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Synthetic biology and the development of tools for metabolic engineering

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ABSTRACT

Synthetic biology can significantly advance metabolic engineering by contributing tools (minimal hosts, vectors, genetic controllers, characterized enzymes). The development of these tools significantly reduced the costs and time to develop the antimalarial drug artemisinin, but the availability of more tools could have reduced these costs substantially.

1. Introduction

Synthetic organic chemistry has dominated the production of chemicals for the past century. When petroleum feedstocks became widely available and inexpensive, synthetic organic chemists found a way to produce many of the products we now use from this feedstock. Even some of the products that were once produced by microbial fermentations were replaced with petrochemical production processes.

There is renewed interest in sourcing chemicals from renewable resources for several reasons. First, commodity chemical production has small margins, and increases in petroleum prices squeeze those margins. Second, many refineries have changed the distribution of products they make, abandoning low margin products for higher margin products. Third, the US has increased the fraction of petroleum it imports over the past few decades, making petroleum products ever more subject to the changes in world politics. Finally, there is a new movement toward green chemistry and renewable feedstocks that makes microbial production of chemicals attractive.

For many years, microbial production of chemicals was limited by nature's chemical repertoire. We made fatty acids, amino acids, organic acids, alcohols, antibiotics, etc. using microorganisms isolated from a variety of environments. Microbes were mutated and selected to produce ever higher titers, and fermentation processes were developed to produce these valuable products.

With the advent of genetic engineering, it became possible to produce heterologous products: products found in nature but not produced by the production host. These products tended to be proteins that could be produced through transformation of a single gene. Genetic engineering also brought the ability to engineer specific genes in natural producers and reduce the time required for mutagenesis and selection. With genome sequencing and better tools came the ability to produce smaller molecules, chemicals that were produced naturally in one organism, but at levels that made extraction economically infeasible. The development of protein engineering and laboratory evolution has delivered enzyme catalysts that can produce chemicals not produced in nature.

Unfortunately, the development of tools to control expression of genes and metabolic pathways has lagged the development of metabolic pathways. And this, in turn, has lead to long development times for biology and high costs. The development of well-characterized gene expression tools and computer-aided-design programs to predict the necessary assembly of these parts to construct highly functional, robust producers has the potential to substantially reduce development times and costs for biological engineering.

2. Our endeavor to develop a robust artemisinin host

The microbial production of the antimalarial drug artemisinin is an example of engineering microorganisms to produce a

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needed chemical. Malaria is a global health problem that threatens 300–500 million people and kills more than one million people annually (World Malaria Report, 2005). Disease control is hampered by the occurrence of multi-drug-resistant strains of the malaria parasite *Plasmodium falciparum* (Korenromp et al., 2003; Marsh, 1998). Synthetic antimalarial drugs and malarial vaccines are currently being developed, but their efficacy against malaria awaits rigorous clinical testing (Hoffman, 2004; Vennerstrom et al., 2004). Artemisinin, a sesquiterpene lactone endoperoxide extracted from *Artemisia annua* L. (family Asteraceae; commonly known as sweet wormwood), is highly effective against multi-drug-resistant *Plasmodium* spp., but is in short supply and unaffordable to most malaria sufferers (Enserink, 2005). Although total synthesis of artemisinin is difficult and costly (Schmid and Hofheinz, 1983), the semi-synthesis of artemisinin or any derivative from microbially sourced artemisinic acid, its immediate precursor, could be a cost-effective, environmentally friendly, high-quality and reliable source of artemisinin (Acton and Roth, 1992; Haynes and Vonwiller, 1994).

