A Modified Cre-*lox* Genetic Switch To Dynamically Control Metabolic Flow in *Saccharomyces cerevisiae*

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S Supporting Information

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. Here, we demonstrated that this flow can be controlled by the exclusive expression of *PDC* and *LDH* with a Cre-*lox* genetic switch. This switch was evaluated with a gene cassette that encoded two different fluorescence proteins and enabled changes in genotype and phenotype within 2 and 10 h, respectively. Transgenic yeast harboring this switch and the *PDC-LDH* cassette showed a specific growth rate (0.45 h^{-1}) that was almost the same as that of wild-type (0.47 h^{-1}). Upon induction of the genetic switch, the transgenic yeast produced lactate from up to 85.4% of the glucose substrate, while 91.7% of glucose went to ethanol before induction. We thus propose a "metabolic shift" concept that can serve as an alternative means to obtain gene products that are currently difficult to obtain by using conventional methodologies.

KEYWORDS: *Cre-lox genetic switch, metabolic engineering, fluorescence protein, flow cytometry, lactic acid-producing yeast, metabolic shift*
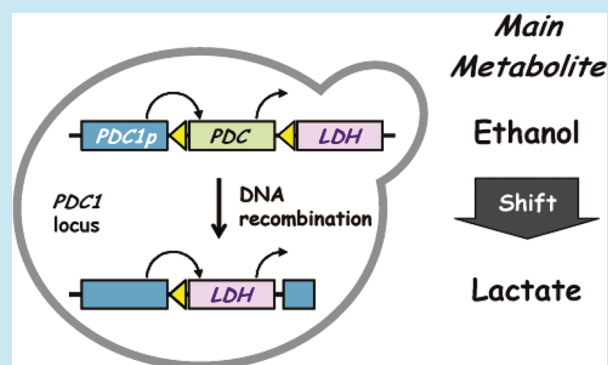
The advent of genetic engineering has allowed microorganisms to produce chemicals for bioplastics and biofuels.[1−5] In these transgenic microorganisms, branching points are inserted into metabolic pathways by the introduction of enzymes that convert intermediates into a target product or its precursors. Since the artificial branching point creates a new end-product and the product of the original metabolic pathway becomes an undesirable byproduct, the flow of the original metabolic pathway from the branching point must be suppressed for efficient production of the target. This suppression often obstructs the growth of the microorganisms, which considerably decreases the efficiency of fermentation. We faced this kind of problem when we developed lactic acid-producing yeast,[6] and to date there is still no effective method to dynamically control the shift from innate to engineered metabolism.

To address this need, we examined the use of the Cre-*lox* system[7] to regulate exclusive gene expression and applied it to metabolic engineering. Stringent and irreversible control of gene expression can be achieved by the inducible removal of a transgene by Cre-*lox* site-specific recombination, which is a well-studied and versatile system.[8,9] In this system, any two transgenes can be controlled exclusively when a promoter is placed in front of the two consecutive open reading frames (ORFs), each of which is prefixed by *lox*P, because the first and second ORFs are independently transcribed before and after DNA recombination, respectively.[10] Although the Cre-*lox* system has been extensively used in studies of eukaryotic cell development, its use has not been thoroughly explored in the fields of metabolic engineering. This may be in part because the primary disadvantage associated with the Cre-*lox* system is that the transgenic phenotype cannot be distinguished, as only low levels of the second protein are produced after recombination.[10]

The yeast *Saccharomyces cerevisiae* has been widely used in food and beverage processing because it ferments glucose to ethanol via pyruvate, the final product of glycolysis (Figure 1, left). Because yeast is more tolerant than lactic acid bacteria to sugar and acid,[11,12] it has potential as a host organism to produce lactate. Dequin and Barre[13] constructed the first lactate-producing transgenic yeast by introducing an exogenous lactate dehydrogenase (*LDH*) gene into yeast (Figure 1, right). Since then, for more efficient lactate production, more genetic
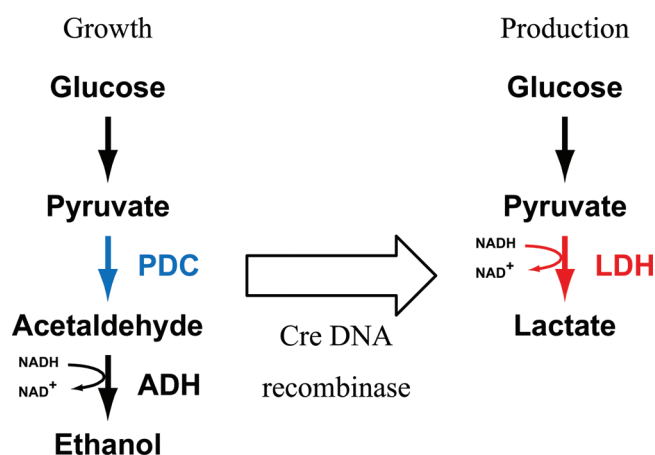
Figure 1. Concept of "metabolic shift". Pyruvate is produced by glycolysis of glucose. Under growth phase conditions, the transgenic yeast converts pyruvate to ethanol via acetaldehyde in the same way as wild-type yeast (left). Cre DNA recombinase changes the *PDC* genotype to the *LDH* genotype of the yeast, and the phenotype of the yeast changes accordingly. Under production phase conditions, pyruvate is now converted to lactate by the acquired LDH (right).

modifications have been introduced to decrease the pyruvate decarboxylase (*PDC*) activity and to increase the LDH activity;[6,14,15] however, repression of *PDC* prevents yeast from growing as efficiently as yeast with *PDC*.[16] For example, the Δ*pdc1/5* lactate-producing yeast, which converts 81.3% of glucose to lactate, requires a 192-h incubation period to grow;[15] the Δ*pdc1* lactate-producing yeast, however, converts 62.2% of glucose in a 72-h period.[6] Although the lactate yield from glucose in Δ*pdc1/5* lactate-producing yeast can reach industrial levels, the rate of production is unsatisfactory. Accordingly, more investment in plants and equipment is required, which raises production costs.

