Trends in Biotechnology

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Review

Metabolic Burden: Cornerstones in Synthetic Biology and Metabolic Engineering Applications

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Engineering cell metabolism for bioproduction not only consumes building blocks and energy molecules (e.g., ATP) but also triggers energetic inefficiency inside the cell. The metabolic burdens on microbial workhorses lead to undesirable physiological changes, placing hidden constraints on host productivity. We discuss cell physiological responses to metabolic burdens, as well as strategies to identify and resolve the carbon and energy burden problems, including metabolic balancing, enhancing respiration, dynamic regulatory systems, chromosomal engineering, decoupling cell growth with production phases, and co-utilization of nutrient resources. To design robust strains with high chances of success in industrial settings, novel genome-scale models (GSMs), ^{13}C-metabolic flux analysis (MFA), and machine-learning approaches are needed for weighting, standardizing, and predicting metabolic costs.

Opportunities and Challenges in Synthetic Biology (SynBio) Applications

Metabolic engineering has created diverse microbial cell factories for applications in the food, pharmaceutical, and biofuel industries, as well as for commodity chemical synthesis. Initially, industrial microbes were developed by random mutations or by expressing only one or two new enzymes. In the past decade new gene sequencing/synthesis/editing techniques have allowed complex genetic manipulations that permit the assembly of new cellular functions, and the International Genetically Engineered Machine (iGEM) competition promoted the concept of using standard modules (e.g., synthetic pathway and genetic circuits) for facilitating bioproduction [1,2]. Despite great technological advances, engineered microbial platforms cannot cheaply manufacture products because of poor production titer, rate, and yield [3,4]. Moreover, engineered microbes often show unpredictable or unstable physiology, and thus iterative 'build–design–test–learn' cycles need to be employed for strain improvement [5]. In March 2015, representatives from industry, academic institutions, and the National Institute of Standards and Technology (NIST) addressed a key issue preventing SynBio from reaching its potential: 'Unlike silicon-based electronic devices, synthetic organisms assembled from genetic components do not always have predictable properties' [6].

When a complex pathway is introduced into the host but shows low performance, one perspective is that the new pathway has 'intermediate toxicity' or 'low enzyme activity'. Therefore, strain improvement often focuses on searching for 'bottleneck' steps and tuning enzyme functions. Another possibility for low productivity in engineered strains is **metabolic burden** (see Glossary), a longstanding problem in biotechnology. Metabolic burden is defined

Trends

To commercialize recombinant organisms for renewable chemical production, it is essential to characterize the cost and benefit of metabolic burden using metabolic flux analysis tools.

Genome-scale modeling can incorporate ^{13}C-fluxome information and machine learning to predict the metabolic burden of synthetic biology modules.

Modularized expression of native or recombinant pathways using a variety of experimental tools for controlling expression can substantially reduce the metabolic burden introduced by these pathways.

The development of a standard synthetic-biology publication database may allow the use of machine learning or artificial intelligence to harness past knowledge for future rational design.

Detailed computational methods have been developed to model macromolecule synthesis (DNA, RNA, proteins) to account for the maintenance costs associated with basal cellular function.

Systems-level dynamic simulations and design algorithms can inform new approaches to engineering microbial production strains.

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as the proportion of the resources of a host cell – either energy molecules [e.g., NAD(P)H and ATP] or carbon building blocks – that are used to construct and operate engineered pathways [7]. Because intracellular carbon and energy resource distribution in healthy cells have been 'optimized' by natural evolution, hijacking cell resources for pathway overexpression, plasmid maintenance, and product synthesis may upset normal cellular processes.

Metabolic engineers first noticed metabolic burden. With the advance of recombinant DNA technology in the 1970s and 1980s, overexpression of proteins to produce desired products has been attempted, but this often results in reduced cell growth and increased mutation rates [7–9]. Metabolic burden can also cause inefficiency in cellular ATP supply, affecting broad cellular functions. A typical example is that *Azotobacter vinelandii* transformed with plasmids gradually lost its ATP-dependent N_2 fixation ability when the plasmid copy-number increased [7,10]. In another case, *Pseudomonas putida* completely lost its ability to synthesize siderophores after transformation with high copy-number plasmids [11]. Therefore, metabolic burden is a key factor leading to undesirable physiological changes, and the cost/benefit of SynBio strategies needs to be evaluated in the light of metabolic burden.

A 'Cliff' of Host Productivity under Metabolic Burden

ATP powers all cellular functions, and cells must produce sufficient ATP for both biosynthesis requirements and cellular maintenance. However, biological/physical factors restrict the capabilities of cellular energy metabolism [12]. For example, the ratio of cell membrane surface area to cell volume influences the overall mass exchange efficiency and the upper limit of nutrient and oxygen uptake rates [13]. For *Escherichia coli* cultures [14], the upper limits of glucose uptake rates are ~18 mmol/gDW/h (gDW: grams dry weight) in anaerobic conditions and 11 mmol/gDW/h in aerobic conditions, with 5.3 ATP molecules per glucose being consumed in aerobic conditions compared with 1.96 ATP per glucose in anaerobic conditions. Nutrient uptake places a hard constraint on cell catabolic rates. Moreover, ATP synthesis is a thermodynamically inefficient process (part of substrate energy is lost as heat) [15]. Under optimal conditions, oxidation of one NADH generates 2.5 ATP (i.e., P/O = 2.5) [15]. For engineered strains, proton gradients are dissipated through the cell membrane instead of charging ATP synthase, causing poor **phosphate/oxygen (P/O) ratios** (<2) [16–18]. Therefore, cell physiology and bioproduction are particularly sensitive to metabolic burden from ATP consumption.

