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Current Opinion in
Biotechnology

Metabolic reconstruction, constraint-based analysis and game theory to probe genome-scale metabolic networks

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With the advent of modern omics technologies, it has become feasible to reconstruct (quasi-) whole-cell metabolic networks and characterize them in more and more detail. Computer simulations of the dynamic behavior of such networks are difficult due to a lack of kinetic data and to computational limitations. In contrast, network analysis based on appropriate constraints such as the steady-state condition (constraint-based analysis) is feasible and allows one to derive conclusions about the system's metabolic capabilities. Here, we review methods for the reconstruction of metabolic networks, modeling techniques such as flux balance analysis and elementary flux modes and current progress in their development and applications. Game-theoretical methods for studying metabolic networks are discussed as well.

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Current Opinion in Biotechnology 2010, 21:1–9

This review comes from a themed issue on
Systems biology
Edited by Vitor Martins dos Santos and Jiri Damborsky

0958-1669/\$ – see front matter
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DOI [10.1016/j.copbio.2010.07.002](https://doi.org/10.1016/j.copbio.2010.07.002)

Introduction

The study of metabolism has changed drastically during the last century. The concept of metabolic pathways was molded by the experimental methods available in the beginning of the 20th century resulting in a stepwise elucidation of metabolism (cf. [1[•]]). In the second half of the 20th century, the discovery of the structure and information coding of DNA laid the foundations for recombinant technology, making microorganisms more amenable to metabolic engineering [2].

The mathematical modeling and computer simulation of metabolic systems started with dynamic modeling [3[•]]. This is useful, for example, to simulate the occasional metabolic oscillations in bioreactors. Later, it was realized that the knowledge of kinetic parameters was insufficient in many cases and that a detailed dynamic simulation is

often unnecessary [4,5]. Many specific questions such as the effect of the activation or overexpression of an enzyme can be tackled by specially tailored techniques such as Metabolic Control Analysis (MCA) (cf. [2,3[•],4]). Moreover, the search for methods to analyze invariants of intracellular networks led to the development of the constraint-based modeling (CBM) approach, also called structural analysis or network analysis [4,5]. This requires even less input data than MCA. A subfield is called Metabolic Pathway Analysis [6,7,8^{••}], in which the structure of pathways (routes) going through the system is detected and/or optimal flux distributions are calculated based on the stoichiometry of the network and the directionality of reactions, the knowledge of which is often available. Elementary flux modes (EFMs) [9^{••},10^{••}] and extreme pathways [11^{••}] were established as unbiased mathematical representations of metabolic pathways.

CBM comprises metabolic flux analysis (MFA) (cf. [2]) by which flux distributions can be predicted using flux measurements in addition to network stoichiometry. The limitations in measurements led to the inclusion of additional constraints coming from the Darwinian theory of optimization in evolution. Notably, optimality principles such as maximizing growth rate or given reaction fluxes at normalized input rate are widely used, thus enabling one to predict fluxes by linear programming [12^{••},13^{••}]. This is the essence of Flux Balance Analysis (FBA), by which phenotypically relevant flux distributions in metabolic networks can be predicted (cf. [14,15]).

At the end of the 20th century, the development of new experimental technologies such as sequencing and chip technologies triggered the explosion of omics data. In metabolomics, several hundreds of metabolite concentrations can be measured simultaneously [16^{••},17]. In fluxomics, in contrast, it is difficult to measure more than a dozen fluxes simultaneously [18^{••}]. After DNA microarrays, transcriptomics has recently reached a higher level by RNA-Seq technologies [19]. A secondary source is bibliomics (text mining). High amounts of data enabled one to reconstruct more complex metabolic networks reaching a genome scale for a rapidly increasing number of species (cf. [20^{••},21^{••}]). In addition, recent efforts to integrate gene expression data [22,23^{••},24], sequencing data [25^{••}], proteomics [17], and other omics data have enabled the generation of reconstructions unique to particular life-cycle stages, environments, and genetic backgrounds.

