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Minireview

Metabolic modelling of microbes: the flux-balance approach

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Where is the Life we have lost in living? . . .
Where is the knowledge we have lost in information?
T.S. Eliot, Choruses from the Rock

Introduction

The history of biological research has witnessed many great successes, and many more are expected in the future. The anticipation of great findings has increased with the development of high-throughput technologies, which enable the rapid generation of sequence, transcript, and proteomic data, to name but a few examples. As a result, it has become clear that further biological discovery will be limited not by the availability of biological data (Blaine Metting and Romine, 1997), but by the lack of available tools to analyse and interpret these data. While the new fields of genomics (Gaasterland and Oprea, 2001), proteomics (Naaby-Hansen *et al*., 2001), transcriptomics (Devaux *et al*., 2001) and metabolomics (Fiehn *et al*., 2000; Raamsdonk *et al*., 2001) are essential and important studies in their own right, they also serve as precursors to the greater goal of using high-throughput information to understand the phenotypic characteristics of a particular organism. The fields of bioinformatics and theoretical biology are now moving to the forefront of biological discovery as scientists attempt to generate new knowledge from the flood of information now readily available, through automated genome annotation, metabolic network reconstruction, protein structure determination and, more recently, regulatory network reconstruction from microarray data. As cellular functions rely on the coordinated activity of multiple gene products and environmental factors, understanding the interrelatedness and connectivity of these elements now becomes critical. In other words, referring to the above quote by T.S. Eliot, we are in a position, to a certain extent, to describe the details of *living*; the task that lies ahead is to integrate these details in such a way that we can better understand *life* as a whole, and not just the sum of its parts.

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Metabolic modelling

One area of active research in this area has focused on bacterial metabolism (van Gulik and Heijnen, 1995; Liao *et al*., 1996; Lee *et al*., 1997; Sauer *et al*., 1998; Edwards and Palsson, 1999; Sauer and Bailey, 1999; Schilling *et al*., 1999; Edwards and Palsson, 2000a, b; Schilling *et al*., 2000; Edwards *et al*., 2001a, b). Genomic information, coupled with biochemical and strain-specific information, has been used to reconstruct whole-cell metabolic networks for sequenced organisms (Edwards and Palsson, 1999; Schilling and Palsson, 2000) (Fig. 1). However, this information is not sufficient to specify completely the metabolic phenotypes that will be expressed under given environmental conditions. Metabolic phenotypes can be defined in terms of flux distributions through a metabolic network. Interpreting and predicting metabolic flux distributions requires the application of mathematical modelling and computer simulation. There exists a long history of quantitative metabolic modelling (Bailey, 1998) that will not be detailed here. Currently, several well developed mathematical approaches exist for the dynamic analysis of cellular metabolism and its regulation (Shuler and Domach, 1983; Liao, 1993; Palsson and Lee, 1993; Fell, 1996; Barkai and Leibler, 1997; Bailey, 1998; Novak *et al*., 1999; Tomita *et al*., 1999; Varner and Ramkrishna, 1999; Vaseghi *et al*., 1999). Most of these methods require detailed kinetic and concentration information about enzymes and various cofactors. Even though biological information is growing rapidly, we still do not have enough information to describe cellular metabolism in mathematical detail for a single cell (Bailey, 2001). The human red blood cell remains the only exception (Holzhutter *et al*., 1985; Schuster *et al*., 1988; Joshi and Palsson, 1989; Rae *et al*., 1990; Lee and Palsson, 1991; Mulquiney and Kuchel, 1999)

Flux-balance analysis (FBA)

To overcome our lack of detailed kinetic information, a paradigm shift in our approach to modelling metabolic