Here, we report a "metabolic shift" in yeast; although ethanol fermentation takes place during growth, the phenotype of the yeast becomes that of lactate-producing. We begin by describing the development of the Cre-*lox* genetic switch and how we overcame the limitations of the current Cre-*lox* system. We then describe how we applied this genetic switch to shift the primary metabolism in yeast by changing the key enzymes from PDC to LDH (Figure 1).

RESULTS AND DISCUSSION

We developed a Cre-*lox* genetic switch that enabled the exclusive expression of two genes, as follows. Since Cre recombinase induced by galactose requires 24 h for complete excision of the floxed DNA region,[17] we applied an enhanced galactose induction system[18] to induce Cre recombinase (Figure 2A and B). Gene expression with the *GAL1* promoter is occasionally leaky,[19] and a very low but noteworthy level of Cre expression was unavoidable with the original *GAL1* promoter even under repressing conditions (data not shown). MIG1, a repressor, binds to the MIG1 binding site in the *GAL1* promoter in the presence of glucose.[20] We converted a pseudo-MIG1 binding site (GGCCCCACAAACCTTCA, -272 to -256 from the beginning of the ORF) in the original *GAL1* promoter to an authentic MIG1 binding sequence (TTCCCC GCATTTTTATT) so that two repressors can bind to the promoter. We termed this modified promoter the *GAL1m1* promoter. To repress the inhibitory effect of Cre recombinase
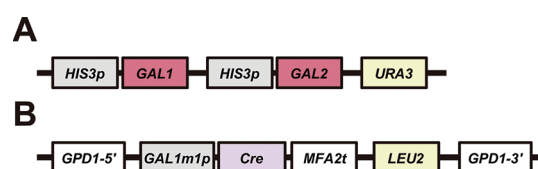
Figure 2. Cre-*lox* genetic switch used in this study. (A) The enhanced galactose induction system[18] enables a gene under the control of the *GAL1* promoter to be expressed within 1 h of induction without altering glucose repression. *HIS3p* signifies the *HIS3* promoter. *URA3* is a selection marker. (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.[22] *LEU2* is a selection marker.

on yeast, we used the *MFA2* terminator, which confers a short mRNA half-life to the upstream ORF.[21]

Fluorescent reporter genes enabled us to qualitatively and quantitatively evaluate any changes in cell phenotype invoked by the Cre genetic switch, which is easier than measuring enzyme activities or metabolites. We employed two fluorescent reporter genes, *GFP* and *mKO2*,[23] and constructed the yeast transformant FS1 harboring the *GFP-mKO2* cassette (Figure 3A, Table 1). These reporter genes were prefixed by *lox*P sequences
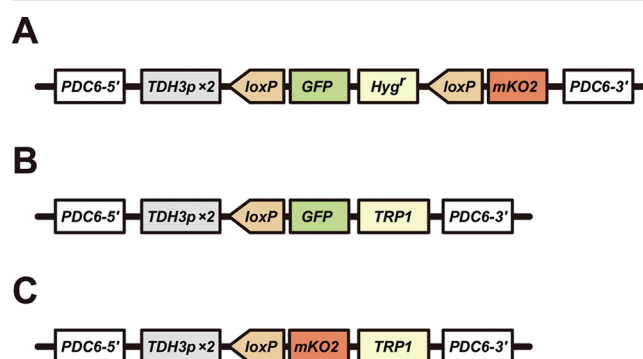
Figure 3. *GFP-mKO2* cassette and its controls. (A) The *GFP-mKO2* cassette gene integrated in the FS1 transformant. (B) The *GFP* control gene integrated in the yeast FC1 transformant. (C) The *mKO2* control gene integrated in the yeast FC2 transformant. *TDH3p*×2 signifies two copies of the upstream activating sequence (-676 to -381 bp from the start codon) of *TDH3*. *TRP1* and *Hyg*[r] are selection markers. *PDC6-5′* and *-3′* signify the 5′ and 3′ regions of the *PDC6* locus.

and placed consecutively in the cassette so that the strong dual-*TDH3* promoter drove expression of the *GFP* gene before the recombination reaction, and the *mKO2* gene after the reaction (Figure 4A). The yeast transformants FC1 (the GFP control, Table 1) and FC2 (the mKO2 control, Table 1) were also constructed as controls mimicking the pre- and post-induction states of FS1 (Figure 3B and 3C, respectively). The *GFP-mKO2* cassette began to recombine within 1 h of galactose induction, and maximum recombination occurred within 2 h, as assessed by using Southern blot analysis (Figure 5), and is consistent with previous reports.[9,10,24] Fluorescence microscopy demonstrated a change in cell phenotype (Figure 4B). Almost all of the FS1 cells emitted green fluorescence from GFP before induction (94% of the total cell number), which changed to red fluorescence from mKO2 at 10 h post-induction (91% of the total cell number). The changes in cell phenotype were followed quantitatively by using flow cytometry

