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Systems biology of microbial metabolism

Matthias Heinemann[1,2] and Uwe Sauer[1]

One current challenge in metabolic systems biology is to map out the regulation networks that control metabolism. From progress in this area, we conclude that non-transcriptional mechanisms (e.g. metabolite–protein interactions and protein phosphorylation) are highly relevant in actually controlling metabolic function. Furthermore, recent results highlight more functions of enzymes and metabolites than currently appreciated in genome-scale metabolic reconstructions, thereby adding another level of complexity. Combining experimental analyses and modeling efforts we are also beginning to understand how metabolic behavior emerges. Particularly, we recognize that metabolism is not simply a dull workhorse process but rather takes very active control of itself and other cellular processes, rendering true system-level understanding of metabolism possibly more difficult than for other cellular systems.

Addresses
[1] ETH Zurich, Institute of Molecular Systems Biology, Wolfgang-Pauli-Str. 16, 8093 Zurich, Switzerland
[2] University of Groningen, Groningen Biomolecular Sciences and Biotechnology Institute, Molecular Systems Biology, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Corresponding author: Heinemann, Matthias (m.heinemann@rug.nl) and Sauer, Uwe (sauer@ethz.ch)

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Introduction

A prerequisite for attaining any system-level understanding is the knowledge of the components of a system and the interactions between those. In contrast to other cellular networks, such topological knowledge is already relatively complete for metabolic networks even in exotic microbes [1], or can be drafted automatically from the genome [2]. At least for organisms with very small genomes, such network reconstructions are nearing completion as judged by the good agreement between *in silico* and *in vivo* growth phenotypes [3••]. In addition, techniques for quantitative analyses of metabolic system components (metabolites and proteins) and output (fluxes) are available [4]. Much less advanced is our knowledge of the topologies and activities of the diverse regulatory networks that control metabolic operation. Moreover, we also lack a true system understanding of metabolism. For instance, most often we even do not know how a metabolite concentration or a metabolic flux emerges from the interactions in the underlying networks or we do not know how cells realize the adaptation of metabolic operation in response to changing nutrient availability.

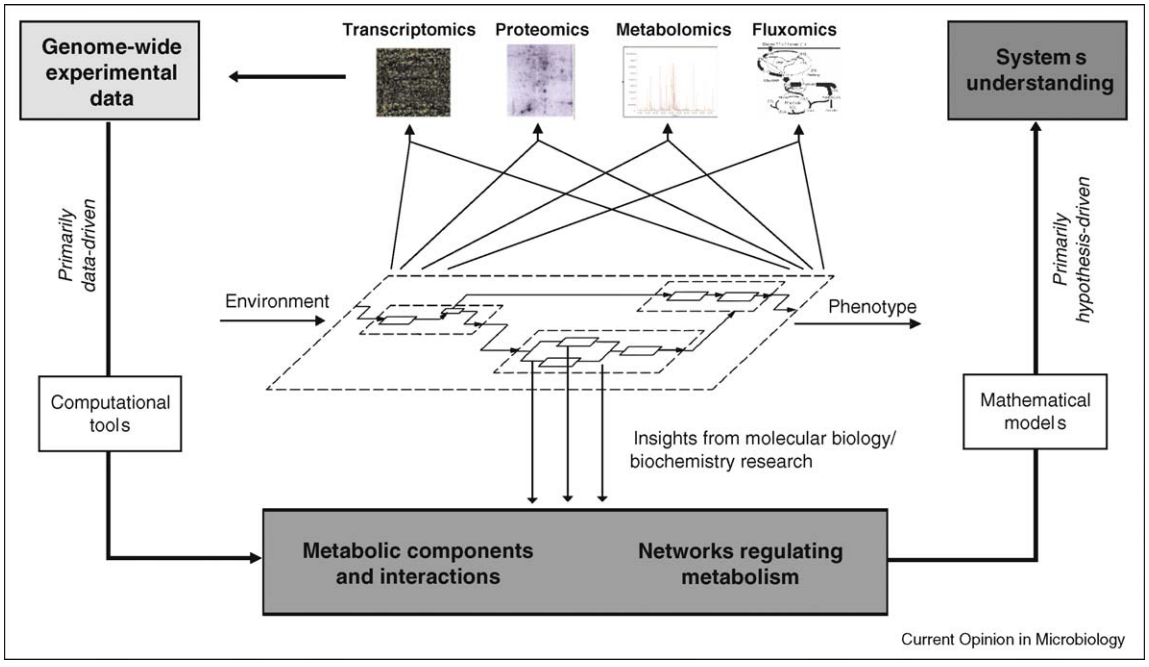
Towards these challenges, systems biology raises great expectation by its novel experimental capabilities and computational approaches that essentially come in three flavors (cf. Figure 1). The first key element is techniques for large-scale, high-throughput experimentation to identify and quantify molecular components, to determine genetic or physical interactions [5•], or to determine the functional network output (*i.e.* metabolic fluxes for metabolism [4,6]). Aiming at extracting actual biological insights from such inherently complex data sets, computational algorithms for the so-called top-down analysis of large-scale data sets are the second key element of systems biology. Such data-driven computational approaches promise to provide us with shortcuts to good hypotheses on, for instance, novel components and molecular interactions. Jointly with classical molecular biology, the first two branches of systems biology identify and quantify the constituents of a system and interactions between them. The third branch of systems biology then aims to understand how a certain biological behavior emerges when the various system components quantitatively interact in time and space. Essential to this task are mathematical models at all levels of complexity to formally describe, to simulate and to ultimately understand such system behavior. It is mostly the third branch that pursues an iterative interplay between experimental and computational analyses.

