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ADVANCES IN ANALYSIS OF MICROBIAL METABOLIC FLUXES VIA ^{13}C ISOTOPIC LABELING

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Metabolic flux analysis via ^{13}C labeling (^{13}C MFA) quantitatively tracks metabolic pathway activity and determines overall enzymatic function in cells. Three core techniques are necessary for ^{13}C MFA: (1) a steady state cell culture in a defined medium with labeled-carbon substrates; (2) precise measurements of the labeling pattern of targeted metabolites; and (3) evaluation of the data sets obtained from mass spectrometry measurements with a computer model to calculate the metabolic fluxes. In this review, we summarize recent advances in the ^{13}C-flux analysis technologies, including mini-bioreactor usage for tracer experiments, isotopomer analysis of metabolites via high resolution mass spectrometry (such as GC-MS, LC-MS, or FT-ICR), high performance and large-scale isotopomer modeling programs for flux analysis, and the integration of fluxomics with other functional genomics studies. It will be shown that there is a significant value for ^{13}C-based metabolic flux analysis in many biological research fields. © 2008 Wiley Periodicals, Inc., Mass Spec Rev 28:362–375, 2009

Keywords: *steady state; mini-bioreactor; mass spectrometry; isotopomer modeling; functional genomics*

I. INTRODUCTION

Microorganisms have evolved complex metabolic pathways that enable them to utilize various nutrients and survive in their local environment. To understand cell metabolism and its response to environmental and genetic changes, an array of genomic and functional genomics tools are now available, including genomic and metagenomic sequencing (Alm et al., 2005; Tringe & Rubin, 2005; Warnecke et al., 2007) and transcript, protein, and metabolite profiling (Sauer, 2004; Wiechert, 2001). However, the most physiologically relevant description of a cell's metabolism remains the set of metabolic fluxes, which represent the final functional output of the interaction of all the molecular machinery studied by the other ''omics'' fields (Fig. 1). Regulation of cellular processes might not always be reflected in the gene annotation, transcript, or protein profiles (Fong et al., 2006; Sauer, 2004; Tang et al., 2007e). The transcription profile, moreover, might have little relationship to the final flux profile of cells due to post-transcriptional regulation of protein synthesis and enzyme activities (Fong et al., 2006; Hua et al., 2007). The metabolic flux profile of a cell, however, reflects the global reaction rates in the cellular metabolic network, and is a key determinant of cellular physiology (Sauer, 2004).

A large number of comprehensive microbial flux studies have been performed with stoichiometric metabolic flux analyses (also referred to as flux balance analysis, FBA) (Varma & Palsson, 1994). As its name implies, stoichiometric MFA uses the stoichiometry of the metabolic reactions (e.g., global metabolite balances of cofactors such as ATP, NADH, and NADPH) in addition to a series of physical, chemical, and biological characteristics (extracellular fluxes, thermodynamic directionality, enzymatic capacity, gene regulation, etc.) to constrain the feasible fluxes for a given physiological condition. If the number of measured extracellular fluxes equals the number of degrees of freedom it is possible to calculate the remaining fluxes, but, typically, the number of constraints is much smaller than the number of reactions in the metabolic network (Vallino & Stephanopoulos, 1993). The system is then underdetermined and it is necessary to postulate an objective function that one assumes the cell uses in its native ''program'' (growth rate maximization, for example) to calculate a set of predicted fluxes (Stephanopoulos, Aristidou, & Nielsen, 1998). The general applicability of this optimization principle has been repeatedly called into question because cellular metabolism in several biological systems seems to display sub-optimal performance (Fischer & Sauer, 2005; Schmidt et al., 1998; Schuetz, Kuepfer, & Sauer, 2007).

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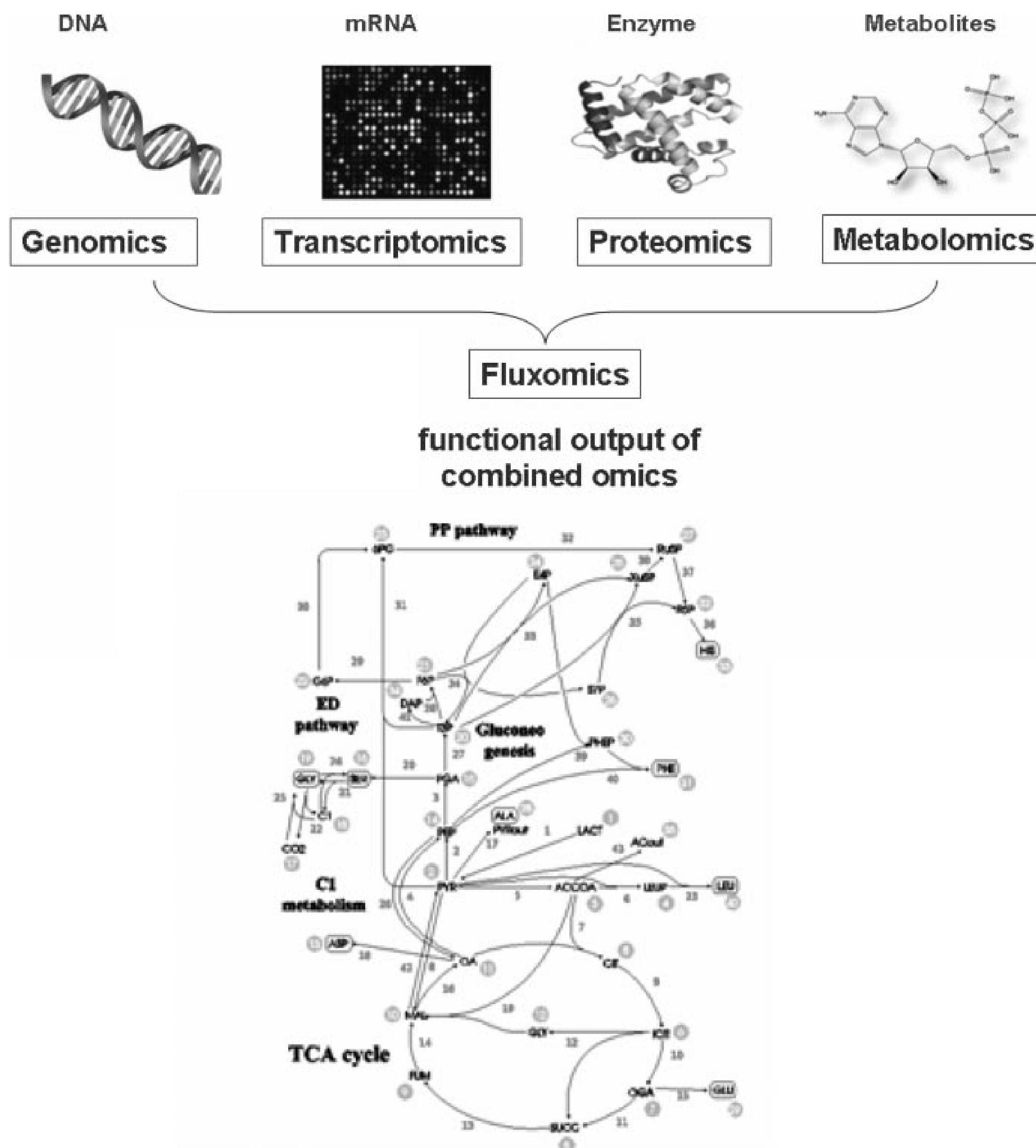