These large models together with CBM methods represent a key foundational advance in Systems Biology [5,14] and

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are essential for seeking comprehension of biological functioning throughout the integration of data with mathematical models. The modeling and computer simulation of metabolism in the genome and post-genome eras have been the subject of a number of reviews [6,7,20^{••},21^{••}]. In this paper we will review recent advancements in the methods for metabolic network reconstruction, the tools available for their analysis and several applications. A special focus will be on game-theoretical methods (cf. [26]). Finally, perspectives on further developments in Systems Biology will be outlined.

Reconstruction of metabolic networks

The process of metabolic network reconstruction begins with the annotation data of the relevant genes (cf. [20^{••},27]). This annotation provides the ‘parts list’ for the network. The metabolic reactions that the associated gene products catalyze are delineated by incorporating data on the metabolites and stoichiometry from databases (e.g. ExPasy and KEGG) and the literature. The stoichiometric coefficients of the metabolites or compounds in the associated reactions are typically represented in a stoichiometric matrix, N (sometimes denoted S) with its rows corresponding to the metabolites and the columns representing the chemical transformations that the gene products (enzymes) catalyze. The usefulness of CBM in the reconstruction process is outlined in Box 1. Two key challenges are firstly, the integration of disparate high-throughput data and secondly, the inclusion of additional constraints to improve the predictive power. Recently, methods for the iterative refinement of the networks using high-throughput transcript data have been devel-

oped and used to significantly improve the reconstruction of *Chlamydomonas reinhardtii* and to identify key genes associated with biofuel production [25^{••}].

For a reconstructed network to be realistic, there must be a flux vector v fulfilling Eqn (1) in Box 1 and covering practically all reactions. While there may be a few reactions that always subsist at thermodynamic equilibrium, the vast majority must be able to operate at non-equilibrium, that is, they must not be blocked due to missing reactions [28[•]] and have to fulfill mass conservation [29]. The coverage of a network by a flux distribution can be tested by a method called flux coupling analysis, which is based on linear programming [30^{••}].

Genome-scale reconstructions have been assembled for organisms from all kingdoms: archaea, eukaryota, and bacteria (Figure 1), and include single-cell and multi-cell organisms (for references to specific reconstructions, see [21^{••}]). Here, we mention only a few: *Escherichia coli* [31^{••}], *Saccharomyces cerevisiae* [32^{••}], *C. reinhardtii* [25^{••},33], *Arabidopsis thaliana* [34,35], mouse [36,37], and human [38^{••},39^{••}]. The goal is to account for all the enzymes encoded in the entire genome. However, the term ‘genome-scale’ is to express the dimension in which this is done, which does not necessarily imply that this difficult task would be accomplished with 100% completeness. Questions such as whether substrate A can be transformed into product B can only be tackled exhaustively in a whole-cell model, which provides a further motivation for their reconstruction [40^{••}].

Some recent efforts are focused on automating the reconstruction process (cf. [20^{••},41]), aided by the development of computational platforms to manage the data associated with gene–enzyme–reaction associations and reaction stoichiometry. This has had some success, although there is typically a recognized need for manual curation efforts [28[•],29,42]. A significant remaining challenge is the visualization of these networks, in spite of some recent efforts in this area [43].

Biotechnological applications of metabolic modeling

With the reconstructions that have been generated, the next important step is the development of analysis tools and frameworks to study functional properties of these networks [30^{••},40^{••},44[•],45[•]]. Various tools for metabolic modeling have been established and refined. These include dynamic modeling, optimization, game-theoretical methods, FBA, Metabolic Pathway Analysis and others (cf. Introduction).

FBA is based on optimality principles (Box 1). It is a matter of debate whether FBA always gives sufficiently correct results [46,47], see below. Various objective functions have been compared by [48[•]]. However, more

Box 1 Most metabolic systems subsist at stationary states. Even if they oscillate (only very few do), the average reaction rates (on a sufficiently long time scale), v , must fulfill the steady-state condition

$$N \cdot v = 0 \quad (1)$$

because, otherwise, the concentrations of metabolic intermediates would accumulate or be depleted in the long run. In addition, for some or all fluxes, inequality constraints can be written:

$$v_{i,\text{inf}} \leq v_i \leq v_{i,\text{sup}} \quad (2)$$

For example, $v_{i,\text{inf}}$ is zero for all irreversible reactions. For all reactions, $v_{i,\text{sup}}$ can be given by the respective maximal velocity if it is known.

