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De novo biosynthetic pathways: rational design of microbial chemical factories

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Increasing interest in the production of organic compounds from non-petroleum-derived feedstocks, especially biomass, is a significant driver for the construction of new recombinant microorganisms for this purpose. As a discipline, Metabolic Engineering has provided a framework for the development of such systems. Efforts have traditionally been focused, first, on the optimization of natural producers, later progressing towards re-construction of natural pathways in heterologous hosts. To maximize the potential of microbes for biosynthetic purposes, new tools and methodologies within Metabolic Engineering are needed for the proposition and construction of *de novo* designed pathways. This review will focus on recent advances towards the design and assembly of biosynthetic pathways, and provide a Synthetic Biology perspective for the construction of microbial chemical factories.

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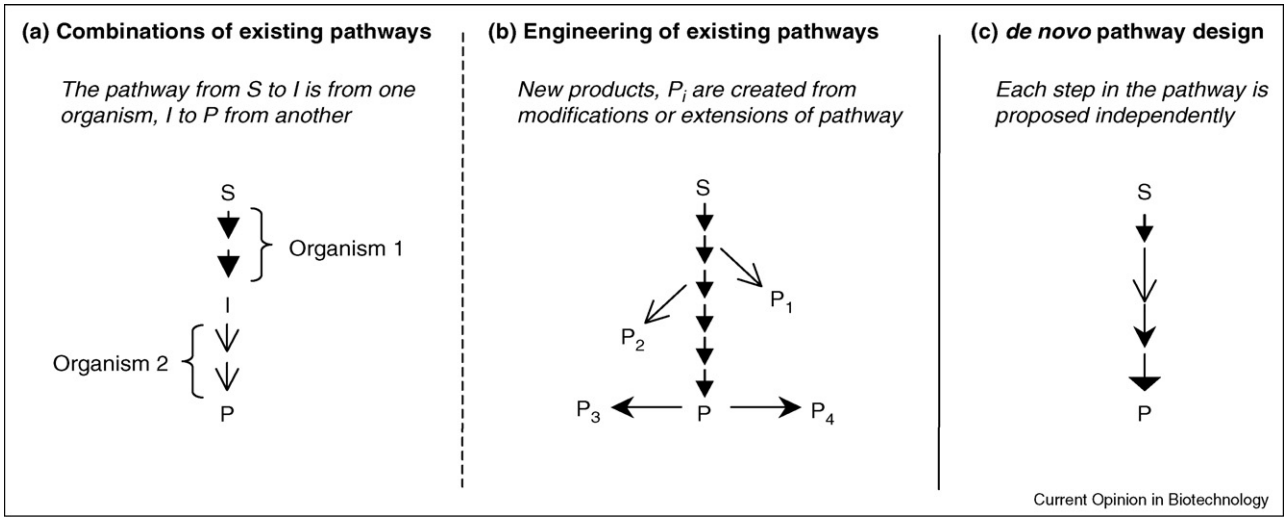
Introduction

Microbes are well established as effective hosts for the biosynthesis of small molecules. A look at the metabolic network of a simple organism such as *Escherichia coli* reveals the inherent potential for the synthesis of hundreds of unique chemical structures. Advances in Metabolic Engineering over roughly the past 15 years have led to high-level production of specific metabolites at commercially viable levels. Classical examples include the production of antibiotics [1,2] and amino acids [3–5] in native hosts. More recently, the development of effective tools for genetic engineering has resulted in the development of microbes that express heterologous biosynthetic pathways, resulting in the production of natural products beyond the genetic confines of the natural host [6–9]. The utility of biological systems to effectively mediate chemical conversions is now an accepted reality. These examples of engineered biological systems are optimizations or reconstructions of existing natural pathways. In order to truly exploit the synthetic capacity of biological systems and broaden the creation of microbial chemical factories, it is necessary to go beyond natural pathways for the synthesis of natural products towards the *de novo* design and assembly of biosynthetic pathways for both natural and unnatural compounds.

