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**Notes:**
- The corrections are marked with symbols in the right-hand margin.
- Some corrections are highlighted in different colors to distinguish between different types of errors.
- The table lists various types of typesetting errors and the corresponding correction marks.
A powerful toolkit for synthetic biology: over 3.8 billion years of evolution

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The combination of evolutionary with engineering principles will enhance synthetic biology. Conversely, synthetic biology has the potential to enrich evolutionary biology by explaining why some adaptive space is empty, on Earth or elsewhere. Synthetic biology, the design and construction of artificial biological systems, substitutes bio-engineering for evolution, which is seen as an obstacle. But because evolution has produced the complexity and diversity of life, it provides a proven toolkit of genetic materials and principles available to synthetic biology. Evolution operates on the population level, with the populations composed of unique individuals that are historical entities. The source of genetic novelty includes mutation, gene regulation, sex, symbiosis, and interspecies gene transfer. At a phenotypic level, variation derives from regulatory control, replication and diversification of components, compartmentalization, sexual selection and speciation, among others. Variation is limited by physical constraints such as diffusion, and chemical constraints such as reaction rates and membrane fluidity. While some of these tools of evolution are currently in use in synthetic biology, all ought to be examined for utility. A hybrid approach of synthetic biology coupled with fine-tuning through evolution is suggested.

Introduction: The promise of synthetic biology

For millennia, mankind has surveyed the natural world and wondered “if only...”. Through breeding, wolves, teosinte and other wild grasses have been transformed into dogs, maize, rice, wheat, and other domesticated species. But artificial selection can be a slow, haphazard affair and, like natural selection, depends on a pre-existing variability. We can increase the pool of variability by mutagens. We can create variability more precisely by genetic engineering. But, what if we could go beyond this to create more complex synthetic biological systems with the ease of plugging in interlocking components that were guaranteed to function in an enclosure of choice, either natural or synthesized?

The “new era of ‘synthetic biology’ where not only existing genes are described and analyzed but also new gene arrangements can be constructed and evaluated” is here.(1) Synthetic biology has acquired several meanings. Knight(2) and Endy(3) see synthetic biology as an engineering challenge with interchangeable parts joined to yield novel pathways. By stripping away the “baggage”(4) of its heritage, a minimal “chassis” organism would be created to provide a blank canvas upon which to build.(4) Church and Venter aim to build completely artificial cells. Venter intends to patent an entirely synthetic free-living organism.(5) The first simplified proteins have been synthesized.(6) Naturally evolved genomes have been recreated. Short segments of the 7,000-base poliovirus genome have been synthesized in vitro, stitched together, and shown to be active.(7) Venter’s team has accomplished a similar feat with a 5,400-base pair phage genome,(8) and the 582,970-base pair Mycoplasma genitalium genome using two methods.(9,10) Endy(3) identified the four challenges to engineering biology as:

(i) biological complexity,
(ii) the tedious and unreliable construction and characterization of synthetic biological systems,
(iii) the apparent spontaneous physical variation of biological system behavior,
(iv) evolution.

Certainly the complexity of biology provides a colossal engineering challenge. Organisms operate not as an engineered system, but as a metabolic ecosystem with extremely complex feedback loops. But until recently it was evolution that created all life that has ever existed on Earth. While some workers try to circumvent the complexity of living organisms, there are various principles and approaches that have been used successfully during evolution, and are worth examining by synthetic biologists. The richness of the evolutionary toolkit should be examined, exploited and, when advisable, overcome. While virtually unknown in nature, wheels are ubiquitous in human cultures. However, biomimetic “limbs” or wind-blown vehicles may be better for traversing Mars.

To develop evolution’s toolkit requires understanding Jacob’s metaphor(11) that natural selection works as a
“tinkerer”, rather than an “engineer” with a pre-conceived plan, tools and materials designed for a particular end. Data on structural(6,12) and regulatory gene evolution have supported this metaphor.(13) For the tinkerer, the premium is on utilizing (exapting) existing components in novel ways.(14) But a certain amount of “preadaptation” – having the ‘‘right’’ parts already available – is helpful. Thus, evolution is constrained by history, creativity, and physical constraints. The result is the Rube Goldberg device we call “life.”

