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Flux analysis and metabolomics for systematic metabolic engineering of microorganisms

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ABSTRACT

Rational engineering of metabolism is important for bio-production using microorganisms. Metabolic design based on *in silico* simulations and experimental validation of the metabolic state in the engineered strain helps in accomplishing systematic metabolic engineering. Flux balance analysis (FBA) is a method for the prediction of metabolic phenotype, and many applications have been developed using FBA to design metabolic networks. Elementary mode analysis (EMA) and ensemble modeling techniques are also useful tools for *in silico* strain design. The metabolome and flux distribution of the metabolic pathways enable us to evaluate the metabolic state and provide useful clues to improve target productivity. Here, we reviewed several computational applications for metabolic engineering by using genome-scale metabolic models of microorganisms. We also discussed the recent progress made in the field of metabolomics and ^{13}C-metabolic flux analysis techniques, and reviewed these applications pertaining to bio-production development. Because these *in silico* or experimental approaches have their respective advantages and disadvantages, the combined usage of these methods is complementary and effective for metabolic engineering.

Contents

1. Introduction

Presently, fuels and numerous chemicals are produced from renewable resources by using microorganisms (Demain and Adrio, 2008; Jang et al., 2012). To enhance the productivity and yield of the target compounds, it is important to improve the cellular system by using metabolic engineering approaches (Becker and Wittmann, 2012; Lee et al., 2012; Stephanopoulos, 1999). A number of successful applications of metabolic engineering have been reported, particularly with the model organisms *Escherichia coli*, *Corynebacterium glutamicum*,

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and *Saccharomyces cerevisiae* as a host cell for bio-productions, for which DNA recombinant techniques have been established. In recent years, stoichiometric metabolic models that include all enzymatic reactions of these organisms have been constructed based on annotated genome sequences, and it has become possible to predict metabolic states under various conditions by using *in silico* simulations. Therefore, rational design of the cellular system based on computational predictions is strongly desired (Blazeck and Alper, 2010; Oberhardt et al., 2009).

Approaches for comprehensive measurement of the intracellular components are also attracting attention (Park et al., 2005; Vemuri and Aristidou, 2005). A cell contains a large number of molecular components such as transcripts, proteins, and metabolites, and the cellular system is highly regulated at various levels, including gene expression, protein transcription, and enzymatic reaction (metabolism). In particular, metabolism is directly linked to cellular activities and it is correlated to target compound production. Thus, accurate evaluation of the metabolic states and understanding the regulatory mechanisms are important. One effective way to evaluate the metabolic state is to measure the abundance of intracellular low-molecular-weight compounds (metabolites) such as sugar phosphates, nucleic acids, amino acids, organic acids, and lipids. Presently, large quantities of metabolites such as amino acids can be measured, and recent advances in analytical techniques have allowed the measurement of various metabolites such as intermediates in central metabolism and secondary metabolites. In other approaches, ^{13}C-metabolic flux analysis can be used to estimate metabolic reaction rates in steady state that cannot be revealed from metabolite concentrations, and it provides quantitative information regarding carbon flow through the pathways.

Here, we reviewed recent techniques and applications of bio-productions related to the "design of metabolic pathways" by *in silico* simulation using genome-scale metabolic models, and the "evaluation of metabolic state" by metabolome measurements and ^{13}C-metabolic flux analysis, and propose a strategy for systematic metabolic engineering for improvement of target productivity.

2. Scheme of systematic metabolic engineering

The systematic metabolic engineering scheme is illustrated in Fig. 1. Currently, genome-scale metabolic models have been developed for a number of microorganisms. *In silico* simulations for rational design of their metabolism to produce target compounds and genetic manipulation of the host organisms can be performed. The properties of the candidate strain should be experimentally evaluated. It is then important to investigate not only the cellular phenotypes such as growth rate and target productivity but also the intracellular metabolic state by using metabolome measurements and ^{13}C-metabolic flux analysis. By measuring accumulation of the intermediates and the activities of each reaction, it is possible to identify the rate-limiting steps in the metabolic pathway and to compare the flux distribution with that predicted by *in silico* simulation. Thus, a more suitable pathway for target production can be created using this experimental information.