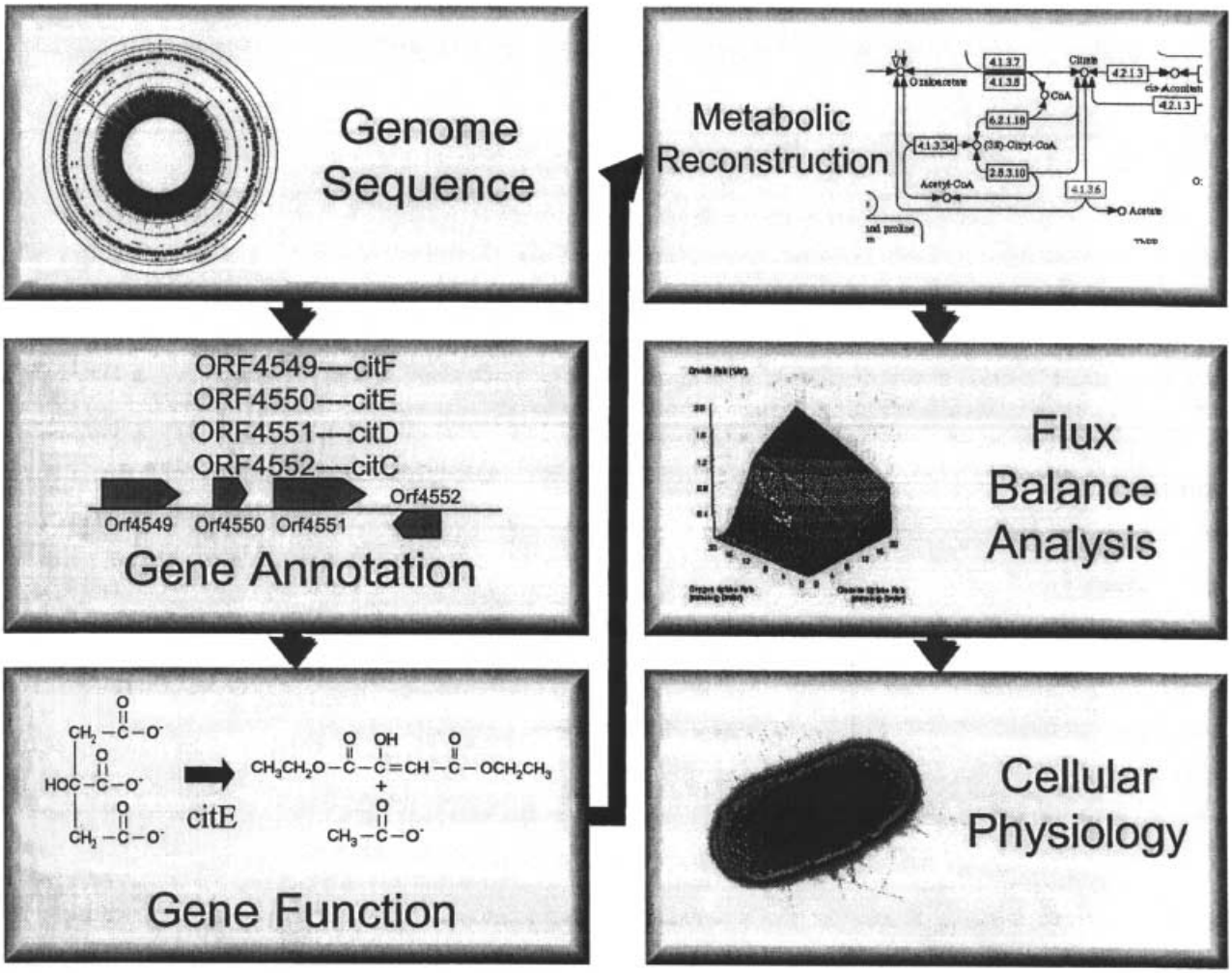

Fig. 1. From a reductionist to a holistic approach in biology. High-throughput sequencing technology and automated genome annotation tools enable identification and functional assignment of most of the metabolic genes in an organism. Once the gene functions are known, they may be integrated into a metabolic network, which can then be subjected to methods such as flux-balance analysis to analyse, interpret and predict cellular behaviour.

systems must be developed. One potential shift in thinking goes as follows: rather than attempting to calculate and predict exactly what a metabolic network does, we should be able to narrow the range of possible phenotypes that a metabolic systems can display based on the successive imposition of governing physicochemical constraints (Palsson, 2000).