Table 1. Yeast Transformants

transformant	genotype[a]	reference
W303-1a (wild-type)	*MAT*a *ade2*-1 *ura3*-1 *his3*-11 *trp1*-1 *leu2*-3 *leu2*-112 *can1*-100	ATCC[b]
FC1 (GFP control)	*PDC6::TDH3p×2-loxP-GFP+PEST+MFA2t-TRP1*	this study
FC2 (mKO2 control)	*PDC6::TDH3p×2-loxP- mKO2-TPS1t-TRP1*	this study
FS1	*ura3-1::HIS3p-GAL1-HIS3p-GAL2-URA3*	18
(before recombination)	*GPD1::GAL1m1p-Cre-MFA2t-LEU2*	this study
	PDC6::TDH3p×2-loxP-GFP+PEST-MFA2t-Hyg^r-loxP-mKO2-TPS1t-TRP1	this study
(after recombination)	*ura3-1::HIS3p-GAL1-HIS3p-GAL2-URA3*	
	GPD1::GAL1m1p-Cre-MFA2t-LEU2	
	PDC6::TDH3p×2-loxP-mKO2-TPS1t -TRP1	
MS1	*ura3-1::HIS3p-GAL1-HIS3p-GAL2-URA3*	18
(before recombination)	*GPD1::GAL1m1p-Cre-MFA2t-LEU2*	this study
	PDC1::PDC1p-loxP-PDC1-Hyg^r-loxP-LDH	this study
	PDC5::TRP1	
(after recombination)	*ura3-1::HIS3p-GAL1-HIS3p-GAL2-URA3*	
	GPD1::GAL1m1p-Cre-MFA2t-LEU2	
	PDC1::PDC1p-loxP-LDH	
	PDC5::TRP1	

[a]All genotypes, except that of W303-1a (wild-type), describe differences from W303-1a. [b]American Type Culture Collection. http://www.atcc.org

(Figure 4C). Before induction, GFP fluorescence in FS1 was the same as that in FC1 and disappeared between 4 and 5 h post-induction. Similarly, mKO2 fluorescence in FS1 was virtually zero before induction and increased to the same level as that in FC2 between 8 and 9 h post-induction. In two-dimensional representations with GFP output versus mKO2 output, the cells formed a tight group that moved in concert, and 89% of the cells shifted from the GFP-expressing phenotype to an mKO2-expressing one (Figure 4C and Supplementary Movie 1). This result was consistent with our fluorescence microscopy observations and indicates that the change in phenotype occurred synchronously in the cell population. We conclude that our genetic construct worked as a genetic switch, as designed.

The transgenic yeast MS1 was designed to shift its main metabolite from ethanol to lactate by switching expression from *PDC* to *LDH* transgenes (Figure 1). There are three *PDC* genes in the yeast genome: *PDC1*, *PDC5*, and *PDC6*. *PDC1* is essential and constitutively expressed.[25] In MS1, the *PDC1* locus was replaced with the *PDC-LDH* gene cassette (Figure 6A), so that the native *PDC1* promoter would drive the *PDC1* gene in the cassette before recombination. After recombination, MS1 acquires the Δ*pdc1* genotype, and the *PDC1* promoter, which is stronger in the Δ*pdc1* mutant than in the wild-type,[26] drives *LDH*. The *PDC5* locus was disrupted by use of a selection marker. *PDC6* was left unchanged because it is a very minor isoform gene under fermentation condition,[27] although *PDC6* in the Δ*pdc1/5* mutant was expressed to some extent after long-term adaptation.[12,29] As anticipated, MS1 grew slightly slower than wild-type (specific growth rate (μ); μ_{MS1} = 0.45 h^{-1} and μ_{WT} = 0.47 h^{-1}).

MS1 was incubated for 12 h post-induction rather than 10 h because higher LDH activity was detected at this time point than at 10 h post-induction (data not shown). The pH of the fermentation medium (initially pH 5.1) went down to pH 4.1 after a 1-h incubation with induced MS1, indicating lactate production. PDC activity is usually determined photometrically by using an alcohol dehydrogenase (ADH)-coupled method in which PDC converts pyruvate to acetaldehyde, and ADH converts acetaldehyde to ethanol by using NADH. The LDH reaction from pyruvate to lactate also requires NADH as a

coenzyme. Since the apparent substrate and coenzyme are the same in both the PDC/ADH and LDH reactions, it was difficult to distinguish the PDC and LDH activities by using conventional methods. We were able to determine the LDH activity alone by using lactate and NAD$^+$ as the substrate and coenzyme, respectively, and by using a coupling method with diaphorase.[30] Because there is no *LDH* gene in yeast, the LDH activity detected in wild-type (0.031 unit/mg) presumably came from other enzymes coupling with diaphorase. The LDH activity of non-induced MS1 (or 0 h post-induction) was 0.068 unit/mg, which was markedly higher than that of wild-type. The LDH activity of induced MS1 was substantially increased in 3 h post-induction and reached a maximum (0.65 unit/mg) at 6 h post-induction (Figure 6B). It then slightly decreased during the 6−12 h post induction but remained about 10 times that of non-induced MS1.