3. Rational design of metabolism based on *in silico* simulation

3.1. Genome-scale models of microorganisms

For systematic metabolic engineering, we begin with a rational design of the metabolic pathway by using computational approaches. Although many methodologies are available to simulate metabolism, flux balance analysis (FBA) is a well-established and versatile method (Orth et al., 2010; Varma and Palsson, 1994). FBA is a constraint-based approach to predict flux distribution in a metabolic network by using only the reaction stoichiometry and an objective function by linear programming methods without considering the enzymatic reaction kinetics. Generally, maximization of biomass synthesis is used as the objective function, according to the assumption that cells use the available nutrient most efficiently for the production of cellular components. It has been reported that metabolic phenotypes such as growth rate and predicted organic acid production rates by FBA correlate well with experimental results (Varma et al., 1993). With the availability of the bacterial genome, metabolic pathway databases such as KEGG (Kanehisa et al., 2008) and BioCyc (Karp et al., 2005), and enzymatic reaction databases such as BRENDA (Scheer et al., 2011) and BKM-react (Lang et al., 2011), reconstruction of whole-cell metabolic networks has become possible (Thiele and Palsson, 2010). Recently, a large number of models for various bacteria have been published (Schellenberger et al., 2010), including model organisms such as *E. coli* (Orth et al., 2011), *C. glutamicum* (Shinfuku et al., 2009), *Aspergillus niger* (Andersen et al., 2008), *S.*

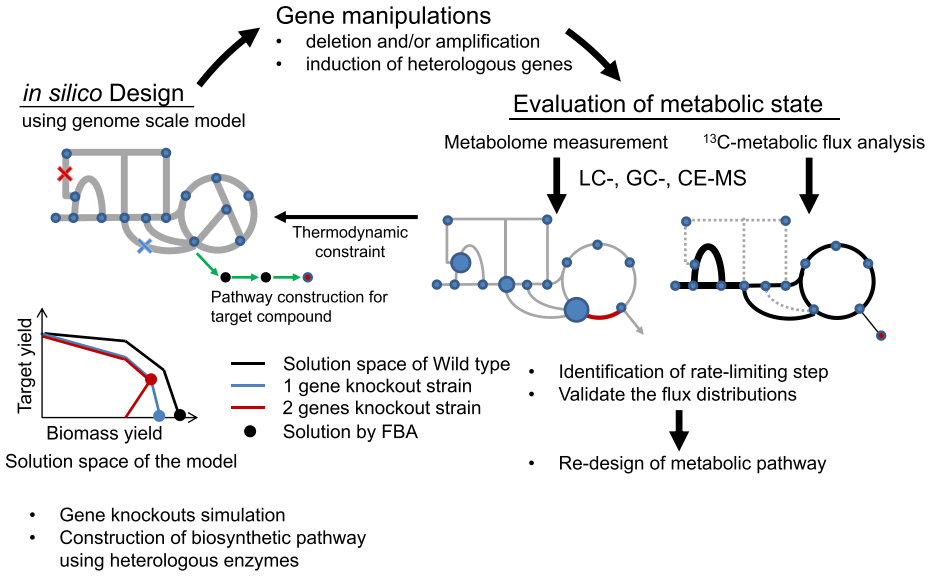

Fig. 1. Scheme of systematic metabolic engineering.

cerevisiae (Mo et al., 2009), and *Synechocystis* sp. (Nogales et al., 2012; Yoshikawa et al., 2011). The accuracy of these models has been confirmed using gene essentialities, nutrient requirements, and growth phenotypes under various conditions (Joyce and Palsson, 2008).

3.2. Metabolic design for microbial productions based on flux balance analysis

Once the stoichiometric model predicting the proper metabolic state is constructed, we can design the metabolic pathway to improve target productivity based on the simulated results by FBA. One advantage of using the stoichiometric model for pathway design is the consideration for cofactor balance. Biosynthetic pathways/reactions for desirable targets often involve NAD(H) and NADP(H), and these cofactors should be balanced in the whole system (Singh et al., 2011). However, due to the complex metabolic network, it is quite difficult to capture the redox balance in whole metabolism without using a model and simulation (Ouzounis and Karp, 2000).

For bio-production coupled with cell growth, one can design the metabolic pathway to produce the target metabolite as a by-product for cellular component synthesis by gene knockout simulation. In the past, many successful applications using this approach were reported, including those with L-valine (Park et al., 2007) and lycopene (Alper et al., 2005) in *E. coli*, and succinic acid in *E. coli* and *A. niger* (Lee et al., 2005; Meijer et al., 2009; Sánchez et al., 2005). In addition, Ohno et al. (2013) demonstrated that many valuable compounds such as 1-butanol, 1-propanol, and 1,3-propanediol can be produced using a triple reaction knockout (Ohno et al., 2013). OptKnock is an efficient framework to search for multiple gene-knockout candidates by bi-level optimization using cell growth (inner problem) and target production (outer problem) (Burgard et al., 2003). OptGene (Patil et al., 2005) and RobustKnock (Tepper and Shlomi, 2010) are also frameworks to identify deletion candidates to achieve growth-associated production of target compounds. OptReg (Pharkya and Maranas, 2006) and EMILiO (Yang et al., 2011) are frameworks to search for target genes not only for deletions but also for up- and down-regulations. Based on these computational tools, not only bacterial engineering such as lactic acid production in *E. coli* (Fong et al., 2005) but also vanillin production in yeast (Brochado et al., 2010) and sesquiterpene production in *S. cerevisiae* (Asadollahi et al., 2009) have been reported. These optimization applications for strain design based on FBA have been summarized by Zomorrodi et al. (2012).

