ACS SyntheticBiology

Review

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Modeling Challenges in the Synthetic Biology of Secondary Metabolism

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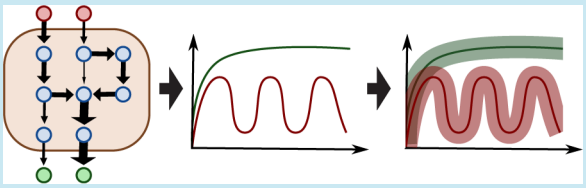
ABSTRACT: The successful engineering of secondary metabolite production relies on the availability of detailed computational models of metabolism. In this brief review we discuss the types of models used for synthetic biology and their application for the engineering of metabolism. We then highlight some of the major modeling challenges, in particular the need to make informative model predictions based on incomplete and uncertain information. This issue is particularly pressing in the synthetic biology of secondary metabolism, due to the genetic diversity of microbial secondary metabolite producers, the difficulty of enzyme-kinetic characterization of the complex biosynthetic machinery, and the need for engineered pathways to function efficiently in heterologous hosts. We argue that an explicit quantitative consideration of the resulting uncertainty of metabolic models can lead to more informative predictions to guide the design of improved production hosts for bioactive secondary metabolites.



KEYWORDS: *synthetic biology, secondary metabolite, metabolism, metabolic modeling, constraint-based modeling, kinetic modeling, uncertainty, Monte Carlo sampling, metabolomics, fluxomics*

COMPUTATIONAL MODELS FOR SYNTHETIC BIOLOGY

Computational modeling has long been an essential tool for the bioengineering of metabolism. Many of the central concepts of systems biology have been developed in the context of metabolic pathway engineering for biotechnology.[1] For example, the unexpected finding that the overexpression of individual "rate-limiting" enzymes failed to lead to massively increased product levels gave support to the idea that the control of metabolic flux can be widely distributed across many enzymes in a pathway; this in turn became one of the central concepts of metabolic control analysis and a major justification for the use of mathematical analysis to understand the behavior of complex biological systems.[1]

As synthetic biology is setting for itself increasingly ambitious aims, the demands on computational modeling approaches are rapidly growing. In the long run, BioCAD approaches, i.e., the comprehensive *in silico* engineering and design of new biological systems, will depend heavily on computational modeling.[2−4]

Two major classes of computational models are commonly used for the synthetic biology of metabolism: constraint-based genome scale models and differential-equation-based dynamic models.[5−7] The former type of model is relatively easy to generate once a genome sequence is available: the enzyme-coding genes in the genome are identified on the basis of homology searches, the catalyzed reactions and their stoichiometry are annotated and combined in a stoichiometric matrix, and finally missing reactions are added in a gap filling and manual curation step.[8] Both the initial genome annotation and the necessary manual curation are not trivial, especially for species that are not closely related to any well-studied model organisms. Nonetheless, an informative working model can usually be created without additional experimentation, and various computational tools are available to automate the individual steps of this model-building pipeline.[9−12] The resulting models can, e.g., be used to predict the relative distribution of metabolic fluxes in varying physiological conditions, such as with and without the production of a desired metabolic end product,[13] and consequently guide the engineering of industrial strains, as has recently been shown for the overproduction of platform chemicals in yeast.[14,15] For an example involving secondary metabolite production, we have used a constraint-based model of the *Streptomyces coelicolor* metabolic network to predict changes in relative flux during the transition from logarithmic growth (when cellular resources are mainly devoted to increasing biomass) to stationary phase (when fluxes are mostly directed toward the production of bioactive secondary metabolites, which are the target compounds of synthetic biology approaches[16]). We could show that the predicted flux changes closely correlated with the gene expression dynamics observed during the same metabolic switch[17] and that discrepancies between flux predictions and