FIGURE 1. Omics tools to investigate cellular metabolism. DNA, microarray and protein images were obtained from Wikimedia Commons (http://commons.wikimedia.org/wiki/main_page). The molecular structure of ATP was obtained from ChemDraw Ultra 8.0. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

^{13}C metabolic flux analysis improves on stoichiometric MFA by using a completely different set of constraints derived from carbon-labeling experiments. These experiments consist of feeding the culture with a defined ^{13}C-labeled substrate, and measuring, through NMR or MS, the isotopic enrichment in intracellular metabolites (typically amino acids). This information is stored in terms of isotopomers (i.e., each of the possible labeling states in which a particular metabolite can be found (Wiechert, 2001)). The resultant ^{13}C-labeling in the intracellular metabolites imposes important constraints on how the labeled carbon substrate is distributed throughout the metabolic network and, hence, on the identity of the metabolic fluxes (Iwatani, Yamada, & Usuda, 2008; Sauer, 2006; Wiechert, 2001). These constraints are enough to solve for the central carbon flux distribution without the need of stoichiometric balances. The general schematic of the procedure is illustrated in Figure 2.

Both approaches of MFA (stoichiometric and ^{13}C based) display advantages and disadvantages. Stoichiometric MFA can be scaled to deal with complete genomes and can be used in a predictive as well as a descriptive fashion but has difficulty in predicting fluxes through reversible reactions or reactions that might form futile cycles (Stephanopoulos, Aristidou, & Nielsen,

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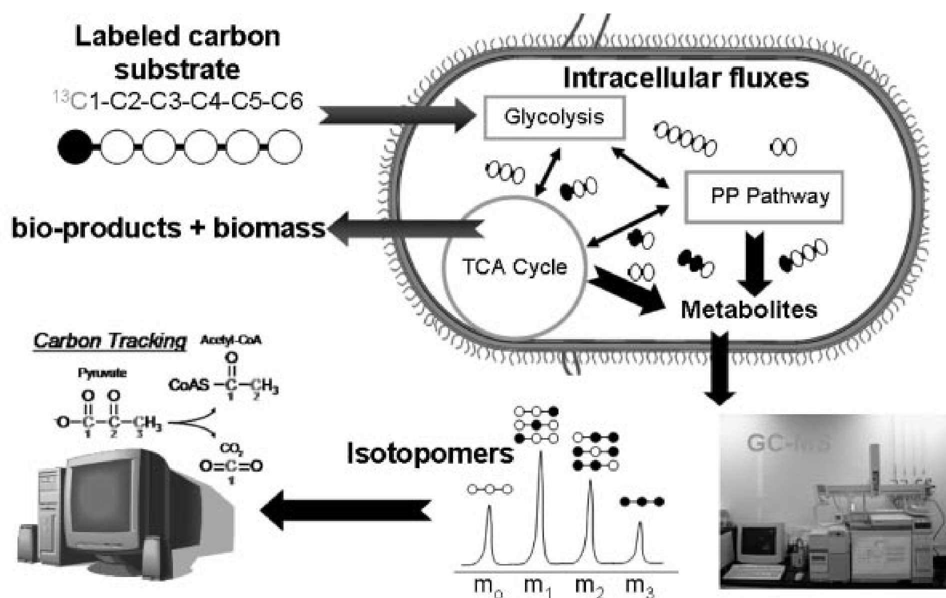

FIGURE 2. Protocol for ^{13}C-based flux analysis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1998; Wiechert & de Graaf, 1997). On the other hand, ^{13}C MFA does not require the assumption of an optimization objective and usually provides more accurate flux estimations since it uses the (highly relevant) isotopomer data; however, ^{13}C MFA can only be used in a descriptive fashion (i.e., it requires carbon labeling data), typically only tackles the central carbon metabolism, and is quite expensive to perform because of the high price of labeled feed.

The ^{14}C-labeling experiments by Blum and Stein (1982) can be seen as the earliest direct precursor of ^{13}C MFA. Since then, ^{13}C-based flux methods have become a key technology to analyze metabolic networks and to provide support information for metabolic engineering applications (Wiechert, 2001). In the last two decades, ^{13}C-based flux analysis has undergone significant developments that range from improvements in measuring the labeling patterns of targeted metabolites to computational algorithms for flux calculation. Such advancements have significantly extended the potential of ^{13}C-based flux analysis for diverse applications in the fields of metabolic engineering, bioremediation, and biomedical research. This review summarizes the recent advances in the ^{13}C-based fluxomics field for microbial systems and points out some possible future directions.

