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Systems-Level Analysis of Genome-Scale *In Silico* Metabolic Models Using MetaFluxNet

Sang Yup Lee[1,2]*, Han Min Woo[1], Dong-Yup Lee[2], Hyung Seok Choi[1], Tae Yong Kim[1], and Hongseok Yun[2]

[1] Metabolic and Biomolecular Engineering National Laboratory, Department of Chemical and Biomolecular, and BioProcess Engineering Research Center, Korea Advanced Institute of Science and Technology, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Korea
[2] Department of BioSystems and Bioinformatics Research Center, Korea Advanced Institute of Science and Technology, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Korea

Abstract The systems-level analysis of microbes with myriad of heterologous data generated by omics technologies has been applied to improve our understanding of cellular function and physiology and consequently to enhance production of various bioproducts. At the heart of this revolution resides *in silico* genome-scale metabolic model. In order to fully exploit the power of genome-scale model, a systematic approach employing user-friendly software is required. Metabolic flux analysis of genome-scale metabolic network is becoming widely employed to quantify the flux distribution and validate model-driven hypotheses. Here we describe the development of an upgraded MetaFluxNet which allows (1) construction of metabolic models connected to metabolic databases, (2) calculation of fluxes by metabolic flux analysis, (3) comparative flux analysis with flux-profile visualization, (4) the use of metabolic flux analysis markup language to enable models to be exchanged efficiently, and (5) the exporting of data from constraints-based flux analysis into various formats. MetaFluxNet also allows cellular physiology to be predicted and strategies for strain improvement to be developed from genome-based information on flux distributions. This integrated software environment promises to enhance our understanding on metabolic network at a whole organism level and to establish novel strategies for improving the properties of organisms for various biotechnological applications.

Keywords: systems biotechnology, MetaFluxNet, metabolic flux analysis, *in silico* simulation

INTRODUCTION

The advent of high-throughput experimental techniques in the postgenomic era has accelerated the accumulation of vast amounts of data at the genome, transcriptome, proteome, metabolome, and fluxome levels. Bioinformatic analysis at each omic level has been aimed at elucidating the cellular functions and physiology of entire systems [1-4]. Diverse disciplines need to be applied to reveal the metabolism of microorganisms at systems level and also to design metabolic pathways for improving strains. One successful approach toward these goals is construction of genome-scale *in silico* metabolic models, followed by metabolic flux analysis to quantify the steady-state flux values of all metabolic reactions under given genetic and environmental conditions [1,5-8]. Considering that the systems level modeling and simulation of complex metabolic network are not straightforward to many biotechnologies, it will be invaluable to develop an integrated software for the efficient construction and analysis of such models. In this regard, we have developed MetaFluxNet program package that allows construction of genome-scale metabolic models, and that provides a better understanding of metabolic network properties, interpretation of the resulting flux maps under different conditions, and design of various metabolic strategies by means of metabolic flux analysis [5,8]. Here we describe development of an upgraded MetaFluxNet with special focus on its new features and its application to constructing genome-scale models followed by its use in systems biotechnology.

***Corresponding author**
Tel: +82-42-869-3930 Fax: +82-42-869-3910
e-mail: leesy@kaist.ac.kr

In Silico Modeling and Simulation of Metabolic Networks

Several approaches have been taken for the quantitative *in silico* modeling and simulation of metabolic systems [9,10], which can be broadly classified into two types: kinetics model-based dynamic analysis and static pseudo-steady state analysis (Fig. 1A and 1B).