Metabolite analysis of MS1 demonstrated a shift in the main metabolic flow; it took 12 h to produce the major product when MS1 was shifted from ethanol to lactate (Figure 6C). The LDH activity of induced MS1 was sufficient to convert more of the glucose than that used in the fermentation test (0.5%) within 1 h; lactate production in induced MS1 was approximately 1 g/OD/L/h. 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 Δ*pdc1/5* lactate-producing yeast, which converted 81.3% of glucose to lactate.[15] The yield would become even higher if several parameters were optimized, in particular, the host strain used. We used W303-1a as the host yeast for all of our experiments. However, this strain may not be suitable to maintain Δ*pdc1/5*. Since we had incorporated the genetic switch, we selected W303-1a. For example, OC-2T strain that we have used previously[6,15,22,28] would be an appropriate next step to developing lactate-producing yeasts that have the characteristics of both high yield and productivity.

Discussion. In this study, the Cre-*lox* genetic switch successfully controlled the exclusive expression of two sets of genes: those encoding two fluorescent proteins and those encoding two metabolic enzymes. We demonstrated changes in
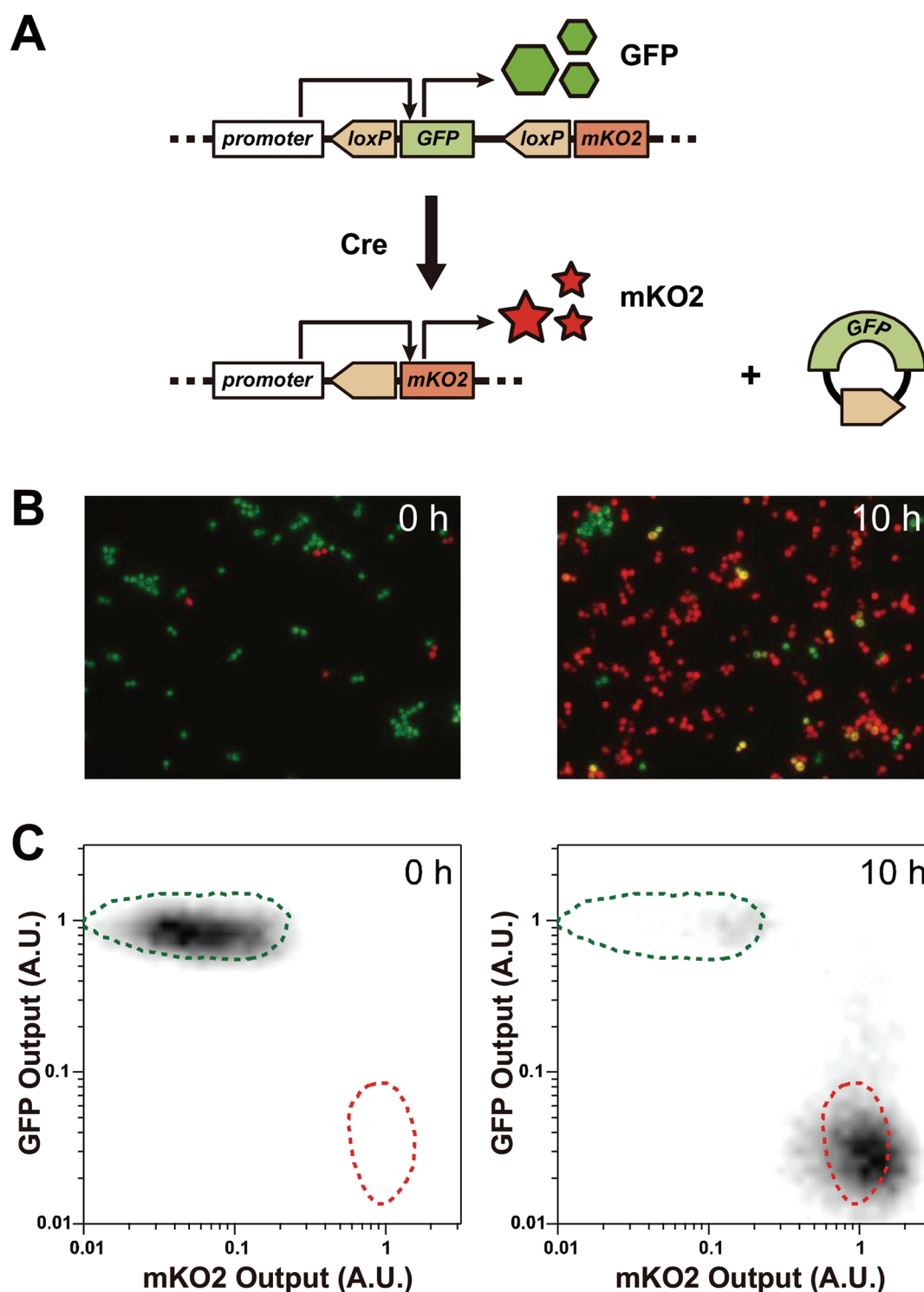
Figure 4. Genetic switch with Cre-*lox* recombination. (A) Schematic diagram of the genetic switch from *GFP* to *mKO2* in the *GFP-mKO2* cassette (FS1 transformant). The top and bottom diagrams show the genetic constructs before and after recombination by Cre, respectively. (B) Fluorescence microscopy 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. (C) Two-dimensional representation of cell distribution. GFP and mKO2 outputs for each cell were plotted on a log scale; the black "clouds" represent areas containing multiple cells. Dotted lines indicate areas where 95% of the cells in the GFP (green) and mKO2 (red) controls (yeast FC1 and FC2 transformants), respectively, are distributed. Two representative images at 0 h (left) and 10 h (right) are shown. See Supplementary Movie 1 for the dynamic changes in fluorescent phenotype.

