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Pathway and protein engineering approaches to produce novel and commodity small molecules

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Nature has provided us the basis of designing the most integrated and efficacious production platforms. Cell factories via their millions of years of evolution have nearly perfected each of their production systems. We have been trying to imitate, utilize and tweak this system to our advantage by using slightly overlapping and greatly interdependent approaches such as metabolic engineering and systems biology to make nature work for us in an efficient and robust way, without producing toxic waste and/or unnecessary side products. Systems biology, metabolic engineering and 'omics' technologies have paved the way for protein and pathway engineering. To this end we will talk about the recent advances in production of novel pharmaceutical and commodity small molecules by designing novel proteins and pathways.

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Introduction

Living organisms have been tuned by millions of years of evolution to efficiently utilize renewable sources of energy to produce a variety of chemicals, many of which have found extensive human applications. The emergence of recombinant DNA technologies and metabolic engineering have allowed the expression of entire metabolic pathways in heterologous hosts and the fine tuning of the resulting recombinant organisms in order to produce such important chemicals at high titers and high yields.

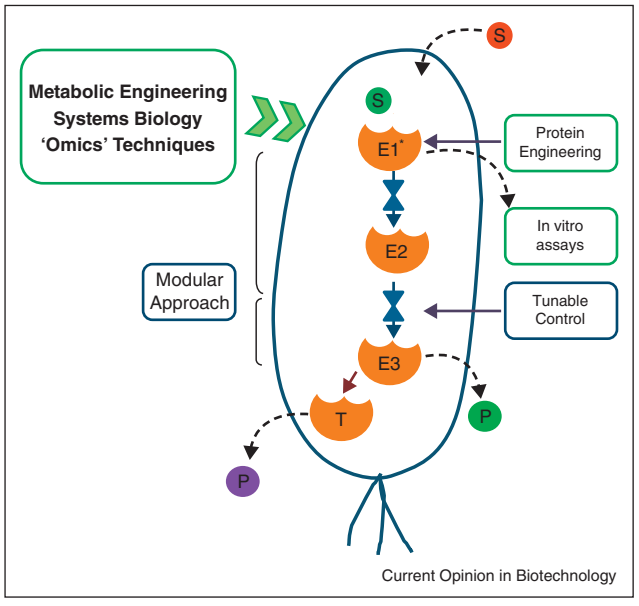
In recent years, the engineering of biological systems has expanded in other directions, beyond making the production of natural compounds economically viable. Metabolic engineers are becoming more and more interested in creating novel metabolic pathways by mixing and matching biosynthetic enzymes from different sources or altering the biochemical properties of enzymes in order to generate novel molecules. Commodity chemicals such as building blocks for biopolymers, aroma chemicals, *etc.* are of significant interest for biological engineering [1] (Figure 1). Extensive work has been done on small molecules such as *n*-butanol, isobutanol and higher alcohols, which are proposed to be the ideal replacement for the conventional fossil-based fuels [2]. In addition, pharmaceutically important small molecules have been diversified using tailoring enzymes, fusion proteins and protein engineering techniques [3]. The different approaches involve *in vitro* or *in vivo* production systems. While *in vitro* production platforms enjoy the benefit of not needing to streamline all the other processes of the cell to enhance yield, assembly of several different enzymes and cascading reactions can be a daunting task. In addition, at an industrial level, optimization of conditions for *in vitro* enzymatic reactions is more difficult than that for whole cell systems. On the other hand, *in vivo* production platforms suffer from difficulty in channeling intermediates intrinsic to the heterologous host into the pathway of interest as well as the potential toxic effects of pathway intermediates or the end product.

The recent advances in understanding cellular metabolism have significantly facilitated the progress of pathway engineering for production of novel and commodity chemicals. In addition, development of more efficient cloning techniques has made it possible to express entire pathways heterologously in genetically tractable organisms, usually bacteria and yeast [4–6]. Metabolomics has also played a pivotal role in the synthetic microbiology of secondary metabolism. It has helped to identify bottlenecks for improving yields and novel compounds in microbes, and the discovery of entirely new pathways, with the assistance of metabolic modeling and genome mining [7,8].