To demonstrate the highly nonlinear impact of energy burden on biosynthesis, **flux balance analysis** (FBA) was used with a constraint-based model to simulate the adverse impacts of ATP metabolism on *E. coli* fatty acid, isobutanol, and acetate yields (Figure 1) [19]. The simulations showed that native cell metabolism can withstand some amount of ATP loss without displaying apparent biosynthetic deficiency (i.e., forming a 'plateau'). If cell metabolism demands more ATP, cells can utilize respiration (increase oxygen influx) to maintain their well-being. When catabolism and respiration become insufficient to accommodate the further increase of ATP expenditure, the biosynthetic yield will suddenly drop, forming a 'cliff' towards the 'death valley' (production yields drop to minimal levels). When the metabolic status of a strain is on the 'cliff', it may show unstable performance and become highly sensitive to suboptimal growth conditions (such as low oxygen level). Moreover, three products show differences in the yield plateau phenomenon. Fatty acid synthesis consumes both NADPH and ATP. Cell productivity is sensitive to oxygen supply, maintenance loss, and P/O ratios (Figure 1A,B). Isobutanol synthesis only consumes NAD(P)H, and it can be produced in the non-growth phase because of competition with amino acid biosynthesis. Therefore, the area of the 'death valley' in Figure 1C,D (showing isobutanol production) is reduced compared with fatty acid production, suggesting that isobutanol has a higher chance of stable industrial production. Acetate synthesis involves net ATP generation; its production has the largest yield plateau and thus is more likely to achieve robust production.

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Use of Flux Analysis To Precisely Measure Metabolic Burdens

Among SynBio tools, fluxomic measurements can provide direct knowledge of metabolic conversion of carbon sources into products. It is the only tool to understand the allocations of cell resources during biosynthesis. FBA uses the stoichiometry of biochemical reactions in addition to the measurement of inflow (uptake) fluxes to predict cellular phenotypes and biosynthetic yields. FBA can characterize cell energy metabolism by dividing maintenance costs into two categories [18]: **non-growth-associated maintenance** (NGAM) by 'resting' cells, and **growth-associated maintenance** (GAM). Owing to the size and underdetermined nature of metabolic networks, FBA has a high degree of uncertainty and requires constraints to analyze the solution spaces. ^{13}C tracing can assist FBA to rigorously measure functional pathways throughout the metabolic network (known as ^{13}C-**metabolic flux analysis**, or ^{13}C-MFA). ^{13}C-MFA can validate the function of genes or genetic circuits, provide knowledge on bottleneck nutrient sources, identify metabolic engineering targets, and directly quantify energy flows (i.e., ATP and cofactor generation and consumption). ^{13}C-MFA is widely used to identify specific flux changes in the network of such mutants.

During pathway engineering, cellular resource overexpenditure for heterologous enzyme synthesis can trigger the reorganization of carbon fluxes and reduce cell well-being [20]. To identify 'the straw that broke the camel's back', ^{13}C-MFA in combination with transcriptomics/proteomics and **genome-scale models** (GSMs) can provide a comprehensive understanding of cell responses to metabolic burdens at different cellular levels (from transcriptome to fluxome). ^{13}C-MFA is particularly useful to link these SynBio components (such as transcriptional regulators) to cell functional outputs. In general, gene expression may have an unpredictable impact on the distribution of fluxes because central metabolic fluxes are rarely regulated at the expression level alone [21]. For example, ^{13}C-MFA and transcriptional analysis for fatty acid production in *E. coli* using overexpression of the free fatty acid pathway and fatty acid transcription regulator revealed complex fluxome responses to fatty acid overproduction, including high ATP maintenance loss, decreased acetate flux, enhanced NADPH-producing pathways (pentose phosphate pathway and Entner–Doudoroff pathway), and strong transhydrogenation activity to achieve cofactor balance [22].

In the SynBio field, ^{13}C-MFA has not become a routine tool because of two bottlenecks. First, ^{13}C-MFA based on amino acid labeling cannot probe cell metabolism in a rich medium, for non-growing cells, or through a genome-scale metabolic network. To resolve such problems, innovative genome-scale MFA and isotopically nonstationary MFA will assist SynBio to gain fast snapshots of cell metabolism for improving strain performance [23–25]. However, the time required to analyze the samples and process the data to calculate fluxes through complex metabolism is still long (often taking months for an experienced lab to quantify cell metabolism correctly), and this method cannot satisfy a fast turnover cycle during build–design–test–learn. Second, ^{13}C-MFA focuses on global fluxes rather than on the activity of individual enzymes. Many SynBio hosts only achieve productivity at mg/l levels, and the priority is thought to be the improvement of heterologous enzyme activities in the production pathway. From this aspect, ^{13}C-MFA is useful when the strain performance is close to industrial performance levels.

