CYC1t, MFA2t, PGK1t, TDH3t, TPS1t, TPS2t, TPS3t, or TSL1t*) were inserted as modules after the *TDH3* promoter (*TDH3p*) and the monomeric Kusabira Orange 2 (mKO2) fluorescent protein gene. Restriction sites (KpnI and BamHI) are indicated. *PDC6-5′* and *PDC6-3′* denote the 5'- and 3'-regions of *PDC6* respectively. The *TRP1* gene was inserted for selection purposes. (B) Comparison of output at the protein level. Relative outputs were calculated as ratios between the fluorescence intensity (FI) of the transformant containing the indicated terminator and the FI of the *CYC1t* transformant. Values are the means of three independent experiments. Error bars represent standard deviation. *p < 0.05, Welch's t-test.

in a tissue-specific manner.[5,23,24] Even though we applied the Cre-*lox* switch, controlling dynamic cellular events is a continuing challenge.

We improved the Cre-*lox* system by employing two components: an enhanced galactose-induction system (**Fig. 4**A, described in section 2) and a codon-optimized Cre gene (Fig. 4B). We employed a modified *GAL1* promoter, *GAL1m1p*, to suppress "leaky" Cre expression under repressing conditions; the pseudo-MIG1 binding site (GGCCCCACAAACCTTCA, –272 to –256 from the beginning of the ORF) in the original *GAL1* promoter[25] was replaced with a MIG1 binding site (TTCCCCGCATTTTTATT) that binds MIG1 in the presence of glucose, thereby suppressing any *GAL1* promoter activity (Fig. 4B). In addition, we introduced the *MFA2* terminator downstream of the Cre gene to reduce the level of transgene expression through labilization of the mRNA (Fig. 4B).[17-19] With these modifications, background (proportion of cells expressing the second transgene without induction) was reduced to 6.6% ± 0.2% (n = 3), whereas that with an unmodified *GAL1* promoter and *CYC1* terminator, background was approximately 30% (data not shown).

In the reporter system, we used a promoter, *TDH3p* × 2, to drive two fluorescent proteins, GFP and mKO2[26]; GFP and mKO2 were individually prefixed by *loxP* and placed consecutively in the same direction (**Fig. 5**A, upper). GFP was exclusively expressed before recombination (Fig. 5A, upper) while mKO2 was expressed after recombination (Fig. 5A, bottom). To enhance mKO2 gene expression, we set the *TPS1* terminator identified as a strong terminator (described in section 3) under the mKO2 ORF.

The changes in cell phenotype were examined qualitatively by fluorescence microscopy. Before induction, most cells emitted green fluorescence from GFP (94% of total cell number). The fluorescent phenotype showed that the first ORF (GFP) and the second ORF (mKO2) indicated the ON and OFF states, respectively (Fig. 5B, left). Ten hours after induction, most cells expressed mKO2 red fluorescent protein (91% of total cell number) and a small number expressed GFP (Fig. 5B, right), indicating that the first and second ORF had become switched to the OFF and ON states, respectively, in most cells. An even smaller number of cells were orange or yellow, suggesting that an unusual recombination event had occurred in these cells. We also performed flow cytometry analyses to quantify the change in the ORF output states, and the results were consistent with the fluorescence microscopy observations.

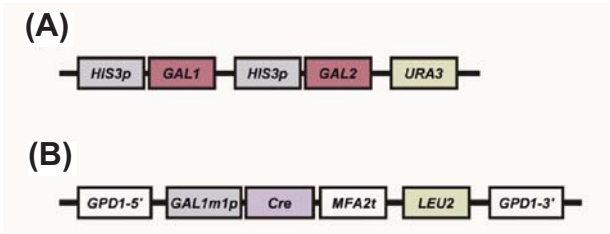

Fig. 4 The Cre-*lox* genetic switch used in this study. (A) The enhanced galactose induction system enables a gene under the control of the *GAL1* promoter to be expressed within 1 h of induction without altering glucose repression. *HIS3p* signifies the *HIS3* promoter. *URA3* is a selection marker. (B) The optimized Cre gene. *GAL1m1p* and *MFA2t* are the *GAL1m1* promoter and *MFA2* terminator, respectively, described in the methods section. *GPD1-5′* and *-3′* signify the *5′* and *3′* regions of the *GPD1* locus, the deactivation of which are reported to give better lactate yields. *LEU2* is a selection marker.