II. RECENT APPLICATION OF ^{13}C-BASED FLUX ANALYSIS

Over the past decade, the high-throughput and high-content analysis of the cellular genome, transcriptome, proteome, and metabolome, commonly referred to as "omics," has been developed to investigate a variety of organisms. What has lagged well behind those omics studies, but might in fact be the most important indicator of cellular physiology, is the study of the cell metabolic fluxes (fluxome). A quick search of the PubMed database (http://www.pubmed.gov) shows that the number of articles on flux analysis, specifically ^{13}C-based flux analysis to measure actual metabolic network, is several orders of magnitudes fewer than those for other omics studies (Table 1), in spite of the fact that flux profiles might provide a more accurate description of cell physiology (Sauer, 2004, 2006). This is due to a variety of reasons, including the high cost of labeled substrates, the requirement of specialized equipment (e.g., MS or NMR) for determination of isotopic labeling and significant mathematical/ statistical analysis (i.e., isotopomer modeling of metabolism). Furthermore, this approach is not amenable to all biological systems, because many organisms cannot grow in a defined minimal medium with labeled carbon substrates. Finally, typical ^{13}C flux analysis provides information about flux distributions for central carbon metabolism. Only recently has^{13}C flux analysis been performed for large-scale metabolic networks (Suthers et al., 2007).

During the last decade, measurement of metabolic fluxes via ^{13}C-labeling has developed quickly across a diverse set of applications (summarized by Table 2), including:

- Pathway bottleneck identification in industrial microorganisms with the final objective of optimizing biomass and metabolite synthesis and, ultimately, providing guidelines for genetic engineering. Rational manipulation of cellular metabolism for product biosynthesis is one of the main drivers for metabolic flux analysis.
- Gene function validation in organisms and development of new insights into active pathways under specific culture conditions. Every year, millions of dollars are spent on sequencing genomes of microorganisms and mammals. Whereas annotated and expressed genes may reflect the potential metabolism of a cell, flux analysis provides a

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TABLE 1. Publications with different omics tools from PubMed database

Omics	Search key words	Total Papers	Review papers	Earliest paper on PubMed
Genomics	Genome sequence	346,859	19,459	~1960s
Transcriptomics	DNA microarray	28,244	3,130	~ 1995
	Transcriptome	32,301	3,366	~1982
Proteomics	Protein analysis	1812,232	89,892	~1910s
	Proteome	10,356	1,921	~1995
Metabolomics	Metabolite analysis	31,581	931	~1950s
Fluxomics	Metabolism+ Flux analysis	8,017	388	~1950s
	^{13}C + flux	651	26	~1980s

Results as of March 24th, 2008.

valuable method to validate the proposed enzyme activities and the physiology of the cell. Flux analysis also reveals the responses of cellular metabolism to different growth conditions or environmental stresses.

- Drug-target search for a variety of diseases: pathogens or aberrant cells (e.g., cancer cells) regulate specific metabolic pathways to benefit their survival inside hosts. Studying the flux distributions in these cells' metabolic networks might reveal specific pathways that are essential for growth, and could be potential drug targets.

III. ADVANCES OF TECHNOLOGIES ASSOCIATED WITH ^{13}C METABOLIC FLUX PROFILING

Over the last few decades, a variety of methods associated with ^{13}C flux analysis have been developed. These methods include high-throughput cell culture experiments, accurate determination of metabolites' labeling patterns by high-resolution mass spectrometry, and new algorithms for flux calculation.

A. High Throughput ^{13}C-Labeled Cell Culture

^{13}C-based flux analysis is most easily done under metabolite and isotopomer steady state, which can be achieved by continuous feeding over three generation times (Tang et al., 2007c). Typical bioreactors with precise chemostat control often have a working volume over 0.5 L. Hence, experiments that use ^{13}C-labeled substrates can become very expensive, because the market price of labeled glucose or other carbon substrates is over \$100/g (www.isotope.com). One way to reduce the cost of labeled medium is to use unlabeled medium to achieve metabolic steady state, and then switch to an identical medium that contains labeled carbon substrates to obtain isotopic data. Because this approach might introduce significant bias from the residual unlabeled carbon, it requires a computational method based on the standard wash-out kinetics of a chemostat culture in steady state to correct the measured isotopomer data (Dauner, Bailey, & Sauer, 2001; Toya et al., 2007; Zhao & Shimizu, 2003). On the other hand, high-resolution mass spectrometry technology can detect the metabolites at the level of nano-moles (i.e., <5 mg biomass is sufficient to measure all proteinogenic amino acids), a large volume of expensive labeled culture is no longer necessary. To have a high-throughput and more economical method, shake flasks or small-scale chemostat systems (<10 mL) are often used for the purpose of reducing experiment costs (Nanchen et al., 2006). Shake flasks, for example, can be used for steady-state flux analysis so long as the cells have maintained exponential growth for a sufficiently long time (Sauer et al., 1999). Even in batch cultures as small as 1 mL, metabolic fluxes are directly comparable to those from cells grown in aerobic bioreactors (Fischer, Zamboni, & Sauer, 2004). As such, deep-well micro-plates can be used to screen the intracellular fluxes of *Escherichia coli* and *Saccharomyces cerevisiae* (Cakar et al., 2005; Fischer, Zamboni, & Sauer, 2004; Sauer, 2004). Besides shake flasks and micro-plates, novel mini-bioreactors with a volume of 1–10 mL have been developed and are commercially available (Kostov et al., 2001; Maharbiz et al., 2004; Puskeiler et al., 2005; Tang et al., 2006). Micro-reactors not only control pH and temperature, but also automatically maintain the oxygen supply (a lack of which is a limitation often observed in shake flasks) and achieve stable dissolved oxygen levels throughout the entire growth period. These high-throughput cultivation systems have been shown to give reproducible results and extraordinary flexibility for tracer experiments under different growth conditions (Weiss et al., 2002; Yang, Wittmann, & Heinzle, 2006).