SynBio Methods To Overcome Metabolic Burden

In traditional fermentation engineering, metabolic burden can be minimized through well-controlled bioreactor conditions (such as pH and oxygen) as well as by the use of different media (including yeast extract or other intermediates to boost cell metabolism). Early metabolic engineers also relied on random mutations or adaptive evolution for improved synthesis of growth-associated products, leveraging natural selection for reducing metabolic burden. For example, strains of *E. coli* can be 'trained' to have fast growth using lactate: over 250 generations of evolution its pathway capacity can be improved, especially for flexible metabolic

Glossary

Cipher of evolutionary design (CiED): an FBA approach that relies on a genetic algorithm to predict gene knockouts for improving fluxes through a metabolic pathway of interest.
Dynamic sensor and regulatory system (DSRS): an approach that uses biosensors for intracellular metabolites to regulate metabolic pathways.
Flux balance analysis (FBA): a mathematical approach for deriving possible metabolic fluxes in metabolic networks.
Genome-scale model (GSM): a metabolic network reconstructed based on genome-scale annotations.
Growth-associated maintenance (GAM): the amount of ATP consumption associated with biosynthesis.
Machine learning: a field of study that gives computers the ability to learn from a large amount of experimental data without being explicitly programmed.
Metabolic burden: the proportion of the resources of a host cell – either energy molecules [e.g., NAD(P)H and ATP] or carbon building blocks – that are used to construct and operate engineered pathways.
Metabolic flux analysis (MFA): an analytical method that uses isotopomers to measure *in vivo* enzyme reaction rates.
Non-growth-associated maintenance (NGAM): the amount of ATP consumption that does not contribute to biomass synthesis.
OptForce, OptStrain series: computational procedures that identify all possible engineering interventions in the metabolic model depending upon whether their flux values must increase, decrease, or become equal to zero to meet a pre-specified overproduction target.
Phosphate/oxygen ratio (P/O ratio): the amount of ATP produced by reduction of an oxygen atom.

