



## Parts plus pipes: Synthetic biology approaches to metabolic engineering

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### ABSTRACT

Synthetic biologists combine modular biological “parts” to create higher-order devices. Metabolic engineers construct biological “pipes” by optimizing the microbial conversion of basic substrates to desired compounds. Many scientists work at the intersection of these two philosophies, employing synthetic devices to enhance metabolic engineering efforts. These integrated approaches promise to do more than simply improve product yields; they can expand the array of products that are tractable to produce biologically. In this review, we explore the application of synthetic biology techniques to next-generation metabolic engineering challenges, as well as the emerging engineering principles for biological design.

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### 1. Introduction

Engineering cellular metabolism requires an understanding of the metabolic reactions involved as well as the regulatory elements that affect metabolic throughput. Our ability as engineers to modulate metabolic pathways has been augmented in recent years by the influx of methods and biological devices from the field of synthetic biology (Fig. 1).

A primary goal of synthetic biology is to develop engineering principles for biology—to translate a quantitative understanding of biological systems into a methodology for building living devices out of standardized biological parts. The advent of cost effective DNA sequencing and de-novo synthesis has resulted in a tremendous increase in the number of potential biological parts available to synthetic biologists (Boyle and Silver, 2009). The development of assembly standards and open databases has facilitated the development and sharing of these parts (Anderson et al., 2010; Knight, 2003; Phillips and Silver, 2006) (<http://partsregistry.org/>). The panoply of synthetic biological devices developed over the last decade has demonstrated that quantitative control over biological systems is possible in many contexts (Agapakis and Silver, 2009; Arkin and Fletcher, 2006; Boyle and Silver, 2009; Drubin et al., 2007; Endy, 2005; Haynes and Silver, 2009; Tyo et al., 2007).

Many synthetic biology endeavors also fall under the umbrella of metabolic engineering. Maximizing the production of a desired metabolite from a given feedstock mandates a quantitative

evaluation and adjustment of cellular metabolism. To achieve this, synthetic biologists and metabolic engineers have sought fundamental engineering principles for biology. These principles have been inspired by traditional engineering disciplines as well as the unique properties of biological systems. In this review, we will explore both rational and evolutionary approaches to improving metabolic pathways.

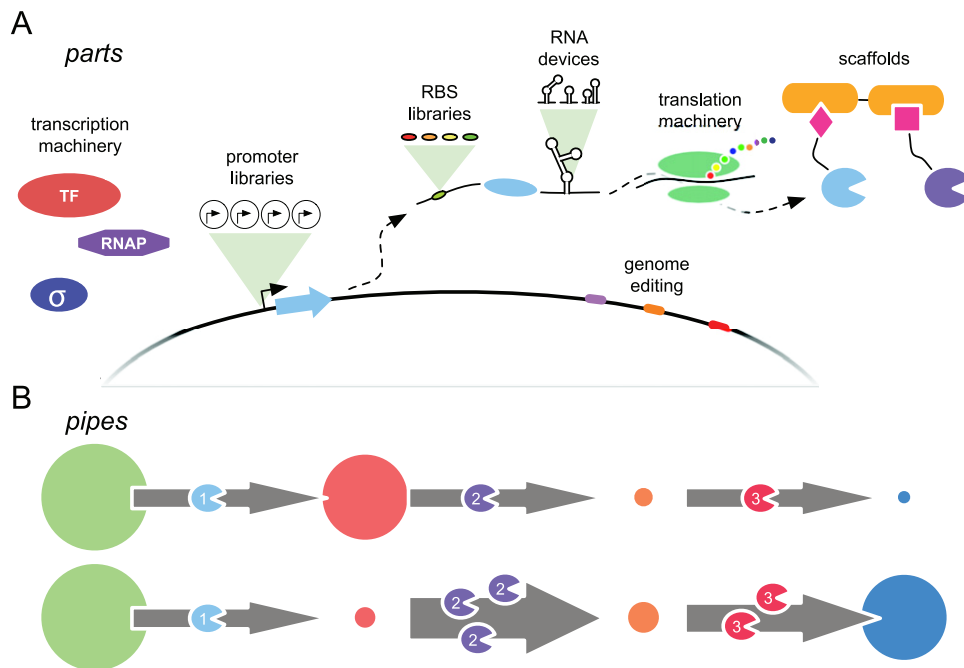
### 2. Transcriptional and translational pathway control

A central challenge for every metabolic engineering project is to maximize product yields through pathway optimization (Keasling, 2010). Natural metabolic pathways are controlled by myriad regulatory systems, for example transcription factors and promoters that can be repurposed by synthetic biologists to modulate pathway components. Ideally, a quantitative understanding of the transcription, translation, interactions, and kinetics of a metabolic pathway as well as how that pathway interfaces with the host cell's metabolism enables the metabolic engineer to tune pathway components to maximize product yields. In practice, our ability to tune pathways has improved as the fundamental principles of metabolism and biological regulation continue to be discovered.

Many synthetic regulatory devices to date have leveraged elements of biology's “central dogma”—transcription, translation, as well as RNA processing—to modulate device behavior (Boyle and Silver, 2009). In the context of metabolic engineering, modifications to biological regulation are intended to maximize metabolic flux to the desired product. In most cases, this is accomplished via adjustments in enzyme expression levels, along

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**Fig. 1.** Parts and pipes for the optimization of metabolic pathways. (A) Synthetic biologists use a variety of parts to adjust the functioning of metabolic pathways. Transcription machinery, enzyme promoters, ribosome binding sites (RBS), and translational machinery can be modified to adjust the concentration of an enzyme. RNA devices can modulate mRNA degradation and translation efficiency. Pathway enzymes can be assembled on scaffolds to optimize the spatial organization of a pathway. Genome editing approaches can be used to adjust host metabolism to improve flux through the target pathway. (B) A “pipe” of key pathway enzymes can be tuned to increase product titers. In this conceptual example, enzyme flux is represented by the size of the gray arrows. Metabolite concentrations are represented by the size of the circles between enzymes. In this example, increasing the concentration of the second and third enzymes in the pathway increases the titer of the product. Note that decreasing the concentration of intermediate metabolites can be beneficial; this is often the case when intermediates are harmful to the host cell. Increasing enzymes does not always improve product titers and can in fact be detrimental. In this review, we present synthetic biological parts that enable optimization of metabolic pipes.

with the elimination of competing pathways via gene knockout (Stephanopoulos, 1999).