Interest has been rising for the production of metabolites that cannot originally be produced by the host organisms because they do not possess the synthetic pathways; such metabolites can be produced using an artificially constructed pathway with several heterologous enzymes (Atsumi et al., 2008; Smith et al., 2010). Such synthetic biotechnology approaches have great potential for the production of various useful targets by using a common bioprocess cell factory. It is also possible to artificially build non-native target compound production pathways by using an *in silico* model. OptStrain constructed a universal reaction model from pathway databases such as KEGG, and identified a maximum yield pathway from substrate to target product, which included heterologous reactions. The number of non-native reactions in this pathway was then minimized, and the non-native reactions were incorporated into the host model (Pharkya et al., 2004). Recently, Chatsurachai et al. (2012) proposed another systematic approach to find an artificial pathway for such compounds. Here, they iteratively expanded the metabolic network of the host organism by adding the connectable heterologous reactions, which were obtained from pathway databases (Chatsurachai et al., 2012).

3.3. Metabolic pathway analysis for engineering microbial production

Metabolic pathway analysis is another useful approach for rational strain design based on a stoichiometric model (Schuster et al., 2000; Trinh et al., 2009). This approach considers metabolism as a network system and reveals properties of its topology. Elementary mode analysis (EMA) is a sophisticated technique to decompose the complex network into the smallest sets of reactions (EMs) that satisfy the mass balances of intermediates and cofactors. The EMs can be calculated by finding a basis for the null space of the stoichiometric matrix of the metabolic network. Every possible flux distribution can be expressed by linear combination of the EMs.

One objective of optimal design for achieving a high target productivity based on EMs is to determine the minimal cell, which includes an efficient pathway for target production (Trinh et al., 2009). To identify deletion candidates, we evaluate the effects of a single reaction deletion on the number of EMs and the maximum target yield. The reaction with the smallest remaining EMs and maintaining a high maximum yield is deleted, and the procedure repeated as many times as needed (Trinh et al., 2009). Many applications of this method have been reported for the production of isobutanol (Trinh et al., 2011) and carotenoids (Unrean et al., 2010). Flux Design is an advanced framework using EMA that enables identification of the target genes to be amplified or down-regulated based on flux correlation (Melzer et al., 2009). In this method, EMs with non-zero target yields were used to calculate the correlation coefficients of fluxes between target production and individual reactions in the metabolic network. The reactions with positive correlations are amplification candidates, whereas those with negative correlations are deletion/attenuation candidates (Melzer et al., 2009). Following Flux Design predictions, Poblete-Castro et al. succeeded in the improvement of polyhydroxyalkanoates production in *Pseudomonas putida* (Poblete-Castro et al., 2013). Neuner and Heinzle also evaluated each reaction using target and biomass productions of EMs, and found amplification targets for the engineering of lysine production by *C. glutamicum* (Neuner and Heinzle, 2011).

4. Microorganism metabolomics for metabolic engineering

4.1. Measurement techniques for microbial metabolomics

In the previous section, we introduced methods to design metabolic pathways by using an informatics approach. Although researchers manipulate the genes of host cells according to the metabolic design, this does not always lead to the expected result. Since the constraint-based FBA and EMA approaches ignore the kinetic mechanisms of enzymatic reactions, the predicted metabolic fluxes may differ from reality. Furthermore, although the metabolic system is regulated by not only enzyme activity levels but also gene expression levels, these factors are not considered by FBA or EMA. This and the subsequent sections introduce experimental approaches for evaluating the metabolic state of a genetically engineered strain based on computational predictions and for investigating the cause of the differences between simulation and experiment.

Cell metabolism includes various categories of metabolites such as sugars, organic acids, amino acids, alcohols, coenzymes, and lipids. In *E. coli* and *S. cerevisiae*, 2708 and 2027 metabolites are registered in the databases, respectively (Guo et al., 2013; Jewison et al., 2012). For microbial metabolome measurements, the cells and the extracellular medium are first separated by centrifugation or rapid filtration, and the metabolites are extracted from the cells after rapidly quenching the enzyme activities (Bolten et al., 2007). Filtration using a membrane filter or centrifugation is performed to harvest the microorganisms (Bolten et al., 2007). Since the turnover rates of intracellular metabolites are much more rapid than gene expression rates in general, the reaction activities should stop quickly after harvesting the cells.