We have engineered *Escherichia coli* to produce high titers of artemisinic acid using a heterologous mevalonate pathway, amorphadiene synthase, and a novel cytochrome P450 monooxygenase (CYP71AV1) from *A. annua* that performs a three-step oxidation of amorpha-4,11-diene to artemisinic acid (Fig. 1) (Chang et al., 2007; Martin et al., 2003b; Newman et al., 2006; Ro et al., 2006). We chose to bypass the native deoxyxylulose-5-phosphate (DXP) pathway and instead import the heterologous mevalonate pathway from *Saccharomyces cerevisiae* (Martin et al., 2003a) to supplement endogenous IPP biosynthesis (Kakinuma et al., 2001). The mevalonate pathway was divided into two synthetic operons. The MevT operon encoded three enzymes – acetoacetyl-CoA synthase (from *E. coli*), hydroxymethylglutaryl-CoA (HMG-CoA) synthase (from *S. cerevisiae*), and HMG-CoA reductase (from *S. cerevisiae*) – to transform acetyl-CoA into mevalonate. The MBIS operon encoded five enzymes – mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase (all from *S. cerevisiae*), and isopentenyl pyrophosphate (IPP) isomerase and FPP synthase (both from *E. coli*) – to transform mevalonate to FPP. The operons were divided around mevalonate because it could be added to the growth medium to select for cells that harbored a functional MBIS pathway (when the DXP pathway was inactivated) (Martin et al., 2003b) or will diffuse out of cells allowing for it to be used as an indicator for MevT activity (Pfleger et al., 2007). Both operons were constructed using the native versions of the genes, since *S. cerevisiae* codon usage is so close to that of *E. coli*, consensus *E. coli* ribosome binding sites and secondary-structure-free spacers between the end of the 5′ gene and the ribosome binding site of the 3′ gene. The operons were placed under the control of *lac* promoters on compatible plasmids (different origins of replication, different antibiotic resistance genes) to ensure their maintenance and expression in the host.

Initial versions of the mevalonate-based pathway suffered from metabolic bottlenecks and accumulation of high levels of the prenyl diphosphates (FPP, IPP and/or DMAPP), which inhibited growth of the host (Martin et al., 2003a). Co-expression of a codon-optimized amorpha-4,11-diene synthase (ADS) (Martin et al., 2001) from *A. annua* (Chang et al., 2000; Merke et al., 2000) alleviated this toxicity. (This same toxicity has been used to identify a phosphatase from *Bacillus subtilis* that hydrolyzes IPP or DMAPP to the corresponding alcohol, isopentenol, which incidentally may be a next generation biofuel (Withers et al., 2007)). Due to limitations in mevalonate production, MevT expression was increased. Imbalances in the MevT pathway resulted in accumulation of HMG-CoA, which inhibited fatty acid

