

Designing metabolic engineering strategies with genome-scale metabolic flux modeling

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Abstract: New in silico tools that make use of genome-scale metabolic flux modeling are improving the design of metabolic engineering strategies. This review highlights the latest developments in this area, explains the interface between these in silico tools and the experimental implementation tools of metabolic engineers, and provides a way forward so that in silico predictions can better mimic reality and more experimental methods can be considered in simulation studies. The several methodologies for solving genome-scale models (eg, flux balance analysis [FBA], parsimonious FBA, flux variability analysis, and minimization of metabolic adjustment) all have unique advantages and applications. There are two basic approaches to designing metabolic engineering strategies in silico, and both have demonstrated success in the literature. The first involves: 1) making a genetic manipulation in a model; 2) testing for improved performance through simulation; and 3) iterating the process. The second approach has been used in more recently designed in silico tools and involves: 1) comparing metabolic flux profiles of a wild-type and ideally engineered state and 2) designing engineering strategies based on the differences in these flux profiles. Improvements in genome-scale modeling are anticipated in areas such as the inclusion of all relevant cellular machinery, the ability to understand and anticipate the results of combinatorial enrichment experiments, and constructing dynamic and flexible biomass equations that can respond to environmental and genetic manipulations.

Keywords: genome-scale modeling, flux balance analysis, flux variability analysis, minimization of metabolic adjustment, metabolic bottleneck, pathway optimization

A brief introduction to genome-scale metabolic flux modeling

A “genome-scale” metabolic flux model (GEM) consists of a network of biochemical reactions that is reconstructed based on the genomic sequence and annotation of a cell. Assuming a “steady-state” metabolism (ie, a snapshot of metabolism at one time point) is reached on a short time-scale, these reactions can be represented by a linear system of equations. Then, problems such as maximizing specific chemical production or growth can be solved efficiently by linear programming. GEMs and their uses have been reviewed thoroughly, and they are most basically used to predict reaction flux, which is the overall rate of metabolite conversion.^{1,2} Often, laboratory measurements including the rates of substrate consumption, product formation, and growth are used as model constraints so calculations coincide with observations. Other model constraints can be derived from reaction thermodynamics,³ cellular regulatory networks,⁴ and -omics datasets.⁵ GEMs have been constructed and utilized for intensively

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studied model organisms with well-annotated genomes (eg, *Escherichia coli* MG1655 [bacteria],⁶ *Saccharomyces cerevisiae* [yeast],⁷ *Mus musculus* [mouse],⁸ and *Arabidopsis thaliana* [plant]⁹). In addition, homology algorithms have enabled GEM construction of the less-studied organisms. For example, the European Bioinformatics Institute has constructed draft GEMs for 2,630 organisms across phylogenetic domains using automated model-building methods,¹⁰ and the Model SEED also contains several GEMs and has the ability to custom-build GEMs for annotated genomes submitted by the user.¹¹ The construction of high-quality models often requires expert-informed manual curation,¹² but automated reconstruction provides foundations for further improvement. Although GEMs have been built for species of all domains, microbes still dominate GEM reconstructions and studies due to their relative genomic simplicity, usefulness in biotechnology, and the pathogenicity of some species.

GEMs have extraordinary utility for biological discovery, and novel computational tools have been developed to predict metabolic engineering strategies, which are then validated in the laboratory. Much research in metabolic engineering is focusing on the synthesis of valuable chemicals, biofuels, and pharmaceuticals. Model-guided metabolic engineering presents significant advantages, notably the minimization of laboratory resource use and time required to develop productive strains. Using GEM predictions to design strains enables researchers to engineer product yield/selectivity, substrate utilization, and growth rate. Future developments are anticipated to allow engineering of toxicity responses, cellular differentiation, culture density, and cellular interactions with other cells and materials. Some of the computational tools for predicting gene targets in GEMs for metabolic engineering have been reviewed.¹ The focuses of this review are: 1) how predictions from different tools have been translated into experimental metabolic engineering strategies and 2) which of the experimental methods available are (or are not) represented in the computational (in silico) tools. Since the experimental toolset for metabolic engineering is expanding, this review also addresses how new tools can be incorporated in the in silico design strategies.

In silico metabolic engineering tools

It has been long believed that cells (especially microbes) maintain optimal growth as their primary objective. It has been shown that an additional objective of a minimal adjustment between initial and engineered states also exists.¹³ Imposing the goal of chemical overproduction by metabolic engineering often conflicts with the optimal growth objective. Thus, genome-scale modeling serves to establish the relationship

between target chemical production and growth. In silico metabolic engineering tools seek to identify genetic manipulations to alter this relationship so that stable strains with high chemical production and growth can be achieved. This section describes the various methods available for solving GEMs, and it highlights those used when metabolism has been engineered. In addition, this section presents the recent advances in in silico tools used with GEMs to generate metabolic engineering strategies for the overproduction of a targeted chemical. In this review, in silico metabolic engineering tools are classified as “top-down” or “bottom-up”. The top-down algorithms generate/apply metabolic modifications in silico and then simulate their effects on the dual objectives (ie, productivity and growth) through genome-scale metabolic flux modeling. The procedure is repeated until optimal metabolic modifications are identified. On the other hand, bottom-up algorithms generate separate flux solutions where: 1) growth is maximized and 2) product formation of interest is maximized. Differences between the two flux distributions are identified as targets to design metabolic engineering strategies. These approaches are reviewed in detail in the following section; however, first the methods for generating metabolic flux solutions of GEMs are summarized.