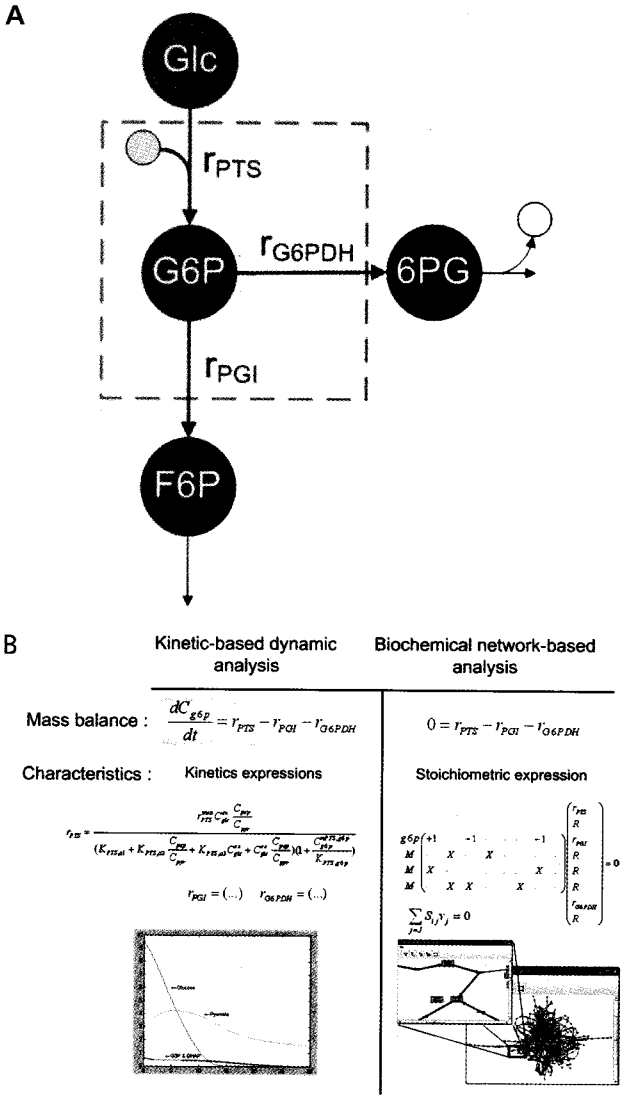

Fig. 1. Modeling metabolic network. A; Example of simple reaction network on central metabolism. This shows that metabolic network can be defined as a set of mass balance equations around each metabolite. B; Comparisons of mass balance, characteristics, and results for kinetics model-based dynamic analysis and static pseudo-steady state analysis. Kinetics models are represented by ordinary differential equations of mass balance equations, which are often expressed in highly non-linear kinetic equations. Static pseudo-steady state analysis is often performed using by matrix operation of stoichiometric matrices which do not contain any kinetic information.

Kinetics Model-Based Dynamic Analysis

Microorganisms adopt new states in response to changes in environmental and genetic conditions. Biological dynamic analysis can be used to determine how cells respond to certain perturbations, and what kind of mechanism can be applied or how it can be expressed mathematically. A kinetics model-based dynamic system can be represented as a set of mass-balanced rate equations with kinetics expressions that incorporate regulatory information. Such rate equations are expressed by ordinary differential equations describing the concentrations of intracellular metabolites. Even when the set of mass-balanced rate equations is a linear system, the metabolic kinetics expressions including many parameters and inhibitors cause the system to be highly nonlinear. Thus, diverse and complicated algorithms are required to solve the differential equations and estimate parameters adjusted by *in vitro* experiments or extracted from available kinetics databases such as BRENDA [11,12].

The limited information on kinetic mechanisms and their parameters significantly restricts dynamic analyses. In addition, the number of known kinetics equations is too small to analyze the whole-cell system, especially considering that the entire genome sequences of some microorganisms are now known. The possibility of inaccurate parameters brings into the question for true reliability of the results of dynamic simulations. This has led to wide adoption of steady-state modeling, which does not need kinetics information.

Static Pseudo-Steady State Analysis

Assuming a pseudo-steady state, a kinetics model can be simplified to a static representation. In contrast to dynamic approaches, a steady-state model considers the biochemical network topology and thermodynamic characteristics as time-invariant properties of the metabolic system. The steady state approaches include extreme pathway analysis and elementary mode analysis in structural or topological pathway analysis, and constraints-based flux analysis [13-15].