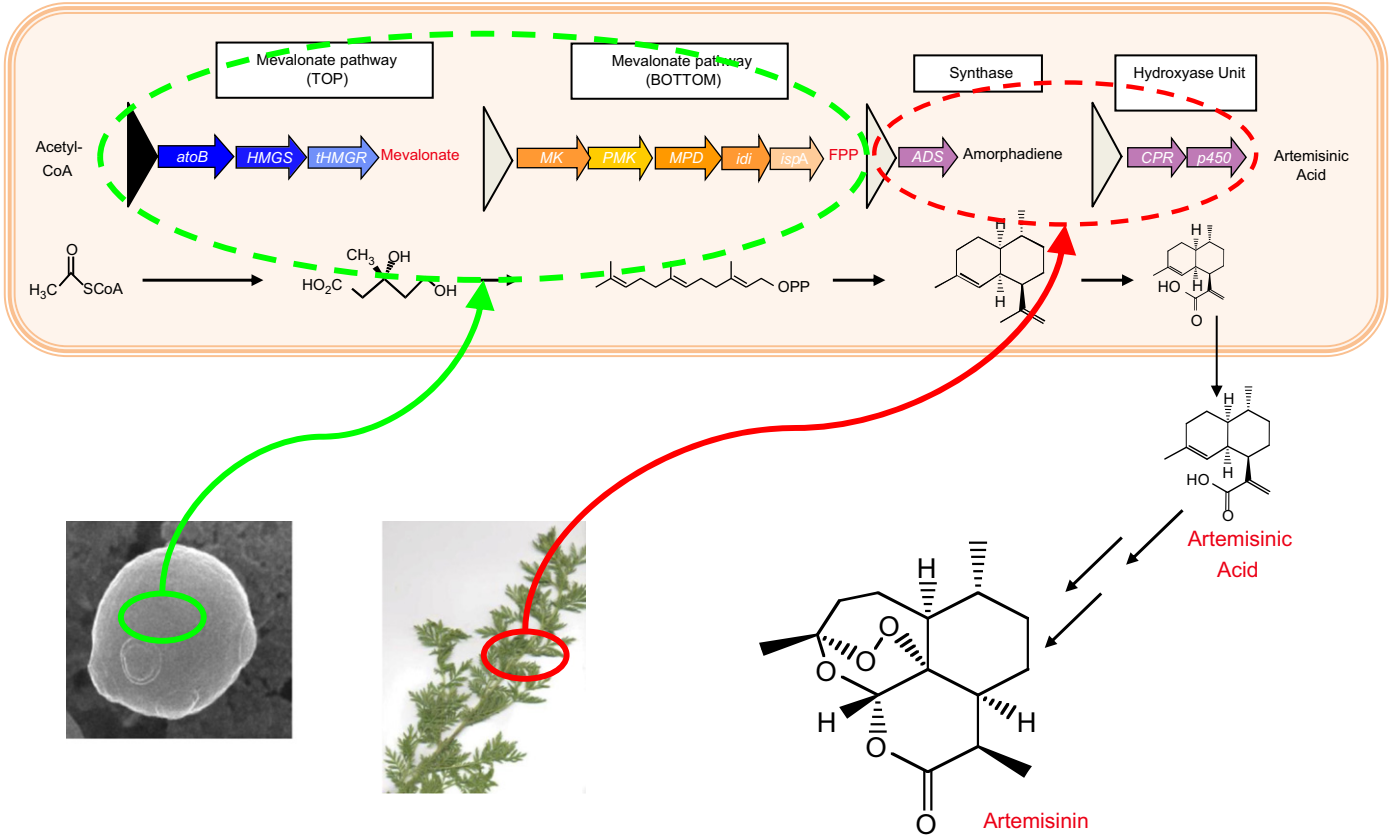

Fig. 1. Overall scheme for engineering artemisinic acid production into *Escherichia coli*. Genes from *Saccharomyces cerevisiae* and *Artemisia annua* were expressed in *E. coli* to transform acetyl-CoA, energy, and reducing equivalents into artemisinic acid, which was then chemically transformed into artemisinin.

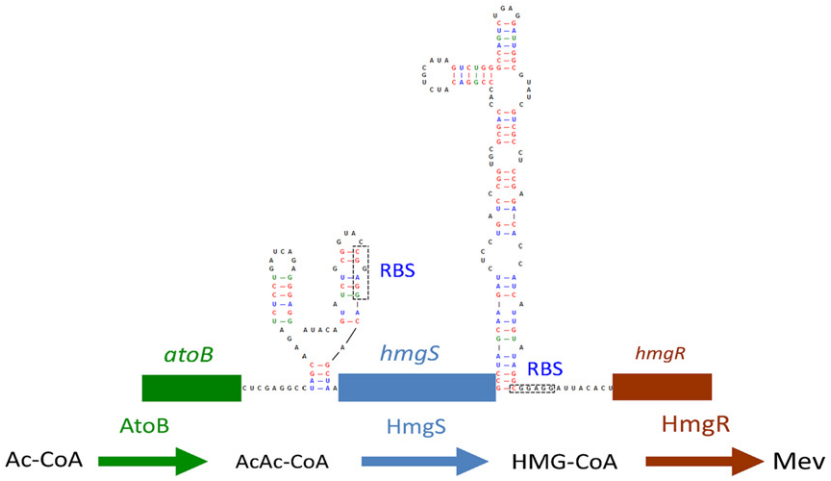

Fig. 2. Use of mRNA secondary structures to control production of the first three enzymes in the mevalonate pathway.