Flux balance analysis and its variants

The fundamental approaches of constraint-based modeling have been reviewed,^{1,2} and a subset of these applicable to metabolic engineering are described here. The essential base of almost all predictive tools is flux balance analysis (FBA), which solves the linear system of biological reactions given the “pseudo-” steady-state assumption and an objective function (eg, maximize growth or chemical production rate) using linear programming. The flux balance equation is now commonly written as $S \cdot v = 0$, where S is an m -by- n matrix containing stoichiometric coefficients for each biochemical reaction. Each compound is represented by a row of the matrix, and each reaction is represented in a column. The vector v contains flux values for all n reactions of the system. The system also contains a “biomass equation” that describes cell growth. This is often composed of stoichiometric amounts of macromolecules (eg, protein, DNA, RNA, lipids, cell wall), small molecules, and adenosine triphosphate (ATP) hydrolysis required for growth “maintenance”.¹⁴ FBA solves the system of equations given an objective function and constraints (upper and lower) for each flux contained in v . Flux constraints are imposed from laboratory measurements, thermodynamic predictions, and regulatory rules; many reaction fluxes are left unconstrained.

Two other useful approaches are parsimonious FBA (pFBA)¹⁵ and flux variability analysis (FVA).¹⁶ Since the number of reactions is typically greater than the number of compounds in GEMs, multiple FBA solutions exist, and techniques that explore this solution space have been reviewed. The pFBA algorithm was developed to provide the FBA solution that meets optimality with a minimized total flux in the system. In addition, FVA serves the purpose of calculating the possible flux distributions of all reactions.

As mentioned earlier, cellular metabolism changes when a genetic manipulation is introduced in vivo. However, dramatic shifts in metabolism, on a global level, toward optimality are not immediate.^{17,18} Thus, a flux distribution predicted in silico that captures this initial response of a cell, instead of one that describes massive flux reorganization toward optimality, provides a better description of the cellular response to genetic changes. For this reason, the minimization of metabolic adjustment (MOMA) algorithm was developed to predict the optimal flux distribution of altered metabolism that would require the smallest change from that of wild-type metabolism.¹⁷ This concept has since been validated by ¹³C-isotope tracing studies.¹³ Similar to MOMA, the regulatory on/off minimization (ROOM) tool hypothesizes that a cell attempts to compensate for genetic manipulations through the fewest number

of enzymatic reactions by gene regulation.¹⁸ Additional studies have shown that, in time, cells will evolve from this minimized flux redistribution state to the FBA solution.¹⁹ This concept, introduced over a decade ago,²⁰ is shown in Figure 1. The goal of metabolic engineering is to alter the metabolic network of a cell so that optimal growth and target chemical production are coupled (meaning a product must be formed as the cell reaches an optimum growth rate). This approach leads to stable strains capable of industrial production. As a cell is engineered, MOMA/ROOM can predict the immediate outcome of genetic manipulations, and FBA (or pFBA) predicts the long-term evolved state of the cell. In the following sections, the top-down and bottom-up in silico metabolic engineering tools are discussed, and a summary of these tools is given in Table 1. However, it is important to note that not all tools are designed to consider evolution and long-term strain stability, which are critically important if an industrial process is going to consider chemostat cultivation over batch processing in which the microbe is replaced frequently.

Top-down in silico tools for designing metabolic engineering strategies

As mentioned previously, a top-down approach is defined here as one in which genetic manipulations are made in

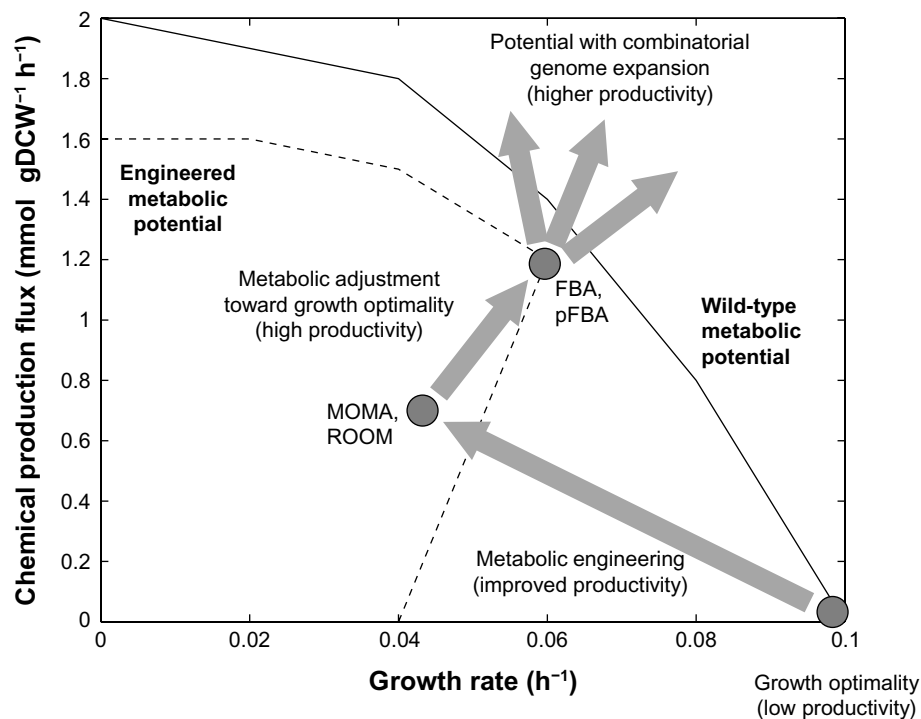


Figure 1 The relationship between the target chemical production flux and the growth rate for wild-type (solid line) and an engineered strain (dash line). The initial wild-type optima determined by FBA (bottom right) can be engineered and the resulting state predicted with MOMA/ROOM. Evolution will eventually optimize growth, which can be predicted by FBA/pFBA. Combinatorial addition of metabolic capabilities can expand the solution space beyond the wild-type potential.