Biosynthetic pathways that are not wholly recruited from a single source can be assembled in one of three ways (Figure 1). First, partial pathways can be recruited from independent sources and co-localized in a single host. Recent examples of these types of pathways include the well-known case of 1,3-propanediol biosynthesis, in which pathways from *Saccharomyces cerevisiae* and *Klebsiella pneumonia* were assembled in *E. coli* [10], and the more recent production of artemisinic acid, a precursor to the plant-based anti-malarial drug artemisinin in yeast [11•]. Pathways can also be constructed for the production of new, non-natural products by engineering existing routes [12–14]. In this manner, structurally novel molecules have been produced by employing enzymes with broad or altered substrate specificity to act upon intermediates or end metabolites in functional pathways.

The combination of these two approaches, the recruitment of partial pathways from discrete species and the use of engineered or promiscuous enzymes for extensions of pathways, leads to a third means of specifying biosynthetic routes, namely *de novo* design. This approach differs philosophically from the other two by lifting the inherent restriction of beginning with known pathways. Instead, an approach analogous to the practices of retrosynthesis used by organic chemists to specify synthesis schemes towards a target compound can be used to specify biosynthetic pathways, by considering the biotransformation of functional groups rather than the entire structure. This 'retro-biosynthetic' approach has many challenges that are unique to the limitations of biology; however, it also presents an opportunity to exploit the tremendous natural diversity of enzyme-catalyzed reactions across many species. Indeed, a methodology such as this significantly expands the possibilities for biological synthesis, especially for the production of compounds for which natural pathways have not been elucidated.

Figure 1



Strategies for biosynthetic pathway design. The third approach for *de novo* pathway specification is of particular interest.

The evolving discipline of Synthetic Biology overlaps with these efforts in Metabolic Engineering and provides a complementary framework for the *de novo* design of new biosynthetic pathways. Synthetic Biology aims to frame the engineering of biology in a manner that is analogous to other engineering disciplines and relies on core principles of design and characterization to facilitate the rapid and reproducible deployment of biological machines [15–18]. In this context, machine specification relies upon sets of well-characterized Parts, consisting of genetically encoded, inherent biological functions (e.g., a promoter), and the ability to physically and functionally compose those Parts into Devices that perform human-defined functions. Multiple Devices can be assembled together into Systems that perform higher-level functions [19–24]. The pathway design problem is therefore an attempt to specify a System for the production of a target molecule. Key to this is the specification of gene sequences encoding enzymes, that is, Parts that catalyze each reaction in the pathway and whose DNA sequences can be incorporated into Devices that lead to functional expression of the proteins of interest.

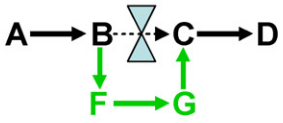
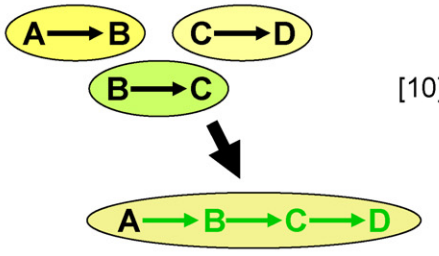
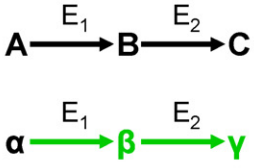
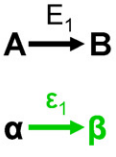
Few examples of *de novo* pathways currently exist, and efforts to assemble them are hampered by both the large number of theoretical possibilities for biotransformations and the limited availability of desired enzymes to mediate specific biological conversions. It is our intent in this article to (i) present recent significant developments that have advanced a methodology for retro-biosynthesis (Table 1), (ii) highlight computational tools that facilitate the specification of pathway designs, and (iii) discuss the very real challenges that impede the wide-scale construction of rationally designed microbial chemical factories.