Central to FBA is an optimization principle

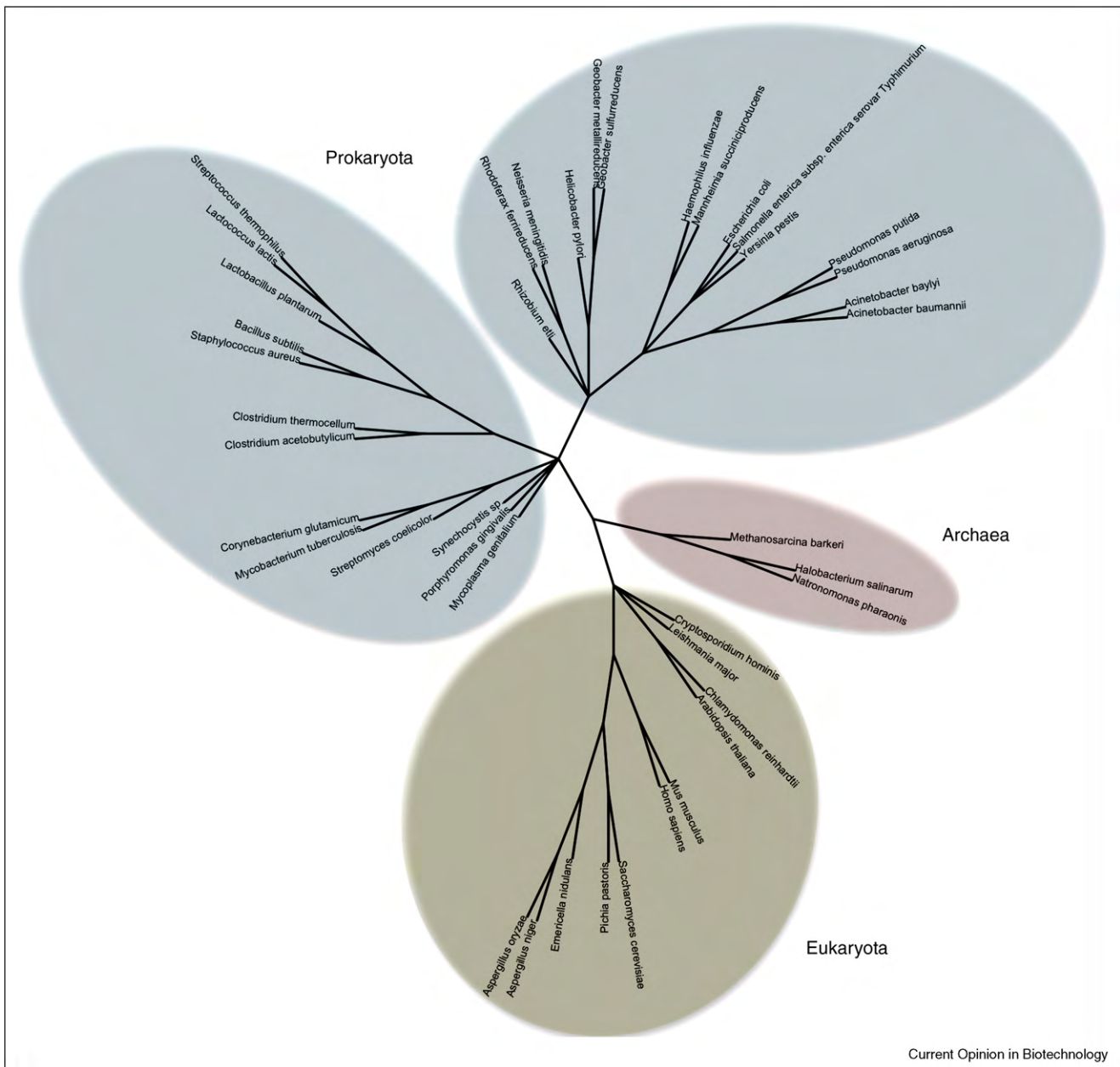
$$\text{maximize } \sum c_j v_j \quad (3)$$

subject to relations (1) and (2) [12^{••},13^{••},15]. The coefficients c_j denote the weights of the particular fluxes in the objective function, for example, the production of biomass. Typically, relation (2) includes one constraint that fixes or limits a relevant input flux, for example, glucose uptake.