Abbreviations: FBA, flux balance analysis; MOMA, minimization of metabolic adjustment; pFBA, parsimonious FBA; ROOM, regulatory on/off minimization.

Table 1 Summary of in silico tools for generating metabolic engineering strategies and the experimental tools that can be used for implementation

In silico tool	Description	Experimental tools ^{§,†}	Reference(s)
Top-down approaches			
Randomized gene knockouts	Fluxes associated with reactions catalyzed by one or more genes are set to zero. Simulation performed by FBA, pFBA, MOMA, or ROOM	Gene knockout [‡]	21, 22
OptKnock	A bilevel optimization to find gene knockout candidates leading to product formation at optimal growth (FBA)	Gene knockout [‡]	19, 20, 23
OptGene	Explores the feasible solution region using a genetic algorithm to identify the necessary gene deletions for the desired phenotype	Gene knockout [‡]	24
Cipher of evolutionary design (CiED)	Uses a genetic algorithm to identify optimal mutations to maximize product formation	Gene knockout [‡] Gene overexpression [§]	25
ReacKnock	Inspired by OptKnock and enables up to 20 gene deletions	Gene knockout [‡]	26
OptReg	An expansion of OptKnock designed to predict up- and downregulation of reactions to achieve a desired phenotype	Gene knockout [‡] Gene overexpression [§] Gene expression knockdown [¶]	27
OptStrain	Uses a universal database of known enzyme-catalyzed reactions to determine the minimal pathway modification required to maximize product formation	Gene knockout [‡] Gene overexpression [§]	28
MOMAKnock	A similar bilevel programming framework to OptKnock except MOMA assumption was adapted to determine flux redistribution	Gene knockout [‡]	29
Ant colony optimization with MOMA (ACOMoMA)	A hybrid of ant colony optimization and MOMA to predict gene knockout strategies	Gene knockout [‡]	30
Bees Algorithm and FBA (BAFBA)	Similar to ACOMoMA, except Bees Algorithm is used to search gene knockouts and FBA is used to determine fitness	Gene knockout [‡]	31
Flux balance analysis with flux ratios (FBrAtio)	Flux ratios serve as constraints to redirect metabolism to a desired product. Resulting flux ratio constraints can be translated to metabolic engineering strategies directly	Gene knockout [‡] Gene overexpression [§] Gene expression knockdown [¶]	32, 33
Bottom-up approaches			
Flux distribution comparison analysis (FDCA)	Incremental solutions are compared to identify genes of reactions with significant flux changes	Gene knockout [‡] Gene overexpression [§] Gene expression knockdown [¶]	35, 36
Flux scanning based on enforce objective flux (FSEOF)	Identifies genes of reactions with increased flux upon maximizing product formation	Gene overexpression [§]	37, 38
OptForce	Flux variability analysis of wild-type and mutants (with a desired phenotype) are compared to identify genes of reactions with significant flux change	Gene knockout [‡] Gene overexpression [§] Gene expression knockdown [¶]	39–41
k-OptForce	An expansion of OptForce with the integration of enzyme kinetic constants to allow optimal solutions to arise from metabolic and/or enzyme engineering	Gene knockout [‡] Gene overexpression [§] Gene expression knockdown [¶]	42
Continuous modifications (CosMos)	A continuous modification to flux bounds is used to identify upper and lower bounds that can guarantee product formation. The solution space is sampled randomly to find optimum solutions	Gene knockout [‡] Gene overexpression [§] Gene expression knockdown [¶]	43
Redirector	Iteratively identifies all reactions with flux changes that accommodate for the progressive change in biomass and desired product	Gene knockout [‡] Gene overexpression [§] Gene expression knockdown [¶]	44

Notes: [§]Not all experimental tools apply to all species; [†]direct genome editing is likely to eventually apply to all cases; [‡]gene knockout can be accomplished through insertion mutagenesis using homologous recombination (ie, λ red recombineering), transposable elements, or by genome editing with the aid of CRISPR-Cas systems; [§]gene overexpression can be performed using plasmids or genome knock-in/editing procedures, which are accomplished by inserting gene-of-interest with high expression promoter element using homologous recombination, transposable elements, or CRISPR-Cas systems. RBS and promoter engineering are recommended methods for modulating expression levels. The RBS calculator is a valuable tool for RBS design; [¶]gene expression knockdown can be achieved through posttranscriptional gene silencing with sRNA, siRNA, antisense RNA, and/or microRNA. RBS redesign using the RBS calculator is also an effective strategy for gene expression knockdown.