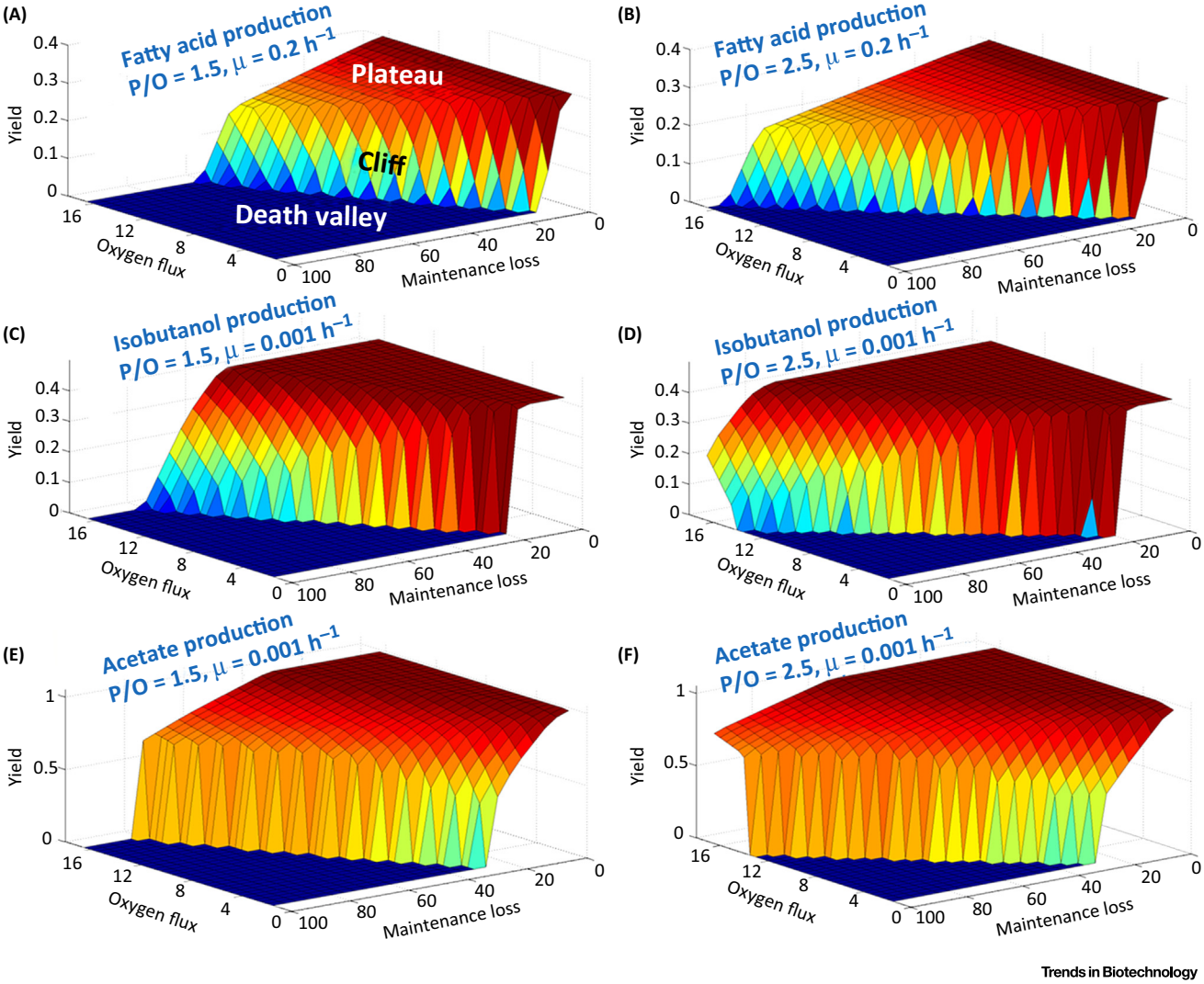

Figure 1. Biosynthesis Yields (g Product/g Substrate) in Engineered *Escherichia coli* in Response to Phosphate/Oxygen (P/O) Ratio, ATP Burden from Maintenance Loss (mmol ATP/g Dry Cell Weight/h), and O_2 Uptake Fluxes (mmol O_2/g Dry Cell Weight/h). A genome-scale metabolic model (iJO1366) is used for simulations [19].

nodes (such as phosphoenolpyruvate, oxaloacetate, and acetyl-CoA) [26]. In addition, co-cultures can also be a possible solution that divides a biosynthetic pathway into two sections, and then uses two species to share the metabolic burdens.

When SynBio tools became available, they allowed more elegant strategies to control cell metabolism at different cellular levels to reduce metabolic burden. Transcriptional regulators have attracted interest because alteration of some regulators may alleviate metabolic repression and unlock cellular carbon and energy fluxes [27]. Catabolite repression is present in various microorganisms and influences carbon, energy, and cofactor metabolism in response to carbon sources. For example, glucose is the preferred carbon source in *E. coli*. Deletion of catabolite repression regulators and overexpression of the genes necessary for pentose metabolism allow fast cellular growth using glucose and xylose simultaneously [28]. Moreover, Smolke and Keasling studied the effect of mRNA stability and DNA copy-number on protein production, and discovered that the correlations between DNA, mRNA, and protein levels are strongly

nonlinear [29]. Their results indicate that optimally tuning both plasmid copy-number and mRNA processing is essential for effective pathway construction. Currently, SynBio has already offered broad strategies to reduce energy burdens. Table 1 shows the common strategies to reduce metabolic burden and balance cell bioproduction, including copy-number optimization, transcriptional optimization, translational optimization, post-translational optimization, dynamic balancing, compartmentalization, and co-culture strain ratio optimization.

In SynBio, transcriptional/protein-level regulation of cell production has become a focal point. Among these strategies, the **dynamic sensor and regulatory system** (DSRS) approach employs a biosensor to detect the level of a metabolic intermediate and to control enzyme expression levels, which may be useful to prevent the biosynthesis of unnecessary RNAs/proteins/metabolites and increase the efficiencies of energy and carbon usage [30–32]. Unlike static control, DSRSs can promote or repress pathways according to cell growth conditions or intermediate metabolite concentrations. Thereby, DSRSs may also be used to decouple cell biomass growth and production phases (e.g., quorum sensing) such that cell resources can be focused on one major task at a time. Recently, CRISPR systems have been implemented for both chromosomal engineering and systematic downregulation of gene expression [33] (drawbacks of plasmid engineering include instability and burdens due to DNA/enzyme synthesis). Over the long term, SynBio may reprogram the entire cell genome to create a 'minimal or smart' cell of best energy fitness [34–36].

Promotion of Metabolic Capacity in the Microbial Chassis

SynBio has attempted to reduce metabolic burden by removing unnecessary genes from the microbial chassis. However, such efforts did not show significant benefits. For example, genome reduction of 332 dispensable genes in *Bacillus subtilis* did not affect the fluxome of the cell [37]. This result is consistent with the fact that DNA synthesis accounts for a very small usage of cellular resources. By contrast, cell energy metabolism has shown thermodynamic constraints for both biomass growth and bioproductivity, which are often limited and difficult to improve. For heterotrophic microbes, ATP generation is coupled with cell catabolism, and the outputs from ATP synthase are limited by the cell membrane space. In addition, SynBio components may interfere with the proton motive force and reduce ATP generation capacity [38]. Therefore, improving intracellular energy generation and catabolic metabolism can allow the hosts to carry more SynBio modules. Three directions can be pursued.

First, ATP is mainly synthesized through oxidative phosphorylation in aerobic metabolism. However, respiration rates in many strains are far below theoretical maxima in bioreactors. Respiration efficiency can be successfully increased using *Vitreoscilla* hemoglobin, which was first recombinantly expressed in *E. coli* [39]. This enzyme can improve oxygen membrane transfer and the P/O ratio, and reduce waste byproducts. Moreover, Zamboni and Sauer enhanced riboflavin production in *B. subtilis* by knockout of cytochrome *bd* oxidase to reduce cell ATP maintenance costs [40]. Engineering of respiration may apply to species with several sets of respiratory chains with different efficiencies [41,42], and successful application of this strategy is also closely related to other factors, such as oxygen concentration and the composition of the medium [43].