After the cells are collected by filtration, the quenching and extraction are simultaneously performed using solvents. The conditions for numerous model organisms have been examined by many researchers, and cold methanol, liquid nitrogen, and perchloric acid have been used for microbial quenching/extraction (Bolten et al., 2007; Mashego et al.,

2007; Wellerdiek et al., 2009). In the case of collection of the cells by centrifugation, the culture is directly mixed with cold aqueous methanol (<60%) for quenching. Although this method can promptly stop cellular activities, metabolites may leak from the cells. In the case of *S. cerevisiae*, methanol was used below −40 °C for sample treatment to ensure leakage-free quenching (Canelas et al., 2009).

The extracted metabolite can be detected by several methods, such as mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, infrared absorption spectrometry (IR), and ultraviolet spectroscopy (UV). In recent years, the development of MS has been astonishing, and measurements with high resolution and high sensitivity have been realized using time-of-flight MS (TOFMS) and tandem MS (MS/MS). These MSs are combined with gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) to increase selectivity of the measurements (Büscher et al., 2009; Han et al., 2009; Soga et al., 2003; Zhang et al., 2012).

4.2. The thermodynamic feasibility of metabolome data

When metabolite pool sizes in pathways are measured, the net direction of each reaction can be thermodynamically predicted. According to the second law of thermodynamics, a reaction should progress in the direction of a negative change in Gibbs free energy (ΔG). The ΔG for a reaction is calculated from the standard free energy and the concentration of metabolites involved in the reaction. In Eq. (1), we show an example of ΔG calculation of a reaction that catalyzes S1 and S2 to P1 and P2.

$$\Delta G = \Delta G_0 + RT \cdot \ln\left(\frac{[P_1][P_2]}{[S_1][S_2]}\right) \quad (1)$$

where ΔG_0 represents the standard Gibbs free energy, and R and T represent the gas constant and the temperature.

Hoppe et al. (2007) proposed a method for the prediction of a thermodynamically feasible flux solution by FBA using experimentally measured metabolite concentrations as an additional constraint. Zamboni et al. (2008) developed a software anNet to test the thermodynamic consistency of the measured metabolome dataset and for prediction of unmeasurable metabolite concentrations.

4.3. Identification of metabolic bottlenecks based on metabolome analysis

For metabolic engineering, the important information from the metabolome measurements is regarding the rate-limiting steps in the metabolic networks. Since the metabolic pathway is a system consisting of a sequence of reactions, the overall pathway flow is restricted by the slowest reaction (bottleneck). Therefore, the gene encoding the identified bottleneck enzyme is the target of metabolic engineering, and we expect to improve the target production rate by manipulation of expression and regulation of the gene. For example, Juminaga et al. (2012) identified the bottleneck steps in the synthetic pathway of L-tyrosine in *E. coli* from accumulation of the pathway intermediates such as dehydroquinate, dehydroshikimate, and quinate. They attained production of 80% of the theoretical yield of L-tyrosine by optimization of copy numbers, promoter strengths, and codon usages of target genes based on the navigation of metabolome measurements. Yamamoto et al. (2012) released the accumulation of the intermediates in the upper part of glycolysis by overexpression of the glycolytic enzyme GapA for alanine production in *C. glutamicum*. Hasunuma et al. (2011) investigated the metabolic profiles in the xylose-fermenting strain of *S. cerevisiae* to improve the weak acid tolerance. They predicted the restriction of the non-oxidative pentose phosphate pathway fluxes from the significant accumulation of intermediates such as sedoheptulose-7-phosphate, ribulose-5-phosphate, ribose-5-phosphate, and erythrose-4-phosphate, and improved ethanol productivity in the presence of acetic and formic acids by overexpression of a gene encoding transaldolase or transketolase.

Although some studies succeeded in improving target productivity by predicting a rate-limiting reaction, the accumulation of intermediates does not always occur because of metabolic regulation. Ishii et al. (2007) compared the metabolome of various gene-knockout mutants for central carbon metabolism. Unexpectedly, the metabolite levels were stable for most mutants because of the robustness of the metabolic system. This indicates that the metabolic system functions to alleviate intermediate accumulation by using an alternative pathway or feedback regulation of enzyme(s). For example, in *E. coli* central carbon metabolism, there are well-known mechanisms for allosteric inhibition by phosphoenolpyruvate and ATP concentrations of phosphofructokinase (Kotlarz et al., 1975) and the phosphorylation regulation of isocitrate dehydrogenase by oxaloacetate concentrations (Walsh and Koshland, 1985).

5. Evaluation of metabolic state based on ^{13}C-metabolic flux analysis

5.1. A brief review of ^{13}C-metabolic flux analysis

Although metabolome measurements are important for observing intermediate accumulation in pathways, they are insufficient to evaluate reaction activities. The reaction rate depends not only on substrate concentrations but also on activator/inhibitor concentrations, enzyme concentrations, and the rate constants. If all the factors involved in the reaction were measured, the reaction rate could be determined. However, in reality, there are many unmeasurable metabolites, and absolute enzyme quantification is difficult.