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; FBA, flux balance analysis; MOMA, minimization of metabolic adjustment; pFBA, parsimonious FBA; RBS, ribosomal binding site; ROOM, regulatory on/off minimization.

in silico, and then genome-scale modeling is used to determine whether the strategy is beneficial. The concept is shown in Figure 2. The simplest strategy to employ is creating single-gene knockouts. This is done in silico by constraining all reactions associated with a gene of interest to zero and performing

FBA or MOMA/ROOM to look for knockouts that enhance target chemical production without compromising growth. This method was used in a well-known study to identify gene knockouts in *E. coli*, resulting in the overproduction of L-valine.²¹ Here, single-, double-, and triple-gene knockouts

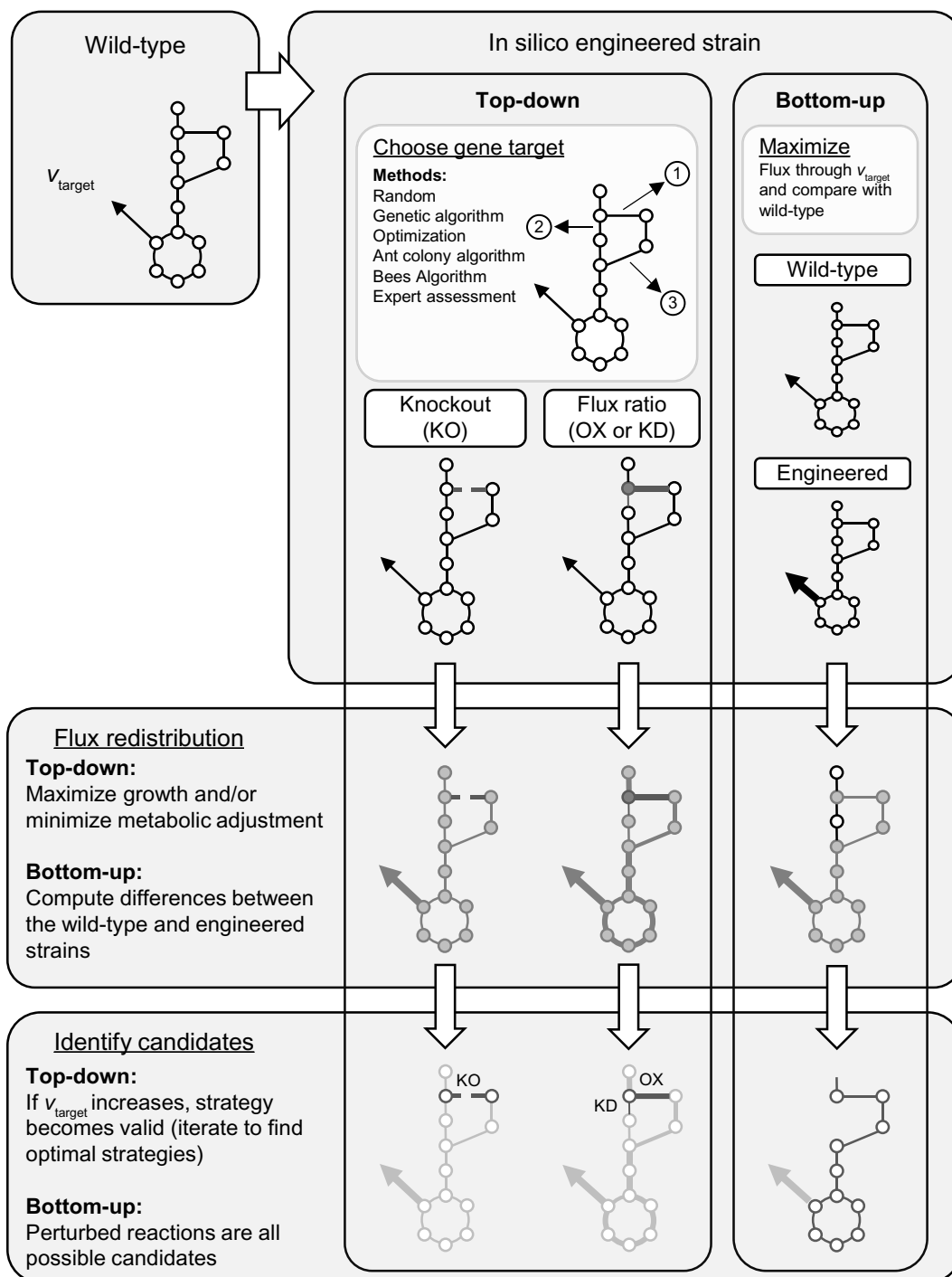


Figure 2 Example workflow to design metabolic engineering strategies using “top-down” and “bottom-up” approaches. Several different in silico tools apply these strategies in different forms. In all cases, the objective is to maximize production of a target chemical (shown here as v_{target}). The following metabolic engineering strategies are shown: (KO) gene knockout, (OX) gene overexpression, and (KD) gene expression knockdown.

were investigated in silico using MOMA to predict resulting phenotypes. Using a multifaceted approach that included the in silico gene deletion study, an industrially relevant strain capable of producing over 7.5 g/L of L-valine (2.27-fold improvement over wild-type) was engineered. This method was also used to generate all single- and double-gene knockout combinations in *S. cerevisiae* in an effort

to overproduce succinate.²² FBA was used in calculations, and three single knockouts (Δmdh , $\Delta oac1$, and $\Delta dic1$) were selected for experimental validation. The $\Delta dic1$ strategy was successful, yet non-intuitive for succinate production, and this study demonstrated an important proof-of-concept for designing strains by in silico predictions followed by experimental validations. OptKnock was one of the first