The goal of this paper is to categorize and survey evolution’s toolkit as a way to point to potential approaches for synthetic biology. Some are already in use. Others are promising future prospects. Some are impractical. I conclude with an even less explored perspective, the potential for synthetic biology to inform evolutionary studies.

How nature took its course

What is different about evolution? First, evolution is a populational phenomenon whereas synthetic biology focuses on constructing single organisms that give rise to a clone. Where a clonal population has genetic homogeneity, a natural population has genetic variation as a foundation upon which phenotypic variation and the potential for selection is built. Natural populations obtain genetic novelty through recombination and mutation. A clonal population could undergo mutation as well. But, without an evolutionary approach, such spontaneous variability would be seen as a liability rather than a source of novelty.

Second, organisms are historical entities. Hydrogen produced during the Big Bang is interchangeable with hydrogen produced today. In contrast, organisms carry the mark of their developmental history, their interaction with the physical and biological environment, and their genetic history. Even if created synthetically,(5–8) from that moment onwards each organism will undergo a unique history which may effect its phenotype and genotype.

Third, natural selection(15) is a very powerful principle in its logic and explanatory power. Offspring show a range of heritable variation. More offspring are born than can survive. Those that are in some way better adapted to their environment produce offspring with the greatest chance of reproductive success. The illusion of directionality and purpose stems from consistent selective criteria over generations. And when the environment fluctuates, selection is for adaptability itself rather than increasing adaptation to a single environment.

Thus, even synthetically created biological systems will, once created, operate under the rules of evolution. Artificial selection is easily designed and applied. Thus, where evolution has the most to teach synthetic biology is in its diverse toolkit of ways to generate heritable novelty, and its core principles.

Origin of heritable novelty

Mayr(16) defined novelty as “any newly acquired structure or property that permits the assumption of a new function.” Various mechanisms for innovation are listed in Table 1, including Mendelian and non-Mendelian inheritance.

Usually heritable variation stems from genetic changes. Endogenous sources include alterations in DNA sequence caused by mutations and genetic recombination. Duplication and diversification of parts is a common form of novelty, from the gene to the cellular and higher levels (reviewed in Refs.(17,18)). Exogenous sources of variation include environmental mutagens, sex, viral-mediated gene transfer, and

<table>
<thead>
<tr>
<th>Source of novelty</th>
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<tr>
<td>Genomic</td>
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<tr>
<td>Endogenous</td>
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<td>Point mutations, gene/genome duplication,</td>
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<td>gene loss/loss of function, altered mutation rates,</td>
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<td>sexual recombination (autogamy), transfer of genetic material within a cell</td>
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<td>Exogenous – gene transfer</td>
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<td>Uptake of DNA from the environment, viral transfer, interspecies gene transfer, sexual and parasexual processes, symbiosis</td>
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<td>Regulatory</td>
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<tr>
<td>Internal</td>
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<td>Environmental mutagens (e.g., UV radiation)</td>
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<td>External</td>
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<td>Regulatory genes, nc (noncoding) RNA</td>
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<td>Physical</td>
<td>Compartmentalization</td>
<td>Organelles, multicellularity</td>
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<td>Non-genetic template</td>
<td>Cortical inheritance (e.g., in ciliates)</td>
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symbiosis. Heritable novelty can arise from changes in structure, regulation, or development.

There are also non-genetic heritable processes that rely on templating to structure. For example, the cortex in ciliates may have an anterior-posterior and dorsal-ventral asymmetry. After conjugation, the exconjugant protozoan may be left with a reversed row or so of cortex derived from their mate. The reversed rows are heritable. (19) Further, there are some physical changes such as compartmentalization in organelles that can be a source of heritable novelty for evolution.

Genomic novelty

Heritable novelty is the raw material for evolution, although much is deleterious or neutral. Changes may occur within or between generations. Novelty may be the result of the transfer of genes within a cell. Plant mitochondrial genes in particular have been transferred to the nucleus at a high rate. (20,21) Alternatively, novelty can be induced by environmental mutagens, and of these, solar ultraviolet radiation is arguably the most important. It is nearly ubiquitous, and was more intense during the early evolution of life, and still acts as a mutagen today. (22,23) Besides its direct mutagenic effect, UV radiation photo-produces reactive oxygen species; these cause oxidative damage including the production of mutagenic 8-hydroxyguanosine.