The structure and function of evolved metabolic networks suggests that this process of pathway optimization requires an understanding of how control is distributed across the entire pathway (Dekel and Alon, 2005; Fell, 1997; Zaslaver et al., 2004). In essence, pathway optimization is a multivariate problem, with no single “rate limiting step” to target. Furthermore, simple overexpression of pathway enzymes is often detrimental to product yields, through both the depletion of essential cellular reserves and the buildup of toxic metabolic intermediates (Alper et al., 2005; Jones et al., 2000; Raab et al., 2005). Efforts to model synthetic biological circuits have also revealed that desired device behavior is highly dependent on the concentration of the device components within cells (Ajo-Franklin et al., 2007; Anderson et al., 2007; Elowitz and Leibler, 2000). As a consequence, methods for the control of protein expression levels are essential to metabolic engineering and synthetic biology in general.

### 2.1. The rational approach

Ideally, a quantitative understanding of the pathway to be engineered can allow metabolic engineers to determine optimal expression level of pathway elements *a priori*. Synthetic biologists have found that tight control of protein concentrations is required to achieve robust behavior of genetic circuits (Ajo-Franklin et al., 2007; Anderson et al., 2007; Boyle and Silver, 2009). Forward engineering of metabolic pathways can be facilitated by a variety of standardized and characterized control elements available to the metabolic engineering community.

For decades, promoter elements have been used to modify gene expression (Reznikoff et al., 1969). In recent years, a number of groups have assembled and characterized promoter libraries

for common industrial hosts, such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris* (Alper et al., 2005; Cox et al., 2007; Davis et al., 2010; Hartner et al., 2008; Nevoigt et al., 2006). In each case, native promoters were mutated or recombined to generate a group of promoters of varying strengths. Work has begun to develop standard metrics for promoter characterization, but remains dependent on high-throughput screening of promoter libraries rather than *in silico* prediction (Bayer, 2010; Kelly et al., 2009). This issue is compounded by the contextual variability of expression levels in response to environmental factors such as temperature or carbon source (Kelly et al., 2009).

Ribosome Binding Sites (RBS) mediate translation initiation, with variation in RBS sequence directly affecting translation efficiency. Thermodynamic models of translation initiation have been generated that now allow *a priori* design of RBS appropriate for a desired expression level. The RBS Calculator (<http://salis.psu.edu/software/>) generates a customized RBS for a given gene based on the desired translation initiation rate, gene sequence, and host organism. The RBS Calculator was successfully utilized to predict RBS combinations that would permit the desired operation of a synthetic AND gate (Salis et al., 2009), a device that is highly dependent on the expression levels of the inputs to produce AND gate output (Anderson et al., 2007).

Modification of RNA degradation rates can also control steady-state expression levels. In *S. cerevisiae*, the Rnt1p RNase recognizes and cleaves a specific class of RNA hairpin (Lamontagne et al., 2003). When Rnt1p target hairpins are placed in the untranslated region (UTR) of an mRNA transcript, Rnt1p degradation lowers the effective expression level of the target gene. A library of variable Rnt1p target hairpins has been constructed that permits quantitative control of *S. cerevisiae* gene expression (Babiskin and Smolke, 2011). A subset of this library was inserted into the 3' UTR of GFP, mCherry, and squalene synthase (*ERG9*).

The strong rank order correlation of expression level between the GFP and mCherry variants ( $\rho=0.848$ ) and between the GFP and ERG9 variants ( $\rho=0.844$ ) indicates that Rnt1p elements are largely modular (Babiskin and Smolke, 2011).

Constitutive promoters and RNA elements are useful for maintaining a steady-state expression level. In a non-steady-state environment, cells maintain homeostasis by reacting to environmental changes; endogenous metabolic pathways dynamically respond to changes in intracellular metabolite concentrations. Regulated gene expression (Beckwith, 1967; Jacob and Monod, 1961), RNA riboswitches (Mandal and Breaker, 2004), and allosteric control of enzyme activity (Monod et al., 1963) provide this control over a wide range of contexts and timescales. Designing similar dynamics into engineered pathways could improve the performance of engineered strains at industrial scales, where reactor conditions are not always uniform (Holtz and Keasling, 2010).

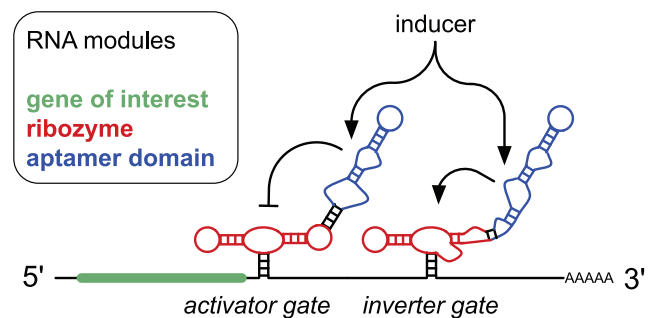
Modular RNA elements can be designed to provide a dynamic response to intracellular metabolite levels. Riboswitches are natural RNA elements that undergo a conformational change in response to a small-molecule ligand. When riboswitches are part of an mRNA molecule, this conformational change modulates the translation of the mRNA sequence. (Nahvi et al., 2002; Stoddard and Batey, 2006). Many riboswitches have been discovered in untranslated regions of mRNAs encoding for metabolic enzymes, offering a post-transcriptional layer of control over enzyme levels.