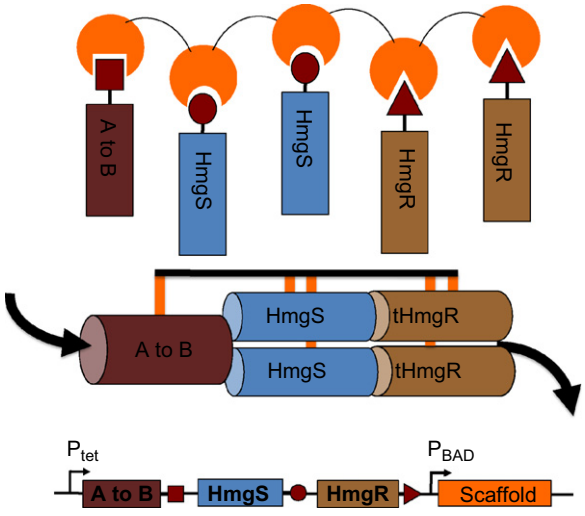

Fig. 3. Scaffold structure and genetic construct used to scaffold the three first enzymes of the mevalonate-based IPP biosynthetic pathway.

biosynthesis and host growth (Kizer et al., 2008; Pitera et al., submitted). Expression of an additional copy of *HMGR* alleviated accumulation of HMG-CoA and increased productivity of mevalonate by almost twofold by relieving growth inhibition (Pitera et al., submitted).

With the goal of balancing expression of genes in the MevT operon, several general approaches have been developed. The first approach exploited mRNA secondary structures and RNase sites to differentially stabilize specific mRNA segments encoding the various enzymes in the upper part of the pathway in order to produce the enzymes at different levels and thereby balance the pathway (Fig. 2) (Pfleger et al., 2006b). Mevalonate titers were improved sevenfold overall and cell growth was improved due to reduced accumulation of HMG-CoA.

The second approach increased flux through the metabolic pathway by tethering the enzymes in the pathway to a synthetic protein scaffold (Fig. 3) (Dueber et al., 2009). Multiple copies of the GBD, SH3 and PDZ binding domains were linked together in a synthetic protein scaffold with non-structure forming amino acids between the domains. Their cognate ligands were placed at the C-terminus of AtoB, HMGS and HMGR. The best combination of one AtoB, two HMGSs and two HMGRs resulted in 77-fold improved production of mevalonate over the untethered pathway.

Yet a third approach involving mass spectrometric analysis of enzyme levels was used to balance expression of genes in the lower part of the mevalonate pathway. Targeted proteomics, using single reaction monitoring (SRM) mass spectrometry, identified enzymes that were produced at low levels (Redding-Johanson et al., 2011). The expression of rate-limiting enzymes was improved by removing an internal promoter from one gene and incorporating a second promoter in the operon to improve expression of all genes.

These efforts show that carbon can be effectively channeled for production of exogenous isoprenoids in *E. coli* even though the flux through native isoprenoid biosynthesis is relatively low. Over several years, production of amorphadiene was increased over one-million-fold (Fig. 4). The combination of several of these techniques as well as optimizing fermentation conditions increased the titers of amorphadiene to well over 27 g/L (Newman et al., 2006).