The non-decomposability of elementary flux modes can be expressed by a constraint saying that the support of the flux vector is not a proper superset of the support of any other steady-state flux vector, v' :

$$S(v) = \{i : v_i \neq 0\} \text{ not proper superset of } S(v') \quad (4)$$

Figure 1



Phylogenetic relationship between the organisms for which metabolic network reconstructions exist, generated with iTOL [84].

sophisticated methods usually require kinetic data, with the above-mentioned problem of incomplete data availability. Some current approaches attempt to guess flux values from thermodynamics [49], purely structural properties [45^{*}] or RNA transcript data [23^{**}].

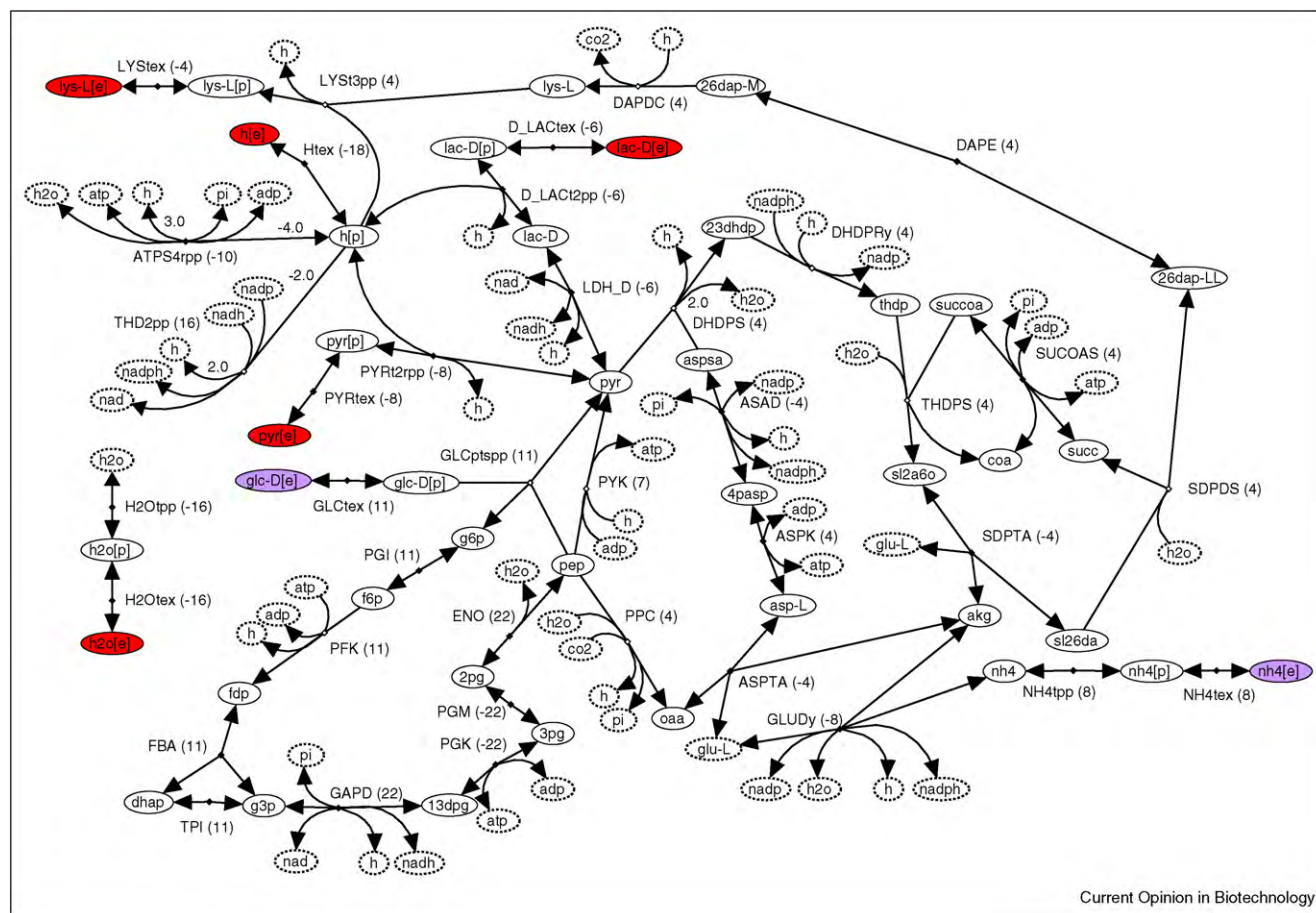
A useful tool in Metabolic Pathway Analysis is based on the concept of EFMs [9^{**},10^{**}]. An EFM is a minimal set of enzymes that can operate at steady-state such that all irreversible reactions involved proceed in the thermodynamically favored direction (Box 1). The related concept