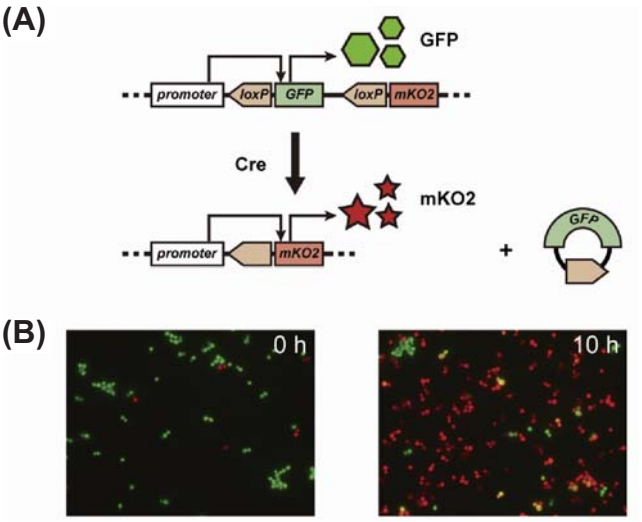

Fig. 5 Genetic switch with Cre-*lox* recombination. (A) Schematic diagram of the genetic switch from GFP to mKO2 in the GFP-mKO2 cassette. The top and bottom diagrams show the genetic constructs before and after recombination by Cre, respectively. (B) Fluorescence microscopy images of the change in fluorescent phenotype. Fluorescence from GFP and mKO2 were recorded independently in green and red channels, respectively, and then merged. Two representative images at 0 h (left) and 10 h (right) are shown.

5. Metabolic Shift from Ethanol to Lactate

With the present genetic switch, we realized the "metabolic shift" concept. A transgenic yeast was prepared to shift its main metabolite from ethanol to lactate by switching expression from *PDC* to *LDH* transgenes (**Fig. 6**A). Lactate-producing yeasts have been developed and improved to the industrial level in our company.[27-33] There are three *PDC* genes in the yeast genome: *PDC1*, *PDC5* and *PDC6*. *PDC1* is indispensable and constitutively expressed.[34] In this transformant, the *PDC-LDH* gene cassette was substituted for the *PDC1* locus (Fig. 6A), allowing the native *PDC1* promoter to drive the *PDC1* gene in the cassette before recombination. After recombination, it acquires the *Δpdc1* genotype, and the *PDC1* promoter, which is stronger in the *Δpdc1* mutant than in the wild-type,[35] drives *LDH*. The *PDC5* locus was disrupted by use of a selection marker. *PDC6* was left unchanged because it is a very minor isoform.[36] As anticipated, the transformant yeast cells grew slightly more slowly than wild-type (specific growth rate (μ): $\mu_{MS1} = 0.45\ h^{-1}$ and $\mu_{WT} = 0.47\ h^{-1}$).