Similar efforts were undertaken to create an amorphadiene-producing yeast strain (Fig. 5), which was used to discover the amorphadiene oxidase and associated redox partners (Paradise et al., 2008; Ro et al., 2006; Shiba et al., 2007). To increase FPP production in *S. cerevisiae*, the expression of several genes responsible for FPP synthesis was upregulated, and one gene responsible for FPP conversion to sterols was downregulated. All of these modifications to the host strain were made by chromosomal integration to ensure the genetic stability of the host strain. Overexpression of a truncated, soluble form of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*tHMGR*) improved amorphadiene production approximately fivefold. Downregulation of *ERG9*, which encodes squalene synthase (the first step after FPP in the sterol biosynthetic pathway), using a methionine-repressible promoter (P_{MET3}) increased amorphadiene production an additional twofold. Although *upc2-1*, a semi-dominant mutant allele that enhances the activity of *UPC2* (a global transcription factor regulating the biosynthesis of sterols in *S. cerevisiae*), had only a modest effect on amorphadiene production when overexpressed in the EPY208 background, the combination of downregulating *ERG9* and overexpressing *upc2-1* increased amorphadiene production to 105 mg l^{-1}. Integration of an additional copy of *tHMGR* into the chromosome further increased amorphadiene production by 50% to 149 mg l^{-1}. Although overexpression of the gene encoding FPP synthase (*ERG20*) had little effect on total amorphadiene production, the specific production increased about 10% owing to a decrease in cell density. Combining all of these modifications resulted in a strain able to produce 153 mg l^{-1} amorphadiene.

The starting point for the mevalonate pathway is acetyl-CoA. In order to enhance the supply of acetyl-CoA to the mevalonate pathway and achieve high-level production of amorphadiene, we also engineered the pyruvate dehydrogenase bypass in *S. cerevisiae* (Shiba et al., 2007). Overproduction of acetaldehyde dehydrogenase and introduction of a *Salmonella enterica* acetyl-CoA synthetase variant increased the carbon flux into the mevalonate pathway resulting in increased amorphadiene production.

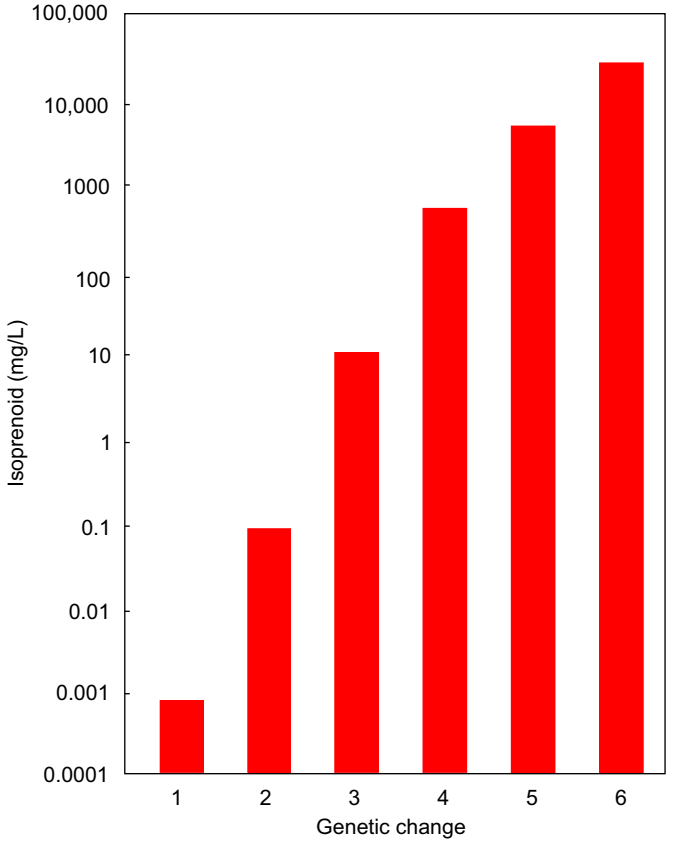

Fig. 4. Improvements in *E. coli*-based isoprenoid production through several genetic constructs and fermentation improvements.

To produce artemisinic acid, which can be converted directly into the antimalarial drug artemisinin in two chemical steps (Roth and Acton, 1989), an amorphadiene oxidase (AMO) and associated redox partners from *A. annua* were introduced into the amorphadiene producer (Chang et al., 2007; Ro et al., 2006) (Figs. 1 and 5). Although the native gene (nAMO) had no detectable *in vivo* or *in vitro* activity, codon-optimization coupled with N-terminal transmembrane domain engineering generated two constructs that were competent to carry out the first oxidation step in vivo to generate the alcohol congener of artemisinic acid at low levels (0.18–0.45 mg L^{-1}). Use of the redox partners with those from *A. annua* increased productivity 12-fold to 5.6 mg L^{-1} of alcohol. Finally, use of the most appropriate promoters and expression vector allowed much higher in vivo productivity of fully oxidized artemisinic acid.