Elementary modes are a set of vectors calculated from a biochemical reaction network using convex analysis, which consists of non-decomposable reactions, while extreme pathways are a set of convex vectors from a stoichiometric matrix, and are an independent subset of the elementary modes. They can be used to calculate the product yields and minimal reaction sets, evaluate pathway redundancy, and determine correlated reaction sets. Flux balance analysis with constraints can be used to determine the intracellular fluxes under certain measured values based on a stoichiometric matrix. Multiple flux distributions and multiple metabolic pathways have been applied to determine the adaptability and robustness of complex cellular networks using linear programming (LP) and p-graph theory in structural pathway analysis [16].

Integrated Software Environment for Systems-Level Aanaysis of Metabolic Networks

Metabolic flux analysis is now widely employed in systems biotechnology and used by researchers with diverse backgrounds, and hence a user-friendly computer program for quantitatively analyzing metabolic fluxes is needed for those researchers who are less familiar with the underlying computational methods. To this end, we have developed the software package MetaFluxNet, which allows metabolic flux analysis. In particular, various constraints ranging from thermodynamic and mass con-

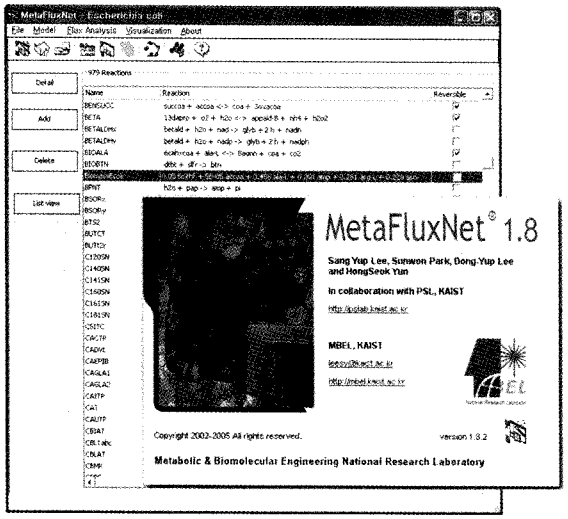

Fig. 2. Overview of MetaFluxNet version 1.8 (available at http://mbel.kaist.ac.kr/mfn). MetaFluxNet package is developed for metabolic flux analysis of local and genome-scale metabolic network models and built on Microsoft .NET environment with C#.

straints to externally measured and user defined constraints can be easily incorporated into the systems to carry out so called constraints-based flux analysis [2,14].

MetaFluxNet

MetaFluxNet (version 1.8; http://mbel.kaist.ac.kr/mfn) is designed to simulate and manage data on metabolic reaction networks and quantitatively analyze metabolic fluxes in an interactive and customizable manner (Fig. 2) [8]. Users can interpret and examine metabolic behavior and changes in response to genetic and/or environmental modifications. Consequently, quantitative *in silico* simulations of metabolic pathways can be used to understand the metabolic status and to design metabolic engineering strategies. The main features of MetaFluxNet include a customized model-construction environment, an interface for interacting with internet-based databases (BioSilico; http://biosilico.org) [17], a user-friendly interface for constraints-based flux analysis, comparative flux analysis of different strains under varying environmental conditions, several types of numerical solver, systems biology markup language (SBML) [18] supporting for communicating with other systems-biology platforms, an XML-based metabolic flux analysis markup language (MFAML) supporting for the formal representation of metabolic flux models, and an automated method for the creation of metabolic pathways [19]. Thus, MetaFluxNet enables the efficient construction and analysis of genome-scale as well as local metabolic networks.