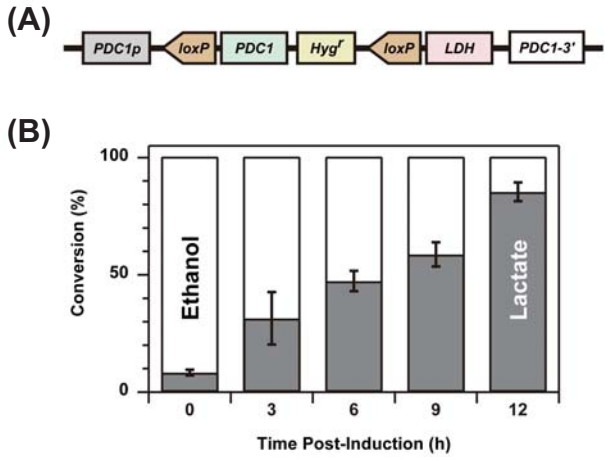

Fig. 6 Metabolic shift from ethanol to lactate production. (A) Schematic representation of the *PDC-LDH* cassette gene. *PDC1p* represents the original *PDC1* promoter and *PDC1-3′* represents its 3' region. Hyg^r signifies the hygromycin resistance marker gene. (B) Metabolite analysis. Open and closed boxes represent the conversion % for ethanol and lactate, respectively. For ethanol, measured values were converted to glucose equivalents, which were then used as the ethanol concentrations. The sum of the ethanol and lactate was taken as 100%, and lactate conversion % was calculated, because glucose was fermented almost to ethanol or lactate. The average of three independent measurements ± SD is shown; 8.3 ± 1.2, 31.5 ± 11.2, 47.4 ± 4.3, 58.7 ± 5.1, and 85.4 ± 4.0 in MS1 at 0 h (non-induced), 3 h, 6 h, 9 h, and 12 h post-induction, respectively. The lactate yield of the wild-type was below the detection limit (data not shown).

While the LDH activity of non-induced cells was 0.068 unit/mg, which was markedly higher than that of wild-type, the LDH activity of the induced cells was substantially elevated at 3 h post-induction, and reached a maximum (0.65 unit/mg) at 6 h post-induction. It then decreased slightly for 6–12 h post-induction, but remained about 10-fold higher than that in non-induced cells. The PDC activity was not determined because of a technical problem, which is discussed in the original report.

Metabolite analysis demonstrated a shift in the main metabolic flow; it took 12 h to produce the major product when the transformant yeast was shifted from ethanol to lactate (Fig. 6B). The lactate yields were increased from 8.3% of glucose converted in non-induced MS1 to 85.4% in MS1 at 12 h post-induction. The lactate productivity in the metabolic-shifted MS1 was as efficient as that of *Δpdc1/5* lactate-producing yeast, which converted 81.3% of glucose to lactate.[30]

In conclusion, we successfully developed a Cre-*lox* genetic switch to control the exclusive expression of two genes: a fluorescent gene set (GFP and mKO2) and a metabolic enzyme gene set (PDC and LDH). We demonstrated that this switch was useful for the shifting of main carbon flow in yeast from ethanol to lactic acid, and we proposed this type of shift as a "metabolic shift". Because the Cre-*lox* system works well in both prokaryotes and eukaryotes, it should be applicable to most organisms and contribute to the production of bio-fuels and bio-plastics.

References

(1) Luengo, J. M., Garcia, B., Sandoval, A., Naharro, G. and Olivera, E. R., "Bioplastics from Microorganisms", *Curr. Opin. Microbiol.*, Vol.6, No.3 (2003), pp.251-260.

(2) Vemuri, G. N. and Aristidou, A. A., "Metabolic Engineering in the -omics Era: Elucidating and Modulating Regulatory Networks", *Microbiol. Mol. Biol. Rev.*, Vol.69, No.2 (2005), pp.197-216.

(3) Suriyamongkol, P., Weselake, R., Narine, S., Moloney, M. and Shah, S., "Biotechnological Approaches for the Production of Polyhydroxyalkanoates in Microorganisms and Plants - A Review", *Biotechnol. Adv.*, Vol.25, No.2 (2007), pp.148-175.