The construction of an industrial-strength artemisinic acid producer required substantially more work than was described above (approximately 150 person years) and funding. Relatively little of the money and effort was devoted to discovery of the metabolic pathway. The remainder of the time and money was devoted to pathway balancing (eliminating bottlenecks and accumulation of metabolic intermediates, balancing redox, etc.), increasing flux of precursors to the heterologous pathway, balancing reducing equivalents, stabilizing the pathway, etc.

3. Synthetic biology and tools to regulate metabolism

Future applications would be facilitated by the availability of tools to engineer metabolism. Unlike production of pharmaceutical proteins in which the genes are highly expressed to maximize production of the target protein, the genes encoding the transformational enzymes in metabolic engineering do not need to be highly expressed; rather the enzymes need to be produced in catalytic amounts only sufficient to adequately transform the

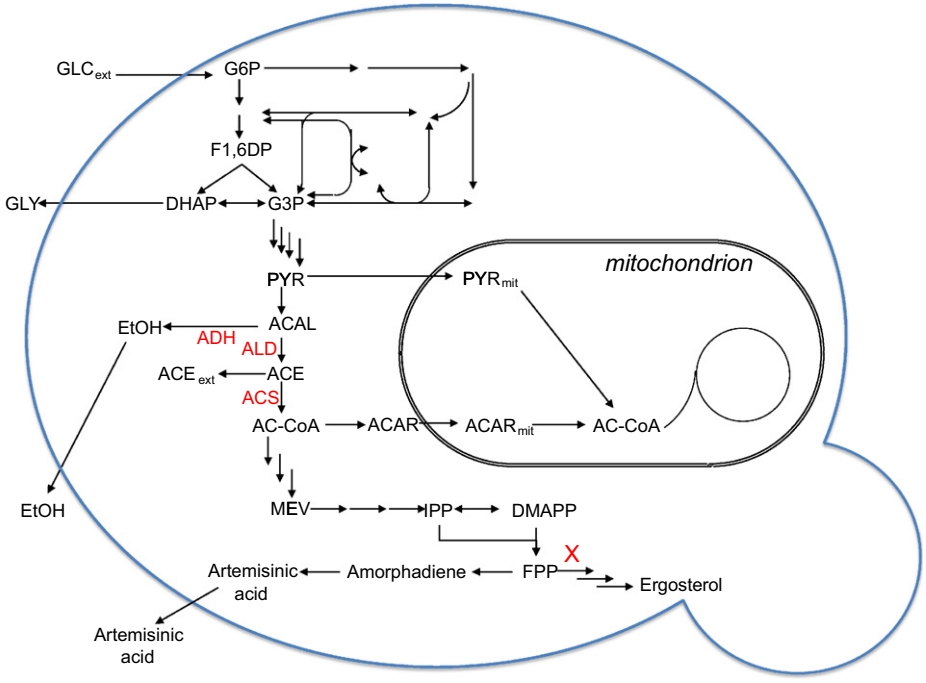

Fig. 5. Genetic manipulation of yeast for production of artemisinic acid.

metabolic intermediates into the desired products at a sufficient rate. Expression of the desired genes at too high a level will rob the cell of metabolites (nucleotides for the excess mRNA, amino acids for the excess protein, etc.) that might be otherwise used to produce the desired molecule of interest. Furthermore, because intermediates of a foreign metabolic pathway can be toxic to a heterologous host (Martin et al., 2003b), which can result in decreased production of the desired final compound, it is essential that the relative levels of the enzymes be coordinated in such a way that no intermediate in the pathway accumulates to toxic levels. Thus, the metabolic engineer needs a toolset that will allow him/her to accurately control the native and heterologous reactions inside the cell.