Point mutations are the canonical source of new genetic material. Transitions (e.g., the replacement of a purine with another purine) are an order of magnitude more common than transversions (the replacement of a purine with a pyrimidine or visa versa). These changes might be beneficial, but are more likely to be silent (have no functional affect on the protein), missense (code for a different amino acid) or nonsense (produce a stop codon). The addition or deletion of a base pair in a protein-coding gene causes a frameshift resulting in a radically altered protein downstream from the mutation.

How important is a point mutation? With only a single point mutation, a highly promiscuous restriction enzyme can radically altered protein downstream from the mutation. After conjugation, the exconjugant protozoan may be left with a reversed row or so of cortex derived from their mate. The reversed rows are heritable. (19) Further, there are some physical changes such as compartmentalization in organelles that can be a source of heritable novelty for evolution.
Classically, genes are the property of an individual that can then be shared with other members of the species. But combining genes from different species – as practiced in nature and synthetic biology – is a very powerful tool to create novelty as it circumvents the need to evolve genes de novo. Interspecies or “horizontal” gene transfer seems to have midwifed major steps in evolution, from the origin of eukaryotes to the symbiotic origin of chloroplasts and mitochondria. The number of documented cases of gene transfer among bacterial and archaeal hyperthermophiles. Wolf and colleagues identified 37 cases of transfer of genes among eukaryotes. For example, genes have been transferred between parasitic flowering plants and their plant hosts. Of the 31 mitochondrial protein genes in the basal angiosperm Amborella, 20 are from other land plants (primarily other angiosperms), but 6 are from mosses. Amazingly, gene transfer occurs across the three domains of life. Wolf and colleagues identified 37 cases of transfer of genes among domains, which subsequently joined endogenous genes resulting in a multi-domain fusion protein. About 24% of the genes of bacterium Thermotoga maritima MSB8 108 are very similar to archaeal genes. The bdelloid rotifer, an invertebrate, has genes that appear to be from bacteria, fungi, and plants. There are several mechanisms for obtaining foreign DNA. Competent bacteria and archaea may incorporate DNA from the environment by transformation. Eukaryotes may also take up environmental DNA by phagocytosis or symbiosis. In animals exogenous DNA may be transported through sperm-mediated transfer. Viral-mediated gene transfer is called transduction. An example of natural transduction is the photosystem I gene cassette present in the phages of marine cyanobacteria. The cassette may increase gene dosage in their new hosts.

Interspecies gene transfer is facilitated by physical proximity, for example between hosts and parasites, and among bacterial and archaeal hyperthermophiles. Other factors predictive of the likelihood of gene transfer include environmental temperature, genome size, genomic G/C composition, the type of carbon utilization (e.g., heterotroph or autotroph), and oxygen tolerance. Exogenous DNA may remain epigenetic or integrated into the genome. DNA uptake may represent a source of pristine genes to replace those that have accumulated mutations, or possibly DNA uptake is a way for the host to explore fitness space. However, new functions are unlikely to arise from this mechanism in nature since the chance of incorporation of a full gene and regulatory sequences is low.

Clearly sexual processes are a major source of genetic novelty, but are not reviewed here. Autogamy is a usual sexual process in some protists, notably ciliates and foraminifera. After meiosis a haploid nucleus undergoes a further mitotic division. The two nuclei then fuse, creating a completely homozygous diploid. Parasexual processes differ from sexual ones in that they do not involve meiosis and formation of a zygote by fertilization. In bacteria, conjugation is a parasexual process where a plasmid is transferred to the recipient cell through a mating bridge. In nature, the soil bacterium Agrobacterium tumefaciens transfers genes to plants resulting in the induction of crown gall tumor. Through biotechnology Agrobacterium’s target range has been extended to yeast and filamentous fungi. In the lab, conjugation has been observed between E. coli and a species of Saccharomyces, and between E. coli and Chinese hamster ovary cells.

Symbiosis also results in genetic transfer between species, with the best-known cases being the evolution of mitochondria and chloroplasts. Endosymbiosis is common among the protists and invertebrates including corals. Some associations, such as the dinoflagellates, appear in transition from containing heritable symbionts to organelles. Parasitic plants are known to transfer mitochondrial genes to their hosts, and vice versa.