(4) Radakovits, R., Jinkerson, R. E., Darzins, A. and Posewitz, M. C., "Genetic Engineering of Algae for Enhanced Biofuel Production", *Eukaryot. Cell*, Vol.9, No.4 (2010), pp.486-501.
(5) Weber, W. and Fussenegger, M., "Novel Gene Switches", *Handb. Exp. Pharmacol.*, No.178 (2007), pp.73-105.
(6) Matsuyama, T., Yamanishi, M. and Takahashi, H., "Improvement of Galactose Induction System in *Saccharomyces cerevisiae*", *J. Biosci. Bioeng.*, Vol.111, No.2 (2011), pp.175-177.
(7) Yamanishi, M., Katahira, S. and Matsuyama, T., "*TPS1* Terminator Increases mRNA and Protein Yield in a *Saccharomyces cerevisiae* Expression System", *Biosci. Biotechnol. Biochem.*, Vol.75, No.11 (2011), pp.2234-2236.
(8) Yamanishi, M. and Matsuyama, T., "A Modified Cre-*lox* Genetic Switch to Dynamically Control Metabolic Flow in *Saccharomyces cerevisiae*", *ACS Synth. Biol.*, Vol.1, No.5 (2012), pp.172-180., <http://dx.doi.org/10.1021/sb200017p>
(9) Lohr, D., Venkov, P. and Zlatanova, J., "Transcriptional Regulation in the Yeast GAL Gene Family: a Complex Genetic Network", *FASEB Journal*, Vol.9, No.9 (1995), pp.777-787.
(10) Gancedo, J. M., "Yeast Carbon Catabolite Repression", *Microbiol. Mol. Biol. Rev.*, Vol.62, No.2 (1998), pp.334-361.
(11) Johnston, M., Flick, J. S. and Pexton, T., "Multiple Mechanisms Provide Rapid and Stringent Glucose Repression of GAL Gene Expression in *Saccharomyces cerevisiae*", *Mol. Cell. Biol.*, Vol.14, No.6 (1994), pp.3834-3841.
(12) Nolden, L., Edenhofer, F., Haupt, S., Koch, P., Wunderlich, F. T., Siemen, H. and Brustle, O., "Site-specific Recombination in Human Embryonic Stem Cells Induced by Cell-permeant Cre Recombinase", *Nat. Methods*, Vol.3, No.6 (2006), pp.461-467.
(13) Carswell, S. and Alwine, J. C., "Efficiency of Utilization of the Simian Virus 40 Late Polyadenylation Site: Effects of Upstream Sequences", *Mol. Cell. Biol.*, Vol.9, No.10 (1989), pp.4248-4258.
(14) Ingelbrecht, I. L., Herman, L. M., Dekeyser, R. A., Van Montagu, M. C. and Depicker, A. G., "Different 3' end Regions Strongly Influence the Level of Gene Expression in Plant Cells", *Plant Cell*, Vol.1, No.7 (1989), pp.671-680.
(15) Kuersten, S. and Goodwin, E. B., "The Power of the 3' UTR: Translational Control and Development", *Nat. Rev. Genet.*, Vol.4, No.8 (2003), pp.626-637.
(16) Zhao, J., Hyman, L. and Moore, C., "Formation of mRNA 3' ends in Eukaryotes: Mechanism, Regulation, and Interrelationships with Other Steps in mRNA Synthesis", *Microbiol. Mol. Biol. Rev.*, Vol.63, No.2 (1999), pp.405-455.
(17) Decker, C. J. and Parker, R., "A Turnover Pathway for Both Stable and Unstable mRNAs in Yeast: Evidence for a Requirement for Deadenylation", *Genes Dev*., Vol.7, No.8 (1993), pp.1632-1643.
(18) Herrick, D., Parker, R. and Jacobson, A., "Identification and Comparison of Stable and Unstable mRNAs in *Saccharomyces cerevisiae*", *Mol. Cell. Biol.*, Vol.10, No.5 (1990), pp.2269-2284.
(19) Muhlrad, D. and Parker, R., "Mutations Affecting Stability and Deadenylation of the Yeast MFA2 Transcript", *Genes Dev*, Vol.6, No.11 (1992), pp.2100-2111.
(20) Wang, Y., Liu, C. L., Storey, J. D., Tibshirani, R. J., Herschlag, D. and Brown, P. O., "Precision and Functional Specificity in mRNA Decay", *Proc. Natl. Acad. Sci. USA*, Vol.99, No.9 (2002), pp.5860-5865.
(21) Zitomer, R. S., Montgomery, D. L., Nichols, D. L. and Hall, B. D., "Transcriptional Regulation of the Yeast Cytochrome c Gene", *Proc. Natl. Acad. Sci. USA*, Vol.76, No.8 (1979), pp.3627-3631.
(23) Orban, P. C., Chui, D. and Marth, J. D., "Tissue- and Site-specific DNA Recombination in Transgenic Mice", *Proc. Natl. Acad. Sci. USA*, Vol.89, No.15 (1992), pp.6861-6865.
(24) Livet, J., Weissman, T. A., Kang, H., Draft, R. W., Lu, J., Bennis, R. A., Sanes, J. R. and Lichtman, J. W., "Transgenic Strategies for Combinatorial Expression of Fluorescent Proteins in the Nervous System", *Nature*, Vol.450, No.7166 (2007), pp.56-62.
(25) Nehlin, J. O., Carlberg, M. and Ronne, H., "Control of Yeast GAL Genes by MIG1 Repressor: a Transcriptional Cascade in the Glucose Response", *EMBO J.*, Vol.10, No.11 (1991), p.3373-3377.
(26) Sakaue-Sawano, A., Kurokawa, H., Morimura, T., Hanyu, A., Hama, H., Osawa, H., Kashiwagi, S., Fukami, K., Miyata, T., Miyoshi, H., Imamura, T., Ogawa, M., Masai, H. and Miyawaki, A., "Visualizing Spatiotemporal Dynamics of Multicellular Cell-cycle Progression", *Cell*, Vol.132, No.3 (2008), pp.487-498.
(27) Tokuhiro, K., Ishida, N., Kondo, A. and Takahashi, H., "Lactic Fermentation of Cellobiose by a Yeast Strain Displaying Beta-glucosidase on the Cell Surface", *Appl. Microbiol. Biotechnol.*, Vol.79, No.3 (2008), pp.481-488.
(28) Ishida, N., Saitoh, S., Onishi, T., Tokuhiro, K., Nagamori, E., Kitamoto, K. and Takahashi, H., "The Effect of Pyruvate Decarboxylase Gene Knockout in *Saccharomyces cerevisiae* on L-lactic Acid Production", *Biosci. Biotechnol. Biochem.*, Vol.70, No.5 (2006), pp.1148-1153.
(29) Ishida, N., Saitoh, S., Tokuhiro, K., Nagamori, E., Matsuyama, T., Kitamoto, K. and Takahashi, H., "Efficient Production of L-Lactic acid by Metabolically Engineered *Saccharomyces cerevisiae* with a Genome-integrated L-lactate Dehydrogenase Gene", *Appl. Environ. Microbiol.*, Vol.71, No.4 (2005), pp.1964-1970.
(30) Saitoh, S., Ishida, N., Onishi, T., Tokuhiro, K., Nagamori, E., Kitamoto, K. and Takahashi, H.,