This constraints-based approach provides a basis for understanding the structure and function of biochemical reaction networks through an incremental process. This incremental refinement presently occurs as shown in Fig. 2, involving the consideration of fundamentally different physicochemical constraints. Each step provides an increasing amount of information that can be used to reduce further the range of feasible flux distributions and phenotypes that a metabolic network can display. Additionally, each of these constraints can be described mathematically, offering a concise geometric interpretation of the effect that each successive constraint places on metabolic function.

First, the dynamics of integrated metabolic networks are described in the form of dynamic mass balances. These balances simply state that the concentration change of a metabolite over time is equal to the difference between the rates at which the metabolite is produced and consumed. In a steady state, these equations are represented by a matrix equation as shown in Fig. 2 (formally analogous to Kirchhoff's first law for electrical circuits).

Once the system has been defined in terms of these mass balance constraints, the constraints imposed by the thermodynamics (e.g. effective reversibility or irreversibility of reactions) and enzyme or transporter capacities (e.g. maximum uptake or reaction rates) are considered

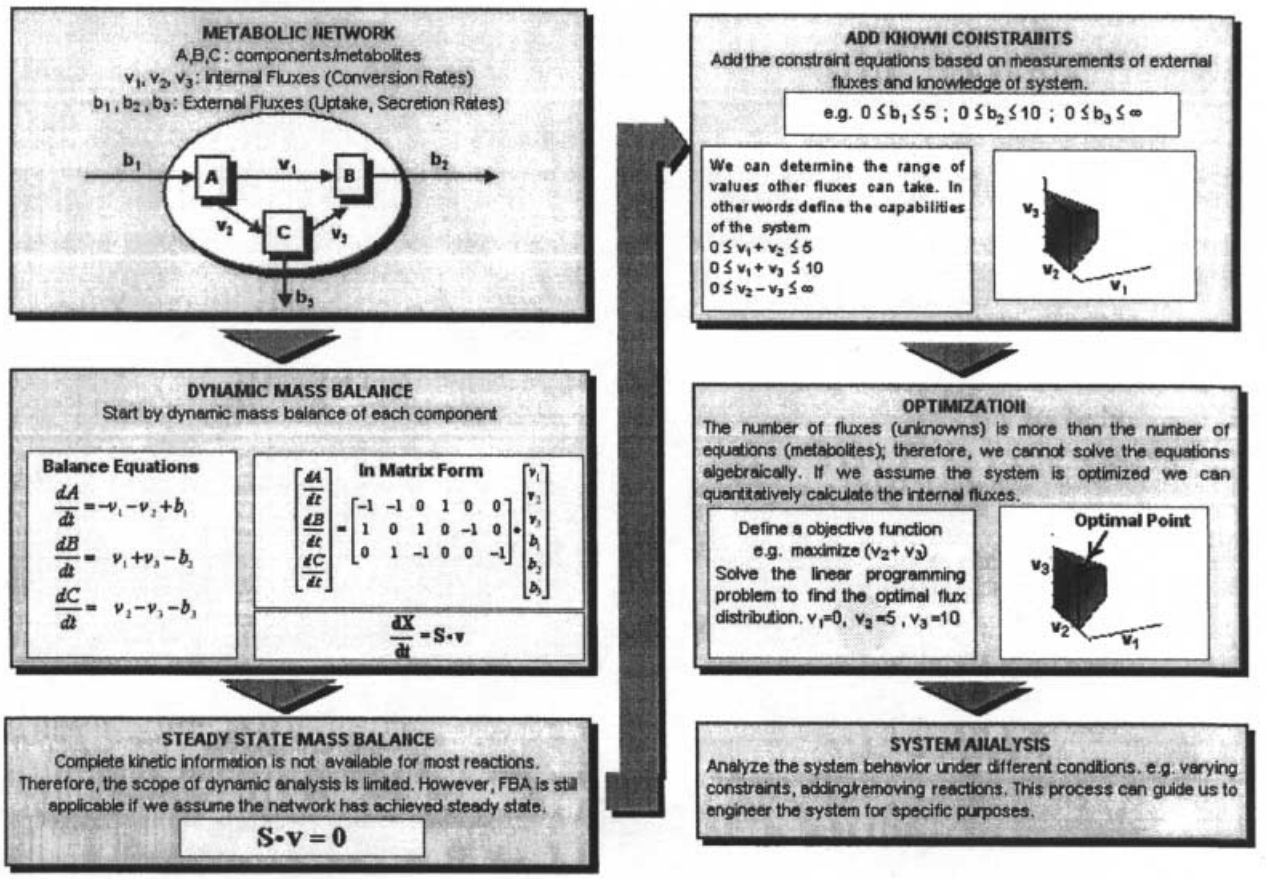

Fig. 2. Flux-balance analysis. Once the metabolic network has been reconstructed, mass balances are written around every metabolite in the network. Known physicochemical constraints are then applied to the system and linear programming is used to determine optimal flux distributions based on objectives such as cell growth and metabolic by-product secretion.