Hybridization does not invariably result in sterile offspring, and thus is a source of genetic novelty. While best known in plants, it also occurs in diversity of animals including vertebrates. Introggression is of practical importance as wild plants have incorporated genes from many domesticated species.

Regulatory changes

Changes in structural genes permanently alter the gene product, whereas regulatory changes leave the structural genes intact, permitting more evolutionary flexibility. Further, a regulatory element may control multiple genes. For example, a single base pair substitution in the coding sequence of a signaling pathway gene in Pseudomonas fluorescens resulted in 52 proteomic changes, corresponding to 46 identified proteins.

There is a discrepancy between the rates of morphological and genetic evolution (e.g., ), which suggests that regulatory genes provide the link between the two. Such an
approach has revealed homologies that have their origins much farther back in evolutionary history than previously apparent. Regulation can occur in a variety of ways, from metabolic loops and promoters to base modification. All are potentially useful to synthetic biology.

Non-coding RNA (ncRNA) are potent regulatory elements that can control chromosome architecture, mRNA turnover and developmental protein expression, and may control other factors as well. Their importance in humans is clear: 97–98% of human transcriptional output is ncRNA. Another form of post-transcriptional regulation is that of the modification of adenosine to inosine editing in the RNA transcript. The inosine is interpreted as guanosine by the translational machinery. Only a single nucleotide is changed, but the ramifications include change in amino acid sequence, or potentially altered splicing or gene silencing.

Regulatory genes can duplicate and diverge. In multicellular organisms, the duplicate genes could then regulate transcription in different tissues as probably occurred after the whole genome duplication in teleost fish soon after their divergence from tetrapods, where nearly all duplicate gene pairs diverged in spatial and/or temporal expression during embryogenesis.

The duplication and subsequent diversification of parts as a source of novelty were recognized by Darwin. It is responsible for, among others, segmentation, digits, cilia, leaves, and multicellularity. For example, multiple similar teeth evolved early in vertebrate evolution. But in some reptiles (e.g., pterosaurs, lizards, and dinosaurs) and Synapsida (which includes mammals), teeth have differentiated, allowing more efficient food capture and access to a diversity of foods.

The duplication and diversification of cells resulting in multicellularity must be a relatively simply evolutionary transition as it has occurred in the plants, animals, and fungi separately and repeatedly. Among the protists, this transition is common and ongoing. For example, in the green algal family Volvocaceae, there are about 40 multicellular species in a variety of genera that differ in the number of Chlamydomonas-like cells they contain (e.g., Fig. 1). In spite of problems of delayed reproduction and coordination, there are advantages to multicellularity such as cell specialization and larger size to avoid ingestion by filter feeders. Among prokaryotes, multicellularity also has arisen in myxobacteria, which form fruiting bodies, and multicellular cyanobacteria.

Figure 1. Sources of genetic novelty in evolution. This figure uses as an example the evolution of multicellularity in the Volvocaceae from a Chlamydomonas-like ancestor (left) to a multicellular, differentiated Volvox (right). Examples of sources of genetic novelty are indicated in the text that may have contributed to this evolution. Although not illustrated, other multicellular genera in the Volvocaceae include Gonium, Pandorina, Eudorina, and Pleodorina.

Developmental changes leading to evolution can be based on regulatory rather than structural gene change. Neoteny – the retention of traits in an adult that formerly were only found in juveniles – is one example. Adult dogs are more like juvenile wolves, and the naked ape called Homo sapiens also evolved by neoteny. The relationship between the size of a part of an organism with respect to its overall size can provide evolutionary novelty. The classic case of allometry was the increasing size of the antlers of the male Irish Elk relative to its body size over time.

Physical compartmentalization separates components, and includes the encapsulation a primitive genetic endowment in a lipid vesicle, allowing for the evolution of individuals. From a biochemical point of view, it allowed the separation of different environments distinguished by such factors as pH and oxygen tension. Compartmentalization of genetic material allows differential activities. For example, most plant mitochondrial genes have low nucleotide substitution rates, whereas those of most animals are high. The physical partitioning of genetic material in the nucleus and organelles was critical to eukaryotic evolution.