"Genetically Engineered Wine Yeast Produces a High Concentration of L-lactic Acid of Extremely High Optical Purity", *Appl. Environ. Microbiol.*, Vol.71, No.5 (2005), pp.2789-2792.
(31) Ishida, N., Suzuki, T., Tokuhiro, K., Nagamori, E., Onishi, T., Saitoh, S., Kitamoto, K. and Takahashi, H., "D-lactic Acid Production by Metabolically Engineered *Saccharomyces cerevisiae*", *J. Biosci. Bioeng.*, Vol.101, No.2 (2006), pp.172-177.
(32) Ishida, N., Saitoh, S., Ohnishi, T., Tokuhiro, K., Nagamori, E., Kitamoto, K. and Takahashi, H., "Metabolic Engineering of *Saccharomyces cerevisiae* for Efficient Production of Pure L-(+)-lactic Acid", *Appl. Biochem. Biotechnol.*, Vol.129-132 (2006), pp.795-807.
(33) Tokuhiro, K., Ishida, N., Nagamori, E., Saitoh, S., Onishi, T., Kondo, A. and Takahashi, H., "Double Mutation of the PDC1 and ADH1 Genes Improves Lactate Production in the Yeast *Saccharomyces cerevisiae* Expressing the Bovine Lactate Dehydrogenase Gene", *Appl. Microbiol. Biotechnol.*, Vol.82, No.5 (2009), pp.883-890.
(34) Kellermann, E., Seeboth, P. G. and Hollenberg, C. P., "Analysis of the Primary Structure and Promoter Function of a Pyruvate Decarboxylase Gene (*PDC1*) from *Saccharomyces cerevisiae*", *Nucleic Acids Res.*, Vol.14, No.22 (1986), p.8963-8977.
(35) Eberhardt, I., Cederberg, H., Li, H., Konig, S., Jordan, F. and Hohmann, S., "Autoregulation of Yeast Pyruvate Decarboxylase Gene Expression Requires the Enzyme but not Its Catalytic Activity", *Eur. J. Biochem.*, Vol.262, No.1 (1999), pp.191-201.
(36) Hohmann, S., "Characterization of PDC6, a Third Structural Gene for Pyruvate Decarboxylase in *Saccharomyces cerevisiae*", *J. Bacteriol.*, Vol.173, No.24 (1991), pp.7963-7969.

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