of minimal T-invariants had been established earlier in Petri net theory (cf. [50]).

Maximal yields can be computed by EFMs or by FBA. The latter methodology is particularly suitable in large-scale networks, in which EFM analysis meets the problem of combinatorial explosion. In small-sized and moderate-sized networks, in contrast, the set of EFMs provides a more comprehensive overview of the network's metabolic capabilities because it also comprises suboptimal pathways and pathways optimal with respect to other

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Figure 2



The shortest EFM producing lysine from glucose in *Escherichia coli* [53**]. The calculation was performed in the genome-scale network from [31**]. Violet nodes belong to the *in silico* growth medium, red nodes denote external metabolites. Duplicate nodes are dashed. Values in parentheses indicate reaction fluxes. For abbreviations, see [31**].

substrate-product pairs. Moreover, knockouts can easily be assessed by considering the remaining subset of EFMs. This sometimes leads to the counter-intuitive result that the average yield increases, as has been confirmed experimentally after the *in silico* analysis [8**].

By EFM analysis, previously unrecognized pathways can be detected (cf. [10**,55*], see also Box 2). Recently, we proposed two methods for pathway prediction in large-scale networks [40**,53**]. We computed the 10 shortest EFMs producing lysine in the genome-scale networks of *E. coli* and *Corynebacterium glutamicum* [53**] (Figure 2). Moreover, EFM analysis allows the quantification of robustness (see below).

There is a growing effort to use network models to identify drug targets and characterize mechanisms of disease. A recent study reconstructed and analyzed the metabolic networks of multiple strains of *Staphylococcus aureus* to identify novel drug targets [56]. A network-based pipeline

Box 2 The EFM method has manifold applications in biotechnology. First, it allows one to compute maximal molar yields (product-to-substrate ratios). For example, a previously undescribed pathway of efficient conversion of carbohydrate to oil in developing green plant seeds was detected [51**]. That pathway involves the pentose-phosphate pathway and the RUBISCO enzyme and provides 20% more acetyl-CoA for fatty acid synthesis than glycolysis. Trinh *et al.* [52*] designed, initially *in silico*, an *E. coli* strain with eight gene KO mutations. By EFM analysis, four pathways with non-growth-associated conversion of pentoses and hexoses into ethanol (important for biofuel production) at maximum theoretical yields and two pathways with tight coupling of growth with ethanol formation at high yields were obtained. Thereafter, they verified in experiment that the ethanol yields of the engineered strains closely matched the theoretical predictions. A third example is the EFM analysis of the synthesis of the commercially important amino acid, lysine (Figure 2) [7,53**,54*]. Depending on the bacterial species and on whether ATP was assumed to be sufficiently available, different maximum lysine-over-glucose yield values have been computed, for example 9:11 in *Corynebacterium glutamicum* when ATP must be regenerated by part of the glucose resource [54*]. There are many more studies in which EFMs were used, see [8**] for a recent review.

for identifying potential antimicrobials is being developed [57]. The human metabolic network reconstruction was analyzed to identify alternative enzyme targets for treating hyperlipidemia [39**]. It has also been recently used to predict biomarker changes characterizing a large set of different genetically inherited metabolic disorders [58].