Exploitation of known Parts

The simplest pathway designs employ existing, 'off-the-shelf' Parts. In an example related to one previously mentioned, a recombinant strain of *E. coli* was constructed to synthesize amorphadiene, a precursor to artemisinin [25]. Of note here is the use of genes from the yeast *Saccharomyces cerevisiae* for the production of isopentenyl diphosphate (IPP), the common precursor for all isoprenoids; endogenous *E. coli* genes for the conversion of IPP to farnesyl disphosphate (FPP), a C_{15} intermediate; and a synthetic plant-based amorphadiene synthase (*ADS*) gene for the conversion of FPP to amorphadiene. Although each of these enzymes was performing its native function in a heterologous host, it is worthwhile to note the successful combinations of enzymes from disparate sources. From a design perspective, the strategy employed in the selection of the mevalonate pathway for the synthesis of IPP in place of upregulation of the inherent non-mevalonate (DXP) pathway highlights the benefits of employing orthogonal pathways as a means of circumventing native regulation. A similar strategy has been applied to increase the yield of L-glutamic acid from glucose in a natural producer [26]. In a more recent example, a pathway was assembled to produce isopropanol in *E. coli* [27•]. The 4-step pathway originating from acetyl-CoA is native to several strains of *Clostridia*, most notably *C. beijernickii*. The most productive strain consisted of enzymes obtained from three bacteria, *E. coli*, *C. acetobutylicum* and *C. beijerinckii*, and outperformed the natural producer. The strain similarly produced high levels of acetone, the precursor to isopropanol. Here, the authors chose a strategy of screening multiple enzymes at each step, selecting the ones yielding the highest productivity, rather than relying only on the enzymes of the natural pathway. This emphasizes

Table 1

Objectives for biosynthetic pathway design

Design Objective	Pathway Schematic	References	Notes
Engineering Unnatural Bypasses within Existing Pathways		[25], [26], [35]	• Bypasses may be implemented with either natural ([25],[26]) or engineered Parts ([35])
Composing Hybrid Pathways from Individual Native Enzyme Activities		[10], [11], [27], [29]	• Hybrid pathways may be assembled to co-localize partial pathways from distinct sources (e.g., [10]) or to exploit homologous enzymes with higher activity (e.g., [27]) • [29] is a special case of polyketide synthesis in which a single, multi-fucntional polypeptide is produced from discrete domains assembled in a combinatorial fashion
Exploiting Enzymatic Promiscuity to Create New Pathways and Products		[28], [45]	• The products of promiscuous enzymes may be from known or designed pathways. This design objective can result in truly *de novo* biosynthetic pathways.
Creating New Products and/or Pathways using Engineered Enzymes		[31-33]	• Engineered enzymes have been used to create unnatural compounds ([31]) or as more productive alternatives to existing enzymes, performing their natural functions ([32,33]).

Products and arrows in green represent the designed alternatives to natural pathways.

the characterization of enzymes as interchangeable Parts with a core function of catalyzing a specific functional group transformation, irrespective of the original location in a specific pathway.

In both examples described above, the designed pathways employed enzymes from distinct sources; however, the end products were known to exist as the result of enzyme-catalyzed reactions. In a departure from this

theme, consider the biological synthesis of 1,2,4-butanetriol from xylose [28]. This system employed two different microbes, starting with *Pseudomonas fragi*, which was known to oxidize xylose to produce xylonate. Enzymes native to *E. coli*, the second microbe, were able to transport and dehydrate this substrate to produce the keto-acid 3-deoxy-D-*glycero*-pentulosonic acid. Six different decarboxylases were screened to identify one, benzoylformate decarboxylase from *P. putida*, capable of acting upon this substrate, and an endogenous *E. coli* dehydrogenase produced the final alcohol. This pathway, consisting ultimately of enzymes from three different organisms, resulted in the production of a compound without a previously identified biosynthetic route. This early example of true *de novo* pathway design illustrates the potential that exists for the biological synthesis of small molecules not previously identified as natural products.

Utilization of engineered Parts

Use of natural Parts has produced much success; however, access to greater structural diversity can be gained using engineered enzyme Parts. This principle has been illustrated in both the previously cited example of the production of structurally diverse carotenoids [12–14], and for the production of a variety of novel polyketides [29,30]. (Several recent reviews obviate the need for further discussion of these compounds.) Consider in this case the design of a microbial pathway for the production of 3-hydroxypropionic acid [31]. The pathway originated from alanine, presumed to be synthesized from endogenous amino acid biosynthetic pathways. The first step involved the isomerization of the α-amino acid to produce β-alanine; however, there was no naturally occurring enzyme possessing this activity. Instead, a lysine 2,3-aminomutase was evolved to act on alanine. The terminal amine group could then be converted to an aldehyde using an enzyme with known β-alanine aminotrasferase activity, and reduction of the aldehyde to an alcohol was facilitated by endogenous *E. coli* activity. Implementation of this *de novo* pathway was only possible as the result of a successful enzyme evolution effort.