Rate of production of genetic novelty

The rates of the production of genetic novelty can vary with increase in transposition, chromosomal rearrangements, mutation, or increasing the frequency of sexual processes. Bacteria are known to adapt their mutation rate by mechanisms such as weakening of mismatch repair when there is insufficient variability for natural selection, e.g., in a fluctuat-
Eukaryotes can also change their mutation rate in response to environmental pressure. For example, 1 month starvation of the budding yeast *S. cerevisiae* induced high rates of genomic rearrangements. Changes in mutation rates are not necessarily random, but can focus on “contingency” genes that are involved in environmental interactions rather than “housekeeping” genes.

**Evolutionary constraints**

The diversity of life is limited by formal, historical, and developmental constraints where surface area to volume ratio affects rates of exchanges with the environment in processes such as nutrient uptake, release of waste products, and intake of gasses and toxins. Similar principles apply to the shapes of animal cells, e.g., red blood versus nerve cells. Surface to volume ratio considerations are the basis for Bergmann’s rule that related animals have a larger body size in colder areas, and Allen’s rule that endotherms have shorter limbs in colder climates relative to their body size than their warm climate relatives. A smaller surface to volume ratio reduces heat loss, which is advantageous in cold climates but detrimental in warm climates.

Formal constraints are particularly noticeable in extremophilic organisms where adaptations are required to live near the physical and chemical limits for a carbon-based life form. For example, chlorophyll-based photosynthesis does not occur at temperatures above ~72 °C as chlorophyll denatures at those temperatures. At low temperatures, membrane fluidity decreases and thus the addition of unsaturated lipids to the membrane is used to restore fluidity.

Historical constraints are the results of the evolutionary history of a taxon. Because of this, the same solution can be converged upon but the differences in history will impart differences. To use a well-worn example, bird, bat, and pterydactyl wings all arose independently through a different historical sequence and thus are not interchangeable in the way that two water molecules are.

Both formal and historical constraints place developmental constraints on organisms. Ontogeny reveals evolution as a tinkerer.

Synthetic biology is limited primarily by formal constraints. In theory, historical and developmental constraints may be irrelevant, but in practice until we can literally “dial an organism” from basic components rather than modify existing ones, historical and developmental constraints must also be considered.

**Synthetic biology: where the principles of evolution can propel the field forward**

The history of molecular genetics shows that sources of novelty exploited by evolution are also of use to synthetic biology. But what about the rest of evolution's toolkit, as outlined in Table 1? The field moves so quickly it would foolhardy to pronounce a technique untested, so rather here I point out areas that are currently not in widespread use to identify the potential for new or expanded approaches.

**Genomic**

For thousand of years, humans have bred living organisms. Over 16 millennia ago, dogs were domesticated, and cereal crops followed within 5,000 years. This breeding took advantage of natural genetic variation coupled with artificial selection. The artificial production of novel DNA sequences came in 1926 when Muller discovered that X-rays could induce mutations. The introduction of foreign DNA into cells via transformation was demonstrated by Griffith in 1928, but it was not until Avery, MacLeod and McCarty showed that Griffith’s “transforming principle” was DNA, did it become the basis for modern molecular – and synthetic – biology. Viral transfer via transduction was shown in 1951 by Lederberg and Zinder. Thus, by the early 1950s, the production of endogenous genomic novelty by the random production of point mutations, gene loss and rearrangement, transduction, and exogenous production of genomic variability through the use of environmental mutagens were known and in use.

Many of the other possible genomic means of variation are utilized in some way, including gene and genome duplication, altered mutation rates, induction of autogamy, uptake of DNA from the environment, interspecies gene transfer, and parasexual gene transfer. Transfer of genetic material within

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**Table 2. Constraints on evolution.**

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<thead>
<tr>
<th>Category</th>
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<th>Examples</th>
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<tr>
<td>Formal</td>
<td>Result of physical laws</td>
<td>Temperature, diffusion rates, gravity</td>
</tr>
<tr>
<td>Historical</td>
<td>Result of evolutionary history</td>
<td>Wing design in insects, birds, and bats</td>
</tr>
<tr>
<td>Developmental</td>
<td>Result of developmental program</td>
<td>Differentiation leading to commitment, e.g., nerve cells</td>
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the cell by the introduction of plasmids and their subsequent incorporation into the genome is widely used, but not so the transfer from an organelle or symbiont into a host cell, or its genome. Some forms of gene transfer have the potential to radically alter the physiology of the host, e.g., when a themophilic phosphoglycerate kinase was transferred to and expressed in a mesophile (yeast). Similarly, symbiosis is extremely widespread in nature, often including a protistan partner, but acquisition of novelty by that means has been ignored by synthetic biology.