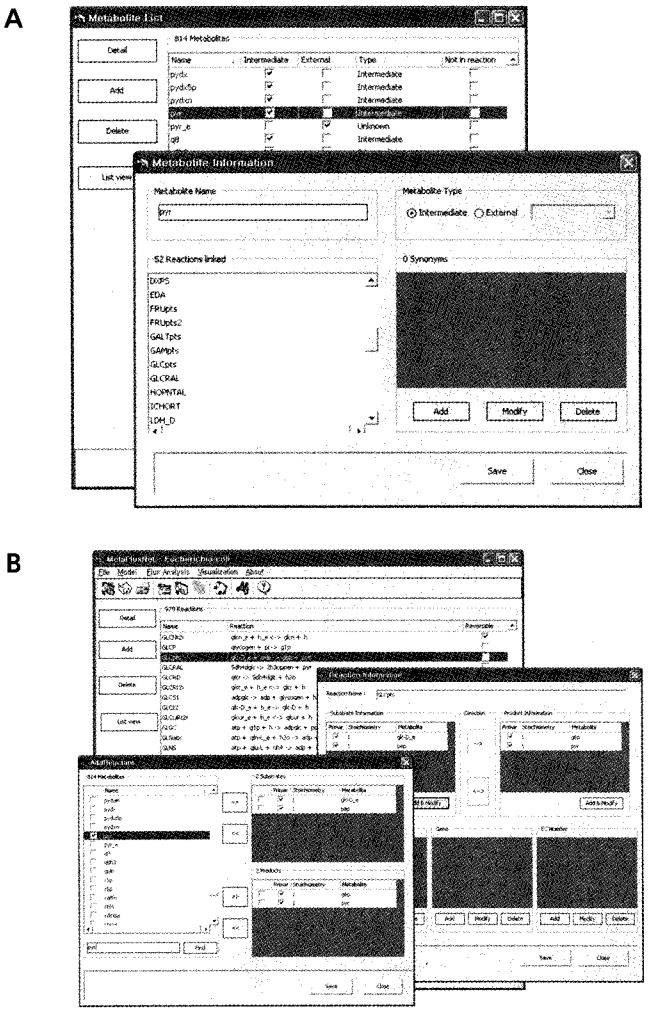

Fig. 3. Feature of MetaFluxNet. A; Metabolite List Panel. Metabolite list panel consists of two windows: metabolite list and metabolite information. Each metabolite is recorded with its name, type and synonyms, and all metabolites are added to the metabolite list. Users add all metabolites as nodes for metabolic networks. For example, 'pyr' on Metabolite List stands for pyruvate as an intermediate, which is involved in 52 reactions linked. When metabolites are known as extracellular metabolites, users select 'External' instead of 'Intermediate'. Here our group has 805 metabolites for *E. coli*. B; Reaction Information Panel. Reaction Information contains all information about enzymatic reactions such as reactants and products, EC number, and reaction reversibility. User can select proper reactants and products with their stoichiometric coefficients from already constructed Metabolite List by clicking arrow buttons. That regenerates stoichiometric matrix for metabolic flux analysis. GLCpts, for example, stands for glucose transport reaction by irreversible phosphotranferase system.

Construction of Metabolic Network Models

Constraints-based flux analysis requires genome-scale models composed of metabolic reactions that are available from biochemical textbooks and public databases [20,21]. The stoichiometric matrix of the models can be constructed from full genome sequences on the basis of physicochemical constraints representing mass conserva-