Certain considerations need to be accounted for when choosing the position of the labeled carbon substrate and its composition in the cell culture medium. Generally, for fully

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TABLE 2. Examples of recent application of ^{13}C-based flux analysis

Authors	Organisms	Culture method/ Measurement	Findings
Model Industrial Microorganisms			
(Suthers et al. 2007)	*Escherichia coli*	Chemostat culture; GC-MS	Used a large scale flux model to analyze engineered amorphadiene producing strain.
(Fischer and Sauer 2003b)	*E. coli*	Chemostat culture; GC-MS	Discovered a novel metabolic cycle catalyzes glucose oxidation and anaplerosis under carbon limited conditions.
(Nanchen et al. 2006)	*E. coli*	Chemostat culture; GC-MS	Revealed the distribution of almost all major fluxes varied nonlinearly with dilution rate in chemostat culture.
(Dauner et al. 2001; Dauner et al. 2002; Fischer and Sauer 2005)	*Bacillus subtilis*	Chemostat or shaking flask culture; NMR or GC-MS	Revealed the fluxes through central metabolism when different carbon substrates are used in carbon-limited chemostat cultures. The robustness of central metabolism was proposed.
(Blank et al. 2005a)	*Saccharomyces cerevisiae*	Mini-batch (deep-well plates) culture; GC-MS	Revealed mechanistic principles of metabolic network robustness to null mutations in yeast, i.e., 75% network reactions have redundancy through duplicate genes.
Novel Microorganisms			
(Tang et al. 2007c)	*Shewanella oneidensis*	Chemostat culture; GC-MS + NMR	Revealed the regulation of central metabolism under various oxygen conditions.
(Tang et al. 2007a)	*Shewanella oneidensis*	Batch culture; GC-MS	Revealed the effect of fullerene nano-particles on cellular metabolism
(Risso et al. 2008; Tang et al. 2007b)	*Geobacter metallireducens*	Anaerobic batch culture; GC-MS	Confirmed a complete TCA cycle under Fe^{3+} reducing condition and found an unusual isoleucine biosynthesis pathway.
(Tang et al. 2007e)	*Desulfovibrio vulgaris*	Anaerobic batch culture; GC-MS + FTICR	Revealed an incomplete central pathway and found the R-type citrate synthase.
(McKinlay et al. 2007)	*Actinobacillus succinogenes*	Anaerobic batch culture; GC-MS + NMR; in vitro enzyme assay performed	Studied carbon flux distributions and redox balance for succinate production.
(Yang et al. 2002)	*Cyanobacterial Synechocystis*	Photosynthetic bioreactor, GC-MS + NMR	Calculated cyanobacterial central carbon metabolism in both heterotrophic and mixotrophic conditions

(*Continued*)

TABLE 2. (*Continued*)

(Fuhrer et al. 2005)	*Agrobacterium tumefaciens, Sinorhizobium meliloti, Rhodobacter sphaeroides, Zymomonas mobilis, Paracoccus versutus,*	Batch cultures and GC-MS	By comparing seven different species , it was shown that the Entner-Doudoroff pathway and pyruvate bypass are commonly used. All aerobes exhibited fully respiratory metabolism without significant overflow metabolism.
(Blank et al. 2005b)	fourteen *Hemiascomycetous yeasts*	Shaking flask and GC-MS	Findings include: compartmentation of amino acid biosynthesis in most species was identical to that in *Saccharomyces cerevisiae*. The flux through the pentose phosphate (PP) pathway was correlated to the yield of biomass, but the operation of a yet unidentified mechanism for NADPH reoxidation in *Pichia angusta* is suggested.
Plant or mammalian cells			
(Sriram et al. 2007)	*Catharanthus roseus*	Liquid medium batch culture; NMR	Fluxes analysis of plant hairy root system quantifies the carbon flows through three-compartments: plastid, cytosol and mitochondrion.
(Allen et al. 2007)	Soybeans *(Glycine max)*	Grown in a greenhouse with labeled glucose medium; GC-MS and MNR	Measurements of labeling of monomers from starch, cell wall and protein glycans estimate key carbon fluxes in the compartmentalized flux network of plant cells.
(Forbes et al. 2006)	breast cancer cells	roller bottles with rich medium ; NMR	The observed dependence of breast cancer cells on pentose phosphate pathway activity and glutamine consumption for estradiol stimulated biosynthesis suggests that these pathways may be targets for estrogen-independent breast cancer therapies.
(Yang et al. 2008a)	mammary carcinoma cells	Culture dishes with rich medium; NMR and GC-MS	An integrated approach for the analysis of metabolome and fluxomics to understand fluxes through the key central metabolic pathways and biosynthetic pathways of fatty acids / amino acids in cancer cells.
(Meadows et al. 2008)	human epithelial breast cells	Culture dishes with rich medium; GC-MS	The unique metabolic characteristics of cancerous breast cells are revealed by a simple flux model based on isotopic Enrichment in free metabolites

labeled carbon substrates (also called [U-^{13}C]), a mixture of 10–20% with unlabeled substrates is used for cell culture. This type of mixture has been proved to give more reliable flux results if NMR is used for isotopomer analysis rather than GC-MS, because NMR can better determine the labeled carbon positions (Tang et al., 2007c). On the other hand, if a substrate with the proper position labeled is chosen, the GC-MS data of resulting metabolites can also give confident flux results through most pathways. For example, the reactions of the pentose phosphate and Entner–Doudoroff pathways can be particularly well

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differentiated using 1st position labeled glucose but not with fully labeled glucose (Fischer, Zamboni, & Sauer, 2004; Suthers et al., 2007). To achieve the most reliable metabolic fluxes computed based on the isotopomer distribution measured using NMR and GC-MS, mixture of singly labeled, doubly labeled, and fully labeled carbon substrates are recommended. A statistical analysis showed that the best feasible mixture of labeled carbon substrates for cultivation of *Synechocystis* sp. is 70% unlabeled, 10% [U-^{13}C] and 20% [1,2-$^{13}C_2$] labeled glucose (Arauzo-Bravo & Shimizu, 2003). Finally, the labeled carbon substrates purchased from commercial companies often contain a small amount of impure substrates (purity is $<$99%), which may adversely affect cell growth (Tang et al., 2007e). Therefore, if large amounts of labeled carbon sources are used in the culture, the purchased labeled substrates have to be further purified to remove the potential toxic compounds.