The potential for RNA-based multisite pathway modulation is exemplified in the 11 known S-adenosylmethionine (SAM) dependent riboswitches of *Bacillus subtilis*. In *B. subtilis*, much of the methionine biosynthesis pathway is regulated by SAM dependent riboswitches. These riboswitches function primarily through SAM-dependent conformational changes that trigger premature transcriptional termination, although a smaller subset disrupts translation initiation instead. Remarkably, each of the 11 riboswitches is independently tuned to a different SAM concentration. Furthermore, the termination efficiency of each SAM riboswitch in both the ligand bound and unbound conformations are different for each gene (Tomsic et al., 2008). Augmenting engineered metabolic pathways with small-molecule responsive RNA regulators could offer similarly distributed control (Beisel and Smolke, 2009).

A variety of synthetic RNA regulators have been designed to control gene expression. Synthetic RNA regulators can interact in *cis* with mRNA via aptamer domains to respond to small molecules (Bayer and Smolke, 2005; Win and Smolke, 2008), or make use of *trans*-acting RNA elements expressed off of an inducible promoter (Callura et al., 2010; Isaacs et al., 2004). Robust methods have been developed for the selection of RNA aptamer domains (Gilbert and Batey, 2005), and modular RNA elements can be combined to generate higher-order behaviors. For example, pairs of RNA aptamer domains alternately promoting or inhibiting translation of a transcript can serve as “bandpass filters,” permitting mRNA translation between the range of concentrations set by the aptamer domains (Win and Smolke, 2008) (Fig. 2). Combining promoters and RBS tuned for steady-state performance with dynamically regulated RNA regulators may improve the robustness of engineered pathways.

## 2.2. The rationally irrational approach

Synthetic biologists strive to make biology “engineerable”—to discover modular biological elements that can be predictably assembled and designed to function robustly. Efforts to produce and characterize libraries of standardized parts have made progress towards this goal, yet the complexity of biological systems has kept biological engineering firmly in the trial and error stage.



inducer concentration	ribozyme activity	ribozyme activity	mRNA translation
low	on	off	off
medium	off	off	on
high	off	on	off

**Fig. 2.** An RNA-regulated bandpass filter. Two modular RNA regulators added to the 3' untranslated region of an mRNA can be used to control mRNA translation in response to the concentration of a small molecule. Each regulator contains a self-cleaving ribozyme, coupled to an RNA aptamer domain. The activator gate ribozyme is repressed by the inducer, while the inverter gate ribozyme is activated by the same inducer. If the inducer concentration is between the ligand-binding thresholds of the two gates, both ribozymes are inactive and translation of the transcript is permitted (Win and Smolke, 2008).

Even synthetic devices with well-defined parameters for desired behavior require exhaustive characterization of the biological components to achieve functionality (Ajo-Franklin et al., 2007; Anderson et al., 2007). However, trial and error through the process of evolution has generated the biological diversity that synthetic biologists seek to redesign. In addition to traditional engineering principles, engineers of biological systems have access to the tools of selection and evolution; these tools can be leveraged to discover improvements to metabolic pathways. The ability to use these “rationally irrational” approaches is a core advantage to engineering biological systems.

Early metabolic engineering efforts relied on genomic mutagenesis to generate strains with desired properties (Stephanopoulos, 1999). If the phenotype of interest is accessible via a single mutation, mutagenesis is an acceptable approach. If the desired phenotype requires multiple mutations, however, the combinatorial expansion of the library size required to identify that phenotype makes untargeted mutagenesis practically infeasible (Dietrich et al., 2010b). Generating variation in a targeted subset of the genome enriches the resulting library for mutants with relevant phenotypes (Carr and Church, 2009).

Mutagenesis of the cellular transcriptional machinery can be used to adjust gene expression levels. In engineered cells, endogenous regulation often interferes with the functioning of heterologous pathways. Global Transcription Machinery Engineering (gTME) is an approach that modifies relative transcription rates across all genes simultaneously by selectively mutagenizing genes involved in the initiation of transcription. For example, mutagenesis of the *S. cerevisiae* TATA-binding protein *SPT15* and selection for improved ethanol tolerance yielded a mutant with a 20% higher biomass yield than the parent strain (Alper et al., 2006). GTME in *E. coli*, targeting the primary sigma factor  $\sigma^{70}$ , saw similar gains when applied to ethanol tolerance as well as 50% gains when applied to lycopene production (Alper and Stephanopoulos, 2007).

A more targeted approach to pathway adjustment is to selectively alter the regulation of pathway genes. Introducing RNAse cleavage sites or hairpin structures that alter mRNA stability into intergenic regions can result in different translation rates for two ORFs on the same mRNA (Smolke et al., 2000). Tunable Intergenic Regions (TIGR) are synthetic RNA constructs that include two hairpins joined by a RNAse cleavage site, and can be used to connect co-transcribed ORFs. Libraries of TIGR elements with a wide variety hairpin structures can be inserted between two co-transcribed genes to screen for optimal translation ratios (Pfleger et al., 2006).