The FBA, introduced in Section 3.1, is a method to predict flux distribution that satisfies an objective function such as biomass production. Contrary to this, ^{13}C-metabolic flux analysis is a method to determine flux distribution based on experimental data (Wiechert, 2001; Wittmann, 2007). The procedure for ^{13}C-metabolic flux analysis is shown in Fig. 2. For ^{13}C-metabolic flux analysis, the cells are cultured using labeled substrate. These carbons were metabolized through various pathways, and they finally reached the amino acids constituting the cellular proteins. Therefore, flux distribution is reflected in the ^{13}C-enrichment of the proteinogenic amino acids, and these were measured by MS and/or NMR. Generally, the ^{13}C experiment is conducted at metabolic steady state conditions, to maintain the cellular metabolism until the ^{13}C atoms, which are taken up by the cell, are completely distributed to all the proteins. The mathematical model simulating the ^{13}C-enrichment of the metabolites from an arbitrary flux distribution is constructed to estimate the flux distribution using the measured ^{13}C-enrichments. The isotopomer and cumomer methods have been used for modeling isotopic mass balances (Schmidt et al., 1997; Wiechert et al., 1999). In recent years, elementary metabolite units (EMU) based on the decomposition of the isotopomer network have been proposed (Antoniewicz et al., 2007a), and the sophisticated framework drastically alleviates the computational cost for the ^{13}C-metabolic flux analysis. Using this model, a flux distribution, in which it is possible to explain the measured ^{13}C-enrichments, is estimated by optimization.

^{13}C-MFA results strongly depend on the analytical metabolic pathways in the model. Thus, a reliable solution is not expected when the wrong model is used for analysis, especially in the case that an active reaction is not included in the model. The model therefore should be carefully set up according to biological clues such as enzyme activities and gene expression. If the solution does not change when an inactive reaction is considered in the model, the solution becomes more reliable. Furthermore, whether a unique solution is present can be evaluated by calculating a confidence interval for each flux. The preliminary simulations are rather effective for designing the proper

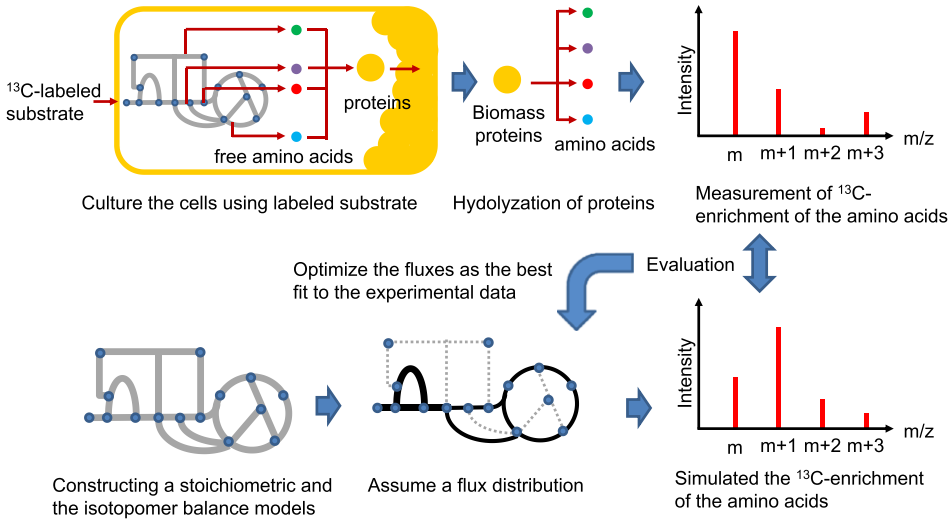

Fig. 2. Overview of flux estimation using ^{13}C-metabolic flux analysis.

substrate-labeling pattern. For example, the labeling pattern of metabolites is calculated using an assumed flux distribution and a substrate-labeling pattern. The fluxes are then estimated from the generated labeling pattern of metabolites. The substrate-labeling pattern can be assessed by comparing the initially assumed fluxes and the estimated fluxes. By comparison of the initially assumed fluxes and the estimated fluxes, the substrate-labeling pattern can be assessed. Recently, Crown and Antoniewicz (2012) proposed the methodology of the optimal tracer experiment design for high-resolution ^{13}C-metabolic flux analysis using EMU decomposition. Furthermore, Leighty and Antoniewicz (2012) performed parallel ^{13}C-labeling experiments by using several different labeling patterns of glucose; their experiment provided a statistically unfeasible solution and produced consistent flux for the parallel experiments when an inappropriate model was used. These findings highlight the importance of the model validation.