and incorporated into the model. It should be emphasized that these constraints are based on what may be considered 'hard-wired' physicochemical constraints that the metabolic system must obey. The addition of these constraints results in the definition of a bounded solution space wherein every possible flux distribution, or every possible metabolic phenotype of the cell, must lie. The solution to the flux balance equations must lie in the closed solution space. It should also be noted that experimental measurements can be incorporated as constraints to aid in the calculation of the entire metabolic flux distribution (Vallino and Stephanopoulos, 1993; Zupke and Stephanopoulos, 1994; Wiechert and de Graaf, 1996; Sauer *et al*., 1997; Klapa *et al*., 1999); however, the measurements do not depict 'hard-wired' constraints, but rather constraints to the specific condition.

Thus, we can calculate what the metabolic network cannot do, although we cannot calculate the exact flux distribution. We can, however, study the properties of the constraint-defined solution spaces. An approach based on linear optimization principles for studying convex systems, commonly termed flux balance analysis (FBA), has been developed (Varma and Palsson, 1994a; Bonarius *et al*., 1997; Pramanik and Keasling, 1997; Sauer *et al*., 1998; Edwards, 1999; Edwards *et al*., 1999). FBA can be used to calculate, interpret and predict metabolic flux distributions and to analyse the capabilities of a metabolic network based on the systemic stoichiometric, thermodynamic and reaction capacity constraints. Subject to these constraints, optimal metabolic flux distributions can be calculated using linear programming. By calculating and examining optimal flux distributions under various conditions, it is possible to generate quantitative hypotheses *in silico* that may be tested experimentally.

There are several important issues that arise when analysing the optimal metabolic flux distribution. First, the optimal solution may not correspond to the *actual* flux distribution. To make statements regarding cellular behaviour based on the optimal solution we must hypothesize that the cell has identified the optimal solution through evolution and that the objective function we have mathematically imposed is consistent with the evolutionary objective. Although other objective functions can be utilized (Savinell and Palsson, 1992a; Bonarius *et al*., 1996;