Here, we review papers since 2008 that used any of the above mentioned systems biology approaches. By focusing on work that has provided novel biological insight (in contrast to advocating new methods), we ask whether systems biology actually holds up against its own promises. For this purpose, papers are grouped into the categories (i) identifying metabolic components and interactions, (ii) connecting regulatory networks with metabolism, and (iii) generating molecular and higher level systems understanding (cf. Figure 1).

Identifying metabolic components and interactions

Metabolic networks are constituted by enzymes and metabolites, and a reaction is considered an 'interaction'

Figure 1



Various systems biology approaches (*i.e.* large-scale omics techniques, data-driven computational approaches and hypothesis-driven mathematical modeling approaches) generate knowledge and understanding about metabolism. The first two approaches primarily support identification of network components and their interactions, the latter approach is suited to generate system understanding; often being described as the ultimate goal of systems biology.

between these components. Today's metabolomics efforts often detect metabolites that were so far not considered in network reconstructions. Likewise enzymatic reactions are often added to an organism's metabolic network on the basis of stoichiometric considerations within genome-scale reconstructions [1] and, more recently, also by computational prediction of novel pathways based on enzyme reaction rules and thermodynamics, as was demonstrated for biodegradation pathways [7•]. Once the topology of a metabolic network is reconstructed, it is often exploited to develop targeted experimental methods for comprehensive component quantification with significant progress towards coverage, resolution, dynamic range, and accuracy of detection, in particular for the proteome [8] and the metabolome [4,9,10•].

Reactions of the metabolic network are conceptually connected via enzymes to genes. Despite extensive genome-scale modeling [1,5•], a surprisingly constant 20% of all enzymatic reactions remain without a link ('interaction') to a gene (*i.e.* are orphans) in network reconstructions of model organisms and exotic species alike. Several systematic computational and experimental approaches to unravel novel enzymatic functions and links to genes were recently reviewed [4]. An alternative approach is the combination of large-scale protein structures with genome-scale modeling, although most inferred gene–enzyme relationships in the so far largest study with 478 enzyme structures were only confirmatory [11]. Beyond identifying new reaction-gene links [12], we need to assure and maintain high quality of the existing functional annotations, in particular given the relatively high error rate in data bases such as KEGG [3••,5•]. To this end, a new and experimentally validated computational method can recognize errors in existing annotations and make specific predictions for better annotations by systematic comparison of function (*i.e.* enzyme position in the metabolic network) and context genomic correlations [13•].

A somewhat rediscovered topic with potentially significant impact on metabolic network connectivity is enzyme promiscuity; *i.e.* latent catalytic side-activity of the reaction an enzyme was presumably evolved for. Such relaxed substrate and reaction specificity facilitates evolution of novel biodegradation pathways [14] but can also have biochemical relevance [4], thereby leading to much higher connectivity of metabolic networks than current genome-scale models represent [15]. Promiscuity does not only concern the peripheral network but is very real even in central metabolism, as was convincingly shown by systematic comparison of stoichiometric model predictions with growth phenotypes of 217 *Escherichia coli*

double enzyme deletion mutants under 13 conditions [16••]. Discrepancies between prediction and data indicated unknown reactions, leading to the discovery of a novel sedoheptulose-7-phosphate breakdown pathway that is catalyzed by side activities of phosphofructokinase and aldolase, rather than the normal transaldolase in the pentose phosphate pathway. Triggered simply by intracellular accumulation of a metabolite, alternative pathways can thus modify the connectivity of the network and possibly its robustness. Beyond novel reactions, promiscuity also concerns cofactor specificity. In particular bacterial enzymes appear to be less specific to energy [17] or redox cofactors [18] than their mammalian homologs. Since these cofactors connect many reactions, flexible cofactor usage significantly increases a network's capability to balance its various demand and supply fluxes.