B. Accurate Isotopomer Determination

Isotopomer analysis of metabolites allows branching (e.g., the pentose phosphate pathway vs. glycolysis) and circular (e.g., TCA cycle) pathway fluxes to be determined, because the labeling pattern of metabolites in these pathways is very sensitive to the amount of flux through them (Stephanopoulos, Aristidou, & Nielsen, 1998). Proteinogenic amino acids are often used for isotopomer analysis because they acquire the labeling pattern of their central metabolic precursors and are abundant and stable. There are a total of 20 amino acids, but only 16 of them can be accessed after protein hydrolysis via 6 M HCl at high temperature (cysteine and tryptophan are degraded; glutamine and asparagines are converted to glutamate and aspartate respectively) (Daunder & Sauer, 2000). The 16 amino acids can provide the isotopic labeling information of 8 crucial precursor metabolites: pyruvate, acetyl-CoA, 3-P-glycerate, phospho*enol*pyruvate, erythrose-4-P, oxaloacetate, 2-oxo-glutarate, and ribose-5-P. Therefore, knowing the isotopomer distributions in these key metabolites provides enough constraints on the central carbon metabolic network model to confidently calculate the flux.

The experimental measurements of labeled carbon in amino acids or other metabolites can be done either by nuclear magnetic resonance (NMR) spectroscopy or by gas chromatography-mass spectrometry (GC-MS). NMR spectroscopy was a common technique for the early period of modern ^{13}C-flux analysis, because it can be used to determine the position of the labeled carbon atoms in metabolites (de Graaf, 2000; Malloy, Sherry, & Jeffrey, 1988; Sauer et al., 1997; Szyperski, 1995, 1998). However, the overall sensitivity of NMR is significantly lower than that of GC-MS. GC-MS, currently the most popular technique, detects the mass distributions, which are the fractions of the total population of any particular molecule or molecular fragment that are unlabeled, singly labeled, doubly labeled, etc. (Christensen & Nielsen, 1999; Daunder & Sauer, 2000; Wittmann, 2007). Although this type of data offers no information about the position of all labeled atoms, some of this information can be determined by fragmenting molecules to provide additional labeling information at certain carbon positions (mostly the carboxyl group) (Wahl, Dauner, & Wiechert, 2004).

Analysis of amino acids or charged/highly polar metabolites via gas chromatography requires that these metabolites are derivatized, commonly with silylation reagents, to render the molecules volatile enough to enter the GC column (Fig. 3). *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (TBDMS method) is most common derivatizing agent (Antoniewicz, Kelleher, & Stephanopoulos, 2007a; Daunder & Sauer, 2000; Wahl, Dauner, & Wiechert, 2004). To measure the extent of labeling in free metabolic acids, such as pyruvate or succinate, a more gentle and sensitive derivatization agent, *N*,*O*-bis-(trimetylsilyl)trifluoroacetamide (BSTFA), can also been used (Meadows et al., 2008; Tang et al., 2007d) (Fig. 3). The derivatization step introduces significant amounts of naturally labeled isotopes (''noise''), including ^{13}C (1.13%), ^{18}O (0.20%), ^{29}Si (4.70%), and ^{30}Si (3.09%), such that the raw mass isotopomer spectrum must be corrected prior to calculation of metabolic fluxes (Daunder & Sauer, 2000; Hellerstein & Neese, 1999; Lee, Bergner, & Guo, 1992; van Winden et al., 2002; Wahl, Dauner, & Wiechert, 2004; Wiechert & de Graaf, 1996). Another disadvantage of GC-MS is that the accuracy of isotopomer measurement can be affected by the choice of the GC-MS spectrum integration algorithm, sample concentration, and overlapping fragments (Antoniewicz, Kelleher, & Stephanopoulos, 2007a).

High-resolution and highly sensitive mass spectrometers can be used to precisely measure the labeling pattern of amino acids and metabolites in central metabolic pathways (at concentrations as low as nM). Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) has been used to determine intracellular free amino acids to profile metabolic flux changes during fed-batch cultivation (Iwatani et al., 2007; Nöh

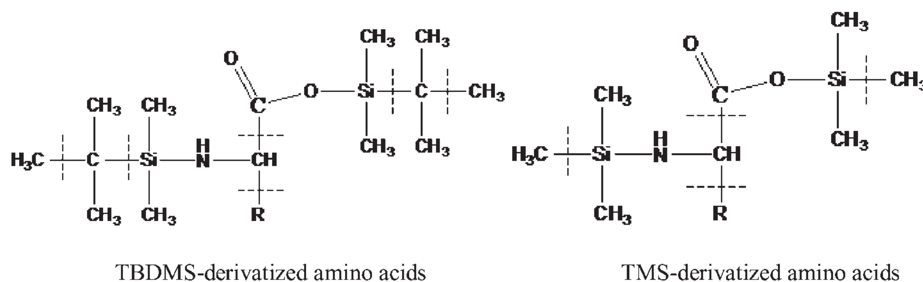

FIGURE 3. The molecular structure and the bond fragmentation positions between two silyation-derivatized amino acids. The dotted line represents the cracking position during ionization.

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et al., 2007). This approach, which involves the labeling of free metabolites with fast-turnover times, has great potential to investigate metabolism during various cell growth phases, because the phase dependent metabolism is difficult to accomplish using proteinogenic amino acids. In a related fashion, GC-MS, LC-MS, and NMR have been combined to extract maximal isotopomer information from amino acids. The information has been used to accurately determine the metabolism in mitochondria and the cytosol in *S. cerevisiae* strains (Kleijn et al., 2007). Capillary electrophoresis time-of-flight mass spectrometry (CE-TOF MS) has been used to measure isotopomers of thirteen unstable metabolites in central metabolism, including some unstable phosphorylated molecules such as 3-P-glycerate, phospho*enol*pyruvate, and ribose-5-P (Toya et al., 2007). Thus, CE-MS allows metabolic flux analysis directly from metabolites without any measurement of amino acids. Recently, Fourier transform-ion cyclotron resonance mass spectrometry (FT-ICR MS), with direct infusion via electrospray ionization, was used to measure the metabolite isotopomer distribution in a biomass hydrolysate of *Desulfovibrio vulgaris* Hildenborough to unveil an unusual citrate synthase activity (Pingitore et al., 2007). This method can determine the ^{13}C positions in the skeleton of the amino acid based on specific fragmentation patterns.