Over time, many studies have been conducted in order to investigate the control mechanisms of cellular metabolism by using ^{13}C-metabolic flux analysis. For example, one study tested how the division of the metabolic pathway affected whole metabolism by comparing the flux distributions between the wild type and the gene-knockout mutant (Canonaco et al., 2001; Shimizu, 2004). Since the changes in the metabolic fluxes reflected the changes in the gene expression, protein expression, and metabolite concentrations, it is important to integrate these varying levels of information to understand metabolic regulation. Toya et al. (2010) cultured *E. coli* wild type and *pykFA*-knockout mutant by using glucose as a carbon source in batch mode, and measured the intermediate metabolite concentrations and the fluxes in central carbon metabolism (Fig. 3). Although the glycolytic intermediates drastically accumulated because of the dumping and feedback inhibition caused by the accumulation of phosphoenolpyruvate (PEP) in the *pykFA* mutant, the overall flux distribution, including glycolysis and the pentose phosphate pathway, changed little due to rerouting *via* the anaplerotic reactions such as PEP carboxylase and malic enzyme. Usui et al. (2012) constructed both genetic deletion and conditional mutant strains of *pgi* and *eno*, in which gene expression was controllable, and they examined how perturbations in gene expression affected fluxes in the central metabolic pathways. Furthermore, external conditions such as the carbon source and oxygen levels affect metabolism *via* various global regulators (Matsuoka and Shimizu, 2011; Perrenoud and Sauer, 2005; Toya et al., 2012). Investigation of the metabolic changes associated with these environmental changes provides important clues for understanding the regulatory mechanisms of the cell.

5.2. Applications of ^{13}C-metabolic flux analysis to bio-production processes

^{13}C-metabolic flux analysis has been applied to many bio-productions, including alcohols (Antoniewicz et al., 2007b), amino acids (Bartek et al., 2011; Becker et al., 2011; Iwatani et al., 2008; Shirai et al., 2007), organic acids (Lu et al., 2009), and proteins (Jordà et al., 2012; Umakoshi et al., 2011). Shirai et al. (2007) performed ^{13}C-metabolic flux analysis of *C. glutamicum* in the growth phase (not producing glutamate) and in the glutamate production phase. They demonstrated that the conversion of pyruvate to oxaloacetate catalyzed by pyruvate carboxylase is the most important reaction in the several anaplerotic pathways for glutamate production.

Once the flux distribution has been estimated, the total production rates of cofactors, including NADH, NADPH, and ATP, can be calculated. Particularly, the redox balance is important for metabolic engineering, as in many cases, it becomes the driving force for target biosynthesis. Some bacteria contain transhydrogenases, which convert NADH to NADPH, and the *in vivo* activities can be estimated from the flux distributions. Sauer et al. (2004) identified the physiological function of the *E. coli* transhydrogenases PntAB and UdhA based on ^{13}C-metabolic flux analysis. For the production of L-valine in *C. glutamicum*, Bartek et al. (2011) revealed that NADPH demand for excess L-valine formation was compensated by the oxidative pentose phosphate pathway (PPP) based on ^{13}C-fluxes. As a result of introduction of *E. coli* transhydrogenase to supply NADPH in the alternative reaction, a significant increase in L-valine yield was achieved by a decrease in the formation of carbon dioxide in the PPP. Furthermore, Umakoshi et al. (2011) performed ^{13}C-metabolic flux analysis of *C. glutamicum* producing a heterologous transglutaminase. They predicted the increase in the NADH/NAD(+) ratio from the increase in the TCA cycle flux, and speculated that the rate of glucose uptake was decreased by this high NADH/NAD(+) ratio. Therefore, they enhanced lactate formation associated with NADH oxidation by controlling the pH in the culture broth, and achieved a 1.4-fold increase in transglutaminase yield (Umakoshi et al., 2011).

The computational framework OptForce, has been developed to predict engineering genes for the overproduction of target compounds by using experimentally measured ^{13}C-fluxes (Ranganathan et al., 2010). OptForce calculates the range of each flux when producing a specified yield of desirable target by FBA. From the difference between ^{13}C-flux and the calculated range for each reaction, the