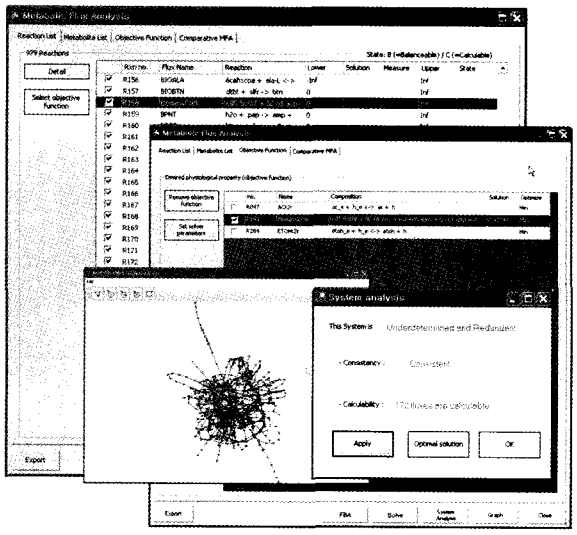

Fig. 4. Metabolic Flux Analysis Panel. After the system analysis is performed, the system can be identified through determinacy and redundancy. Because not enough measured data are available for the constraints-based analysis, the characteristics of most of genome-scale model is the underdetermined system which can be calculated by linear programming optimization. Optimal fluxes can be calculated by maximizing the objective function such as cellular growth (highlighted line). Finally, the calculated fluxes can be listed in solution column of reaction tab and analyzed further through MetaFluxNet-Visualization Panel and comparative metabolic flux analysis.

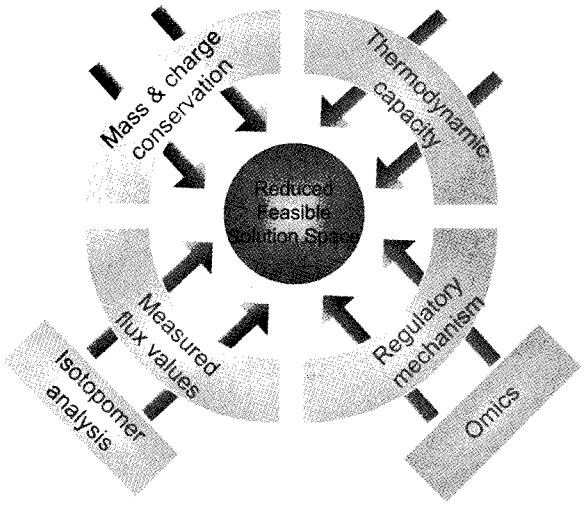

Fig. 5. Concept of constraints-based flux analysis. The number of feasible solutions is reduced by mass and charge conservation, thermodynamic capacity, measured flux data from external measurements or isotopomer analysis, and regulatory mechanism obtained from omics technologies.

tion and charge balances of the reactants and products that have negative and positive values, respectively.

MetaFluxNet provides a user-friendly interface for constructing such a metabolic network from a myriad of genome data through reaction and metabolite panels (Fig. 3A and 3B) [22]. Three integrated approaches for constructing a genome-scale model are available in MetaFluxNet: (1) adding reactions directly from original sources, (2) importing metabolic models in SBML formats, and (3) selecting reactions from searches of the name of a reaction or enzyme on the Database Query panel. The first approach-involving manual addition from diverse sources-can be tedious, but it can be used to guarantee the proper connections among metabolites. The second approach is applied to small metabolic networks because of the easy construction and modification. However, there is the possibility that metabolic models in different formats will not be applicable to metabolic flux analysis. This is a major the reason why we developed MFAML and incorporated it into MetaFluxNet (see below). The third approach is widely used because several full genome sequences have been elucidated, and it is possible to construct large-scale genome models from open public or in-house databases. Additional information such as gene names, EC number, or isozyme and multienzyme complexes allows the system to be expanded and analyzed further. The linkability of MetaFluxNet to the BioSilico DB reduces the effort required to compare the names of metabolites and reactions by providing the ability to systematically compare those from heterogeneous metabolic databases including LIGAND, ENZYME, EcoCyc, and MetaCyc [20,21].

Analysis of Metabolic Network Models

Constraints-Based Flux Analysis

The constraints-based flux analysis can be implemented using linear programming (LP) on the basis of mass balance and stoichiometric equations constrained in a user defined manner, thus yielding internal flux distributions (Fig. 4) [23,24]. This LP-based approach has been applied to genome-scale microbial models including *Escherichia coli* K-12, *Saccharomyces cerevisiae* and *Mannheimia succiniciproducens* [22,25-27].