Special Issue: Natural Products: Tools and More

Received: March 7, 2013
Published: May 9, 2013

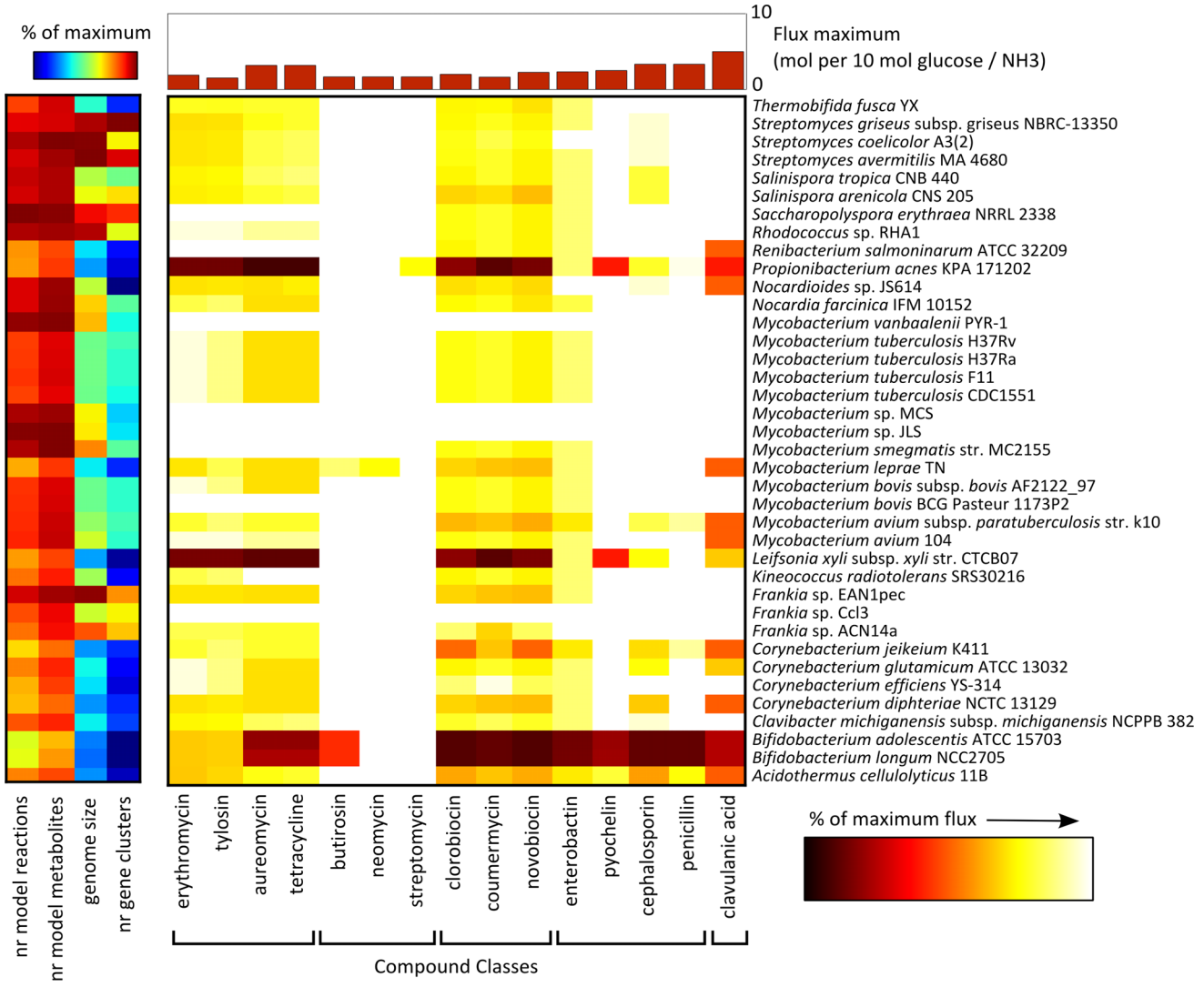


Figure 1. Heat map showing the predicted maximal flux toward 15 different secondary metabolites, in genome-scale metabolic models of 41 species of actinobacteria. Highest relative flux levels are shown in white, lower fluxes in progressively darker shades of red. The maximum flux for each compound across all species is indicated at the top. The heat map on the left indicates the number of model reactions and metabolites, the genome size, and the number of secondary metabolite biosynthesis gene clusters in each species. This shows that high predicted flux does not necessarily correlate with a high complexity of the native secondary metabolism in a species. (Figure based on Zakrzewski et al.[23])

gene expression could be used to identify misannotations of individual gene products in the model (e.g., specialized enzymes for the production of secondary metabolite precursors, which had been assigned to functions in primary metabolism, from which they had originally evolved).