in silico metabolic engineering tools, and it guides the selection of gene knockouts in order to couple maximized product formation to growth.²⁰ It uses FBA and identifies a limited number of gene knockouts, which serve to reshape the growth and product formation relationship as shown in Figure 1. Successful identification of gene knockout targets, followed by adaptive evolution to achieve FBA predictions, have led to industrially relevant strains capable of producing lactic acid,¹⁹ 1,4-butanediol,²³ and others. Since its introduction, other inspired approaches have attempted to extend its capabilities (ie, increase the potential number of gene candidates for knockout) by reconsidering the bilevel optimization framework. Approaches such as OptGene²⁴ and the cipher of evolutionary design (CiED)²⁵ relied on an evolutionary algorithm to select gene targets, and improved functionality was noted. ReacKnock has emerged recently with a new approach to the mixed integer bilevel optimization problem and enables up to 20 gene deletion predictions in a short amount of computational time.²⁶ In their publication, the authors provide ReacKnock- and OptKnock-designed gene knockout strategies to produce succinate, ethanol, acetate, hydrogen, formate, glycolate, D-lactate, fumarate, and threonine from *E. coli*.²⁶ OptReg extended OptKnock to include gene overexpressions,²⁷ and OptStrain allowed incorporation of non-native metabolic pathways for the production of new chemicals.²⁸ Other approaches, such as MOMAKnock²⁹ have focused on the limitations of FBA and have sought to implement MOMA in the automated design of gene knockout strategies. Further modifications have combined ant colony optimization (ACO) methods with MOMA in an algorithm called ACOMoMA. The ACOMoMA approach was applied to produce an improved gene knockout strategy for succinate production from *E. coli*.³⁰ Another development achieved significant results using a hybrid of Bees Algorithm and FBA (BAFBA; a metaheuristic procedure) to design gene knockouts for succinate and lactate production.³¹

While most in silico designs rely on gene knockouts, others infer gene overexpression and partial gene knockdowns as metabolic engineering strategies. In general, the flux change of a reaction may be the result of: 1) directly engineering genes of the catalyzing enzymes; 2) engineering the availability of reaction precursors and substrates upstream; or 3) eliminating bottlenecks downstream. Thus, these strategies are all major contributors to a metabolic adjustment. A recent approach called FBA with flux ratios (FBrAtio) considers strategies of gene overexpression, knockout, and partial knockdown for designing metabolic engineering strategies.^{32,33} FBrAtio examines how multiple enzymes

compete for the same substrate and allow the distribution of this substrate to be modified and included as a flux ratio constraint in a GEM. Flux ratio constraints can be modified, and pFBA is used to predict global flux distributions. This procedure has been used to design metabolic engineering strategies for several chemicals by different organisms. The concept of the flux ratio constraint was first introduced for two enzymes that compete for the same compound.³² However, this was later expanded to include all enzymes competing for the same compound.³³ FBrAtio has been used to model the metabolic shift in *Clostridium acetobutylicum* from acids to solvents production as well as predict a high-ethanol-producing phenotype.³² In addition, it has been used to examine metabolic engineering strategies for: 1) cellulose overproduction by *A. thaliana*; 2) isobutanol production by yeast; 3) acetone production by *Synechocystis*; 4) hydrogen production by *E. coli*; and 5) mixed solvents production by *C. acetobutylicum*.³³ The purpose of this study was to demonstrate further improvements of experimental implementations where possible with “fine-tuned” metabolic engineering strategies derived by FBrAtio. With *Arabidopsis*, it was shown experimentally that the overexpression of a heterologous uridine diphosphate (UDP)–glucose pyrophosphorylase (UGPase) increased cellulose production by approximately 25%.³⁴ The FBrAtio approach predicted that further increased uridine triphosphate (UTP) consumption by the UGPase could continue to increase cellulose production up to 30%–50% (compared to wild-type) before UTP depletion impacted the growth of the plants negatively.

Bottom-up in silico tools for designing metabolic engineering strategies

The tools classified as bottom-up approaches rely on multiple objective functions in genome-scale modeling to design metabolic engineering strategies. The flux distribution comparison analysis (FDCA) provides a good example of this. First, a GEM is solved by FBA to maximize growth. Then, the GEM is solved (by linear MOMA [lMOMA]) to maximize the production of a chemical of interest. The differences between the flux distributions are considered, and rules for up- or downregulation of genes are determined based on significant changes between the flux distributions.³⁵ FDCA has been used to improve lycopene production by 174% in an *E. coli* strain already capable of high lycopene production,³⁶ and it identified 51 potential gene targets, including five novel gene knockout targets and four novel gene overexpression targets. The flux scanning based on enforced objective flux (FSEOF) approach was also developed to

enhance lycopene production.³⁷ This approach also begins with maximizing biomass formation of a GEM with FBA, but the flux of product formation is constrained to be equal to the experimentally observed flux in the wild-type organism. Then, the theoretical maximum product formation rate is calculated in a new simulation by setting this as the objective function. FSEOF works by maximizing the cell growth rate while the target product formation rate is increased gradually from its initial value toward its theoretical maximum. Targets for gene overexpression are identified as fluxes that increase throughout simulations without changing direction. This method identified 35 gene overexpression targets for lycopene production by *E. coli*. FVA was then employed to narrow these potential targets by selecting those showing increases outside of the ranges due to flux variability.³⁷ This approach can also be used with an altered biomass equation to accommodate intracellular target (eg, protein) accumulation. For example, the human superoxide dismutase (hSOD) enzyme was overproduced in *Pichia pastoris* using predicted gene knockout and overexpression strategies from MOMA and FSEOF, respectively.³⁸