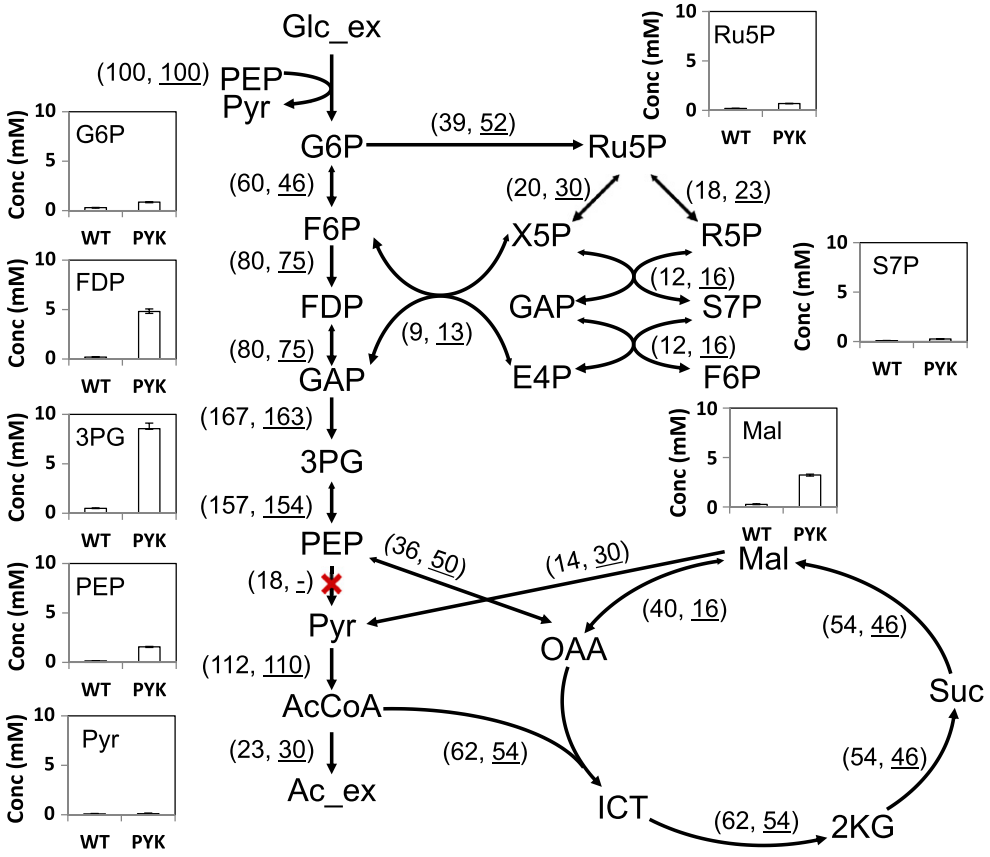

Fig. 3. Intermediate concentrations and flux distribution of *E. coli* wild type and Pyk mutant (underlined) at 6 h (mid-log phase) in batch culture. All fluxes are given as relative values to the specific glucose uptake rate.

targets for up- or down-regulation can be identified (Ranganathan et al., 2010).

5.3. Comparison between flux distributions by FBA and ^{13}C-MFA

Here, we introduced 2 methods (FBA and ^{13}C-MFA) used to determine the flux distribution on metabolism; both FBA and ^{13}C-MFA can provide a unique solution in an underdetermined metabolic system. FBA narrows down the solution space by using an assumed objective function such as a maximization of biomass yield, while ^{13}C-MFA determines the flux distribution based on experimental data. Therefore, if the predicted fluxes by FBA do not coincide with the ^{13}C fluxes, the ^{13}C-MFA result should be trusted as the flux distribution in actual cells. In practice, some specific gene-knockout mutants gave different metabolic states between the FBA solution and the experimental phenotype. Thus, minimization of metabolic adjustment (MOMA) (Segrè et al., 2002) and regulatory on/off minimization (ROOM) (Shlomi et al., 2005) were developed to improve the prediction abilities for such gene-knockout mutants by FBA. Both methods use the wild-type fluxes predicted by FBA as normal. MOMA then predicts the mutant fluxes to minimize the Euclidean distance of fluxes between the mutant and wild type, and ROOM prediction minimizes the number of changed fluxes from wild-type fluxes. It has been confirmed that both MOMA and ROOM improve the prediction accuracy for deletion mutants (Segrè et al., 2002). Furthermore, since general FBA does not involve regulations at the level of gene expression, it is difficult to predict the metabolic state with regulation of gene expression. To overcome this problem, regulatory FBA (rFBA), which enables one to describe the given gene expression controls by transcription factors using Boolean rules, has been proposed (Covert et al., 2001). However, rFBA can only apply on/off constraints in each regulated reaction. More recently, the probabilistic regulation of metabolism (PROM) algorithm has been developed to apply continuous flux restriction based on gene expression data (Chandrasekaran and Price, 2010). PROM successfully identified lethality with accuracies as high as 95% in *E. coli* and *Mycobacterium tuberculosis* metabolism.