The ability to engineer enzymes to act upon new substrates significantly expands the potential to design and assemble new biosynthetic pathways. Simply put, enzymes acting upon unnatural substrates will produce unnatural products, thereby facilitating the creation of diverse chemical structures. This diversity can be used to produce compounds with no known natural route as described above, or it can be exploited to create either more productive or orthogonal pathways for alternative synthetic schemes. In one example of this, a mutant form of 6-methylsalycilic acid synthase (6-MSAS) was found to be more effective in producing triacetic acid lactone than a native 2-pyrone synthase enzyme known to possess the desired activity [32•,33]. As an illustration of the desire to create new pathways for orthogonality, an evolved 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) aldolase was used to produce shikimate, a metabolite endogenous to *E. coli* [34,35•]. The natural pathway utilizes phosphoenolpyruvate (PEP), a required component of the PTS sugar transport system. The designed pathway circumvents the limitation of PEP availability and results in higher yields of the desired compound on glucose. Thus, designed pathways need not produce unnatural products to be of significant value.

Computational tools for pathway design: specification of Parts

The examples discussed above illustrate the potential of *de novo* pathway design to expand the synthetic capacity of biological systems. The availability of computational tools will be of great value in proposing pathways that can then be selected for implementation. Many algorithms currently exist to propose biosynthetic pathways in the form of metabolic networks extracted from genome-wide data sets [36–38]. For the *de novo* design of biosynthetic pathways, two algorithms are of particular note. The user-guided metabolic 'pathway prediction system' (PPS) of the University of Minnesota Biocatalysis and Biodegradation Database (UM-BBD) is designed to predict routes for the biodegradation of xenobiotic compounds [39–41]. Using a set of previously defined Biotransformation Rules, the PPS guides the user through potential pathways one step at a time, requiring the selection of a new target metabolite at each step. Although this algorithm is specific to catabolic pathways, the principles employed are amenable to biosynthesis. Hatzimanikatis and co-workers have developed the BNICE algorithm (*B*iochemical *N*etwork *I*ntegrated *C*omputational *E*xplorer) specifically for the proposition of *de novo* pathways [42,43]. The algorithm uses the E.C. nomenclature of enzymes to specify generalized enzymatic reactions in graph-theory matrices that act as operators on each reactant. BNICE was utilized to explore theoretical diversity in polyketide synthesis, predicting more than one billion linear structures [44]. We have developed a database, ReBiT (*R*etro-*B*iosynthesis *T*ool, http://www.retro-biosynthesis.com), to facilitate the identification of enzymes for use in pathway designs. ReBiT is organized by the functional groups that participate in the reactions, accepting as input a molecular structure and returning as output all enzyme groups capable of either generating or consuming that structure.

Challenges towards broader implementation of biosynthetic pathway designs

The assembly of large numbers of *de novo* designed pathways for unnatural product synthesis is impeded by several obstacles. The ReBiT database contains 605 unique structures and 637 chemical conversions, and algorithms such as BNICE highlight the vast number of possibilities for biosynthetic pathways if one considers the full biotransformation potential of enzymes. To successfully implement designed pathways, it is necessary to

choose from among the myriad possibilities (for example, 75,000 theoretical routes from chorismate to phenylalanine [42]). Establishing a set of guidelines to filter through the various pathways presents a significant challenge. One such filter, as implemented in BNICE, considers thermodynamic feasibility [42], but this still leaves too many pathways to implement experimentally. Ultimately, the choice of a pathway is the recursive selection of the enzyme Parts that comprise it.