A “next generation” approach at exploiting the function of mobile elements is the creation of an artificial retrotransposon. Boeke’s lab engineered an artificial form of the LINE-1 mobile elements is the creation of an artificial retrotranspo-son. Similarly, symbiosis is extremely widespread in nature, often including a protistan partner, but acquisition of novelty by that means has been ignored by synthetic biology.

A different approach is to abandon the bilayer concept, and design a novel type of organelle. A functional prototype Golgi organelle has been created using digital microfluidics, recombinant enzyme technology, and magnetic nanoparticles.

Conclusions and prospects

As Bromham pointed out the need to put “the ‘bio’ into bioinformatics”, so too it is time to put the (evolutionary) biology into synthetic biology.

What evolution can do for synthetic biology

Synthetic biology is beginning to exploit some of the more esoteric components of nature’s toolkit. As novel evolutionary mechanisms, such as ncRNA, become better understood in nature, they will be used to a greater extent since their advantage of maintaining structural genes applies to synthetic systems. Encapsulation technology will improve with nanotechnology. Others seem to be curiously ignored, such as duplication and diversification. Non-Mendelian inheritance also provides untapped riches, such as creating novel membranes.

But the success of evolution relied on other principles that should be embraced by synthetic biology. Evolution operates on a population of individuals that vary in some heritable fashion and are historical entities. So why not create variation around an engineered solution and let evolution lead the engineer to the optimal solution? For example, why not engineer a template for a part, and let evolution search for a better solution by exploring fitness space? Or use the template as starting point for duplication and diversification? Perhaps this could include genetic exchange among individuals. The design of microbial consortia should be a powerful approach. Thus, instead of fearing evolution, synthetic biologists will do well to learn and adopt where fruitful.

What synthetic biology can do for evolution

For all evolution has taught synthetic biologists, the latter field has the potential to answer three types of questions for evolutionary and astrobiologists.

First, organisms and their components, from the gene up, do not form a continuum of all possible combinations but rather occupy discrete pinnacles. At the organismal level, these are recognized, more or less, as species. The
amino acids were found to be functional tion factor Max. However, by exploring the total potential amino acids in the five DNA-contacting residues of transcrip-
universe. Synthetic biology could produce new organ-
starting point to define a minimum envelope for life in the biological world cannot reveal the answer since its distribution in adaptive space is patchy.

assumption is that species, and genes and genomes, are in adaptive peaks to optimize fitness (Fig. 2). But this Panglossian view is unlikely to be true unless evolution has tried all permutations. With the help of synthetic biology, there is the potential to alter each nucleotide in a transcript and then measure fitness, thus supporting one or more of these hypotheses. Maerkl and Quake systematically substituted amino acids in the five DNA-contacting residues of transcription factor Max. However, by exploring the total potential diversity for only these five residues, several potential amino acids were found to be functional in vitro, but did not occur in nature. Clearly not every residue occupies an adaptive peak.

Second, synthetic biology could play a parallel role in the search for life in Ref. The range of life on earth is taken as an adaptive peak in vitro. However, it is likely that life will be created soon. Even then, it will be impossible to prove that the artificial life replicated the pathway primordial life form(s) followed on earth. It may well suggest one, or perhaps many, ways for life to originate and evolve. And then the proverbial ball will be back in the evolutionary biologists’ court, back to the territory that is their province: Why?

Acknowledgments: I thank Victor de Lorenzo for his kind invitation to contribute to the field, S. Pete Worden for making it possible, and John Cumbers for his infectious enthusiasm for synthetic biology. The comments of an anonymous review and Andrew Moore greatly enhanced the manuscript.

References


**Q1:** Author: Please provide three author names with et al if there are more than three authors else provide three authors through.