Measurement of labeling in central metabolites, other than proteinogenic amino acids, can significantly extend the scope of ^{13}C-based flux analysis applications and improve the accuracy of flux determination. Because ^{13}C MFA derives flux distributions from the labeling of amino acids produced by the labeled feed once it percolates through the metabolic network, it requires a minimal medium to avoid the introduction of a bias into the isotopomer measurements. However, many pure cultures are non-viable without nutrient supplements. When cells are grown in a rich medium that contains amino acids, only those proteinogenic amino acids synthesized from the central metabolism (e.g., alanine, aspartate, and glutamate) and not taken up from the medium can be used for flux determination using standard methods (Christiansen, Christensen, & Nielsen, 2002). To circumvent this problem, highly sensitive mass spectrometers can be used to directly obtain the isotopomer information from free central metabolites (e.g., acids in the TCA cycle). Provided that the amino acids are not metabolized, use of isotopomers of central metabolic intermediates will allow calculation of metabolic fluxes even when amino acids are supplemented into the medium. Additionally, this strategy avoids the possible mistakes of acquiring the labeling pattern of metabolites (e.g., amino acids) for organisms that are not well-known or where holes in genomic annotation are present (e.g., the alternative isoleucine pathway in *Geobacter* spp. contain holes in the annotation that might introduce errors in metabolic flux calculations) (Risso et al., 2008; Tang et al., 2007b).

C. High-Performance Flux Calculation Algorithms

Metabolic fluxes cannot be measured directly but, rather, must be inferred through model based data evaluation from the knowledge of the reactions involved, the information contained in the amino acid/metabolite labeling, and the external fluxes (Fig. 4).

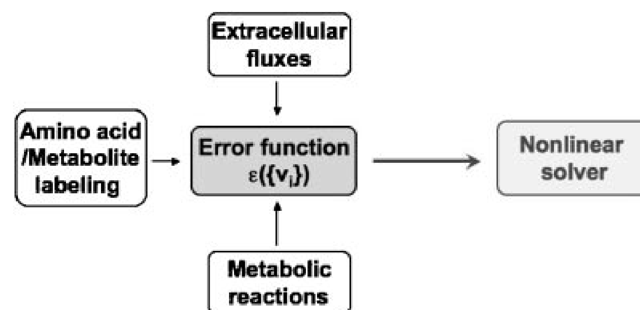

FIGURE 4. Inputs for metabolic flux analysis. The information on the metabolic reactions, amino acid/metabolite labeling and extracellular fluxes is combined to produce the error function ε (average difference between measured and computed labeling patterns). The predicted labeling can be computed following different methods (see main text) and then coupled to a chosen nonlinear solver (see main text) to solve for the fluxes following the iterative procedure in Figure 5.

Hence, computational algorithms are a key component of ^{13}C-based flux analysis (Sauer, 2006; Schmidt, Nielsen, & Villadsen, 1999a; Schmidt et al., 1999b; Wiechert, 2001). Because finding analytical expressions for the internal fluxes as a function of carbon-labeling data is, for all practical cases, impossible, the determination of fluxes is usually achieved through a heuristic recursive procedure (Fig. 5). The computational bottlenecks for this procedure are: (1) how to best choose the new set of fluxes based on the past information on the error function $\varepsilon(\{v_i\})$ so as to minimize the number of steps necessary to reach its global minimum without getting trapped in the relative minima, and (2) calculating the amino acid/metabolite labeling pattern from the assumed set of fluxes $\{v_i\}$.

Finding the input that optimizes a function (function optimization) has been an intensely studied challenge in numerical analysis. An array of tools (Floudas & Pardalos,

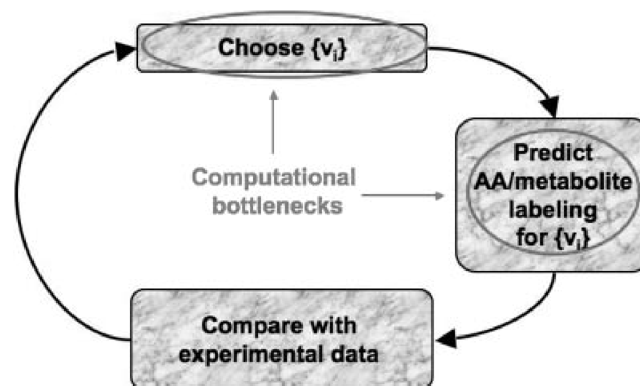

FIGURE 5. Recursive procedure to obtain fluxes from amino acid/metabolite labeling information. A set of fluxes $\{v_i\}$ is initially chosen and the expected amino acid/metabolite labeling is calculated under the assumed fluxes $\{v_i\}$. This computationally generated labeling is compared with the labeling obtained experimentally and the difference is quantified as the error function $\varepsilon(\{v_i\})$. A new set of fluxes $\{v_i\}$ is then chosen so as to try to decrease $\varepsilon(\{v_i\})$. This procedure is repeated until the calculated labeling and the experimental data are within the experimental error. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1992; Goldberg, 1989; Nocedal & Wright, 1999; Press et al., 1992) is available to tackle the first bottleneck. Among those methods used in ^{13}C-based flux analysis are global search algorithms such as simulated annealing and evolutionary algorithms (Dauner, Bailey, & Sauer, 2001; Forbes, Clark, & Blanch, 2001; Schmidt et al., 1999b; Zhao & Shimizu, 2003); and local search algorithms such as the Levenberg–Marquardt method (Yang et al., 2008a; Zhao & Shimizu, 2003), the Nelder–Mead method (Wiechert et al., 2001), Sequential Quadratic Programming (SQP) (Wiechert et al., 2001), or a hybrid SQP/Newton algorithm (Yang, Frick, & Heinzle, 2008b). Although local search algorithms might easily become trapped in local minima, they are typically much faster than global search algorithms. Hence, they can be run many times with different initial points and have the best solution chosen, in the same amount of time that a single global search would be performed. Although no systematic comparison between methods has been published, the Levenberg–Marquardt method (Young et al., 2008) and a range-restricted evolutionary algorithm (Möllney et al., 1999; Nöh et al., 2007; Nöh & Wiechert, 2006) are used for the most computationally demanding flux analysis: non-stationary metabolic flux analysis.