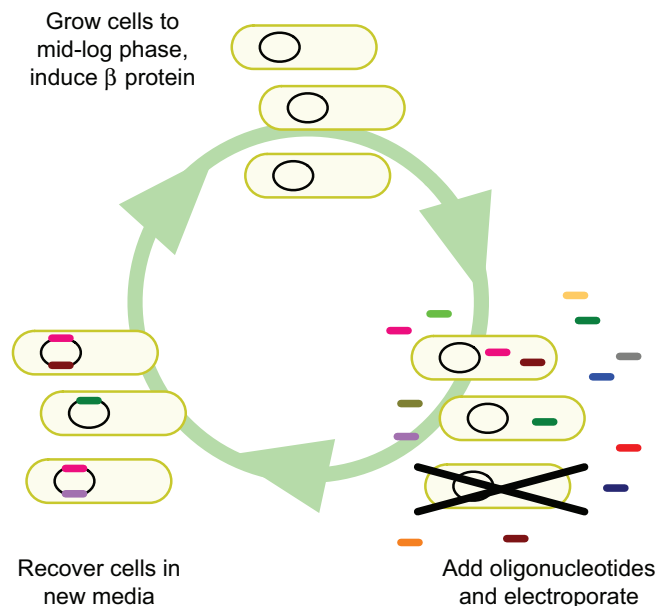
TIGR elements have been used to improve the production of mevalonate in *E. coli* (Pfleger et al., 2006). This pathway requires the expression of acetoacetyl-CoA thiolase (AtoB), as well as the heterologous expression of hydroxy-methylglutaryl-CoA synthase (HMGS) and hydroxy-methylglutaryl-CoA reductase (HMGR). Inserting a library of TIGR elements into the AtoB-HMGS-HMGR operon identified a combination that increased mevalonate titers seven-fold over the initial AtoB-HMGS-HMGR operon. Each of the four best mevalonate producers identified in the screen lowered the expression levels of HMGS and HMGR relative to AtoB (Pfleger et al., 2006). HMG-CoA, the product of HMGS, was later shown to be cytotoxic to *E. coli* (Kizer et al., 2008); the best operons identified in the TIGR-mevalonate screen maintained or lowered HMG-CoA concentrations 11 h post-induction versus the parent strain (Pfleger et al., 2006).

New mutagenesis strategies are enabling iterative and simultaneous mutation of gene regulatory elements. Multiplex Automated Genome Engineering (MAGE) is a high throughput technique for the directed evolution of microbial genomes (Wang et al., 2009). MAGE combines both rational design and directed evolution approaches; specific genomic targets are selected for mutagenesis. For each genomic target, pools of degenerate oligonucleotides that retain homology to the target sequence are electroporated into the cells to be engineered. Multiple pools of oligonucleotides can be combined in a single electroporation step, allowing multiple genomic loci to be modified simultaneously. Iterative rounds of electroporation and growth generate a mixed population of cells with a variety of mutations at loci of interest (Fig. 3).

The utility of MAGE in improving pathway flux was evaluated in the context of engineered lycopene production in *E. coli*. Twenty endogenous genes known to affect lycopene yields were targeted with alternative RBS oligos. Simultaneously, four genes known to direct flux to competing pathways were targeted with oligos harboring nonsense mutations. Over 35 MAGE cycles, approximately 15 billion genetic variants were generated. Screening colonies based on the red pigmentation of lycopene identified a variant that produced fivefold more lycopene than the parent strain (Wang et al., 2009).

Combinatorial approaches are powerful tools for pathway optimization because they can adjust multiple gene levels simultaneously. Iterative pathway improvement, in which a single gene level is adjusted at a time, can fail to identify global maxima accessible by simultaneous perturbation (Alper and Stephanopoulos, 2007). Both gene knockout and upregulation studies have shown that mutations often interact in a cooperative and non-linear manner with regards to metabolite production (Kennedy et al., 2009). As a further complication, many modern metabolic engineering efforts involve the heterologous expression of enzymes from several different species in an unoptimized host (Agapakis et al., 2010; Bayer et al., 2009; Martin et al., 2003; Ro et al., 2006). Engineering these chimeric pathways to interface with host metabolism demands many factors be adjusted simultaneously.

Generating genomic or pathway-specific variation in gene regulation is only the first step in pathway optimization. Each approach outlined in this section was paired with a screening or



**Fig. 3.** The Multiplex Automated Genome Engineering (MAGE) cycle. MAGE incorporates oligonucleotides into *E. coli* by electroporation, with the  $\lambda$ -Red  $\beta$  protein integrating the oligonucleotides into the genome. Oligonucleotides can be synthesized to introduce mutations at precise genomic loci. Iterated rounds of MAGE introduce increasing amounts of diversity at these loci, although many cells are killed at the electroporation step (Wang et al., 2009).

selection strategy to identify improved product yields. Pathways that are not observable via high-throughput assays are less amenable to screening approaches. Selection strategies that connect pathway output to cell viability are designed ad hoc, and success is not guaranteed (Dietrich et al., 2010a). The lack of generalized methods for pathway screening and selection currently limits the broad application of combinatorial pathway optimization methods.