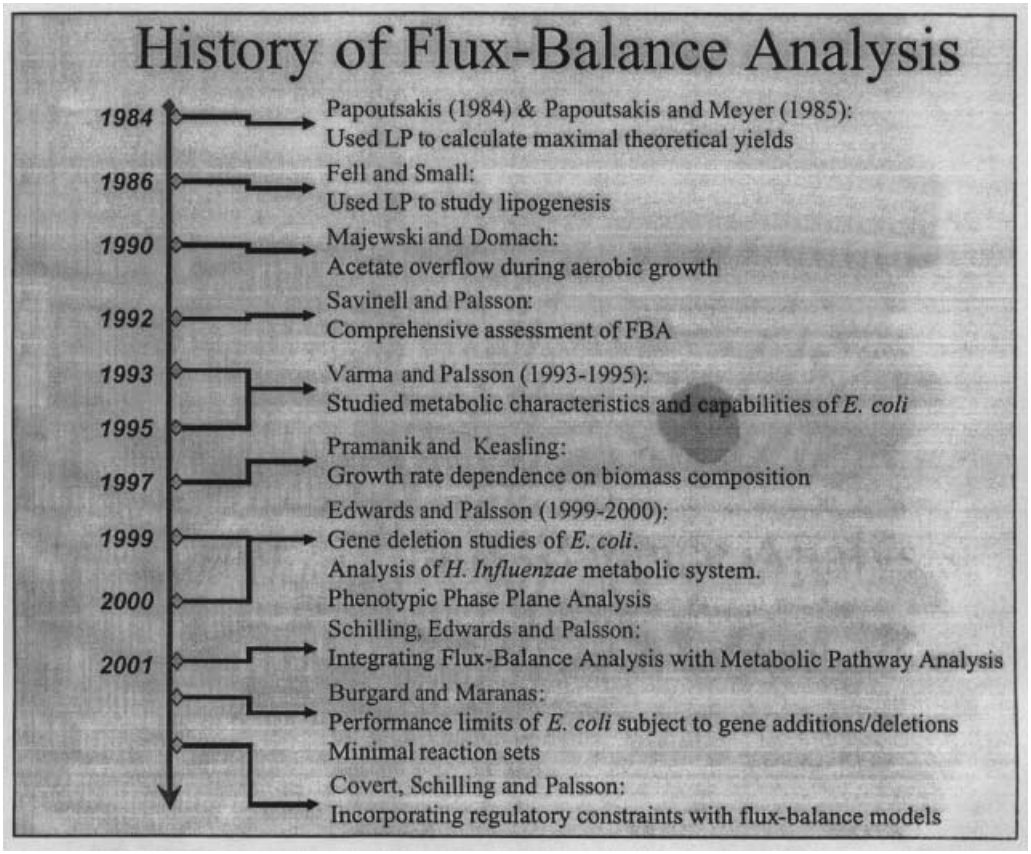

Fig. 3. A timeline depicting some highlights of the development of flux-balance analysis (Papoutsakis, 1984; Papoutsakis and Meyer, 1985a,b; Fell and Small, 1986; Majewski and Domach, 1990; Savinell and Palsson, 1992a,b; Varma *et al*., 1993; Varma and Palsson, 1993a,b, 1994a,b,c, 1995; Pramanik and Keasling, 1997, 1998; Pramanik *et al*., 1998, 1999; Burgard and Maranas, 2001; Covert *et al*., 2001).

Bonarius *et al*., 1997), experimental data have shown that optimal solutions are consistent with cellular behaviour for growth on acetate and succinate (Edwards *et al*., 2001b). The other important issue involves the uniqueness of the optimal solution, and methods are needed to assess the uniqueness of the optimal solution and identify all alternative optima when applicable. Lee *et al*. (2000) have developed a recursive mixed-integer linear programming algorithm to find all alternative optima. When one is cognizant of these, and other issues, FBA can be used in many different applications and greatly contribute to the biological sciences.

Applications of FBA

FBA has been used for over 15 years to study cellular metabolism extensively (Fig. 3), most thoroughly for *Escherichia coli.* The *E. coli* metabolic genotype was generated using its annotated genome sequence (Blattner *et al*., 1997), metabolic databases (Karp *et al*., 1998; Ogata *et al*., 1998; Selkov *et al*., 1998) and biochemical information (Neidhardt, 1996). This metabolic genotype includes 695 genes encoding metabolic enzymes; 47 of these genes do not currently have open reading frame (ORF) assignments but are included based on biochemical data. Based on the annotated genetic sequence and biochemical data, the *E. coli* metabolic genotype catalyses 720 internal metabolic reactions and transport processes operating on a network of 436 internal metabolites. The research performed in this study was based on the assumption that microbial organisms optimize for the production of biomass precursors in relative quantities that correspond to measured values (i.e. growth) (Neidhardt and Umbarger, 1996).

Alterations of the metabolic network

E. coli MG1655 *in silico* has been used to examine the systemic effects of altering the genotype, in terms of both gene deletions (Edwards and Palsson, 2000a, c; Badarinarayana *et al*., 2001; Burgard and Maranas, 2001) and additions (Burgard and Maranas, 2001). In one study, the *in silico* organism was subjected to deletion of each individual gene product in the central metabolic pathways (glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, respiration processes), and the maximal capability

of each '*in silico* mutant' metabolic network to support growth was assessed with FBA. Gene deletions (or equivalently, loss of gene product function) are simulated by restricting the flux through a particular reaction to zero. Equivalently, the column(s) corresponding to the gene in question can be removed from the stoichiometric matrix. Genes that code for isozymes or genes that code for components of the same enzyme complex were simultaneously removed (e.g. *aceEF*, *sucCD*).