The last two years also brought a typically ignored feature of metabolism to the general attention—moonlighting proteins [19–21]. Beyond their well-characterized metabolic function, a steadily growing number of enzymes perform a second function ('only in secret under the moonlight') that is not metabolic in nature and not caused by gene fusions, etc. Such second functions establish interactions between metabolism and other cellular networks. A large class of these second functions is control of gene expression, exerted by so-called trigger enzymes [22]. Such 'moonlighting' of enzymes is particularly abundant in central metabolism, where for example glycolytic enzymes function also in DNA replication, mRNA processing, apoptosis, and transcriptional regulation [23]. Multifunctionality is still in a discovery phase where the few tens of so far identified moonlighting proteins are most likely the tip of an iceberg, and systematic protein–protein interaction analyses are expected to be of key importance in identification [23]. So far, systems biology has not yet tackled the added complexity that arises from different biochemical networks that are functionally intertwined by such protein multifunctionality.

Connecting regulatory networks with metabolism

Despite promiscuity and some missing links to the genome, metabolism is currently still the most comprehensively known biological network and therefore an ideal playground for systems biology. The major thrust of top-down systems biology now attempts to connect metabolic networks with the regulatory networks that control them. Currently, transcriptional regulation attracts the major attention, primarily because of mature experimental methods for transcriptomics and physical DNA–protein interaction analyses. On the basis of such data, the main focus is to reconstruct the architecture of transcriptional regulation networks [24,25]. Such reconstructed regulation networks already provided hints on how environmental cues are linked to a cell's transcriptional state [26], in some cases explicitly including metabolite concentrations as regulatory feedbacks [27].

After having inferred the topology of metabolic regulation networks the next quest is then to determine those parts of the network that actively control metabolism under a given condition [10•,28••,29••]. Here, ^{13}C flux analysis is being used to identify whether certain transcription factors actually control the distribution of metabolic flux [30–32]. Further, it seems important to analyze data from multiple cellular levels as otherwise major mechanisms can be missed. This is illustrated by the different conclusions derived from dynamic metabolite data [33] compared to dynamic mRNA-based expression data [34] for yeast's response to oxidative stress. The potential of multiple data integration is illustrated by the recent identification of novel regulatory gene–metabolite interactions upon nutrient deprivation in yeast that were obtained from context-dependent correlations of metabolite and transcript data using a probabilistic framework [35]. Another example of how regulation can control a metabolic function comes from correlating metabolite, transcript and enzyme levels that revealed a passive homeostasis mechanism, which is also a mechanistic explanation for why decreased enzyme abundance, and thus to some extent transcript abundance, cause increased substrate metabolite concentrations [36]. By correlating fluxes and metabolite concentrations with expression data in a global transcription factor mutant, specific hypotheses could be derived on active regulation processes and functional pathway usage [37]. As a mechanistic formalism for data integration, time-dependent regulation analysis is a particularly pertinent method to quantify the actual control exerted by metabolic *versus* expression regulation mechanisms [38].

While much efforts in connecting metabolic with regulatory networks still concentrate on transcriptional mechanisms [26,27,39], a pertinent question is how relevant transcriptional regulation actually is for metabolic operation? A comprehensive omics data set from the reduced-genome *Mycoplasma pneumoniae*, which lacks the majority of metabolic transcription factors [25], clearly shows that complex metabolic regulation can be achieved with a reduced transcription factor network [3••]. This conclusion is further corroborated by empirical observations from bacteria to yeast showing (i) that only a small number of transcription factors actually control the distribution of metabolic flux [31,32], (ii) that flux control is distributed between the metabolic and the other layers of regulation [38,40], and (iii) that dynamic responses of metabolism are dominated by metabolic and not by transcriptional regulation [33]. Collectively, these arguments suggest that other regulatory mechanisms, such as post-translational modifications and metabolite–protein interactions are possibly even more important.

One such potentially underestimated regulation process for metabolism is enzyme phosphorylation. Although follow-ups to prove functional relevance of phosphorylation events are clearly missing, surprisingly many central metabolic enzymes appear to be phosphorylated in many microbes as identified by mass spectrometric analyses [41,42]. How kinases thereby achieve control of metabolism has elegantly been demonstrated for the global energy regulator kinase Snf1 in yeast by integrating large-scale data sets with various computational network analysis methods [43•].