At the level of Parts selection, the use of enzymes with naturally broad substrate range presents the greatest opportunity for implementation of a *de novo* designed pathway. Two examples of such enzymes are keto-acid decarboxylases and alcohol dehydrogenases. The former were employed in the 1,2,4-butanetriol example presented previously as well as in the recent design of biosynthetic pathways for a variety of alcohols derived from intermediates in amino acid biosynthesis [45•]. The ability to use these enzymes against a wide variety of structurally diverse substrates, as well as the availability of several members of the enzyme class makes them attractive for deployment in *de novo* designed pathways. Similarly, endogenous as well as heterologous alcohol dehydrogenases have been utilized in several pathways, including those for 1,3-propanediol, 1,2,4-butanetriol and 3-hydroxypropionic acid [10,28,31]. The dehydrogenases are particularly well known for use in single-step biocatalytic transformations [46]. However, most pathways will not be limited to the use of one or two steps that can take advantage of readily available, promiscuous enzymes. While additional work is needed to determine an appropriate set of criteria for Part selection, we propose as first steps that reaction diversity (i.e., the diversity of structures with similar functional group transformations) and genetic diversity (i.e., the diversity of species exhibiting that chemistry) in addition to the previously described substrate diversity provide a means for assessing the potential of a reaction step to be successfully implemented in a designed pathway.

The continued availability of new or newly engineered enzyme Parts is also a critical challenge. Retro-biosynthetic specification of pathways ultimately assumes the availability of an enzyme capable of acting upon a specific substrate. In the absence of a naturally occurring variant, one may need to be created. Recent advances in protein engineering make this an increasingly likely proposition [47••,48]. It is also important to recognize that protein engineering may be necessary to facilitate 'chassis' compatibility, that is, functional expression of the enzyme in the host organism [49•,50,51]. Finally, engineering of the specific enzyme or of the broader System may be needed for functional composability, that is, to utilize proteins whose optimal conditions, such as pH, may be inherently incompatible with other enzymes in the pathway and the host itself.

These challenges highlight the reality that while Synthetic Biology provides a complementary framework for *de novo* pathway design, it is unclear how well some of the core principles, for example, Abstraction, can be implemented. The gene sequence for an enzyme does not merely represent a biotransformation function, but also encodes its optimal operating conditions, as well as its limitations. Nevertheless, our efforts to capture critical information and to abstract others will represent progress towards the development of a sound framework for the assembly of designed pathways.

Conclusion and perspectives

Many examples from the discipline of Metabolic Engineering have proven that the capacity of microorganisms for synthesis of biochemicals is extensive and highly amenable to modification. The progression of demonstrable examples from the optimization of natural hosts for the overproduction of a metabolite to the re-construction of pathways in heterologous hosts indicates that movement of enzymes between species is an effective and accessible technology. The current challenge is to expand the biosynthetic capacity of microbes through the *de novo* design of biosynthetic pathways towards compounds for which natural pathways either do not exist or are intractable. The opportunity to do so is increasing, particularly in the efforts to displace compounds produced from petroleum-derived feedstocks, including fuels and both commodity and fine chemicals, with those derived from biomass [52•,53]. Under the umbrella of Synthetic Biology, one can exploit enabling technologies, such as DNA synthesis and design of enzymes with novel functions, as well as a framework for proposing the pathway design problem as a challenge involving both the best means for specifying and selecting appropriate enzymatic Parts and the availability and characterization of those Parts. Simultaneously, it is important to recognize that the ultimate goal is the construction of a microbial factory that would be implemented in a large-scale facility, that is, a *real* factory. Accordingly, the tools of Metabolic Engineering for assembly, analysis and optimization of complex biological systems – and its long history of successes in this arena – will continue to have prominence and broad applicability [54–56].

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This landmark paper describes the creation of an enzyme that catalyzes a novel reaction. Computational methods were used to design protein folds predicted to bind to the desired transition state intermediates, and screening of several dozen variants resulted in the synthesis of several with the desired activity. This represents a first step towards the creation of new enzymes to either catalyze known biochemical reactions but on structurally distant substrates, or to expand the overall biosynthetic potential of enzymes, both of which would find unlimited applicability towards implementation in pathway designs.

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