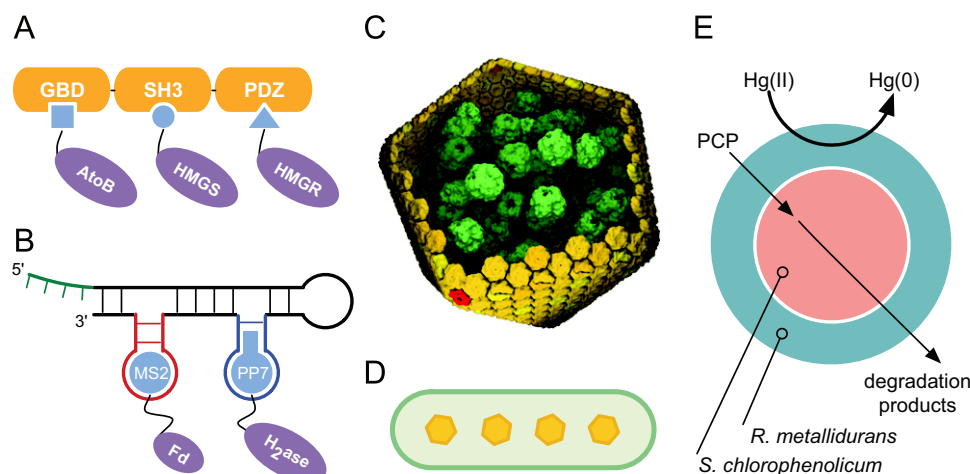
### 3. Spatial pathway control

The spatial organization of cellular components is tightly controlled in all organisms, including prokaryotes. For example, many cyanobacteria target photosystems and electron transport machinery to a highly ordered thylakoid membrane (Nelson and Yocum, 2006), while maintaining carbon fixation enzymes in separate protein-bound compartments positioned along the cell axis (Savage et al., 2010) (Fig. 4C and D). Co-localization of pathway enzymes to the same subcellular organelle or compartment can increase the local concentration of pathway intermediates and exclude competing cytosolic pathways. Multienzyme complexes often arrange enzymes in defined stoichiometric ratios to improve enzyme saturation (Zhou et al., 2001). Direct linkage of enzyme active sites via substrate tunnels has also been observed in nature, such as in the synthesis of tryptophan (Hyde et al., 1988). The success of spatial pathway control in nature has led synthetic biologists to develop methods for adjusting the physical arrangement of metabolic pathways.

#### 3.1. Scaffolds

Co-localization of related enzymes via direct linkage or scaffold proteins is an evolutionary development that has inspired new approaches to metabolic engineering. Polyketide synthases are modular enzymes that pass the growing polyketide chain from one enzymatic module to the next, much like an assembly line (Menzella et al., 2005). Cellulosomes are massive bacterial





**Fig. 4.** Spatial optimization in natural and synthetic pathways across multiple scales. (A) At the protein scale, synthetic scaffolds can be used to bind mevalonate pathway enzymes in close proximity to each other. The scaffold contains binding domains for the GBD, SH3, and PDZ protein tags (Dueber et al., 2009). (B) Synthetic scaffolds also work for electron-transfer proteins, such as ferredoxin (Fd) and an [FeFe]-hydrogenase ( $H_2ase$ ). In this example, an RNA scaffold was used to coordinate hydrogen production. RNA aptamers specific for the MS2 and PP7 protein tags allow control over enzyme binding. A 5' extension (shown in green) allows attachment of this RNA building block onto extended scaffold architectures (Delebecque et al., 2011). (C) The carboxysome coordinates enzymes at a larger subcellular scale. RuBisCO (green) is tightly packed inside the icosahedral carboxysome shell (yellow). Carbonic anhydrase (not shown) provides RuBisCO with gaseous  $CO_2$  inside the carboxysome (Savage et al., 2010). Carboxysome image used with permission from Bruno Afonso and David Savage. (D) At the cellular scale, carboxysomes in *S. elongatus* are evenly spaced across the length of the cell (Savage et al., 2010). (E) At the multicellular scale, microbial consortia can be assembled via microfluidic devices. Fibers of the PCP-degrading *S. chlorophenicum* can be protected from Hg inhibition when coated with the Hg-reducing *R. metallidurans* (Kim et al., 2011).

complexes that arrange enzymes on scaffolds on the cell surface. The highly ordered arrangement of cellulosome enzymes facilitates the breakdown of plant cellulose, and work to heterologously express cellulosome assemblies has demonstrated that scaffolding increases enzyme activity in a cooperative manner (Gilbert, 2007; Mitsuzawa et al., 2009; Morais et al., 2010a, 2010b; Tsai et al., 2009).

Synthetic scaffolds have been constructed to improve the production of mevalonate (Dueber et al., 2009). As with the application of TIGR elements to the mevalonate pathway, the enzymes AtoB, HMGS, and HMGR were co-expressed in *E. coli*. Attachment of these enzymes to a scaffold protein was achieved by fusing the enzymes to the metazoan protein–protein interaction ligands GBD, SH3, and PDZ, respectively. Co-expressing a synthetic scaffold protein with cognate binding domains for the protein tags permitted all three enzymes to co-localize on the scaffold (Fig. 4A). Varying the number and order of binding sites for each tag allowed the enzyme stoichiometry on the scaffold to be tuned, as well as the relative positioning of each enzyme (Dueber et al., 2009).

Attaching the mevalonate pathway to the scaffold resulted in a striking 77-fold improvement in mevalonate yield over the unscaffolded pathway (Dueber et al., 2009). The scaffolding system appears to be generalizable to other metabolic pathways, as demonstrated by the use of the same scaffolding system for the production of glucaric acid in *E. coli*. Once again, the scaffold boosted product titers, with adjustments to scaffold binding site ratios increasing titers fivefold over the parent strain (Dueber et al., 2009; Moon et al., 2010).

The GBD/SH3/PDZ scaffolding system has been extended to a heterologous hydrogen production pathway (Agapakis et al., 2010). Pyruvate:ferredoxin oxidoreductase (PFOR) from *Desulfovibrio africanus* and ferredoxin and an [FeFe]-hydrogenase from *Clostridium acetobutylicum* were heterologously coexpressed with a scaffold. Unlike the mevalonate or glucaric acid pathways, the key intermediate for hydrogen production is not a small molecule. Instead, PFOR reduces the ferredoxin protein, which delivers the electrons to the hydrogenase. Despite this important difference, placing pathway components on a synthetic scaffold also resulted

in increased product yields. The length of the linker between each component and the scaffold-binding ligand, the distance between binding sites on the scaffold, and the ratio and order of ligand binding sites all affected pathway yields. The study saw a threefold increase in hydrogen production with the best scaffold configuration over an unscaffolded parent strain. This work, along with evidence from natural and engineered signaling pathways (Bashor et al., 2008), demonstrate the utility of scaffolding in improving the specificity of protein–protein interactions.