If we wanted to make metabolic engineering easier and faster, what standard biological components and other tools would we need? To answer this question, it is most useful to consider the essential characteristics of a microbial chemical factory. First, the microbial chemical factory should be genetically stable for long periods of cultivation, at least from scale-up to the large-scale production phases; that is, the vector used to carry the genes into and harbor the genes within the cell should be genetically stable, and the host should have minimal ability (or need) to inactivate these heterologous genes. Second, the microbial chassis should be able to grow with minimal supplements to the growth medium to minimize production costs and should be robust to industrial processing conditions (e.g., from small, laboratory-scale reactors to extremely large bioreactors). As the desired chemical or intermediate in its biosynthesis pathway might be toxic to the microbial factory, the third requirement is the ability to switch on the entire biosynthetic pathway at the correct time in the production process while having the biosynthetic pathway completely repressed during the non-productive (growth) stages. Fourth, as most chemicals will require multiple enzymatic transformations, it is important to be able to coordinate the expression of many genes simultaneously. Fifth, a computer-aided-design (CAD) system and accurate descriptions of biological components would make the design of the entire systems from off-the-shelf components much easier. Finally, biological debugging routines would make it faster and easier to identify and correct problems in engineered microorganisms.

3.1. Chassis

A stable, well-behaved chassis (host cell) is essential for large-scale, and even laboratory-scale, production of the desired molecule. Although minimal, bacterial chassis may have significant scientific interest (Gibson et al., 2008; Gibson et al., 2010; Posfai et al., 2006), industrial production of chemicals, particularly fuels and commodity chemicals, where cost of goods is most critical, requires the use of a chassis capable of synthesizing all biological macromolecules necessary for growth from a simple, inexpensive carbon source and salts of N, P, S and the like. Hence, minimal hosts that require addition of many complex nutrients or that cannot cope with potential stresses in processing will probably not find a niche in industrial chemical/fuel production. Robust, well-characterized organisms capable of growing on minimal, inexpensive carbon sources have been and will continue to be of enormous practical use. In this case, the development of methods to rapidly engineer the chromosome of an existing organism is essential (Wang et al., 2009). Nonetheless, one can envision a future where the chromosome of a particular host is tailor-made for production of a desired chemical.

3.2. Vectors

Central to any genetic manipulation is the vector used to carry and/or harbor the transforming DNA in the host. Important features of the cloning vector include (1) segregational stability to ensure that all cells in the culture have the plasmid and produce the desired product, (2) minimal and consistent copy number in all cells of a culture, and (3) the ability to carry large sequences of DNA and (4) have consistent copy number in all cells of the culture to ensure product consistency. There is growing recognition of the viability of low copy plasmids (Jones et al., 2000) and chromosomes for integration of genes (Court et al., 2002; Datsenko and Wanner, 2000; de Lorenzo et al., 1990). With the ability to vary promoter and ribosome binding strength as well as the stability of the mRNA and the resulting protein produced from it, there are many levers other than copy number to alter protein expression. In cases where copy number limits protein production, one can amplify genes on the chromosome to increase copy number (Tyo et al., 2009).

3.3. Promoters

The first line of control for a biosynthetic pathway is transcription and transcript processing, and as such promoters play an essential role in controlling biosynthetic pathways. Inducible promoters are one of the easiest and most effective ways to turn on gene expression. Several inducible expression systems are now available for use in bacteria. Although many of these promoters do not have consistent expression in all cells of the culture, there are at least three ways to ensure (or engineer) consistent, regulatable control in all cells of a culture: uncouple transporter expression from inducer control, which has been done for the arabinose expression system (Khlebnikov et al., 2001); synthesize an inducer that can readily diffuses across the cell membrane without a transporter, like IPTG; or use a natural inducer whose transport is not autoregulated, such as propionate (Lee and Keasling, 2005). Constitutive promoters (Alper and Stephanopoulos, 2007; Jensen and Hammer, 1998), promoters induced by starvation of an essential nutrient, and promoters induced upon entry into stationary phase allow for inexpensive, inducer-free gene expression, particularly important in large-scale fermentations for production of chemicals and fuels where cost is an issue.