Second, photosynthetic microorganisms convert CO_2 to useful products using light as the energy source [44]. However, the photoautotrophic process has been hindered by CO_2 and light availability inside photobioreactors, which leads to low cell density and cost-inefficient harvesting. To circumvent these problems, a photomixotrophic strategy is advantageous [45]. Under conditions of sufficient light and glucose, microalgae can consume both CO_2 and glucose for biomass production, and this potentially leads to higher biomass density. The ability to utilize light as an energy source can be enabled for non-photosynthetic species through heterologous

Table 1. Summary of Experimental Metabolic Balancing Efforts[a]

Host	Product	Titer	Type of Pathway Optimization	Refs
Escherichia coli	Amorphadiene	293 mg/l/OD600	CN, TS	[74]
E. coli	Amorphadiene	N.r.	CN, TS	[75]
E. coli	Amorphadiene	1.6 g/l	PTL	[76]
E. coli	Amorphadiene	3.6 g/l	TL	[77]
Saccharomyces cerevisiae	Amorphadiene	20 mg/l	CP	[78]
S. cerevisiae	Valencene	1.5 mg/l	CP	[78]
E. coli	Poly-3-hydroxybutyrate	70% DCW	CN	[79]
E. coli	Lycopene	11 000 ppm	CN	[79]
E. coli	Neurosporene	4.2 mg/gDCW	TS, TL	[80]
E. coli	Mevalonate	740 mg/l	PTL	[81]
E. coli	Glucaric acid	1.7 g/l	PTL	[81]
E. coli	Glucaric acid	2.5 g/l	PTL	[82]
E. coli	Glucaric acid	1.2 g/l	DB	[83]
E. coli	*Cis,cis* muconic acid	2 g/l	SR	[84]
E. coli	*Cis,cis* muconic acid	4.7 g/l	SR	[85]
E. coli	4-Hydroxybenzoic acid	2.3 g/l	SR	[85]
E. coli	Myo-inositol	1.31 g/l	DB	[86]
E. coli	Riboflavin	2.70 g/l	TL	[87]
Aspergillus nidulans	Penicillin	N.r.	CP	[88]
S. cerevisiae	Xylose utilization	N.a.	TS	[89]
E. coli	Chondroitin	2.4 g/l	TS	[90]
E. coli	Violacein	1.83 g/l	TS	[91]
S. cerevisiae	Violacein and derivatives	N.r.	TS	[92]
E. coli	(+)-Catechin	911 mg/l	CN, PTL	[93]
E. coli	Caffeic acid	3.8 g/l	TS	[94]
E. coli	Caffeic acid	106 mg/l	CN	[95]
E. coli	Resveratrol	35 mg/l	CN, TS	[96]
E. coli and S. cerevisiae	Oxygenated taxanes	33 mg/l	SR	[97]
E. coli	Taxadiene	1.02 ± 0.08 g/l	CN, TS	[98]
E. coli	Fatty acids	8.6 g/l	CN, TL	[99]
E. coli	Fatty acids	3.9 g/l	DB	[30]
E. coli	Fatty acid ethyl esters	1.5 g/l	DB	[31]
E. coli	Butyrate	7.2 g/l	PTL	[100]
E. coli	Butanol	6.2 g/l	SR	[101]
S. cerevisiae	Isobutanol	635 mg/l	CP	[102]
S. cerevisiae	Isopentanol	95 mg/l	CP	[102]
S. cerevisiae	2-Methyl-1-butanol	118 mg/l	CP	[102]

[a]Abbreviations: CN, DNA copy-number optimization; CP, compartmentalization; DB, dynamic balancing; n.a., not applicable; n.r., not reported; PTL, post-translational optimization; SR, co-culture strain ratio optimization; TL, translational optimization; TS, transcriptional optimization.

expression of proteorhodopsins [46–48]. Proteorhodopsins are a class of membrane proteins distributed in diverse species ranging from bacteria to archaea and fungi [49]. They can absorb energy from light of different wavelengths and generate a proton gradient for use in ATP synthesis. Compared with photosystems, a distinctive advantage for proteorhodopsin is its simplicity for heterologous photosynthesis [50]: the function of proteorhodopsin relies on a single protein of 249 amino acids. Hence, the metabolic costs associated with heterologous expression of proteorhodopsins will be much smaller than of intact photosystems. An added potential benefit is that species with proteorhodopsin have demonstrated increased tolerance to environmental stress [46,48].