Constraint-based models can also be used to identify essential enzymes in a metabolic network and, in reverse, to identify those reactions that are possibly redundant and could be removed in a genome-minimization strategy. For example, in Medema et al.[18] we used a constraint-based model of *Streptomyces clavuligerus*, an industrial producer of secondary metabolites, to show that a large 1.8 Mb megaplasmid detected in the genome of this species did not contain any essential enzyme-coding genes and could be safely deleted to streamline the genome size dramatically. The feasibility of removing the entire plasmid was subsequently experimentally confirmed.[19] Subsequently, we performed *in silico* single, double, and triple enzyme knockouts on the model to identify more than 100 presumably redundant metabolic reactions in the remainder of the genome;[20] together with gene expression data available for wild-type and industrially optimized *S. clavuligerus* strains,[21] these predictions can be used to inform the iterative further minimization of this genome, with the aim to create an optimized superhost for the industrial production of secondary metabolites, not in the least removing the complex background of potentially interfering native compounds.[22]

On a larger scale, the comparative analysis of constraint-based models can be used to identify bacterial species that are metabolically preadapted to the overproduction of compounds of biotechnological interest. To explore this possibility, we developed a computational tool to compare the theoretical production limits for a series of secondary metabolite classes in about 40 different species of actinomycetes.[23] We not only found that there are large variations in the predicted capacities of different species but also could show that the species that are predicted to be most productive and versatile are not always those that are typically used in biotechnology and that predicted suitability for overproduction does not correlate with genome size or the complexity of native secondary metabolism (Figure 1).

dx.doi.org/10.1021/sb4000228 | *ACS Synth. Biol.* 2013, 2, 373–378

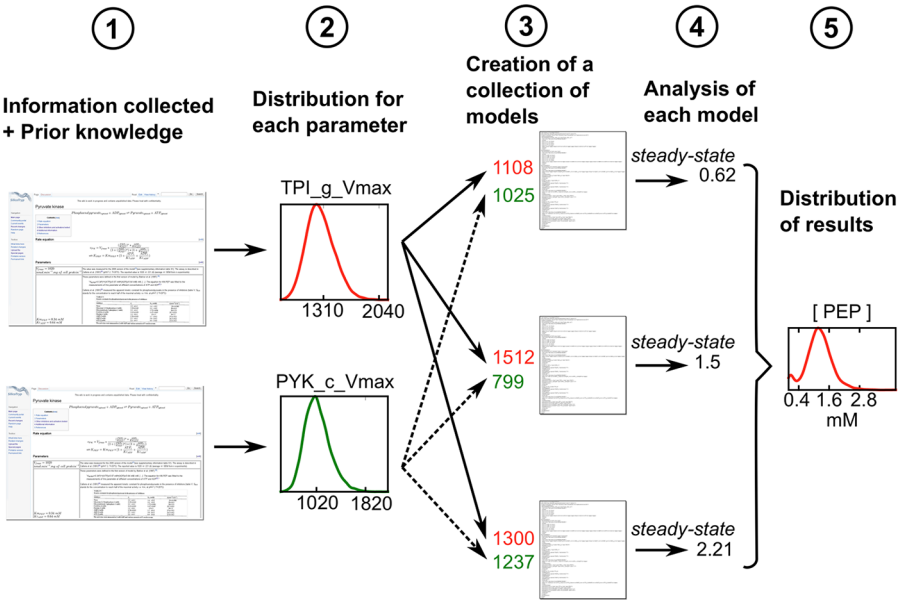


Figure 2. Schematic representation of dynamic modeling with an explicit quantitative consideration of parameter uncertainty. See text for details. (Figure based on Achcar et al.[39])

These results are a promising first step toward the computational identification of a suitable chassis for the synthetic biology of secondary metabolism. The ability of the underlying algorithm to analyze the system under multiple, potentially conflicting, physiological objectives will also be helpful for understanding the evolutionary trade-offs that lead to specific system properties that now may be constraining the industrial optimization of hosts for synthetic biology.[24]

However, the results also reveal a number of challenges affecting constraint-based models, which make any conclusions based on this modeling exercise very preliminary. First, the underlying models are purely genome-based and, with the exception of the secondary metabolite biosynthesis pathways, which were added manually, do not incorporate experimental information. Second, being constraint-based, the models are lacking quantitative information on reaction rates and do not allow predictions on intermediary metabolite levels, which would be particularly valuable for "debugging" engineered bacterial strains, which might accumulate toxic intermediates or deplete critical precursors.[25]