The *in silico* gene deletion study results were compared with growth data from known mutants (Edwards and Palsson, 2000a). The growth characteristics of a series of *E. coli* mutants on several different carbon sources were examined and compared with the *in silico* deletion results. From this analysis, 68 of 79 cases or 86% of the *in silico* predictions were consistent with the experimental observations. Although the degree of agreement is remarkable, the failures are of more interest. Further analysis reveals that there are three failure modes:

1 failure to incorporate gene regulatory events (four genes: aceEF, eno, pfk, ppc);
2 failure to specify metabolic network demands correctly (one gene: pgi);
3 failure to account for toxic intermediate build-up (two genes: fba, tpi).

In the first case, the *in silico* strain utilizes genes repressed by glucose. The second case is simply a matter of re-examining the model definition for required biomass components, as the production of certain components by the network is probably not required for growth. The model can be adjusted in both cases to reconcile the predicted and experimental results. In the third case, however, FBA fundamentally fails. It cannot predict build-up of toxic metabolic intermediates, and this failure helps define the limitations of FBA.

Phenotype phase plane analysis

Metabolic flux maps are typically calculated under single growth conditions, giving a limited view of the metabolic genotype–phenotype relation. However, an approach that provides a global perspective on the genotype–phenotype relation is imperative. To this end, we have developed an analysis methodology that allows us to map all the growth conditions represented by two environmental variables into a single plane. This methodology is called phenotypic phase plane (PhPP) analysis (Edwards *et al*., 2002). PhPP analysis was developed to consider all possible variations in two constraining environmental variables, i.e. the carbon substrate and oxygen uptake rates.

Using an *in silico* representation of *E. coli* MG1655 metabolism, experimentally testable hypotheses were formulated describing the quantitative relationship between the uptake rate of the primary carbon source (acetate or succinate), oxygen uptake rate and cellular growth rate. Experiments with *E. coli* were performed and the membrane transport fluxes were measured to test the *in silico*-derived hypotheses. The experimental data were consistent with our hypothesis: the *E. coli* metabolic network is optimized to maximize growth under the experimental conditions considered (Edwards *et al*., 2001b). Additional data for malate and several other substrates show a similar agreement to predicted optimal growth performance (unpublished results).

Further constraining the solution space

In summary, FBA, based on the principles of constraints-based analysis, is one approach to modelling cellular systemic behaviour which can make quantitative predictions in the absence of detailed kinetic information. FBA has been used in many applications, some of which were highlighted here using *E. coli* as an example. The use of the *E. coli* model has expanded our ability to interpret and even predict the behaviour of this organism, and it is to be expected that, as genome annotation updates (Serres *et al*., 2001) and new experimental data are made public, the predictive capabilities of these models will increase.

The next step in developing these models is to determine, define and incorporate other constraints which effect cellular behaviour. Although these FBA models of *E. coli* and other organisms have enjoyed success in many instances, there are also notable instances where they fail. These failures are generally due to the presence of as an unknown constraint on the cell that change its range of allowable behaviours.

For example, the models have assumed that all gene products in the metabolic reaction network are available to contribute to an optimal solution, unrestricted by regulatory processes. This assumption leads to some false predictions, as the gene deletion study shows. For FBA to be used effectively to predict cell behaviour on a more general scale, the effects of regulation must be incorporated. It has recently been demonstrated that the FBA models can be integrated with a set of transcriptional regulatory rules that are represented by Boolean logic equations (Covert *et al*., 2001). The resulting regulatory constraints differ from the constraints previously described in that they are (1) self-imposed, as the cell responds to changing environmental conditions; and (2) time dependent, as opposed to the fixed invariant constraints described above. As these and other constraints are considered and integrated to the current metabolic models, the scope of such prediction may be expected to increase.

Constraints-based cellular modelling, such as FBA,

represents a new modelling philosophy. FBA is particularly applicable for studying cellular metabolism because of the lack of complete kinetic data required for traditional mathematical modelling. The first generation of FBA models have been successful, but many questions remain unanswered.

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