Structural RNA and DNA devices are powerful alternatives to protein structures for spatially arranging biological parts. Tools for predicting secondary structures from the primary nucleic acid sequence are becoming sufficiently robust to enable the design of new RNA and DNA structures ab initio (Andronescu et al., 2004; Douglas et al., 2009). The field of DNA nanotechnology has demonstrated the versatility of DNA as a structural molecule (Aldaye et al., 2008; Shih and Lin, 2010), and RNA-based designs are beginning to be constructed in much the same manner (Chworos et al., 2004; Guo, 2010).

Functional in vivo RNA architectures were recently developed to scaffold a metabolic pathway. These RNA assemblies were used to coordinate the PFOR/ferredoxin/hydrogenase system in *E. coli*. Hydrogenase and ferredoxin proteins linked to PP7 and MS2 aptamer proteins were spatially organized onto an extended RNA scaffold bearing the corresponding RNA aptamers (Fig. 4B). As with the GBD/SH3/PDZ scaffold, hydrogen production benefited from scaffolding: the RNA scaffold increased hydrogen yield by 48-fold over the unscaffolded system (Delebecque et al., 2011). Since a vast array of multidimensional structures has already been designed with nucleic acids (Aldaye et al., 2008; Shih and Lin, 2010), there is great potential in the exploration of complex geometries for spatial optimization of metabolic pathways.

The exact mechanism behind yield improvements seen with scaffolds in various applications remains unclear (DeLisa and Conrado, 2009). Protein intermediates such as ferredoxin, which diffuse more slowly than small molecules, are likely to benefit from the local increase in concentration afforded by tethering to the scaffold (Agapakis et al., 2010; Cironi et al., 2008). For pathways with small molecule intermediates, it is more difficult

to prove that the local concentration of metabolic intermediates is increased near the scaffold. In the case of the mevalonate, the results are certainly indicative of increased pathway flux, as buildup of HMG-CoA is toxic to *E. coli* (Dueber et al., 2009). Importantly, synthetic scaffolds offer an elegant mechanism for balancing pathway flux through the precise adjustment of enzyme stoichiometries.

### 3.2. Subcellular compartments

Membrane- and protein-bound compartments encapsulate metabolic processes in prokaryotes and eukaryotes. Active transport or selective diffusion can boost substrate concentrations as well as protect a pathway from competing reactions. Compartments often host metabolic reactions that are harmful to the rest of the cell, or are thermodynamically infeasible in the cytoplasm (Chance et al., 1979; Feldman and Sigman, 1983; Page et al., 1998).

Many prokaryotes target critical metabolic reactions to protein-bound microcompartments. Perhaps the most well-studied of these compartments is the bacterial carboxysome, a structure that contains the key carbon fixation enzymes ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) and carbonic anhydrase (Fig. 4C). The carboxysome is an evolutionary adaptation for a difficult metabolic task: to promote the fixation of gaseous carbon dioxide by RuBisCO while preventing oxygen from interfering with the reaction. The intricate organization of carboxysomes within *Synechococcus elongatus* suggests that the spatial positioning of carbon fixation is vital, even at the micron scale (Savage et al., 2010). Given the need for carbon-neutral or carbon-negative fuel sources (Savage et al., 2008), the bacterial carbon fixation machinery is a likely target for metabolic engineering.

Recent work has raised the possibility of repurposing prokaryotic microcompartments for metabolic engineering. The propanediol utilization (*pdu*) machinery of several bacterial species is enclosed in a proteinaceous shell much like a carboxysome (Parsons et al., 2008; Yeates et al., 2011). The *pdu* “metabolosome” of *Citrobacter freundii* was shown to confer the ability to metabolize propanediol when expressed heterologously in *E. coli*. Furthermore, electron micrographs appear to show successful assembly of the *pdu* shell proteins to form microcompartments in *E. coli* (Parsons et al., 2008). Intriguingly, there is evidence that the N-terminal domains of non-shell *pdu* proteins can be appropriated to target heterologous proteins to the metabolosome interior (Fan et al., 2010; Parsons et al., 2010). The pairing of microcompartment shells with novel biosynthetic pathways may expand the reach of bacterial metabolic engineering.

In eukaryotes, methods have been developed for the targeting of heterologous proteins to many membrane-bound organelles (Hood and Silver, 1999; Léon et al., 2006; Soll and Schleiff, 2004; Truscott et al., 2003). Efforts to synthesize methyl halides in *S. cerevisiae* have demonstrated the utility of localizing exogenous enzymes in appropriate subcellular environments. Researchers noted that the primary substrates of methyl halide transferases (MHT), SAM, and halide ions are sequestered in the yeast vacuole. Targeting the MHT from *Batis maritima* to the vacuole increased yields of methyl iodide by nearly 50 mg/L-h over targeting the identical MHT to the cytosol (Bayer et al., 2009). The benefits of compartmentalization are likely to be even greater for the heterologous expression of biosynthetic pathways that require an organelle, such as penicillin synthesis (Gidijala et al., 2009; Meijer et al., 2010).

### 3.3. Microbial consortia

The natural world has demonstrated that consortia and communities of organisms are capable of performing metabolic

conversions that are difficult or thermodynamically unfavorable to do in a single cell (Wintermute and Silver, 2010a). From an engineering perspective, co-culture offers many of the same advantages as subcellular compartmentalization: incompatible metabolic reactions can be conducted in separate organelles or cells. The bovine rumen, which is itself compartmentalized, harbors a rich assortment of microorganisms that together metabolize cellulose (Annison and Bryden, 1998; Hungate, 1947; McAllister et al., 1994). Even communities of soil bacteria appear to be highly ordered (Young and Crawford, 2004). The success of microbial cooperation in the natural world has inspired efforts to engineer synthetic microbial consortia (Brenner et al., 2007, 2008; Eiteman et al., 2008; Kim et al., 2008; Shou et al., 2007; Wintermute and Silver, 2010b).

