Evolutionary metabolic engineering

In *The Selfish Gene*, Dr Richard Dawkins writes:

If superior creatures from space ever visit earth, the first question they will ask, in order to assess the level of our civilization is: “Have they discovered evolution yet?” (Dawkins, 1989)

The fact that modern biology relies on the Darwin’s theory leaves little space for debating this question at the scientific level. Thus, the practical follow-up question is “Have they discovered how to use evolution as a tool?” The collection of papers in this special issue of *Metabolic Engineering* provide a compelling case that the answer to question is a resounding “yes” and that evolutionary engineering (Sauer, 2001; Sonderegger and Sauer, 2003) is an established tool that continues to develop both technically and in the breadth of its application.

Evolution through adaptation and natural selection is an iterative process of genetic diversification and functional selection. It is nature’s design algorithm, and it is believed that all natural biological structures were “designed” through this process so that they would best “fit” in certain conditions. Evolutionary engineering is simply the application of this first principle of biological design (Tobin et al., 2000) and involves controlling all aspects of the genetic diversification and functional selection of biological systems in the lab. A given system, for example, a gene encoding an enzyme, and a desired engineering performance goal, such as catalytic turnover, are identified. The gene encoding the enzyme is then diversified by any of a variety of techniques. The resulting “gene library” is then screened for those genes that encode new versions of the enzyme having the desired improvements in catalytic performance. This process is iterated as required until a final performance or economic criterion has been met. The application of evolutionary engineering will almost always result in new systems that have improved performance under the screening conditions. However, the true success of this approach requires the engineer to clearly understand the important performance criteria, to correctly choose the gene(s) responsible for controlling this criteria, to design a high-throughput screen that truly measures the desired performance criteria such that identified “hits” perform as originally envisioned (i.e., under scaled-up industrial conditions), and then to implement the evolutionary algorithm using best practices. The success of evolutionary engineering in the academic and industrial literature emphasizes the robust nature of this approach and its general importance to biosystems engineering. The papers in this issue provide further examples of this importance and illustrate how the technology can be used to successfully address the technical challenges of metabolic engineering.

Evolutionary engineering is the original application of biotechnology and has been practiced for thousands of years through the purposeful breeding of plants and animals (Brooks, 1999). For centuries, strain selection has resulted in improved yeast strains for baking and spirit production, and for more than half a century classical strain improvement has produced countless high-productivity fermentation organisms (Demain and Solomon, 1986; Demain and Davies, 1999). Modern molecular biological techniques have now enabled evolutionary engineering to be applied to isolated genes. To this end, Barry Hall “evolved” the galactosidase gene in *Escherichia coli* mutant cultures, providing solid experimental evidence that mutation and laboratory selection in concert could support the directed evolution of a new metabolic function (Hall, 1982). Molecular evolutionary engineering now includes a portfolio of tools for controlling the genetic diversification of a targeted biological system. These include error-prone polymerase chain reaction (Goldman and Youvan, 1992; Harlow et al., 1994; Jones and Howard, 1991) and cassette mutagenesis (Delagrave and Youvan, 1993; Kluget al., 1991; Krishnan et al., 1991) that result in the gradual generation of a “mutant cloud” around a specific gene sequence; gene, family, and genome shuffling that rely on recursive sequence recombination of diverse populations having useful function to generate a combinatorial population of those traits (Crameri et al., 1998; Stemmer, 1994); and sequence homology-independent technologies, such as exon shuffling, which sparsely populate larger regions of sequence space (Kolkman and Stemmer, 2001; Lutz et al., 2001;
Ostermeier et al., 1999; Sieber et al., 2001; Zhang et al., 2002. Computational tools, such as SCHEMA (Voigt et al., 2002) and FamClash (Saraf et al., 2004), are now routinely used to bias genetic diversification based on protein structure–function criteria. All of these methods in combination with laboratory screening or selection continue to support the successful generation of individual genes, multigene pathways, plasmids, viruses, and microbial genomes having improved performance and desired characteristics. Indeed, the promise of this approach has now led to an industry dedicated to its commercial development and application.

Evolutionary and metabolic engineering have an obvious synergy, as both strive to manipulate biological systems to perform in a desired fashion. A primary goal of metabolic engineering is the genetic manipulation of whole cell catalysts to perform in a truly useful fashion. This requires identification of a desired set of chemical transformations to be catalyzed (i.e., conversion of simple feedstocks to desired primary, secondary, or protein metabolites), the selection of an appropriate host organism, the identification and subsequent genetic manipulation of that host to engender the desired transformations, followed by further genetic and process manipulations to enable a truly useful process. To this end, nature’s diversity must be initially sampled to identify a host organism and additional genetic elements required to compile the desired catalytic activities. Indeed, “bioprospecting,” the discovery of new organisms and genes from nature, remains a critical component of cellular, genetic, metabolic, and evolutionary engineering. Once an active biocatalyst has been constructed, it usually will require significant improvement to be of practical value (i.e., perform at economically defined conditions). Native organisms and their recombinant progeny containing naturally derived genes seldom meet these rigid commercial criteria, and further genetic modifications of the host genome, sometimes through the introduction of heterologous genes, are required to achieve properties beyond what nature has provided. It is here that evolutionary engineering can provide advantages over the rational approach, because there are no robust first principles defining the relationship between biological structure and function.