Using metabolic models to study basic biological questions

Metabolic games

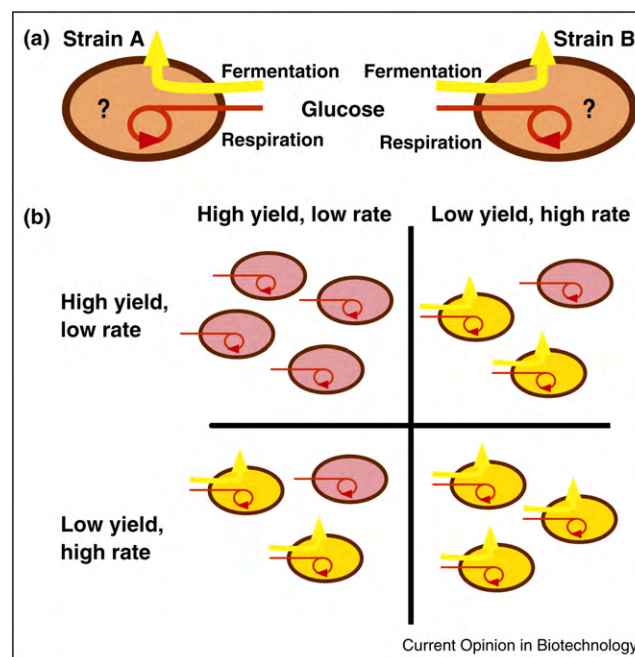
As has been seen above with FBA and EFMs, the concept of optimality has turned out to be extremely useful in understanding biological systems. Traditional optimization is, however, often insufficient for a deeper understanding of evolution. It usually neglects that the properties of the environment can change, and this in turn can change the optimal strategy. This is particularly important if the environment includes coevolving competitors that optimize their own metabolic capabilities. A mathematical framework to describe coevolution is provided by game theory (cf. [59]).

An illustrative example of the importance of competition in pathway evolution is the interplay between fermentation and respiration in ATP production [60**]. Several organisms and cell types such as *S. cerevisiae* and *E. coli* use respiro-fermentation at high glucose levels for degrading glucose to produce ATP, that is, respiration at maximum rate and fermentation in addition (Figure 3a), while many wild yeasts such as *Kluyveromyces marxianus* do fully respire glucose under aerobic conditions [61]. Respiration has a higher ATP-over-glucose yield but a relatively low rate in comparison to fermentation. When two species or strains compete for the same substrate, a typical game-theoretical situation arises (Figure 3b). The fitness of either organism depends not only on its own strategy (pathway usage in this case) but also on that of the other because both strategies affect the common nutrient pool.

To apply game theory requires little kinetic information. Not much more than the constraints (1) and (2) in Box 1 are necessary. In the example of fermentation and respiration, the upper limits given by relation (2) have to be chosen appropriately. In addition, the different yields of the pathways must be considered.

In order to use external glucose as economically as possible, it would be best if all organisms in a given habitat opted for respiration. The evolutionary reason for the profligate utilization of glucose by baker's yeast is that it thus out-competes organisms that operate more economically. In the terminology of game theory (cf. [59]), yeast cells are trapped in a Nash equilibrium (stable solution of a game) of a Prisoner's Dilemma (cf. [62*]). In the light of these results, FBA should be critically

Figure 3



Game played by *S. cerevisiae* when growing at high glucose level under aerobic conditions. (a) The strains face the decision as to whether they use respiration (red arrow) or respiro-fermentation (red and yellow arrows). The higher rate of fermentation is indicated by a thicker arrow. (b) Schematic picture of the payoff matrix for this game. The best solution is to use respiration in order to have a higher yield. However, the respiro-fermenters would grow faster.

re-visited [46,47] although it was very successful in many cases [14,15,63**]. If maximum yield were the relevant criterion for the choice of pathway, respiration would always be chosen by *S. cerevisiae*. Fermentation was predicted only by FBA when additional constraints or specially tailored optimization principles were used [64]. Game-theoretical approaches can help predict flux distributions without additional corrections.