Development of computational methods for ^{13}C-based flux analysis has, therefore, focused on speeding up the calculation of amino acid/metabolite labeling from an assumed set of fluxes. Several methods have been proposed, including the iterative averaging isotopomer method (Schmidt, Nielsen, & Villadsen, 1999a), the cumomer method (Wiechert et al., 1999), the Elementary Metabolic Unit (EMU) method (Antoniewicz, Kelleher, & Stephanopoulos, 2007b), the isotopomer path tracing method (Forbes, Clark, & Blanch, 2001), and the fractional labeling method (Riascos, Gombert, & Pinto, 2005). In practice, the only methods under continuous development are the cumomer and EMU method. The cumomer method was developed as a more efficient strategy to solve metabolite labeling than the iterative averaging isotopomer method: by casting the problem in terms of the new concept of cumomers (cumulated isotopomers fractions), the isotopomer labeling can be obtained from the solution to a cascade of linear equations. The EMU method uses the knowledge of atomic transitions in the reactions network to identify a set of variables containing the minimum amount of information necessary to simulate isotopic labeling in the system. The models resulting from the use of this set of variables require significantly fewer equations to be solved than for the cumomer case. The EMU method results in computation times that are claimed to be several orders of magnitude lower than for the cumomer method (Young et al., 2008) and, hence, the possibility of tracking other labeling atoms such as ^{2}H, ^{15}N, and ^{18}O in tracer experiments with multiple labeled substrates. Nonetheless, a recent study attributes similar improvements in speed for the cumomer method based on careful study of the labeling network topology (Weitzel, Wiechert, & Nöh, 2007). Because the insights obtained form network topology are applicable to the EMU method, significant increases in performance are expected in the future (Weitzel, Wiechert, & Nöh, 2007).

Not every ^{13}C-based flux analysis strategy follows the scheme shown in Figure 5. There are other alternatives: one of them is to choose a set of amino acids with well-known precursors and to extract the relative ratio of fluxes that contribute to their labeling (Fischer & Sauer, 2003a). This method has been used to perform the first large-scale experimental analysis of intracellular flux distributions for 137 null mutants of *B. subtilis* (Fischer & Sauer, 2005). Although this approach provides direct evidence for the relative magnitude of each flux and has the great benefit of using local network data around single metabolite nodes, it is restricted to 10–15 pre-selected pathways directly accessible from the isotopomer data (Sauer, 2006). Another alternative involves using a nonlinear problem (NLP) solver to search simultaneously among the total number of configurations of fluxes and metabolite labeling patterns to match experimental data (Riascos, Gombert, & Pinto, 2005; Vo & Palsson, 2006). Whereas initial applications of this method were limited to central carbon metabolism reactions, more recent work (Suthers et al., 2007) makes use of much more comprehensive metabolic networks and can, hence, take into account *global* metabolite balances of cofactors (e.g., ATP, NADH, and NADPH), which traditional ^{13}C MFA does not. These studies integrate ^{13}C MFA with FBA and display the benefits of both methods.

As with every measurement, it is desirable to assign a confidence interval to flux estimates. A traditional approach has been to find a general mapping from the experimental data (i.e., isotopomer labeling and extracellular fluxes) to the best flux estimate, linearize that mapping around the actual measured experimental data, and map the confidence intervals from the measured data on to the flux estimates (Arauzo-Bravo & Shimizu, 2003; Dauner, Bailey, & Sauer, 2001; Schmidt et al., 1999b; Wiechert & de Graaf, 1997; Wiechert et al., 1997). Recently, nonlinear statistical methods (Gallant, 1987) have been applied to take into account the intrinsic nonlinear nature of flux analysis (Antoniewicz, Kelleher, & Stephanopoulos, 2006). Under this scenario, each individual flux is increased from its estimated value until the objective function for the recalculated fluxes reaches the maximum value allowed for a given confidence value. This marks the upper confidence limit. The crucial difference with the linear approach is that the flux estimates are recalculated for each individual flux increase. The lower confidence limit is obtained by decreasing each independent flux and following the same procedure. An alternative (but more computationally intensive) way to take into account the nonlinear nature of flux calculation is to use a Monte–Carlo approach in which new experimental data is randomly generated within the measurement errors and new flux estimates are calculated. By doing this a large enough number of times, the probability distribution of estimated fluxes provides the confidence intervals and correlation between individual fluxes (Schmidt et al., 1999b; Zhao & Shimizu, 2003).

D. Non-Stationary Flux Analysis

One of the traditional prerequisites for ^{13}C-based flux analysis is that the system must be in a metabolic and isotopic steady-state; that is, fluxes and amino acid/metabolite labeling does not change in time. This requirement means that the length of the experiment should be significantly longer than the inverse of the growth rate, $1/\mu$ (Nöh & Wiechert, 2006; Wiechert & Nöh, 2005); that requirement leads to impractically long experiments in the case

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of low growth rates, such as in the production phase of typical bioprocesses. A way to circumvent this problem is to use the information of the labeling of intracellular free amino acids as input (Iwatani et al., 2007; Krömer et al., 2004). Intracellular amino acids are continuously renewed, and represent the actual flux state of the cells, as modified by protein turnover, transamination, mRNA degradation and other effects (Grotkjaer et al., 2004). Proteinogenic amino acids, on the other hand, are an accumulation of intracellular amino acids throughout the entire cultivation process and display much longer turnover rates. Another strategy to overcome this difficulty is to use intracellular metabolite labeling and non-stationary flux analysis. This type of ^{13}C MFA assumes a changing metabolite labeling and tracks these changes to estimate fluxes. Cumomer- and EMU-based algorithms have been developed, and their application allows for the quantification of time-resolved metabolite labeling patterns and flux profiles (Antoniewicz et al., 2007c; Nöh, Wahl, & Wiechert, 2006; Young et al., 2008). Those developments extend ^{13}C-flux analysis to non-stationary conditions like batch and fed-batch fermentations, and reduce the cost and duration of labeling experiments.