6. Systematic identification of metabolic bottlenecks based on dynamic modeling

Although ^{13}C-MFA provides information regarding the reaction rate in each pathway, it is difficult to identify a rate-limiting step in a sequence of reactions in a pathway. Integration of metabolite concentrations and metabolic fluxes using a mathematical model is an effective approach. Kinetic modeling of metabolism is a theme that has been studied for a long time, and experimentally measured metabolite concentrations and fluxes are useful sources for model construction. Once a reliable model is constructed, one can perform a metabolic control analysis (MCA) using the model. MCA is a mathematical framework for understanding the control of the metabolic system (Heinrich and Rapoport, 1974; Kacser and Burns, 1995), and the flux control coefficient (FCC) is an important indicator to identify the rate-limiting enzymes, and is defined by Eq. 2.

$$\mathrm{FCC}_{\mathrm{i}}^{J_{\mathrm{k}}} = \frac{dJ_{\mathrm{k}}}{dE_{\mathrm{i}}}\frac{E_{\mathrm{i}}}{J_{\mathrm{k}}} = \frac{d\ln J_{\mathrm{k}}}{d\ln E_{\mathrm{i}}} \tag{2}$$

where E_{i} and J_{k} represent the ith enzyme concentration and jth flux. The FCC represents the change in flux in response to a change in each enzyme concentration. Thus, the flux of a reaction is controlled by the enzyme with the largest FCC value. Oh et al. (2011) performed

MCA using a kinetic model of the lactic acid fermentation pathway of *Lactococcus lactis* and identified the metabolites affecting the fluxes of L-lactate dehydrogenase, pyruvate dehydrogenase, and hexose transporter. Cintolesi et al. (2012) also performed MCA for ethanol fermentation of glycerol in *E. coli*. They revealed that glycolytic flux was almost exclusively controlled by glycerol dehydrogenase and dihydroxy acetone kinase based on the FCCs. Overexpression of these enzymes significantly improved glycerol utilization and ethanol production. However, construction of such a kinetic model is difficult, because it requires considerable information regarding the detailed reaction mechanism, including the kinetic parameters, for each enzyme. Thus, only several models in few microorganisms have been developed (Cintolesi et al., 2012; Kadir et al., 2010; Rizzi et al., 1997; Shinto et al., 2007).

Recently, a new framework for the construction of a dynamic model of the metabolic system, ensemble modeling, has been proposed (Rizk and Liao, 2009). Ensemble modeling does not require the kinetic parameters and time-series data of intermediate concentrations, and uses only phenotypic data such as production rate in enzyme overexpression or deletion strains. In this model, the rate equations of enzyme reactions can be described based on elementary reaction mechanisms (Rizk and Liao, 2009). Briefly, their approach generates a large number of kinetic parameter sets (ensemble models), which satisfies the initial setting fluxes using a Monte Carlo algorithm. These ensemble models are simulated with a perturbation based on experimental conditions of phenotypic data (such as enzyme overexpression or deletion), and are screened by comparison with the experimental phenotypes. The screened models are iteratively trained using various phenotypic data. Based on the ensemble modeling framework, Contador et al. (2009) identified aspartate kinase as the next bottleneck in the *E. coli* L-lysine pathway. Additionally, Rizk and Liao (2009) constructed ensemble models and identified the rate-limiting enzymes transketolase (first bottleneck) and phosphoenolpyruvate synthase (second bottleneck). The ensemble modeling approach can be more effective together with experimentally measured fluxes by ^{13}C-MFA than with phenotypic data alone, since inappropriate ensemble models can be more strictly pruned by flux constraints. Miskovic and Hatzimanikatis (2010) proposed a similar framework, named Optimization and Risk Analysis of Complex Living Entities (ORACLE), which predicts control coefficients based on available -omics information (transcriptomics, proteomics, metabolomics, and fluxomics) using a Monte Carlo approach.

7. Conclusion

In the current microbial metabolic engineering field, gene manipulation does not become a bottleneck step because of advances in molecular biology. Many convenient tools and techniques have been developed specifically for genetic engineering of model organisms (Baba et al., 2006; Breslow et al., 2008). It is now important to design the strategies for metabolic engineering. Here, we summarized the methods for *in silico* design of metabolic pathways by using a genome-scale model and by experimental evaluation of metabolism by metabolomics and ^{13}C-metabolic flux analysis to achieve systematic metabolic engineering for bio-production using microorganisms. Metabolic design using the genome-scale model is already a widely used approach, and it enables prediction of the knockout or amplification target genes for enhancement of productivity. We introduced several successful studies in this review. Furthermore, techniques for metabolome analysis and ^{13}C-metabolic flux analysis have made progress in recent years, and researchers can now rapidly obtain a large amount of data in the cells. Nowadays, it is increasingly important to systematically optimize metabolism by using experimental measurements. To achieve this, it is necessary to understand the detailed regulatory mechanisms of the cellular system. Furthermore, the complementary use of *in silico* and experimental methods will become more important in the field of metabolic engineering.

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