This indicates that successful bioengineering will require a more fine-grained, quantitative prediction of cellular behavior, enabled by a different modeling strategy: differential-equation-based dynamic models of metabolism, usually based on ordinary differential equations, not only can describe relative steady-state flux distributions but also can be used to make predictions about metabolite levels and the rate of change in metabolic fluxes following an intervention, whether the result of genetic variation or molecular engineering, including the use of quantitative expression systems and regulatory RNA. Most importantly, dynamic models allow a quantitative identification of rate-controlling reactions in a metabolic network.

However, dynamic modeling depends on quantitative information about (1) all enzyme kinetic parameters, (2) all enzyme activities (preferably for different cellular states), (3) intracellular concentrations for metabolites, and often (4) the distribution of metabolites and enzymes across subcellular compartments. It is obvious that this detailed information is rarely available for any cellular system, not even for extremely well studied model organisms or subsets of metabolism. It would be unrealistic to expect that sufficient data on enzyme kinetics for classical dynamic modeling on a genomic scale will ever be available for the main workhorses of biotechnology, in particular highly diverse groups, such as actinomycetes or filamentous fungi.

Recently, progress has been made in moving from constraint-based to dynamic models of metabolism on a large scale, by incorporating thermodynamic and regulatory information[26−32] and automatically generating differential equation systems for all reactions, sometimes using simplified kinetic equations, which are then, e.g., explored across a wide range of thermodynamically feasible parameter values[33−35] or are parametrized using parameter estimates collected from the literature or collated from public databases of dynamic models.[36] These models can be used, e.g., to perform metabolic control analysis, identify key reactions that limit flux toward desired end products, and predict the feasible ranges of metabolite concentrations for particular physiological states of the system.

■ DYNAMIC MODELING UNDER UNCERTAINTY

It is obvious that all approaches to generate comprehensive dynamic models of metabolism will have to operate in the context of highly unreliable parameter data,[37] in particular in synthetic biology: even where the kinetics of individual enzymes have been studied, assay conditions will rarely match the *in vivo* situation,[38] and in an engineered system the enzymes will by definition have to function in a new cellular environment. As the parameters will never be known with certainty, principled approaches to handling the resulting parameter uncertainty are needed. In a proof-of-concept study on central energy metabolism in a protozoan parasite, we have recently shown how dynamic models of metabolism can explicitly take the uncertainty about each individual parameter into account (Figure 2[39]).

Starting from a highly curated dynamic model of a small part of energy metabolism, we identified the experimental uncertainty (confidence intervals or standard deviations) for

dx.doi.org/10.1021/sb4000228 | *ACS Synth. Biol.* 2013, 2, 373−378

every parameter in the model. The experimental evidence and the calculations underlying the uncertainty assessment were documented in a dedicated Wiki page for each enzymatic reaction. We then used a Monte Carlo sampling strategy to create a large collection of model variants, each with a different thermodynamically consistent combination of plausible parameter values sampled from the confidence intervals. As the uncertainty of the parameter values was described by a log-normal distribution for most parameters, more plausible values tended to be present more often in the sample. The collection of models could then be subjected to the same types of analysis as traditional dynamic models, e.g., to determine the flux control coefficients for individual enzyme parameters, with the important benefit that the results of these calculations now come with a confidence interval that reflects the experiment-based uncertainty about the parameter values in the system, rather than a general thermodynamic constraint.

This strategy of explicitly incorporating a quantitative and at least partially experiment-based description of parameter uncertainty already in the model building process seems to be of general applicability for synthetic biology and bioCAD approaches, but it will need a considerable extension of our tool box, both for model building and for model analysis and visualization.