In recent years, efforts to model and construct stable engineered co-cultures have intensified in the systems and synthetic biology communities. Synthetic consortia have been established through the exchange of signaling molecules governing quorum-sensing circuits (Brenner et al., 2007) as well as the exchange of essential metabolites among complementary auxotrophs (Shou et al., 2007; Wintermute and Silver, 2010b). Constraint-based modeling frameworks familiar to metabolic engineers have been adapted to multi-organism systems, facilitating the development of compatible synthetic consortia (Klitgord and Segrè, 2010; Wintermute and Silver, 2010b).

As with proteins, the spatial arrangement of microbial consortia can be optimized for greater product yields. In a unique approach, wild-type *Sphingobium chlorophenolicum* and *Ralstonia metallidurans* were used as modular parts to assemble a structure capable of degrading pentachlorophenol (PCP) in the presence of Hg(II) (Kim et al., 2011) (Fig. 4E). PCP and Hg(II) are particularly harsh industrial pollutants that are often produced together. While *S. chlorophenolicum* is capable of degrading PCP, it is inhibited by Hg(II). *R. metallidurans*, a mercuric ion reducer, can reduce Hg(II) to Hg(0). Microfluidic laminar flow devices were used to assemble fibers of *S. chlorophenolicum* wrapped in a protective shell of *R. metallidurans*. The hybrid fibers were capable of fully degrading 120  $\mu$ M PCP in the presence of micromolar Hg(II). A well-mixed solution of both species was essentially incapable of PCP degradation.

Synthetic microbial consortia consisting of wild-type organisms may be well suited for bioremediation and other ecologically sensitive applications. Wild-type organisms are not subject to laws and regulations concerning the deployment of genetically modified organisms into the environment. Ideally, spatially optimized consortia assembled of species native to polluted areas could be administered to accelerate the bioremediation process. The possibility of engineered microbes simultaneously obsolescing harsh industrial processes while cleaning up existing industrial pollution is an attractive vision for the future.

## 4. Modeling and measuring the metabolic network

In many ways, metabolites are the “dark matter” of the cell—their existence, intracellular concentrations, and fluxes are difficult to derive from genomic information and difficult to experimentally measure (Blow, 2008). Combining tools for characterizing the status of the metabolome with robust metabolic models is a foundational mission for systems and synthetic biology.

Genome-scale constraint-based models of cellular metabolism have been invaluable tools for *in silico* screening of mutant backgrounds suitable for metabolic engineering. These models incorporate the stoichiometry of all known metabolic reactions in a cell subject to linear or quadratic constraints, such as mass

balance, media composition, and thermodynamic limits on flux direction (Schellenberger et al., 2011; Segrè et al., 2002; Varma and Palsson, 1994). The result is a steady-state approximation of metabolic fluxes. Metabolic reactions can be added and removed to model heterologous gene expression and gene knockouts, respectively. Models of this type have successfully been employed to identify genetic modifications to improve product yields (Bro et al., 2006; Brochado et al., 2010; Burgard et al., 2003; Kennedy et al., 2009; Pharkya et al., 2004).

In order to model the entire metabolic network, constraint-based models eschew kinetic parameters and do not predict metabolite concentrations (Varma and Palsson, 1994). More quantitative approaches such as Metabolic Control Analysis are also more dependent on experimentally determined parameters; errors in parameter estimation become increasingly problematic as the model size increases (Schuster, 1999). Thus, constraint-based models are preferable for the forward engineering of the metabolic network, while detailed kinetic models of select pathways are beneficial for pathway tuning. As with all biological modeling, experimental analysis is crucial for evaluating the predictive value of metabolic models in a given context.

Multiple studies have attempted to reconcile transcriptome data with proteomic or metabolomic measurements in engineered cells (Bradley et al., 2009; Fendt et al., 2010; Ishii et al., 2007; Moxley et al., 2009). The integration of -omics level data with network-scale metabolic models benefits both *a priori* prediction as well as *post hoc* evaluation of metabolic engineering. In particular, developing quantitative models for the relationship between transcript levels and metabolite pools and fluxes would allow metabolomic data to be inferred from the vast number of microarray datasets that are already available (Yizhak et al., 2010). The chemically uniform nature of mRNA transcripts allows the reliable collection of total mRNA in a single extraction condition. Due to the chemical diversity of small molecules within cells, extraction conditions limit the extent of the metabolome that is observed (Yanes et al., 2011).

Coordination between gene expression and metabolite concentrations appears to be dependent on the type of perturbation. Comparison of the transcriptome and metabolome in *E. coli* over a range of growth rates revealed that enzyme transcript and protein levels increased with increasing growth rates while metabolite pools remained steady (Ishii et al., 2007). It was also noted that gene deletions that reverse the flux direction of the pentose phosphate pathway did not significantly alter enzyme levels or metabolite pool sizes. In addition, it was observed that metabolic enzymes do not appreciably up-regulate to compensate for enzyme knockouts (Ishii et al., 2007). Another study comparing the transcriptome and metabolome of *S. cerevisiae* during carbon and nitrogen starvation observed coordinated changes in expression and metabolite levels. In this study, a network model identified novel coordinated gene-metabolite pairs in this dataset (Bradley et al., 2009).

Further work is required to identify contexts in which transcript levels correlate to metabolite concentrations. Comparison of the above studies suggests a differential metabolic response to enzyme knockouts versus shifting media conditions (Bradley et al., 2009; Ishii et al., 2007); this could be a consequence of evolutionary selection for robustness against condition changes (Cornelius et al., 2011; Segrè et al., 2002). Alternatively, *E. coli* and *S. cerevisiae* may simply respond differently to metabolic perturbations. A significant confounding issue is that major metabolic flux alterations can occur without major shifts in enzyme or metabolite concentrations (Fell, 1997; Ishii et al., 2007).