Another key regulation process occurs at the level of metabolite–protein interactions. There is increasing recognition of the fact that metabolism is not simply a dull workhorse process [29••] but rather takes very active control of itself and other cellular processes through regulatory crosstalk by signaling metabolites that modulate activity of regulatory and other proteins [27,29••,44•]. From dynamic single cell expression data, the consequences of such metabolite–transcription factor interactions were quantified for leucine biosynthesis [45] to parameterize a five ordinary differential equation model that predicts the pathway response and ultimately its flux upon new perturbations. The drastic higher induction of enzymes downstream of the intermediate control point was shown to be crucial for dynamic but not steady-state leucine formation, thus questioning the generality of the previously described just-in-time dynamics of amino acid biosynthesis in *E. coli* [46].

Generating systems understanding

Understanding of a biological system (cf. right part of Figure 1) can come in different flavors. First, one could aim at a mechanistic understanding on the molecular level. Since biological systems are very complex, we still have to make compromises between a system's size that we consider and the level of detail at which we model a system. Second, one could aim at a more global system understanding, where not exact molecular mechanisms but rather the general principles underlying a particular system are in the core of the interest. The following section first highlights important work that generated mechanistic system understanding and then briefly illustrates work that generated a more global system understanding.

On our way towards a true molecular systems understanding, stoichiometric model analyses can provide us with certain relevant information—particularly when combined with experimental efforts [47,48]. Ultimately, however, such models will not be sufficient. Instead, differential equation-type models will be required to describe molecular interactions in mechanistic detail. Lack of appropriate rate expressions and kinetic parameters and persisting difficulty in generating time-course data to fit the model parameters renders development of such models challenging. Thus, until today most of the modeled systems are still rather small but recent examples provided valuable insight about metabolism that could not have been obtained through classical approaches [49,50]. For *E. coli*, several new detailed models have been developed for diverse sub-systems. For instance, models of the PTS-based carbohydrate uptake systems have revealed a set of interesting functional insights [51], such as demonstrating the importance of a forward loop that guarantees robust behavior in carbohydrate uptake [52]. Another model was built for the ammonia assimilation system and validated with a set of environmental and genetic perturbations that were not used for model fitting. In a nice iterative cycle between modeling and experimental efforts, the authors found that the dynamic metabolome data acquired in nutrient perturbation experiments in the wild-type and in mutant strains could only be fitted to the model when competition for the active sites of saturated enzymes was considered [10•]. This sort of regulation mechanism is hardly ever considered and the results indicate that this mechanism might be much more important for the regulation of metabolism than is usually considered. Also another metabolism-relevant regulation mechanism was recently found to be important in a similar model-based experimental study of *Saccharomyces cerevisiae*. Using data from fluorescence reporter genes obtained from dynamically changing environments in microfluidic devices and an adapted comprehensive model of the galactose network, it was found that the experimental data could only be described by the model when active glucose-dependent degradation of the *GAL1* and *GAL3* transcripts was considered [28••]. This model prediction was found to be correct in follow-up experimental analyses, which overall provided insights into the importance of post-transcriptional regulation in this case.

All so far mentioned models only modeled small subsystems. For several system-level questions, for example, how central metabolism and its regulatory machinery ensure metabolic homeostasis in changing environments, however, we will need to use larger mechanistic models. To this end, the field seems to be a bit hampered by the conjecture that exact mechanisms cannot be modeled at large, given the lack of rate expressions and kinetic parameters. Indeed it might take a while until we have a sufficient number of modular models developed to combine them into a large cellular model as was recently suggested [10•]. Thus, in order to answer more comprehensive system-level questions on the molecular functioning of microbial metabolism, it deems necessary to find ways to develop mechanistic-based models despite this uncertainty and still to be able to extract relevant insights from these models. In fact, there is indication that structural features of cellular networks outweigh the potentially critical fine-tuning of rate laws and parameters [53,54]. Two recent approaches exploit this property in

the context of mechanistic metabolic models and make a leap towards larger models without the need for detailed characterization of kinetic parameters.