The flux solution space can be reduced further on the basis of thermodynamic capacity in an energy-balance model incorporating the second law of thermodynamics and chemical potential differences, which is analogous to Kirchhoff's current and voltage laws for electric networks [28,29]. Regulatory constraints under several conditions and known regulatory mechanisms can further reduce the solution space [30]. However, constraints-based flux models have several limitations, including a lack of considering regulatory mechanisms, the existence of suboptimal subspaces, and different optimal solutions due to the inherent formulation and algorithmic procedures. These limitations can be partially solved by developing better genome-scale network model, providing internal flux values or flux split rations at the branch points, and by incorporating regulatory mechanisms deciphered by various omics tools. For example, high-throughput omics data including transcriptome, proteome and metabolome data can provide useful constraints for flux analysis of a

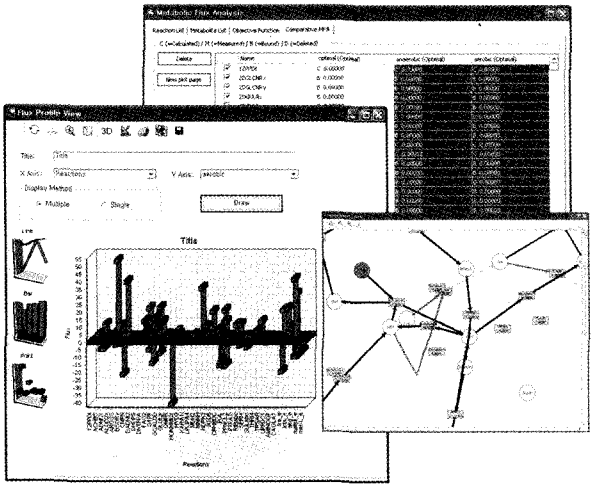

Fig. 6. Comparative flux analysis. Results of calculated fluxes under various conditions can be easily and directly compared. Flux-profiles view can help better understanding of metabolic flux analysis. The example shown is the comparison of metabolic fluxes calculated under aerobic and anaerobic conditions.

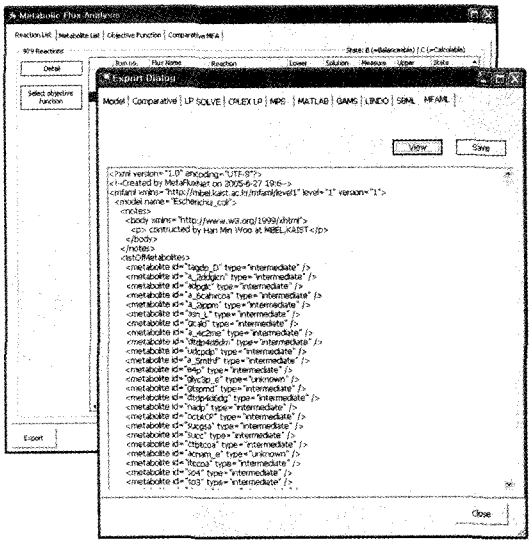

Fig. 7. Export Dialog. MetaFluxNet allows the export of metabolic models to various modeling formats including LP SOLVE, CPLEX LP, MPS, MATLAB, GAMS, LINDO, SBML and MFAML for efficient metabolic model analysis. Especially, MetaFluxNet also provides the standard structures for metabolic flux analysis through MFAML.

genome-scale model, thereby potentially leading to a systems-level understanding of metabolic networks, as illustrated in Fig. 5 [13,31,32].

Comparative Flux Analysis

MetaFluxNet provides comparative flux analysis in one window where specific measurements from fermentation data [33] or isotopomer data [34,35] and results of flux analyses under genetically and/or environmentally perturbed conditions can be compared (Fig. 6). For better understanding of comparative analysis, flux-profiles can be visualized by the use of lines, bars, and points within a Java platform. The thickness of lines and the size of flux name boxes are proportionally rescaled to the calculated flux values for easy understanding of local and global metabolic characteristics of the genome-scale metabolic network.