OptForce is another bottom-up approach that has enabled the incorporation of gene knockouts, overexpressions, and knockdowns as metabolic engineering strategies.³⁹ OptForce also allows (and encourages) the incorporation of experimentally measured metabolic flux data of the wild-type and a strain engineered to overproduce a target chemical. In general, flux variability is calculated for both wild-type and engineered strains, and the flux ranges are compared for each reaction. Candidates for metabolic engineering are identified as those reactions where there is no overlap between possible flux ranges. OptForce then performs a secondary optimization (a top-down procedure) where the minimal set of metabolic interventions is identified to achieve a desired goal. OptForce has been used in several applications, including the overexpression of succinate³⁹ and fatty acids of specified chain length in *E. coli*.⁴⁰ In addition, OptForce was used to design a metabolic engineering strategy leading to a four-fold increase in intracellular malonyl-CoA concentration in *E. coli*, which was then utilized for the production of naringenin (a valuable plant secondary metabolite).⁴¹ The recent extension k-OptForce has enabled the incorporation of enzyme kinetic constants, where possible, and returns metabolic engineering strategies (ie, gene knockout, overexpression, or knockdown) along with kinetic parameters that could be altered by enzyme engineering.⁴² This approach can consider relevant phenomena, such as substrate inhibition, that cannot be modeled using flux-based approaches alone. The continuous modifications (CosMos)

approach significantly differs from OptForce in that changes to flux bounds are modified continuously, rather than by FVA results. CosMos then minimizes product formation given a constrained non-zero growth rate, and looks for modified flux constraints that still yield product formation under these conditions.⁴³

Finally, the Redirector approach is different in that it relies on an artificial objective function consisting of contributions from growth and metabolic flux redirected into a product-forming pathway and does not rely on manipulating flux bounds.⁴⁴ Redirector can also design gene knockout, overexpression, or knockdown metabolic engineering strategies, and the manipulation of algorithm parameters can alter the number of manipulations returned by the algorithm. The production of fatty acids by *E. coli* MG1655 was chosen as a test case of the algorithm. The algorithm designed strategies capable of reaching 80% of the theoretical yield for myristoyl-CoA while maintaining 20% biomass yield.⁴⁴ The global implementation of FBrAtio (currently in press) is also classified as an approach that does not manipulate flux bounds to derive metabolic engineering strategies. The global FBrAtio uses flux distribution maps of maximized growth and product formation using pFBA and designs flux ratio constraints that enable product formation and growth.

Experimental metabolic engineering tools

The available in silico metabolic engineering tools return strategies consisting of gene knockout, overexpression, and/or knockdown (and enzyme engineering for k-OptForce). There are several ways in which these strategies can be implemented, but current in silico tools do not consider this level of detail. In this section, many common (but certainly not all) experimental implementation methods are reviewed along with their relationship to in silico predictions. For example, returning a gene overexpression strategy does not explain how it should be implemented. If it must be encoded on a plasmid, what type and strength of promoter/ribosomal binding site (RBS) combination should be used? What copy number of plasmid should be used? Since plasmid copy number per cell is heterogeneous, what impacts will this have? Will plasmid replication demand cellular resources that influence metabolic flux predictions? What are the impacts of antibiotic resistance genes? Should one or multiple copies of the gene of interest be knocked into the genome? Or, should a native promoter/RBS be tuned instead? If so, to what levels? Finally, what impact will this genetic manipulation have on the resulting phenotype? Will this significantly impact cell

composition, metabolic flux distribution, and predictions? These and more questions will be addressed by in silico tools that returned “fine-tuned” metabolic engineering strategies (eg, overexpress a target gene by 70% relative to wild-type) and take into account changing cell phenotype by updating the GEM biomass equation.

Manipulating gene expression

Here, basic experimental strategies for gene expression manipulations are reviewed in the context of genome-scale modeling. Clearly, not all tools and approaches can be discussed here, but the basics are identified. Manipulations can occur at the transcriptional, translational, and posttranslational levels, with emphasis on the first two in microbes. Several experimental methods exist for generating gene knockouts that involve chromosomal integration for gene disruption. Of course, chromosomal integration can also be used to knock-in useful genes/regulatory elements. One particularly popular method for single-gene targeting is the polymerase chain reaction (PCR)-based version of λ red recombineering.⁴⁵ It has also been used for the introduction of site-directed mutations, promoter tuning/replacement, and reporter genes for promoter tagging experiments.⁴⁶ The knock-in/knockout (KIKO) vectors facilitate the chromosomal integration of large DNA segments (including multigene cassettes and entire pathways) at specific well-characterized loci using λ red recombination.⁴⁷ Other means of gene knockout involve the use of transposons or homologous recombination mediated by phage-derived elements, and more advanced genetic systems are required for other microbes, such as the clostridia.^{48,49} In higher plant species, such as *A. thaliana*, genomic integration is accomplished using an *Agrobacterium*-mediated method that makes use of its ability to transfer DNA from its tumor-inducing plasmid into the plant host genome.^{50,51} This technology has been used for both gene disruption and knock-in in *Arabidopsis*. Gene knockouts (and knock-ins) appear to be the most benign to genome-scale modeling predictions, as long as plasmids and antibiotic resistance markers are removed. Indeed, the presence of plasmids and antibiotics (even with effective antibiotic resistance genes) has been shown to alter cell phenotypes.⁵² The GEM biomass equation describes the cell phenotype, and how this equation should be altered by the presence of plasmids, antibiotics, or other genetic or environmental manipulations remains a subject for research. This makes clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems⁵³ attractive for genome

editing from a genome-scale modeling standpoint. While the mechanisms of plasmid replication are understood, this cellular machinery is not yet encoded in GEMs, creating a divergence between the in silico and experimental systems. In addition, gene knockouts, knock-ins, and genome editing are designed to alter metabolism. When successful, this alters the cellular phenotype; thus, the biomass equation must be updated accordingly. However, this will require predictions or a simplified method of measurement, both of which are discussed later.