Third, H_2 can be utilized by a broad range of microbes. However, the use of hydrogen as the energy supply in industrial fermentations is not preferred. This is due to various undesirable properties of H_2 such as its low solubility, volatility, and explosiveness. Compared with H_2, formate is a better source of energy supply in terms of uptake efficiency. The utilization of formate by microbes as an extra energy source was reported for *Candida utilis* that can uptake formate in the presence of glucose to increase biomass yield [51]. Meanwhile, similar phenomena were observed in *Hansemula polymorpha* and *Pichia pastoris* [52,53]. The strategy of employing formate as an extra energy source has been extended to other species that have a formate dehydrogenase (*fdh*), such as in oleaginous yeasts for improving lipid production [54], *Penicillium chrysogenum* for enhancing penicillin productivity [55], and *Bacillus thuringiensis* for promoting thuringiensin yield [56]. Further, formate can be generated through an electrochemical process to feed engineered *Ralstonia eutropha* to produce isobutanol and 3-methyl-1-butanol [57].

$$\text{Formate} + \text{NAD} \rightarrow CO_2 + \text{NADH}$$

By contrast, heterologous expression of an *fdh* gene enables formate usage in hosts that lack the ability to oxidize formate. For instance, after chromosomal insertion of the *fdh* gene, *C. glutamicum* was able to utilize formate and produce 20% more succinate anaerobically in the presence of glucose (formate was used both to generate NADH and as a carbon donor) [58]. In yet another case, *fdh* was introduced into a succinate-producing *E. coli* strain, leading to reduced formate and improved succinate yield [59].

Current Metabolic Modeling for Rational Metabolic Engineering

Metabolic burden has a direct affect on the biochemical productivity of engineered strains, and thus it will be important to explicitly consider metabolic burden during the design process. Numerous good review papers are available that cover different tools and conceptual approaches to model-driven metabolic engineering design (Box 1), including a review by Medema *et al*. [60] that presents *de novo* design as involving six steps. The steps include: (i) pathway prediction (*de novo* discovery of possible biosynthetic routes), (ii) pathway prioritization (ranking of possible pathways), (iii) metabolic modeling (modeling biochemical function in network context), (iv) pathway selection, (v) pathway refactoring and integration (molecular-level design specification of necessary DNA constructs), and (vi) product synthesis. These design approaches primarily focus on specifying biochemical pathway usage necessary to produce theoretical yields of a desired product, but experimental strains fall short of theoretical yields as a results of unaccounted *in vivo* considerations such as overall metabolic burden.

Cell-Wide GSMs for Complex Cell Systems

GSMs are stoichiometric representations of the biochemical capacity of a metabolic network based upon the genome contents of an organism. A key to this approach is establishing gene–protein–reaction (GPR) relationships, where identifying a gene in the genome implies the possibility of the associated protein and biochemical reaction. However, if details associated

Box 1. Rational Algorithmic Design

In this overall framework, some of the most heavily developed work has focused on the metabolic modeling component that is directly tied to metabolic burden. Metabolic control analysis (MCA) is the earliest approach that quantified the correlations between the flux through a pathway and the kinetics of the constituent enzymes. MCA can describe the different levels of flux regulation through a single pathway, but not yet for the distribution of flux through the entire network [21]. By contrast, GSMs used in conjunction with flux balance analysis constitute the most commonly used approach to estimate optimal growth rates and product yields from different feedstocks and the lethality of gene knockouts. Such GSMs can identify the best biosynthesis pathways through elementary mode analysis [103] or systematic pathway modifications (e.g., OptKnock [23,24] and **OptStrain** [25]).

A methodology for predicting optimal metabolic landscapes for the production of a desired metabolite, termed **cipher of evolutionary design** (CiED) [104], was initially developed by integrating an evolutionary algorithm around constraint-based modeling, allowing one to simultaneously search an unlimited number of gene targets for modifications. Furthermore, another constraint-based modeling approach termed **OptForce** [72,104–106] was utilized to identify genetic perturbations leading to increases in intracellular malonyl-CoA. OptForce suggested the upregulation of glycolytic reactions, namely glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, as well as upregulation of pyruvate dehydrogenase and acetyl-CoA carboxylase. It also suggested downregulation of reactions in the citric acid cycle, namely malate dehydrogenase, fumarase, and aconitase, to reduce the drain of carbon towards TCA cycle products. OptForce also suggested reducing the activity of TCA reactions instead of completely eliminating them.

with the molecular aspects of moving from DNA to mRNA and mature protein are absent (as is the case with basic GSMs), then these models allow any/all reactions to be used as much as needed to fulfill an objective without any restrictions, meaning that there is no explicit cost to expressing a gene.

A substantial amount of work to improve GSMs has been undertaken recently to account for this shortcoming and to allow computational consideration of metabolic costs/burden. Beginning with a careful curation of literature associated with *E. coli*, an expression matrix (E-Matrix) was developed that included 13 694 reactions associated with all facets of transcription and translation [61] to account for the synthetic reactions for DNA, mRNA, proteins, and protein complexes in a sequence-specific manner. The E-Matrix formalism was directly integrated with metabolic networks to create ME (metabolism and expression) models of *Thermoto gamaritime* [62] and *E. coli* [63]. The ME models involved extensive attention to molecular detail and the formulation of new objective functions for simulations, and have resulted in improved predictive power including the ability to predict transcriptomes and proteomes *in silico* [64].

In addition to GSMs, a whole-cell model of *Mycoplasma genitalium* has been generated using 28 modularized subsystems [65]. The approach of this work differed from the development of ME models: in the case of the whole-cell *Mycoplasma* model, different modeling approaches (e.g., ordinary differential equations, Boolean statements, probabilistic, constraint-based) were implemented for the different modules to permit flexibility and to dynamically model each process in an appropriate way. By developing and integrating modules, it was possible to simulate dynamic whole-cell function at a high level of detail. Subsequent to this initial publication, the WholeCellKB database has been established to facilitate development of additional whole-cell models using the modularized approach implemented for *Mycoplasma* [66].