both genotype and phenotype in each transgenic yeast. Ethanol fermentation is a signature metabolic process in yeast, and PDC is an indispensable enzyme in that process. The fact that

we could switch from *PDC* to *LDH* induction in this system suggests that the Cre-*lox* genetic switch could be used to introduce and control the expression of any yeast gene. The
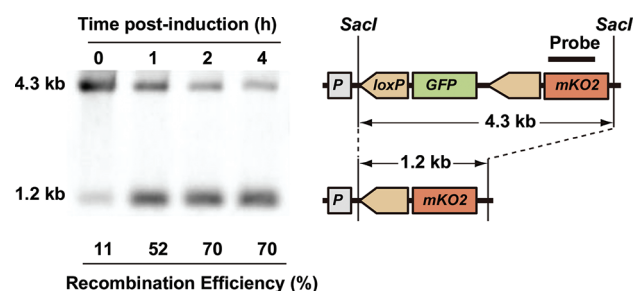
Figure 5. Southern blot image and the recombination efficiency calculated from the image (left panel) and schematic representations of the genes before and after recombination (right panel). The genes before and after recombination produced DNA fragments of length 4.3 and 1.2 kb, respectively. The signal intensity was integrated electrically, and the intensity of the 1.2-kb band was calculated as a percentage of the total intensity. Flow cytometry indicated that more than 90% of the cells changed their phenotype, whereas the genomic Southern analysis demonstrated that the recombination efficiency was approximately 70% at best. This inconsistency has been found in other Cre-*lox* genetic switches.[10,24] One possible explanation is that the total genomic DNA may contain unswitched DNA from yeast that died before the recombination reaction was complete. Such yeast would be excluded in flow cytometry with the appropriate window.

Cre-*lox* system functions in both prokaryotes and eukaryotes[31] and has been a key tool in studies of development in mammalian cells.[32,33] Conditional versions of Cre have been developed in several organisms,[8] and it should be possible to control the exclusive expression of two genes in most microorganisms or in cultured cells.

While the genomic DNA was reorganized as we had anticipated, control at the phenotype level needed careful design. We observed that the metabolic phenotype appeared much faster than the fluorescent phenotype. The LDH activity was observed considerably at 3 h post-induction and reached a maximum at 6 h post-induction, whereas mKO2 became active between 8 and 9 h post-induction. The reason for this apparent difference may be that the enzyme activity and fluorescence came from mature proteins. Fluorescent proteins become active after chromophore formation, which takes a few hours following the synthesis of the protein.[34] On the other hand, LDH requires only protein synthesis. In our case, the delay in the fluorescent reporter was 2−3 h, which might be a reasonable time requirement for chromophore formation. The disappearance of the remaining PDC activity is another issue that must be addressed. The highest lactate yield conversion was achieved not at 6 h post-induction, when the highest LDH activity was detected, but at 12 h post-induction. PDC and LDH have similar affinities (K_m) for pyruvate, 1.09[35] and 1.0 mM,[36] respectively, and both enzymes compete for substrate to produce acetaldehyde or lactate, respectively. In addition, PDC, but not LDH, might be involved in a multienzyme complex of glycolysis, and the substrate might be supplied via substrate channeling.[37] Substrate channeling might be efficient in W303-1a, because the lactate yield of the lactate-producing yeast derived from W303-1a was smaller than that derived from other host strains.[38] To control the remaining activity, we introduced a PEST sequence at the C-terminal of PDC, which successfully reduced GFP levels in the yeast FS1 (ref 39, Table 1). However, we could not obtain transgenic yeast. The active form of PDC is a tetramer, and the C-terminal region of PDC is located at its interface.[40] The introduction of around 30 amino acids may, therefore, have hampered the formation of this
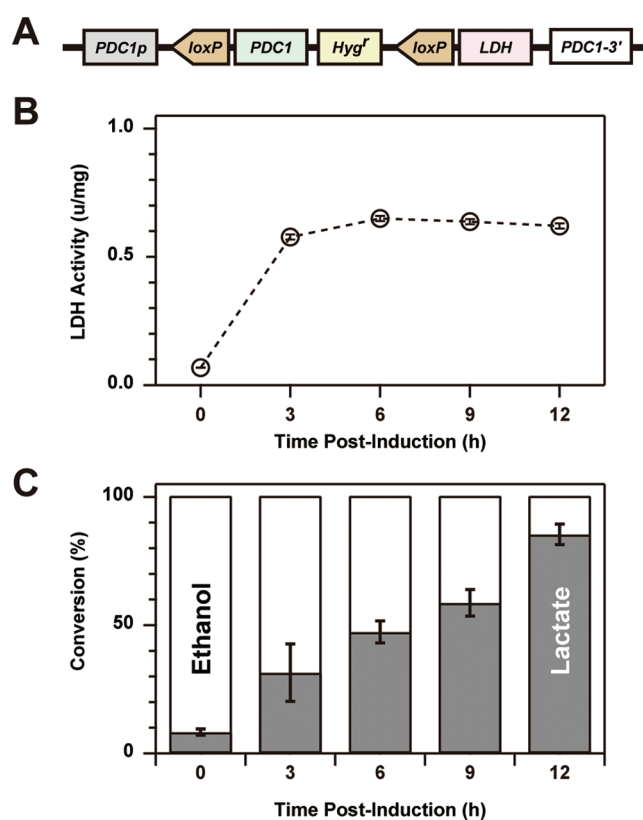
Figure 6. Metabolic shift from ethanol to lactate production. (A) Schematic representation of the *PDC-LDH* cassette gene. *PDC1p* represents the original *PDC1* promoter and *PDC1-3′* represents its 3′ region. *Hyg'* signifies the hygromycin resistance marker gene.[28] (B) LDH activity test. LDH activities were determined by using the coupling method described in Methods. The average of three independent measurements ± standard deviation (SD) is shown: 0.068 ± 0.001, 0.58 ± 0.01, 0.65 ± 0.01, 0.64 ± 0.01, and 0.62 ± 0.01 in MS1 at 0 (non-induced), 3, 6, 9, and 12 h post-induction, respectively. (C) 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 (non-induced), 3, 6, 9, and 12 h post-induction, respectively. The lactate yield of the wild-type was below the detection limit (data not shown).