Representation and Exchange of Metabolic Network Models

Currently the SBML is available for representing several standard data structures for the dynamic simulation of a kinetics model. However, it does not provide a structure for storing data from a steady-state analysis (*e.g.*, constraints-based flux analysis). Thus, MFAML was developed for allowing the exchange of metabolic models, since it provides standard structures for steady-state flux analysis. MetaFluxNet adopted MFAML to allow efficient comparison of steady-state flux analysis models, and is thus useful for studying genome-scale models built on MetaFluxNet and for calculating metabolic fluxes under diverse constraints and conditions such as measured internal or external fluxes, and environmental and genetic modifications (Fig. 7).

MetaFluxNet can transform metabolic models into various modeling formats for LP standards such as a mathematical programming system and AMPL (a modeling language for mathematical programming; http://www.ampl.com), and applications that support LP, such as MATLAB (http://www.mathworks.com) and GAMS (general algebraic modeling system; http://www.gams. com). Thus, MetaFluxNet can provide constraints-based analysis models in different formats to allow comparisons of the results of flux analyses using different solvers.

Modeling and analysis of Genome-Scale Microbial Models

Metabolic flux analysis using MetaFluxNet allows an extensive and quantitative understanding of the metabolic characteristic of various microorganisms. Also, MetaFluxNet can be useful for constructing genome-scale models to aid the development of strategies for improving metabolically engineered strains. In the following, the current status of *in silico* model and its use in metabolic flux analysis are described.

E. coli is one of the best characterized microorganisms, in terms of its physiology, the function of its genes and its regulation. Constraints-based flux analyses have been successfully carried out using the genome-scale *in silico* metabolic network models of *E. coli* over several years [23] and widely applied to the prediction of cellular behaviors and the engineering of metabolically improved strains [36,37]. The iJR904 model developed by Palsson's group consists of 931 reactions involving 625 metabolites in which the elements and charge balances have been considered for a pH of 7.2 and by including water

and protons. Network gap analysis was used when missing links were found in metabolic pathways. Genes-to-proteins-to-reactions associations were used to understand the relationships among the genes and the reactions underlying cellular physiology [26]. The iJR904 model has been successfully applied in many recent studies: integrating high-throughput and computational data to bacterial networks and metabolic gene-deletion strains of *E. coli* for prediction of growth phenotypes [38-40], and incorporating regulatory mechanisms under particular conditions through a Boolean algorithm to reduce feasible solution spaces [41].

Similarly, we developed the EcoMBEL979 model comprising 979 reactions involving 814 metabolites. This *E. coli* model was constructed in MetaFluxNet to investigate the strategies for enhanced succinic acid production. For understanding *E. coli* metabolism at the systems level, MetaFluxNet can simulate the effects of changes in the desired products and biomass production rate by unchecking certain reaction boxes, so as to elucidate the appropriate combinations of gene knockouts for improving strains. When the data from transcriptome and proteome experiments indicate clearly that genes are not expressed, at least they can be used as simple on-off constraints.

CONCLUSION

In the postgenome era, improving our understanding on global cellular function and phenotypes is crucial to improving productivity through omics technologies and metabolic engineering. Metabolic fluxes can be considered as the final output of combined cellular regulation and phenotypes under particular conditions [1,22]. Analyzing metabolic fluxes requires genome-scale models based on public or in-house metabolic databases. Since the calculated fluxes exist at discrete points on the feasible solution space, constraints-based flux analysis can provide more realistic solutions with reasonable constraints such as mass and charge conservation, thermodynamics, measured fluxes, and regulatory mechanisms obtained through diverse omics technologies [24]. To assist constraints-based analysis, MetaFluxNet furnishes several user-friendly interfaces for easy connection to the BioSilico database and calculations for metabolic fluxes. Graphic visualization of comparative fluxes allows easy interpretation of the results. Also, several unique features such as flexible data exchange in various formats and provision of various LP solvers should make MetaFluxNet a versatile program package for metabolic flux analysis at local- and/or genome-scale. Taken together, MetaFluxNet will help understand global metabolic characteristics and develop strategies for metabolic engineering.

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