Liao and co-workers introduced an approach where they capitalized on thermodynamics and on experimentally measured steady states to constrain ensembles of ordinary differential equation models, thereby reducing the candidate ensembles significantly [55]. Models with predictive power were obtained, which were able to guide metabolic engineering efforts [56]. In another work, a recently proposed approach to estimate model structures and parameters from steady-state omics data [57] was used to develop a large-scale model of *E. coli*'s central metabolism, including its allosteric, transcriptional and post-transcriptional regulation. Model analyses revealed that adaptations to fluctuating nutrient availability are enabled by indirect recognition of carbon sources through a distributed sensing of intracellular metabolic fluxes. Molecular flux sensors were found to be embedded in global feedback loop architectures, which realize that metabolic operation adapts itself autonomously to fluctuating carbon sources without requiring any classical sensing and signaling [29••]. This work shows that, despite the inherent uncertainty, a molecular system understanding can be generated even for larger metabolic systems. In fact, recently generated omics data point to the existence of similar metabolism/transcriptional level-overarching regulation mechanisms in yeast [35].

Beyond answering questions that require molecular understanding (as the ones just illustrated), there are also questions in the realm of 'system understanding' that can be addressed at a more abstract level using existing knowledge on biological networks and experimental data. One example of such global system-level problems is the question of how a unicellular organism optimizes its growth rate and fitness in the light of (fluctuating) nutrient availability and competition by other organisms. Here, several recent studies made interesting contributions.

After reconstructing metabolic networks from genomic data for 113 bacterial species, Freilich and colleagues computationally inferred the species' habitable environments and scored these environments according to the number of species that can live in the respective environment (denoting the level of competition that a species encounters in this environment). When correlating these data with the species' growth rates, they found that obviously slow growth rates are used in specialized niches with little co-habitation and high growth rates in competitive environments [58]. Another interesting study shed new light on the phenomenon of overflow metabolism that is usually seen to go against a growth maximization goal. Teusink and colleagues developed a simple self-replicator model that considers several cellular sub-systems. They found that when the system, for instance, also accounts for enzyme synthesis costs, then under certain environmental conditions indeed an overflow metabolism can actually maximize growth [59]. Not only focusing on something completely different, but also advancing our understanding in what contributes to fitness, van Oudenaarden and colleagues investigated how fitness can be optimized in fluctuating environments. Using an experimental approach supported by a population-dynamics model they found that in rapidly fluctuating environments fast-switching populations outcompete slow switchers and that the opposite is the case in rarely changing environments, overall suggesting that cells might be have adapted their inter-phenotype switching rates to the frequency of environmental changes [60]. On the basis of models, computational analyses and experimental data, these studies provided us with novel perspectives on growth rate and fitness as an important cellular (metabolic) behavior and thus global systems understanding.

Conclusions

Does systems biology hold up against its promises? Experimentally, large-scale omics techniques provide unprecedented opportunities and much of the current systems biology activities are concerned with generating data. Several of these experimental efforts deliver data that directly allow us to complete network topologies. Once information on novel components and interactions cannot be directly obtained from omics data, however, we often struggle in translating this data into actual biological insight, arguably because of still lacking theoretical methods or concepts. Even when inference methods are used, the type of obtained biological insight is mostly of a hypothesizing or spotlighting nature and thus distinct from the molecular level insights that are provided by molecular biology. Since the number of such hypotheses can be rather large, a great number of follow-up analyses are typically required to prove functionality and pinpoint molecular mechanisms. The challenge for computational methods is (i) to obtain more 'molecular' insights through top-down analyses of omics data and (ii) to efficiently guide follow-up analyses.

On the more hypothesis-driven side of systems biology, we have recently seen several nice examples of molecular systems understanding in small sub-networks. For small systems, most theoretical methods are in place and we can thus expect to see more of such studies. Before the understanding of larger systems can be tackled, however, several conceptual challenges will have to be solved, most pressing perhaps how to deal with the inherent uncertainty [53].

In our view, the current key challenge for systems biology of microbial metabolism is regulation, in particular through so far not often considered mechanisms of metabolite–protein interactions and enzyme phosphorylation.

Systems biology must provide appropriate experimental means and computational methods of how such regulation can be inferred from large-scale data. Further, we are convinced that mathematical modeling will be the only vehicle capable of generating a true understanding about how biological systems really work. In this context, metabolism is frequently presented as an ideal test case for systems biology, given the wealth of our current knowledge. However, the operation of metabolism is a system-level property that is possibly more complex than any other cellular system. This is because metabolic operation is (i) influenced by a whole arsenal of regulatory actions at various cellular levels, and (ii) metabolism itself feeds back to almost all cellular systems, including itself. Thus, although metabolism is definitely a good starting point for system biology endeavors, it might very well turn out to be the most challenging cellular system to generate a system-level understanding.

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