active conformation. We were also unable to obtain transgenic yeast harboring *PEST*-fused *PDC1* in place of *PDC1* (data not shown).

The main technical problem we encountered was that the presence of Cre inhibited the expression of the second ORF, mKO2 (Figure 7A, C, and D open circles, and Supplementary Movie 2). It was necessary to remove Cre as quickly as possible after the genomic reorganization was complete. In estrogen receptor-fused recombinases,[9,41−43] which undergo active conformation only in the presence of estrogen, the quick removal of Cre activity is brought about by ligand dilution with medium.[24] Dilution is effective in the laboratory setting but is not compatible with industrial processes, where the substrate is exposed to dense microorganisms. In our genetic switch, Cre activity was controlled by the use of the *MFA2* terminator downstream of the *Cre* gene, which reduced the half-life of the mRNA, and by using the "pulse" induction method (Figure 7B).
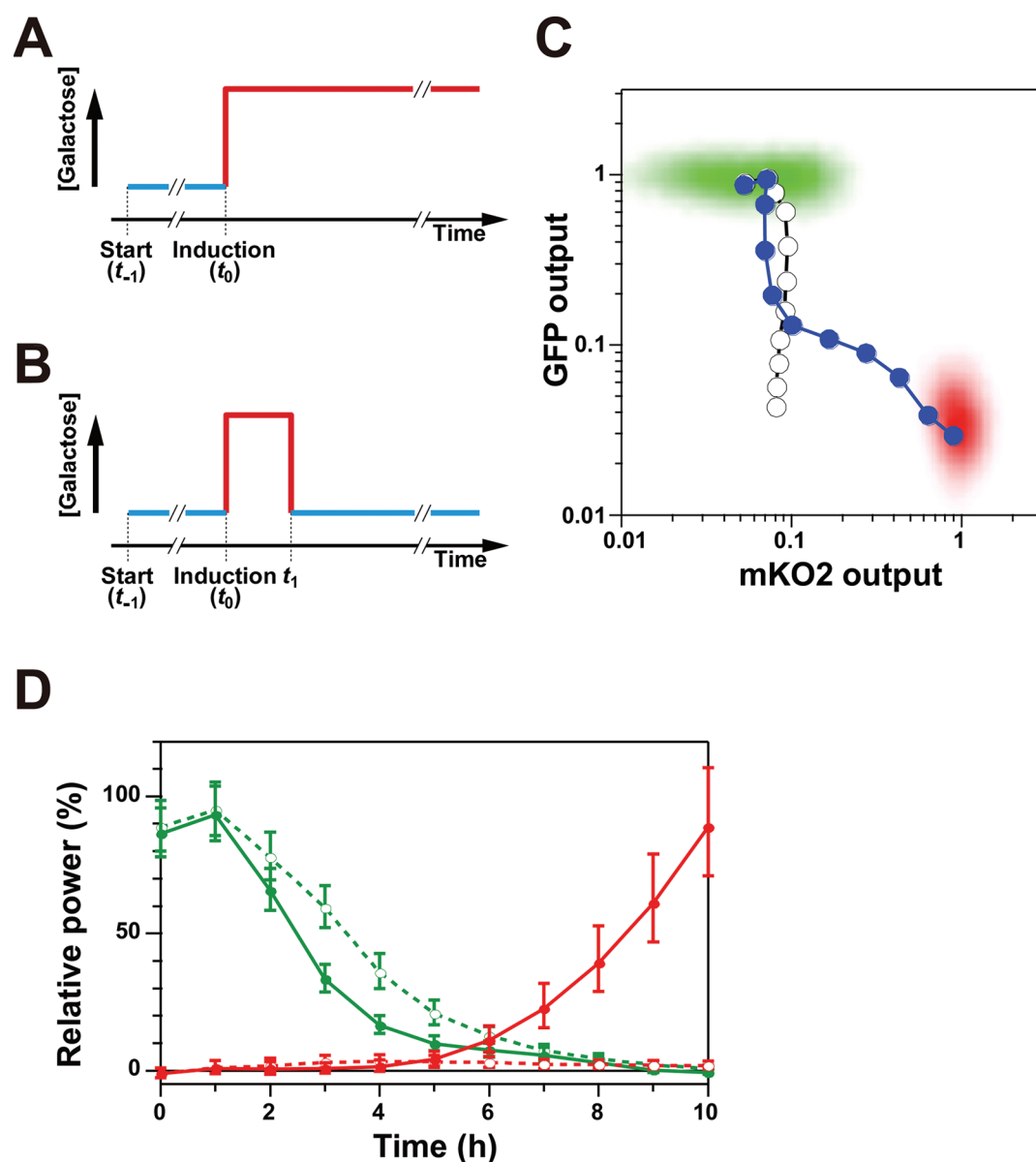
Figure 7. Methods for induction. (A, B) Schematic diagram of the induction methods: the continuous (A) and the pulse (B) methods. The culture is started in glucose medium. Galactose is applied during the period colored in red. With the pulse method, the medium is changed at t_1, and the culture is continued. The t_1 was optimized over several experiments, and $t_1 = 1$ h was used here. (C) Two-dimensional representation of the change in fluorescent phenotype using the continuous method (open circles) and the pulse method (closed circles). Green and red clouds indicate the distribution of values for the GFP and mKO2 controls, which correspond to the areas shown in Figure 4C. Each closed circle represents the center of the cell distribution for that hour. See Supplementary Movies 1 and 2 for the dynamic changes in fluorescent phenotype with the pulse and continuous methods, respectively. (D) Time-dependent changes in fluorescence output. Open circles with dashed lines and closed circles with solid lines represent results obtained for the continuous and pulse methods, respectively. To calculate the relative output of GFP, the FL1/SS values from FC1 and FC2 were taken as 100% and 0%, respectively. Similarly, the FL2/SS values from FC1 and FC2 were taken as 0% and 100% of the relative output of mKO2, respectively.