3.4. Simultaneous engagement of multiple genes

Construction of a metabolic pathway most often involves the introduction of more than one heterologous gene. There are multiple ways to coordinate expression of multiple genes: (1) use different inducible promoters for each gene, (2) use the same inducible promoter for each gene but vary the promoter strength, (3) use a non-native RNA polymerase (Kennedy et al., 2003; Murli et al., 2003; Mutka et al., 2006) or transcription factor (Alper and Stephanopoulos, 2007; Tyo et al., 2007) to control the expression of more than one gene, (4) group multiple, related genes into operons and use internal ribosomal entry sequences (IRESs) in eukaryotes (Chappell and Mauro, 2003; Fernandez-Miragall et al., 2006; Komar and Hatzoglou, 2005; Martin et al., 2006) and (5) vary the ribosome binding strength for the proteins encoded in the operon (Salis et al., 2009). Sequences inserted into the intergenic regions of bacterial operons can direct the processing and segmental stability of a transcript containing multiple coding regions (Pfleger et al., 2006a; Smolke et al., 2000).

3.5. CAD tools

The design of any microbial chemical factory is made difficult by the lack of simulation tools to predict the types and expression levels of genes necessary to engineer the metabolism of any cell for high-level production of a desired molecule. In almost all

other areas of engineering, there are models and simulation tools that allow one to design needed components and to assemble those components into a large, functioning system. Similar biological design tools are in their infancy.

Because biology is so complicated, most "design" tools are largely analytical and focused on a particular aspect of biology. For example, a number of metabolic engineers have developed models for metabolism to analyze fluxes through central metabolic pathways and to predict the impact of changes in a particular metabolic reaction or pathway on chassis growth and product formation (Burgard et al., 2003; Christensen and Nielsen, 2000; Edwards et al., 2001). These models have been very useful for analyzing metabolic fluxes once the changes to metabolism have been made. However, very few models allow one to accurately predict the necessary changes to effect a change in flux. Unfortunately, it is still not possible with any degree of accuracy, to predict the functioning of a promoter or any other component in the chassis and the actual levels of expression needed to achieve a particular flux through a reaction or pathway. Integration of flux balance models with models of genetic regulation could greatly facilitate predictive engineering of cellular metabolism.

3.6. Debugging routines

The development of software has been made possible only through the development and use of debugging software to quickly identify and correct problems with code. The development of similar tools for biological debugging would reduce development times for building and optimizing engineered cells. For the development of microbial chemical factories, functional genomics can serve in the role of debugging routines (Bro and Nielsen, 2004; Park et al., 2007), because the introduction of a metabolic pathway often elicits a stress response (e.g., stringent response, unfolded protein response, etc.) in the host (Gill et al., 2000; Oh and Liao, 2000a; Oh and Liao, 2000b). These stresses are reflected in mRNA and proteins expressed at that time and are thus detectable using DNA arrays or proteomics (Gill et al., 2000). Overexpression of a metabolic pathway might rob the cell of central metabolic intermediates resulting in decreased cell growth, a change in the profiles of intracellular and extracellular metabolites, which would be reflected in the metabolite profile, and a change in metabolic fluxes, which would be reflected in the flux profile. Imbalances in a metabolic pathway will result in an accumulation of potentially toxic metabolites that inhibit growth (Martin et al., 2003b). Information from one or more of these techniques can be used to then modify expression of genes in the metabolic pathway or in the host to improve titers and/or productivity of the final product.

4. Conclusions

The continued development of tools using the developments in synthetic biology is the surest way to reduce the cost and time required to engineer biological systems, such as those engineered to produce pharmaceutical ingredients, fine and commodity chemicals, and fuels. While the development of biological components might be less 'sexy' than the development of solutions to important problems, those components will enable many solutions, not just the ones for which the components were developed.

Acknowledgments

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