In vitro approaches

In vitro enzymatic reactions have the distinct advantage of producing regio-selective compounds usually at high purity. The conversion rate and specificity of the enzymes can be tweaked by modifying the catalytically important residues of the enzymes involved [9–11]. Certain robust enzymes such as polyketide synthase (PKS) type 1 are difficult to functionally express *in vitro*. Recently however Ma *et al.* have reported the isolation of functional lovastatin nonoketide synthase (LovB) by altering the

Figure 1



Schematic representation of strategies to generate novel pharmaceuticals and commodity chemicals. Metabolic engineering, systems biology and 'omics' techniques provide the basis for protein and pathway engineering. The boxes in blue represent *in vivo* approaches. Green box represents *in vitro* and *in vivo* approaches. Tailoring enzymes can be used to decorate the small molecules through glycosylation, acylation, alkylation, *etc. Notations*: E1*: engineered protein; E2, E3: enzymes of engineered pathway; T: tailoring enzymes.

expression host (*Saccharomyces cerevisiae* instead of *Aspergillus*) and formed the nonaketide, dihydromonacolin L acid, by coupling LovB and enoyl reductase (LovC) *in vitro* [12•]. Further fusion of different iterative enzymes like PKSs and nonribosomal peptide synthases (NRPS), which act on similar substrates has resulted in *in vitro* synthesis of the siderophore yersiniabactin [13], tetramic acid-containing macrolactams (antifungal agents) [14], and cyclopiazonic acid intermediates (inhibitors of Ca^{2+}-ATPase) [15]. Type II PKSs are analogous to the bacterial fatty acid synthases (FAS), and catalyze the formation of bacterial aromatic natural products such as actinorhodin, frenolicin and tetracenomycin. Khosla *et al.* have extensively worked on *in vivo* expression of type II PKSs. They have also recently reported *in vitro* analysis of the hedamycin PKS, which has shed some light on the functionality of type II PKSs [16].

Diversification of products of type III PKSs is a classic example of protein engineering. Unlike their type I counterparts, type III PKSs are relatively small proteins with a single polypeptide chain. Site-directed engineering of the active-site architecture of these enzymes has been used to produce unnatural polyketide scaffolds [17]. Abe *et al.* have recently reported producing a novel polyketide-alkaloid scaffold [18]. A precursor-directed approach was applied, where malonyl-CoA synthetase (MCS) in conjunction with chalcone synthase (CHS) was expressed *in vitro* and synthetic starter molecules were used as substrates and malonate as extender. Kwon *et al.* have also showed how the immobilization of the enzymes helps to improve the yields by a great extent (~30% in this case), which can be an important factor when trying to reconstitute an entire pathway *in vitro* [19,20••] (Figure 2).

Tailoring enzymes are nature's own way of diversifying the secondary metabolites by decorating the molecules produced with different chemical moieties such as sugar and alkyl groups and halogen molecules. In a recent study the DesVII, a glycosyltransferase from *S. venezuelae* was coupled with an auxiliary protein, DesVIII, to form a number of unnatural macrolides [21]. In addition, more than 50 novel *O*-glycosides, *S*-glycosides and *N*-glycosides of oleandomycin were produced using the wild-type glycosyltransferase OleD and a triple mutant of the same enzyme (A242V/S132F/P67T) [22]. Various geranyl and dimethylallyl aromatic polyketides were synthesized *in vitro*, after expression and purification of Fur7, a prenyltransferase (PT) from *Escherichia coli* [23].

Terpenoids constitute another class of highly structurally diverse molecules. The substrate flexibility of the terpenoid pathway enzymes is relatively limited and as a result diversification of products is achieved by altering chain length, branching and cyclization. A recent successful attempt was made to form chlorinated analogs of GGPP by supplying chlorinated isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) as substrates for farnesyl pyrophosphatase (FPPase) [24••]. In addition, chimeric enzymes were prepared by fusing catalytic fragments of FFPase and chrysanthemyl pyrophosphatase (CPPase), so that the resulting enzyme could catalyze chain branching and cyclobutanation reaction [25]. Moreover, a novel triterpenoid (13αH)-isomalabarica-14(27),17E,21-trien-3β-ol was created by mutating lanosterol synthase [26]. Promiscuity of various sesquiterpene synthases from *Coprinus cinereus* was tested for different FPP stereoisomers [27••].

In vivo approaches

Construction of new pathways by assembling enzymes from different unrelated organisms or from different pathways from the same organism has enabled the construction of systems that can result in a wide variety of nonnatural or diversified natural products. Tseng and Prather have recently addressed the issue of removing the bottlenecks for different modules in a synthetically designed pathway based on the butanol biosynthetic pathway [28••]. They achieved this by creating a bypass for each module by developing a CoA addition/removal tool kit. All the intermediates of the pathway are CoA derivatives, and are thus trapped inside the cell. The use