Integration of Metabolic Burdens of Pathway Engineering in GSMs

As metabolic engineering design strategies become more sophisticated and involve the implementation of larger DNA constructs, it has become increasingly important to consider the implications of the metabolic burden imposed by the expression of synthetic constructs. The approaches discussed above are possible frameworks for implementing a new level of modeling detail that can account for metabolic burden associated with the expression and activity of proteins. Improvements to computational modeling frameworks to explicitly include metabolic burden considerations should greatly facilitate metabolic engineering design strategies and help to avoid strategies that impose a large non-native metabolic burden. For example, mutants may

have decreased productivity as a result of ATP loss associated with heterologous gene expression. Previous studies have shown that protein synthesis consumes >60% of the ATP from cellular energy metabolism [67], while protein overexpression is a major metabolic burden in engineered cells [7]. Based on experimental proteomic studies in *E. coli* [68], the energy costs for the synthesis of a single protein can be calculated to range from about 400 to 50 000 high-energy phosphate bond equivalents. The average cost per amino acid ranged from 18–38 high-energy phosphate bond equivalents. The unit of phosphate bond equivalents can be related to cellular energy as stored in the phosphate bonds of ATP. Considering protein synthesis costs in terms of phosphate bonds enables a new level of energy-associated analysis within the framework of GSMs that can predict overall metabolic burden. By explicitly considering the protein production cost and amino acid composition of individual proteins, metabolic burden associated with enzymes (and their affiliated biochemical reaction) can be analyzed.

The use of phosphate bond costs associated with each amino acid provides a means to connect computational simulations with experimental ^{13}C-MFA results related to ATP generation and consumption. In addition, FBA may be used to calculate metabolic burdens from synthesis of plasmid DNA (based on sizes and copy-numbers). Moreover, ^{13}C-MFA can be combined to offer comprehensive insights into the intracellular activities responding to the increase in corresponding metabolic reactions [69]. Traditional FBA models lack a precise determination of ATP loss, while ^{13}C-MFA can provide a quantitative measurement of ATP losses: ATP maintenance is the sum of the all of the cellular burdens for all the different metabolic functions