IV. STRATEGIES FOR SYSTEMS-LEVEL ^{13}C-BASED FLUX ANALYSIS

Metabolic flux distributions provide new insights into how metabolism works. However, ^{13}C-based flux analysis, so far, is mainly focused on central metabolic pathways and biomass synthesis pathways (e.g., amino acids). Because each pathway could be controlled by several genes/enzymes, flux analysis alone might be insufficient to reveal exact gene targets or regulatory mechanisms in a complicated biological system. Hence, ^{13}C-based flux measurement has been integrated with other ‘‘omics’’ tools to understand global metabolism.

1. *Genomics combined with flux analysis*: Most flux-analysis studies focus on central metabolism and neglect other flux routes that could contribute to biomass growth and metabolite synthesis. Recently, ^{13}C-based genome-scale flux models of *S. cerevisiae* and *E. coli* have been developed to identify annotated gene functions in more comprehensive metabolic networks (Blank, Kuepfer, & Sauer, 2005a; Suthers et al., 2007). For the *E. coli* case, for example, a reaction network that consisted of 350 fluxes and 184 metabolites in *E. coli*, including global metabolite balances on cofactors such as ATP, NADH, and NADPH (Suthers et al., 2007), was developed. This approach demonstrated possible key genes in an *E. coli* strain engineered to produce amorphadiene (a precursor to the anti-malarial drug artemisinin). Additionally, conventional genome-scale flux-balance analysis (FBA) can determine intracellular fluxes, but it requires choosing proper objective functions to accurately describe certain metabolic conditions. The ^{13}C-based flux analysis approach can be used to verify the FBA model and to provide useful metabolic regulation information. For instance, different objective functions to predict fluxes in the genome-scale FBA have been evaluated via isotopomer flux models (Schuetz, Kuepfer, & Sauer, 2007). The study showed that unlimited growth on glucose in oxygen- or nitrate-respiring batch cultures is best described by nonlinear maximization of the ATP yield with minimal enzyme usage, whereas under nutrient scarcity in continuous cultures, linear maximization of the overall ATP or biomass yields achieved the highest predictive accuracy.
2. *Transcriptomics and flux analysis*: Metabolic fluxes and global mRNA transcript analyses have been used to study the flexibility of the metabolic network of *E. coli* to compensate for genetic perturbations (Fong et al., 2006). Only activation of latent pathways and flux changes in some tricarboxylic acid cycle pathways were found to correlate with molecular changes at the transcriptional level, whereas flux alterations in other central metabolic pathways were not connected to changes in the transcriptional network; those data suggest complex regulatory mechanisms at transcription and enzyme activity levels. Similar observations were reported from the study on *E. coli* strains adapted to growth on lactate and *S. cerevisiae* grown under a variety of carbon sources; that is, no clear qualitative correlations between most transcriptional expression and metabolic flux changes (Hua et al., 2007).
3. *In vitro enzyme chemistry, proteomics, and flux analysis*: ^{13}C-labeling experiments combined with measurements of enzyme activities and intracellular metabolite profiles are often used to clarify the unknown pathways and support the results of the *in vivo* flux measurement (Klapa, Aon, & Stephanopoulos, 2003; McKinlay et al., 2007; McKinlay & Vieille, 2008; Sauer et al., 2004). For example, when the metabolism in a *pykF* mutant of *E. coli* was studied, information on intracellular metabolic flux distributions, enzyme activities, and intracellular metabolite concentrations were integrated to quantitatively reveal the regulation of phospho*enol*pyruvate carboxylase, malic enzyme, phosphofructokinase, acetate formation, and the oxidative pentose phosphate (PP) pathway in the mutant (Al Zaid Siddiquee, Arauzo-Bravo, & Shimizu, 2004). Proteomics tools have also been used to provide labeling constraints for flux analysis of individual strains in microbial communities (Shaikh et al., 2008). This study shows that it is possible to analyze the isotopomer distribution of amino acids from targeted organism via highly expressed His-tagged green fluorescent protein (GFP).

Multiple ‘‘omics’’ analysis, including transcriptomics, proteomics, metabolomics, and fluxomics, are beginning to be integrated to monitor cellular physiology, and those ‘‘omics’’ studies create a new concept of functional genomics. The combination of those high-throughput tools allows for the systematic quantification of the interactions of thousands of metabolic network components under genetic or environmental perturbations (Ishii et al., 2007; Krömer et al., 2004).

V. CONCLUSIONS

We have strived to show that there is a significant value for ^{13}C-based metabolic flux analysis in many fields. A series of new techniques associated with ^{13}C-flux analysis have recently emerged, including: high-throughput cultivation

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systems, high-sensitivity, high-resolution mass spectrometry for isotopomer analysis of metabolites (such as GC-MS, LC-MS, ESI-TOF, or FT-ICR), high-performance modeling programs for isotopomer flux analysis, and integrated flux analysis with other ''omics'' tools. These techniques extend the application of ^{13}C-based flux analysis to diverse applications from microbial to mammalian cells to (1) discover or validate gene functions involved in central metabolic pathways; (2) understand the *in vivo* metabolisms under different culture conditions; (3) provide information of the bottleneck pathways for biomass or metabolite synthesis in engineered microorganisms; (4) and identify pathogen-specific metabolic pathways for drug targets.

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