Some data, for instance, the kinetic parameters, come with a straightforward quantitative estimate of our confidence in their exact values, either from the experimental standard deviation or because they are predicted using statistical approaches that quantify their predictive uncertainty. For other types of data that can inform the model building process, in particular for large genome-scale models, new methods to estimate the associated confidence intervals are needed. Global metabolomics and fluxomics data sets are important examples of information that should be integrated in the model building and model interpretation stage.[40] In the case of metabolomics, often even the chemical identity of the detected metabolites is uncertain.[41] Current analysis tools tend to circumvent this problem by either reporting all possible chemical compounds that could match a detected analyte or by using ranking heuristics and arbitrary thresholds to identify the most plausible match. Such strategies are not easily integrated with a modeling framework that relies on quantitative estimates of the experimental uncertainty. New statistical approaches to generate such quantitative estimates and at the same time provide a rigorous way of assessing the relative merits of alternative interpretations of the same data sets have recently been presented in a proof-of-concept study.[42] In the case of ^{13}C fluxomics, the challenge is even more interesting, as here the interpretation of the data strongly depends on the existing model of metabolism (i.e., which reactions are expected to be active in the system[43]), while the experimental results can also change our view of the correct model (e.g., because strong evidence for the activity of unexpected reactions is found[44]). This mutual dependency will most reliably be captured in a statistical framework where both model and data are associated with explicit representations of uncertainty, which can be updated iteratively as additional evidence becomes available. Adding this functionality to the existing flux analysis software (e.g., ref 45) will be a major challenge, as it entails combining two computationally very expensive sets of algorithms. Addressing this challenge will be necessary, considering that metabolic flux data are not only the most informative input for model construction but also the main engineering target in the synthetic biology of secondary metabolite production.[46,47]

The challenge of parameter uncertainty becomes even more pressing when regulatory interactions in the metabolic network are considered. While constraint-based models can incorporate some regulatory information, e.g., in the form of Boolean constraints on the presence/absence of particular reactions in certain conditions, the detailed modeling of regulation has always been the domain of dynamic differential equation models. These have been used to describe and understand some of the most influential regulatory building blocks for synthetic circuits, including bistable switches and oscillators.[48,49] Being able to quantitatively predict the behavior of the regulatory circuitry controlling an engineered pathway is clearly an important requirement for synthetic biology. The use of libraries of regulatory modules, covering a large part of parameter space, can partially avoid the challenge but is less than satisfactory from an engineering perspective. Refactoring of engineered pathways can remove unknown and uncharacterized regulatory interactions[50] but is currently not feasible for central metabolism, which is a prime target for optimization toward the production of valuable secondary metabolites.[51] Thus, not only are the parameters of regulatory cascades far more difficult to measure than enzyme kinetic parameters, but we face the additional challenge that the topology of the regulatory circuitry is only partially known. In this case, the sampling approach has to be extended to handle explicit uncertainty about alternative regulatory interactions in addition to uncertainty about their strength and dynamics.

The challenge can be illustrated, e.g., by the simple two-gene system implementing the bistable switch controlling antibiotic production in *S. coelicolor*. This system has been studied in detail using differential equations; the model of this very small and relatively abstract system required 21 kinetic parameters (rate constants and equilibrium constants), all based on rough experimental estimates, plus information on the absolute concentration of 8 regulatory metabolites, proteins, and protein complexes.[52] However, the experimental evidence from some of the crucial complexes is lacking, and the system also contains a cis-encoded antisense RNA that could implement an alternative mechanism to ensure robust bistability of the switch.[53] An explicitly uncertainty-aware modeling strategy would quantify the confidence intervals for all kinetic parameters in the model and our initial *a priori* belief in the relative merits of the alternative regulatory circuitries. Depending on the aims of the analysis, one can then sample models from the alternative topologies according to their *a priori* probability, or one can use the relative likelihood of experimental data under the different topologies to update the beliefs and (in the ideal case) discard all but one of them as implausible.

Synthetic biology will always be performed in the context of incomplete and noisy information, in imperfectly insulated systems and with poorly characterized parts.[54] This special situation requires new strategies for computational model building and analysis, which are fully and explicitly aware of the uncertainty that is inherent in the parameters and topologies of the models. Acknowledging the uncertainty and incorporating it in the model building, rather than postponing it to the model interpretation stage, will enable predictions that come with specified confidence intervals and can guide truly robust designs of cellular factories for bioactive secondary metabolites.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Work in the authors' laboratories was supported by SysMO (http://www.sysmo.net/), NWO-Vidi (http://www.nwo.nl/nwohome.nsf/pages/NWOP_5VTGQW_Eng) and SULSA (http://www.sulsa.ac.uk/).

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