Optimization of the pulse duration (1 h) produced an output of 90% ± 20% (n = 3) of the mKO2 control (Figure 7C and D closed circles, and Supplementary Movie 1). We initially tried the PEST sequence downstream of Cre to accelerate the protein degradation, but it did not work as we expected (data not shown). Controlling adequate Cre activity at the protein level by use of other degradation sequences or at the mRNA level by using an appropriate terminator may improve the Cre-lox genetic switch.

On the basis of the fluorescence experiment, at most 10% of the cells did not recombine, continuing to express *PDC* during

the production phase and to produce residual ethanol. The LDH activity in non-induced MS1 appeared to be substantially higher than that of wild-type, and lactate production was evident. This undesirable expression of PDC and LDH during the production and growth phases, respectively, could be problematic for the application of this system. It should be relatively easy to reduce PDC levels during the production phase, for example, by fine-tuning the induction; it is important to induce adequate Cre activity during this phase. LDH activity during the growth phase, however, is much more problematic; if the target product is not lactate but rather a toxic compound,

the leaky induction of this product may kill the host organism. To reduce such leaks during the growth phase, the recombination reaction must be more strictly controlled, for example, by use of other inducible promoters such as the *CUP1* or *MET25* promoter.[44,45]

Here, we propose a "metabolic shift" approach to fermentation and demonstrate that this concept is executable by using PDC and LDH. This metabolic shift is not limited to ethanol and lactate production in yeast. By using the Cre-*lox* genetic switch, it should be possible to invoke metabolic shifts in any microorganism. The "metabolic shift" concept can thus be an alternative means to obtain gene products that are currently difficult or impossible to obtain by using conventional methodologies. We believe that this system could be used to improve, for example, the production of biofuels and bioplastics.[46,47]

■ METHODS

Medium. Yeast transformants were grown in synthetic complete medium (SC medium), which contains a 0.67% Yeast Nitrogen Base without Amino Acids (YNB) (Difco, Franklin Lakes, NJ), 0.082% Complete Supplement Mixture (CSM) (FORMEDIUM, Norfolk, U.K.), and adenine (40 mg/L), supplemented with various carbon sources, or in YPAD medium, which contains 1% yeast extract, 2% peptone, adenine (40 mg/L), and 2% glucose.

For selecting yeast transformant, SC-URA, SC-LEU, and SC-TRP media were prepared by using CSM-URA, CSM-LEU, and CSM-TRP (FORMEDIUM), respectively, supplemented with YNB (0.67%), adenine (40 mg/L), and 2% glucose. Hygromycin B was added at 150 mg/L to select yeast transformant harboring the *Hyg*r gene.[28]

Gene Construction. The following DNA fragments were designed in this study and their sequences are available through GenBank: the *GAL1m1* promoter (GenBank AB594820), the codon-optimized Cre (GenBank AB594821), and the *GFP* gene that includes a modified PEST sequence[39] at its 3′ end (GenBank AB594822). The *MFA2* terminator[21] was used to reduce the level of Cre and GFP transgene expression through labilization of both the protein and the mRNA. The *TPS1* terminator, which is reported to increase the level of transgene expression,[48] was used for mKO2. In the *PDC-LDH* cassette, we used the open reading frames of the original *PDC1* and bovine L-LDH-A (GenBank D90141).[49]

All synthetic DNA fragments were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). PCR primers were purchased from Operon Biotechnologies (Tokyo, Japan). All genetic constructs were constructed onto pSP73 (Promega, Madison, WI) by using standard ligation techniques or an In-Fusion Advantage PCR cloning kit (Clontech, Mountain View, CA).