Two recent studies measured transcriptomic and metabolomic shifts in *S. cerevisiae* in response to the deletion of global regulatory genes rather than enzymes (Fendt et al., 2010;

Moxley et al., 2009). Following the deletion of the Gcn4p, a global stress response regulator, it was observed that flux control was highest among metabolites that were involved in many enzymatic reactions (Moxley et al., 2009). This raises the possibility of utilizing network topology to inform metabolic engineering. In the case of central carbon metabolism, deletion of the glycolysis-activating transcription factor Gcr2p showed a negative correlation between enzyme levels and associated metabolite levels (Fendt et al., 2010). This could be indicative of a buffering phenomenon, in which changes in metabolite pools counteract enzyme concentration changes to maintain a steady pathway flux.

A grand unifying theory of metabolism has not yet arisen from these meta-omics studies. It is possible that a truly general relationship between gene expression and metabolic concentrations does not exist but several important observations about their correlation have been made. Overall, it appears that evolved metabolic networks are quite robust in response to genetic and environmental perturbations. This is corroborated by many of the metabolic engineering efforts that we have reviewed, in which multiple perturbations were required to improve product yields. This could also explain why conservative modeling frameworks such as Minimization of Metabolic Adjustment (MOMA) are often more accurate than models that assume that mutant flux is optimized for maximum growth. Instead, MOMA-derived solutions assume that mutant fluxes are regulated by the cell to approximate the wild-type flux distribution (Segrè et al., 2002). Integrative data from -omics scale datasets may help to identify genes that disrupt the resistance to perturbations in pathways of interest.

## 5. Conclusions

As a practical application of synthetic biology, metabolic engineering has field-tested emerging biological design principles. Through these efforts, it has become increasingly apparent that rational design approaches are limited by our understanding of biological systems. The complexity of living cells far surpasses the complexity of human-made devices. The tremendous improvement in DNA sequencing and assembly techniques is now bringing about an era in which cells themselves can be man-made devices (Benders et al., 2010; Gibson et al., 2010; Lartigue et al., 2009), but our ability to modify cells has outpaced our ability to predict how those modifications will function. Fortunately, the ability of living systems to self-replicate has led animal and plant breeders, geneticists, molecular biologists, metabolic engineers, and now synthetic biologists to utilize selective pressure in their research.

Incorporating irrational design into synthetic biology does not require an abandonment of forward engineering approaches. Instead, the emerging engineering design cycle of synthetic biology and metabolic engineering appears to include both *in silico* modeling and prediction as well as directed evolution and screening. Similarly, experimental analysis remains invaluable for hypothesis generation as well as confirmation. In our own work, we utilized FBA to predict knockout combinations in *S. cerevisiae* likely to increase production of formic acid. Our initial strain produced formic acid as predicted, but at modest levels. Expression analysis and metabolic phenotyping allowed us to identify further genetic interventions that boosted formic acid titers (Kennedy et al., 2009). In the case of the mevalonate-based isoprenoid pathway, transcriptomic and metabolomic methods revealed that the cytotoxicity of HMG-CoA was causing stress responses that negatively impacted isoprenoid titers (Kizer et al., 2008; Martin et al., 2003). Based on these experiments, researchers were able to counteract the effect by varying pathway



expression levels (Pfleger et al., 2006), by the addition of palmitic acid (Kizer et al., 2008), and by the use of engineered scaffolds (Dueber et al., 2009). Undoubtedly genome-scale optimization approaches such as gTME and MAGe can identify further regulatory adjustments that have not been predicted.

The ability to synthesize any DNA sequence has increased the reach of metabolic engineering. Modern metabolic engineering efforts often assemble parts from disparate species into novel pathways. Recent work to produce fatty acids in *E. coli* utilized heterologous genes from *S. cerevisiae*, *M. musculus*, *A. calcoaceticus*, *A. baylyi* ADP1, *Z. mobilis* ZM4, *C. stercorarium*, *B. ovatus*, *U. californica*, *C. hookeriana*, and *A. thaliana*, sampling multiple expression level combinations (Steen et al., 2010). Low-cost synthesis will undoubtedly drive more “synthetic metagenomics” studies, in which libraries of homologous enzymes are synthesized and evaluated (Bayer et al., 2009). Demands for renewable energy have led synthetic biologists to construct more spatially complex pathways, such as the expression of the electron transfer apparatus in *E. coli* (Jensen et al., 2010). Exploring all relevant expression level variants and spatial arrangements becomes increasingly difficult in these complex pathways.

The engineering of complex pathways can be divided into two stages: a proof of concept stage where novel enzyme combinations are determined to produce a desired product, and an optimization stage where regulatory adjustments are made to improve product yields. As engineered biosynthetic pathways become more complex, they also become more difficult to tune. New biofabrication facilities such as the BIOFAB (<http://www.biofab.org/>) seek to abstract pathway optimization from pathway design by developing rapid prototyping services. The BIOFAB is intended to provide new libraries of characterized regulatory elements, as well as facilitate prototyping of collaborator’s synthetic devices via high-throughput cloning and testing (Bayer, 2010). Applying forward engineering and directed evolution approaches in a high-throughput manner will generate parts and data that will improve our ability to rationally engineer cells.

The growth of synthetic biology has often been compared to the personal computer revolution of the late twentieth century. The home personal computer was made possible by the availability of high-quality off-the-shelf electronic components that could be assembled by technically inclined enthusiasts. Molecular biologists have generated a vast assortment of biological parts, but inadequate characterization has limited their general usefulness. In many ways, the current stage of synthetic biology is more analogous to the early days of heavier-than-air flight. Despite our current sophistication regarding the computational design of aircraft, flight principles were first elucidated through exhaustive wind tunnel experiments and test flights (Carlson, 2010). Similarly, the successes and failures of synthetic biology continue to reveal biological design principles.

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