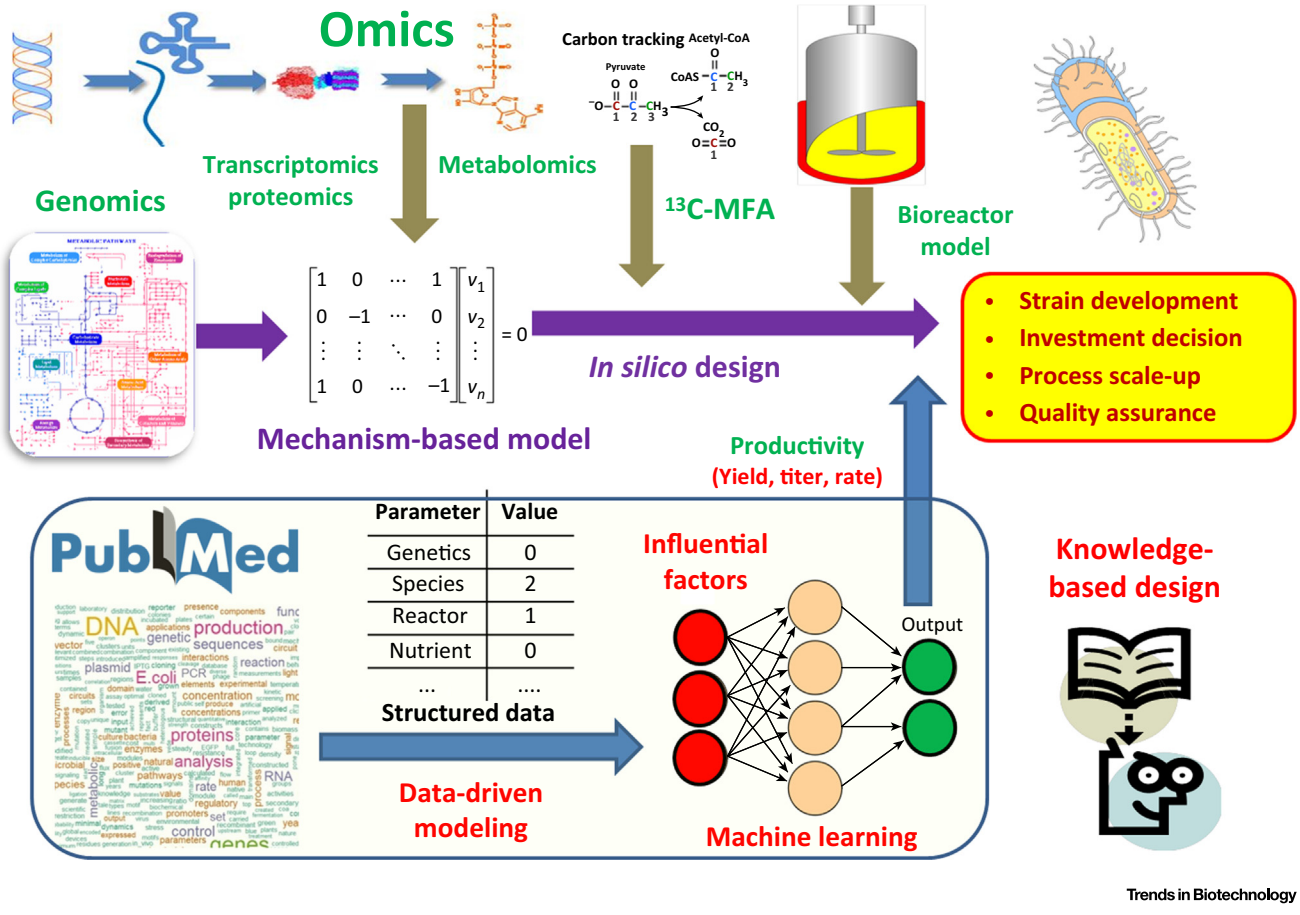

Figure 2. A New Modeling Paradigm for Synthetic Biology.

(protein expression, energy consumption associated with individual reactions, etc.), and thus is a cumulative result of explicitly accounting for burden in individual components of the FBA model. Lastly, RNA profiles can be used with the genome-scale FBA modeling components to provide weighting to expression costs. The integration of high-throughput transcriptomics with ^{13}C-MFA and metabolic models can also be used for the analysis of regulatory networks (such as SynBio circuits).

Innovative Models for Assisting SynBio Applications

First, integrating FBA with process models is important to study scale-up fermentations because large bioreactors are often dynamic and heterogeneous. These multi-scale models can offer knowledge of intracellular function and overall fermentation performance. For example, dynamic FBA (a combination of kinetic models and FBA) simulates the industrial fermentation of *E. coli* to produce recombinant proteins [19]. The hybrid of a constrained FBA model with an agent-based model can successfully simulate highly heterogeneous systems (e.g., biofilm) [70]. Integration of FBA with other information or models will be widely adopted to strengthen the capabilities of modeling tools and to provide guidance when moving laboratory strains to industrial applications [71].

Second, industrial standards play fundamental roles in R&D and communication within their respective disciplines. In the case of SynBio, the lack of a standard for journal publications hinders fast communication among SynBio researchers as well as constraining the reproducibility of published work. We would therefore emphasize the need for standardization of publications. For example, published papers need to clarify their reports on: (i) genetic methodologies; (ii) fermentation conditions such as substrate type, incubation mode, nutrient condition, etc.; (iii) production titer, rate, and yield. For high-profile papers, tracer experiments should be employed to make sure the engineered pathways are actually functional.

Third, data-driven models using published SynBio papers can provide complementary information to mechanism-based models. For example, a linear empirical model with numerical and categorical variables was generated to predict production yields based on dozens of published papers involving *E. coli* and *Saccharomyces cerevisiae* [8,19]. This study identified key factors controlling production yields, including biosynthetic steps, metabolic engineering methods, nutrient supplementation, and fermentation conditions. The large number of papers published on metabolic engineering provides a rich database for performing **machine learning** studies and for capturing microbial outputs (production titer, rate, yield) in response to genetic and fermentation conditions. Lessons from the past can allow us to better evaluate SynBio projects. Published papers can be stored as structural data such that machine-learning methods can be used to extract knowledge for future rational strain development. Machine learning evolved from pattern recognition, statistics, and optimization, and makes data-driven predictions for outcomes of mutant physiologies [72]. It can assist mechanistic-based GSMs for rational metabolic engineering (Figure 2).

Concluding Remarks

The long-held assumption of never-ending rapid growth in biotechnology in general, and of SynBio in particular, has been recently questioned owing to lack of substantial return of investment [73]. One of the main reasons for failures in SynBio is the largely unaccounted for metabolic burden that may offset SynBio benefits, and thus careful consideration is necessary at the stage of strain design (see Outstanding Questions). To avoid inappropriate commercial decisions or investments in such 'perpetual motion machine' types of projects, ^{13}C-MFA provides excellent measures and standards for SynBio that define the limitations of SynBio, leading to rational metabolic or bioprocess engineering strategies to bypass metabolic burdens (e.g., the use of co-cultures or integration of biological conversion with chemical conversions).

Outstanding Questions

From the viewpoint of metabolic burden and the tradeoffs of cell fitness, what types of chemicals (or biosynthetic pathways) are the most suitable to be produced (or engineered) using SynBio and metabolic engineering approaches?

Can we reprogram cell carbon and energy metabolism such that it can support complex SynBio components inside the cell?

Can we rigorously quantify and predict metabolic loads from SynBio modules?

What types of genetic modifications do we need to introduce to minimize metabolic burden and maximize product titers?

Can computational models accurately predict cost/benefit tradeoffs of genetic engineering at a system level?

Will cells engineered with metabolic burden criteria lead to new commercially viable bioprocesses?

More importantly, constructing new genome-scale modeling analyses and novel machine-learning methods can give broad guidelines for strain development and resolve metabolic imbalances arising from SynBio applications.

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