While an engineer may believe that a particular enzyme in a metabolic pathway is rate limiting, the general knowledge required to improve the catalytic proficiency of enzymes through rational design remains elusive. Further, in the complex milieu of the cell, enzyme activities are affected by countless physical, chemical, and biological factors that cannot be accurately modeled or predicted. Overexpression is a crude option that can be successful; however, it is costly to the cell (one peptide bond costs the cell more than 3 ATP molecules). Diversifying the gene encoding the rate-limiting enzyme, expressing that gene in the production host, and screening for new genes that enable improved production should find a molecular solution if the targeted gene is truly rate limiting and the screen is predictive. Once improved catalysts are identified, their analysis can lead to a better understanding of the original limitation and its solution at a molecular level. As mentioned above, individual genes, entire metabolic pathways, or complete host genomes can be targeted for evolutionary engineering; the choice simply relies on the engineer’s confidence that a genetic solution can arise from the targeted DNA.

This special issue of Metabolic Engineering was designed to highlight the synergies of evolutionary and metabolic engineering. To this end, the papers in this issue emphasize current methods for controlling genetic diversification and functional selection in the lab and the application of evolutionary engineering to the improvement of protein and whole cell catalysts. Stutzman-Engwall and colleagues set the paradigm for the state of the art application of evolutionary engineering. The Pfizer and Codexis teams describe the application of evolutionary engineering to improve the purity of Doramectin produced in commercial fermentations. Semi-synthetic DNA shuffling was used to diversify avWC, a gene of unknown biosynthetic function, but which is known to affect the ratio of avermectin B1 to avermectin B2, an unwanted byproduct. The resulting gene library was then expressed in the native host, Streptomyces avermitilis. Extracts from 96-well solid-phase fermentations were then screened by high-throughput mass spectrometry for those containing an improved B1:B2 ratio. Several rounds of evolutionary engineering resulted in a 23-fold improvement in the B1:B2 ratio, and a new process based on the newly engineered gene is now being commercialized. Qiong Cheng and colleagues describe their efforts to develop a high-copy-number broad-host-range plasmid that might be broadly useful for metabolic engineering efforts requiring high gene dosage. To this end, the rec region of a pBBR-based plasmid expressing a β-carotene reporter was diversified by error prone PCR, and Escherichia coli strains harboring these variants were visually screened for strains producing increased β-carotene. Improved strains harbored plasmid variants that supported 3- to 7-fold higher plasmid copy number in E. coli and increased β-carotene production in both E. coli and Agrobacterium tumefaciens. Employing a similar screening method, the Schmidt-Dannert group describes the production of new geranyl geranyl diphosphate synthases from a farnesyl synthase. The ispA gene was diversified by error prone PCR, and then strains expressing the gene library were visually screened for those producing β-carotene. An analysis of the genes encoding the new GGPP synthases identified new structural mechanisms for determining the isoprenoid chain length. The Sauer group demonstrates an elegant
application of the use of evolutionary engineering to improve the complex whole cell phenotype of quiescent metabolic activity. *E. coli* mutants were enriched in continuously and discontinuously fed nitrogen limited chemostat cultures. The enriched mutants were then screened for high specific glucose uptake under ammonium starvation induced stationary phase. The best strains demonstrated up to 2-fold catabolic rate improvements in the stationary phase. Three papers focus on the development of tools to facilitate evolutionary engineering, i.e., genetic diversification and screening. Copley and colleagues describe methods for effecting efficient recombination between protoplasts of *E. coli* auxotrophs that are an $\sim 10^4$ improvement over previous methods. These methods should support the production of genome shuffled libraries of Gram-negative bacteria from which improved strains could be selected. It is anticipated that methods supporting libraries that could be screened for improvements should be forthcoming with additional improvements. Sun and colleagues describe the use of the scintillation proximity assay (SPA) for the identification of protein ligands. The ease and high-throughput nature of this versatile binding assay are also useful for quantifying small and macromolecule production, an important phenotype in metabolic engineering (Carreras et al., 2001). Finally, Kitell et al. describe the use of 96-well parallel capillary electrophoresis for the separation and quantification of lovastatin from complex fermentation broths. This relatively new method of high-throughput screening provides the opportunity to quantify quickly the level of target compound(s) in a complex mixture in a rapid and parallel manner that could eliminate the bottleneck of liquid chromatographic separations.

This issue of *Metabolic Engineering* highlights the importance of evolutionary engineering for the design of useful biological systems. Methods and applications for this technology continue to develop, and it is anticipated that evolutionary engineering will become a default tool in biosystems engineering. The increased development of new and useful biological systems should yield a database of successful structure–function correlations. Perhaps from these evolutionary data, a useful set of design rules shall evolve that could be applied in a truly rational and robust manner.

**References**


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