Strain Construction. Linearized DNA fragments were transformed into the W303-1a strain or its derived yeast by using a Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA). Transformants were grown on suitable selection media. The integration of each DNA fragment into genomic DNA was confirmed by using the colony PCR technique. The genotypes of the yeast transformants used in this study are summarized in Table 1.

Cell Culture and Induction. FS1 was first grown in SC medium containing 2% glucose until the optical density at 660 nm reached 0.7–1.0. The cells were then collected by use of centrifugation and suspended in SC medium containing 0.5%

galactose. One hour later, the cells were collected again and resuspended in SC medium containing 3% glucose (Figure 7B). Time zero was defined as the start of the culture in the galactose medium. FS1 was cultured for 10 h.

Yeast transformant MS1 was grown in the same way with two modifications: the culture was continued for 12 h with three medium exchanges at 4, 7, and 10 h post-induction.

Fermentation Test. Yeast cells were washed twice with 0.84% sodium chloride solution and suspended in SC medium containing 0.5% glucose. After a 1-h incubation, the supernatant was subjected to metabolite analysis. The concentrations (w/v %) of glucose, ethanol, and lactate were determined by using enzyme-coupled electrodes (Bio Flow 5i, Oji Scientific Instruments, Amagasaki, Japan). The detection limits were 0.001 w/v % for ethanol and 0.0001 w/v % for lactate in this experimental setting. The percentage of lactate conversion (conversion %) was the ratio, as a percentage, (w/w %) of the lactate concentration to the consumed glucose in a sample.

Enzyme Activity Test. Authentic bovine muscle LDH and yeast PDC were purchased from Sigma (St. Louis, MO), and activity was assayed at 30 °C as previously described.[16] Reaction rates were calculated with $\Delta\varepsilon = 6.22 \times 10^3$ L/mol/cm. One unit of LDH activity was defined as the amount of enzyme necessary to convert 1 μmol NADH to NAD$^+$ in 1 min.

LDH activity in samples was determined colorimetrically by using the Cytotoxicity Detection Kit (Roche Applied Science, Indianapolis, IN) and a microtiter plate reader (Infinite F500, TECAN, Männedorf, Switzerland) equipped with a 492 nm filter. This assay comprises two coupled enzymatic reactions: in the first step, NAD$^+$ is reduced to NADH/H$^+$ by the LDH-catalyzed conversion of lactate to pyruvate; in the second step, the tetrazolium salt is reduced to give a red pigment by the diaphorase-catalyzed conversion of NADH/H$^+$ to NAD$^+$.[30] The accumulation of the pigment in each sample was compared to that for authentic LDH. The PDC activity in the samples does not affect the results obtained with this kit (data not shown).

Fluorescence Microscopy. A drop of cell suspension was observed through an all-in-one fluorescence microscope (BZ-9000, Keyence, Tokyo, Japan) equipped with a charge-coupled device (CCD) camera, two band-pass filters (475 nm for excitation and 510 nm for emission), and a 500-nm dichroic mirror. Each image was recorded in 8-bit mode. Red and green images were merged with a blank blue image to produce an RGB image by using Adobe Photoshop software (ver. 11, Adobe Inc., CA).

Flow Cytometry. The values for the electric volume (EV), side scattering (SS), and fluorescence channels 1 (FL1, GFP) and 2 (FL2, mKO2) were recorded by using a flow cytometer (Cell Lab Quanta SC MPL, Beckman-Coulter, Brea, CA) equipped with two dichroic mirrors (525 and 590 nm) and two band-pass filters (510 nm for FL1 and 570 nm for FL2). Approximately 10,000 sets of values were processed by using IgorPro software (ver. 6.1, Wavemetrics, Inc., Lake Oswego, OR). Fluorescence output was defined as the FL1/SS and FL2/SS values for GFP and mKO2, respectively. These outputs were log-normally distributed. A two-dimensional histogram was calculated by using the log(GFP output) and log(mKO2 output) values. The center of the cell distribution was calculated for the two-dimensional histogram by means of curve fitting with a two-dimensional Gaussian function.

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ASSOCIATED CONTENT

S Supporting Information

Supplementary Movie 1. Change in fluorescent phenotype with the pulse method. The FS1 transformant was cultured by using the continuous method (Figure 7B). The two-dimensional histogram was converted to an image by using IgorPro software (Wavemetrics, Inc.). A series of images were converted to MPEG animation by using ImageMagick (http://www.imagemagick.org). **Supplementary Movie 2.** Change in fluorescent phenotype with the pulse method. The FS1 transformant was cultured by using the pulse method (Figure. 7A). The movie was created in the same way as Supplementary Movie 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

DNA: deoxy ribonucleotide; GFP: green fluorescent protein; mKO2: monomeric Kusabira orange version 2; LDH: lactate dehydrogenase (EC 1.1.1.27); PDC: pyruvate decarboxylase (EC 4.1.1.1); ADH: alcohol dehydrogenase (EC 1.1.1.1); NADH: nicotine adenine dinucleotide (reduced form); NAD$^+$: nicotine adenine dinucleotide (oxidized form); ORF: open reading frame; SD: standard deviation

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