Several other metabolic systems have been analyzed by game theory, such as distinct regimes of glycolysis [65] and metabolic strategies in biofilms [66]. *S. cerevisiae* is involved in yet another interesting game. It concerns the extracellular enzyme, invertase, which generates glucose [67*]. A cheating strategy is to take up glucose while saving the metabolic costs of production and secretion of invertase. Gore *et al.* [68**] showed by experiments and a mathematical model that a stable coexistence between invertase-secreting and non-secreting yeast cells can be established (for an alternative mathematical model and the biotechnological relevance, see [69]). When the metabolic effort for exoenzyme production is low, all cells cooperate (harmony game); at intermediate costs, cooperators and cheaters coexist (hawk-dove game), while at high costs, all cells use the cheating strategy (Prisoner's Dilemma) [68**,69].

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Several other biochemical examples including photosynthesis have been reviewed in [62[•]]. Moreover, the concept of Shapley value from game theory has been used in quantifying metabolic robustness [70], see below.

Robustness and its evolution

A general feature of living cells is their robustness to varying environmental conditions and genetic mutations. As metabolic network models provide an exciting opportunity to study genotype–phenotype relations on a genome scale, CBM models (and metabolic models in general) have been successfully used to study many facets of this fundamental relationship [71[•],72^{••},73,74]. These studies have mainly asked two basic questions: Firstly, how did genetic robustness evolve? Does it have a direct adaptive value, or is it a consequence of environmental robustness, or perhaps just a side effect of other network properties? Secondly, what network mechanisms underlie the observed robustness—is it primarily due to gene duplications, to alternative metabolic pathways, or related to untested environmental conditions?

Since employing FBA in an exhaustive search of all gene knockout combinations cannot proceed beyond combinations of four knocked out (KO) genes, Deutscher *et al.* [75^{••}] used a probabilistic approach. Thus, gene sets providing mutual functional backup until the depth of eight could be cataloged for *S. cerevisiae*. This has enabled them to characterize the '*k*-robustness' (the depth of backup interactions) of each gene, revealing that almost three quarters of yeast metabolic genes do participate in processes that are essential to growth in a standard laboratory environment, compared with only 13% previously found to be essential using single KOs. Optimization-based procedures for the exhaustive identification of multi-gene backup sets in genome-scale metabolic models have been recently developed [76], revealing new avenues available for redirecting metabolism, and uncovering complex patterns of gene interdependence. On the reverse side, genetic robustness may markedly hamper classical genetic studies using KOs to identify gene functionality, due to backups. Using the concept of Shapley value, Deutscher *et al.* [70] have shown that when assigning gene contributions for individual metabolic functions (such as the production of a given amino acid), the picture arising from single-perturbations is severely lacking and a multiple-perturbations approach turns out to be essential. Metabolic robustness under multiple KOs has also been studied in CBMs of several cell types by developing a robustness measure [73]. That measure is based on the ratio of the number of remaining EFMs after KO and the number of EFMs in the unperturbed situation.

Genetic interactions and network organization

By systematically generating double KOs of nonessential genes and assessing the resulting growth rate (fitness) of the organism, geneticists have traditionally identified

both positive (alleviating) and negative (aggravating) genetic interactions, which has been a traditional tool for discovering functional relationships between genes. A comprehensive experimental screening for this in a whole organism is currently underway for yeast [77^{••}]. Naturally, CBM models offer an opportunity to carry out such screens *in silico*. Segrè *et al.* [78] computed growth phenotypes of all single and double KOs of metabolic genes in *S. cerevisiae*, using FBA. The ensuing genetic interaction network could be clustered into modules composed of genes interacting with each other 'monochromatically' (i.e. with purely aggravating or purely alleviating interactions), emphasizing interactions between, rather than within, functional modules. Harrison *et al.* [79^{••}] investigated the extent to which the functional impact of single and double KOs in yeast changes across different environments, employing FBA across 53 different conditions. The synthetic lethal (SL) predictions of the model were then validated by an *in vivo* double gene KO experiment and by literature search. The strong context dependency of the pattern of SL interactions observed suggests that the environment plays an important role in shaping genetic robustness.