With this knowledge, it is easy to see why gene overexpression methods may lead to greater metabolic burden and uncertainty with genome-scale modeling, especially when a gene is overexpressed from a plasmid. Techniques that minimize the ATP maintenance requirements of a cell are preferred and are more effectively modeled. With gene overexpression, promoter and RBS engineering have enabled significant progress. Controllable gene expression has launched the field of synthetic biology and led to the quest to design genetic circuits.⁵⁴ Furthermore, promoter tuning⁵⁵ with RBS optimization can improve metabolic pathway function.⁵⁶ Tools, such as the RBS calculator,⁵⁷ are enabling RBS design based on thermodynamic principles. In these cases, it becomes clear that synthetic designs are enabling pathway overexpression by orders of magnitude, and at some point, cellular resources are depleted (eg, transfer RNA [tRNA] pools), creating competition between cell growth and pathway expression. This is not yet accounted for by genome-scale modeling and presents a unique opportunity to integrate metabolic pathway tuning with genome-wide metabolic activity.

Gene expression tuning can also be engineered at the posttranslational level, where interactions with mRNA are the major focus. Small RNA (sRNA) bind targeted mRNA (through complementary base-pairing) and modulate its translation.⁵⁸ The majority of sRNAs have been identified as translation repressors, and binding generally occurs at or near the RBS.⁵⁹ Thermodynamic-based design has enabled “fine-tuned” gene expression knockdowns,⁶⁰ and these have proven advantageous in a metabolic engineering strategy to produce phenol from glucose.⁶¹ Similarly, artificial small interfering RNA and microRNA have been widely used in plant systems to reduce gene expression.⁶² Achieving stable gene integration in higher plants can be problematic. An alternative is to employ viral-induced gene silencing approaches.⁶³ While these technologies enable gene knockdown, they are generally operated from plasmid-based systems, which provide the same challenges to genome-scale modeling as mentioned previously.

Combinatorial approaches

Using genome-scale modeling to predict the outcomes of combinatorial metabolic engineering experiments is an area for many future advances. As shown in Figure 1, the addition of new genetic material (either synthetic or from other organisms) can expand the product-forming capabilities of an organism. Combinatorial approaches can involve induced chromosomal mutations, random insertion of transposons, genome shuffling, transcription factor engineering,⁶⁴ or even randomized chromosomal insertion of synthetic DNA.⁶⁵ Lycopene production has been engineered successfully through: 1) a combination of model-driven and transposon-based combinatorial knockouts⁶⁶ and 2) the multiplex automated genome engineering (MAGE) platform, which relies on synthetic DNA insertion.⁶⁵ In addition, gene overexpression libraries offer the opportunity to insert the genomic capabilities of a single organism or a metagenome. This strategy has proven successful in locating genomic sequences to confer tolerance to furfural,⁶⁷ among many others. The simultaneous expression of dual libraries on a plasmid and fosmid led to a unique combination of gene enrichment that increased acid tolerance in *E. coli* by 9,000-fold.⁶⁸ Expanding the genome to confer resistance to toxins or new/improved metabolic capabilities has the potential to redefine the relationship between product formation and culture growth, as shown in Figure 1. In the case of conferring resistance to toxins, often uncharacterized or non-obvious library fragments are selected during enrichment.⁶⁹ This is often because toxicity mechanisms, as well as many cellular interactions, are multigenic and still not understood fully. While genome-scale modeling cannot provide these types of predictions, where the interaction mechanisms are uncharacterized, the metabolic potentials through the completion and addition of new pathways and enzymes are predictable. It is likely that the theoretical limits of metabolic enhancement due to library enrichment can be found through genome-scale modeling, and the emergence of metagenomic GEMs will likely contain the metabolic potentials.

Phenotyping

Phenotyping refers to the monitoring of cell chemical composition and differentiation. This is critical because the GEM biomass equation contains the cell chemical composition and is representative of the cellular phenotype, which is known to change with genetic and environmental perturbations. The role of the biomass equation has been shown to be crucial in genome-scale modeling,^{14,70} creating the need for accurate

and near real-time monitoring techniques to interface with GEMs. In silico optimization methods have shown promising results,⁷⁰ but it is likely that an experimental approach will be needed as a supplement. Traditional methods of biomass equation generation are laborious and involve offline analytical methods. Analysis of heterogeneous populations of differentiating (eg, sporulating) microbes is now possible using flow cytometry.⁷¹ In addition, Raman spectroscopy has recently proven useful for near real-time phenotyping of *E. coli*. Raman spectroscopy also does not require the use of chemical labels and is nondestructive to the sample. In one application, Raman spectroscopy was used to resolve fatty acids (saturated, unsaturated, and cyclopropane), cell membrane fluidity, amino acids, and total protein content of cultures exposed to toxic 1.2% volume per volume 1-butanol (and control cultures) over a 180-minute time course.⁷² In another approach, “chemometric fingerprinting”, a multivariate statistical analysis involving principal component analysis and linear discriminate analysis, was used to classify the *E. coli* phenotypes resulting from exposure to different classes of antibiotics.⁷³ Chemometric fingerprinting is unique in that it uses the entire Raman spectrum to characterize a phenotype, whereas most approaches focus on only a few well-defined characteristic bands of the spectrum. With these types of near real-time analyses, GEM biomass equations can become dynamic and responsive to environmental and genetic changes. With current offline methods of phenotype characterization, this level of detail is not possible. However, with easily accessible phenotyping capabilities, biomass equations can be updated easily, leading to improved genome-scale modeling performance.