From unicellular to multi-cellular organisms

The vast majority of the work on metabolic CBM performed up until now has focused on unicellular organisms. Naturally, in recent years, there have been attempts at extending these methods to study the metabolism of multicellular and multi-tissue organisms, a considerably greater challenge. CBM reconstructions of human metabolic networks were performed up to 2007 only for cell types and organelles with a very limited scope of metabolism [80,81[•],82^{••}]. A fundamental step forward has been presented in recent reconstructions of the global, generic human metabolic network based on an extensive evaluation of genomic and bibliomic data [38^{••},39^{••}]. These networks include ~3000 reactions, ~2000 metabolites, and ~1500 genes mapped to the different reactions over 7 organelles. The generic model of [39^{••}] helped identify a set of functionally related reactions involving glutathione metabolism that were causally related to hemolytic anemia, and another set of functionally related enzymes containing HMG-CoA reductase, a common target for the cholesterol lowering statins. This model, however, is not tissue or cell specific. More recent efforts have presented methods for inferring context-specific networks [23^{••},24], which can be utilized to infer large-scale descriptions of the human tissues' metabolism. Accordingly, Shlomi *et al.* [23^{••}] have integrated tissue-specific gene and protein expression data to predict and validate versus publicly available data for the tissue-specific metabolic activity for 10 human tissues, identifying that post-transcriptional regulation plays a central role in shaping tissue-specific metabolic activity. Very recently, an extended approach of the latter has been used to build and study the first large-scale model of liver metabolism [83^{••}].

Concluding remarks and future directions/challenges

CBM methods are very useful for understanding the complex architecture of metabolism and for manifold biotechnological and medical applications. Even if kinetic parameters were to become available, an analysis of the network properties using tools of FBA and Metabolic Pathway Analysis often provides valuable insight before performing a dynamic simulation. As outlined above, the integration of omics data of different types into metabolic models has had much success. Nevertheless, its refinement and scaling-up certainly remains a challenge. Cell-specific and tissue-specific studies can now be performed for those multi-cellular organisms for which metabolic reconstructions are available, as is already being done for humans. Whole-cell modeling has also raised philosophical issues on what level of completeness can be reached in modeling.

More work in this field is also needed to study emergent properties, which is at the heart of Systems Biology, after the necessary assembly of the network constituents has been done. Another direction is Synthetic Biology. Specific goals are the design of minimal metabolisms (depending very much on the given set of nutrients) and minimal genomes. This could help design efficient microbes for biosyntheses. Game-theoretical methods, in particular, are helpful in assessing the impact of 'cheater' mutants in bioreactors, which may impair productivity.

Overall, one can safely maintain that the field of genome-scale metabolic modeling has undergone a tremendous development and growth in the last decade, in terms of the organisms spanned, the methodologies developed, and the themes covered. Certainly, if there is one specific field in systems biology where we have made significant strides towards the holy grail of generating a working cell *in silico*, this is the one.

Acknowledgements

Financial support from the following agencies is gratefully acknowledged: German-Israeli Foundation to ER and SS, Israeli Science Foundation (ISF) to ER, National Science Foundation CAREER award (0643548) to JP and German Ministry of Education and Research (BMBF) to SS. Financial support from Portuguese entities: Fundação Calouste Gulbenkian, Fundação para a Ciência e a Tecnologia (FCT) and Siemens SA Portugal (PhD grant SFRH/BD/32961/2006) to LF de F. The authors thank Christoph Kaleta and Steffen Klamt for helpful discussions.

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