The path forward

New metabolic engineering targets and opportunities with plants

Deriving metabolic engineering strategies with genome-scale modeling is proving to be efficient and informative. As research continues to derive de novo metabolic pathways to synthesize valuable chemicals, optimization of product yield to meet industrial demands will be inevitable. Still, the variety of potential products from microbes remains limited and may be expanded in the near term by looking into complex eukaryotic species, such as plants. There are many valuable compounds made by plants that are not available elsewhere. For example, oil seed crops (eg, soybeans) produce edible vegetable oil that is used throughout the world. Although the pathways for lipid biosynthesis in higher plants have been studied

for years, understanding of the crucial regulatory mechanisms of these pathways remains limited. Thus, engineering plants to accumulate high levels of healthy omega-3 long-chain polyunsaturated fatty acids,⁷⁴ or modified non-native fatty acids as replacements for petroleum-derived chemicals in industrial processes⁷⁵ is desirable. Similarly, central carbon metabolism is a target for understanding the relationship between the regulation of carbon partitioning and biomass production in plants. Identifying metabolic bottlenecks in the production of cellulose, the energy-rich polymer that is targeted for consolidated bioprocessing,⁷⁶ could enhance efforts to produce more cellulose per plant in the field. Likewise, modeling is being applied to the goal of reducing plant lignin, a phenolic polymer in the secondary wall that limits our use of cellulosic biomass during industrial processing.⁷⁷ One caveat of reducing lignin is that optimal plant growth must also be preserved, and GEMs may be uniquely positioned to tackle this issue because they can theoretically integrate metabolic behavior with plant growth.⁷⁸ Enhancing the vitamin content of edible plants is another active area of research.⁷⁹ For some vitamin synthesis pathways, enough information exists to begin the application of genome-scale modeling to increase the concentration of vitamins to meet minimal requirements for humans.⁸⁰ In the future, it may be possible to use genome-scale modeling to tackle issues such as optimizing plant growth under stressful or poor nutrient growth conditions. In these cases, genome-scale models would have to account for complex interactions between stress, hormone, and other signaling pathways that impact biomass synthesis and composition.⁸¹ In addition, a related application is to understand how to limit plant yield loss due to pests, by engineering known, disease-resistance pathways.⁸¹ All of these approaches will require flexible biomass equations that can respond to manipulations, and a complex multicellular plant will likely require tissue-specific GEMs that will integrate to form an overall plant phenotype.

Enzyme engineering for pathway redirection

The k-OptForce *in silico* tool is among the first to incorporate the concept of enzyme engineering to redirect metabolic flux for the production of target chemicals. Kinetics-based approaches to genome-scale metabolic modeling are emerging,^{82,83} and soon enzyme redesign will be a valid metabolic engineering strategy. Direct genome editing, which is preferred over insertion of plasmids and markers that consume cellular resources, will enable easy implementation. Enzyme engineering is a complex field

itself and beyond the scope of this review, but effective *in silico* methods are emerging and are expected to play a role in enzyme redesign. Improvements in hardware and software performance will continue to expand the range and size of enzyme engineering problems and systems that can be studied. Current computational approaches can be divided into bioinformatics, molecular modeling, and *de novo* design.⁸⁴ Bioinformatics approaches are typically based on analysis of evolutionary data and can be used to change activity, selectivity, and stability within a family of enzymes. Molecular modeling approaches (eg, molecular dynamics, quantum mechanics/molecular mechanics simulations) have considerable potential to address challenges in computational enzyme design and redesign. In particular, advances in these methods may enable improved calculation of binding affinities and energy barriers, which will enhance understanding of enzyme specificity.⁸⁵ *De novo* design is also showing increasing promise in designing enzymes, including those that catalyze reactions for which nature has not designed a catalyst. Notably, these novel methods may be enhanced by the application of molecular modeling approaches.⁸⁶

Increasing modeling accuracy

Finally, the path forward must focus on methods that increase the accuracy of genome-scale metabolic flux modeling and improve agreements with ¹³C-isotopomer tracing studies. In our experience, there are four areas for immediate improvement. The first area includes the incorporation of a more detailed account of cellular machinery in GEMs. As mentioned previously, the ATP maintenance approximation of the GEM biomass equation should be replaced by mechanistic accounts. This must also allow for the identification of metabolic burdens of plasmids and altered metabolic states as a result of genome editing. There are current ongoing efforts of “whole cell modeling” that aim to include cellular machinery in modeling efforts. These models are showing promise of being able to predict phenotypes as well as better integrate and explain -omics datasets.⁸⁷ Second, more accurate biomass equations are needed. Whether these will be derived computationally or experimentally remains to be seen, and there are good arguments for both approaches. Third, a more accurate representation of flux branching at critical metabolic nodes is needed. This occurs when multiple enzymes can consume the same metabolite. Ultimately, the laws of thermodynamics (including enzyme availability) determine how that metabolite is distributed among the competing enzymes. Current methods of FBA, pFBA, FVA, ROOM (etc) do not consider this level of detail. FBrAtio provides this capability, though significant

strides are needed to first translate the biophysical constraints into flux ratio constraints. Finally, the roles of redox states in product secretion profiles and the influx/efflux of protons across the cell membrane need to be included as constraints in GEMs. In addition, efforts in these areas will supplement the many useful emerging tools that are focusing on genomic regulation and -omics dataset integrations. All of these will improve genome-scale modeling accuracy, which is needed for deriving effective metabolic engineering strategies.

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