An engineering theory of evolution

Simeon D. Castle[1], Claire S. Grierson[1,2] and Thomas E. Gorochowski[1,2,*]

[1] School of Biological Sciences, University of Bristol, Tyndall Avenue, Bristol, BS8 1TQ, UK

[2] BrisSynBio, University of Bristol, Tyndall Avenue, Bristol, BS8 1TQ, UK

* Correspondence should be addressed to T.E.G. (thomas.gorochowski@bristol.ac.uk)

Keywords: evolution; evotype; engineering theory; genotype-phenotype maps; directed evolution; selection; synthetic biology; bioengineering; evolvability; self-adaptive systems

1 **Abstract**

2 Biological technologies are fundamentally unlike any other because biology evolves.
3 Bioengineering therefore requires novel design methodologies with evolution at their core.
4 Knowledge about evolution is currently applied to the design of biosystems *ad hoc*. Unless we
5 have a unified engineering theory of evolution, we will neither be able to meet evolution's
6 potential as an engineering tool, nor understand or limit its unintended consequences on our
7 biological designs. Our concept of the evotype offers a conceptual framework for engineering
8 the evolutionary potential of biosystems. We show how a biosystem's evolutionary properties
9 might be designed by engineering genetic variation, the production of function, and selection.
10 This idea could apply to all biosystems – from individual proteins to communities of whole-
11 cells or even entire ecosystems – whether the goal is to direct evolution in the design process,
12 or to limit its impacts during application. These principles could even be used beyond the realm
13 of bioengineering to design entirely synthetic auto-adaptive technologies.

Engineering evolution

The past few decades have seen a revolution in our ability to engineer biology and create living systems with novel functions [1]. Yet, several hurdles still hinder our capability to harness biology's full potential [2]. These predominantly stem from the fact that engineering the stuff of life is not the same as engineering its properties because life evolves. Evolution makes engineering living systems a radically different challenge to engineering other mediums. To be effective, we cannot simply apply traditional engineering design principles to biology and deal with evolution as a secondary thought. If nothing in biology makes sense except in the light of evolution [3], then evolution must be a central part of an engineering theory of biology.

Evolution poses both a challenge and an opportunity when designing biosystems. On one hand it is a detrimental force that can unpick the meticulous plans of an engineer through genetic variation [4]. Designed biosystems cannot escape evolution when used, and loss of function is a particular concern for engineers, especially as there are often selection pressures working against the design's function [5,6]. It is essential that we learn to build *evolutionarily stable* biosystems that can continue to operate under unavoidable evolutionary forces.

On the other hand, evolution is an extremely effective problem solver and engineers have exploited this fact for decades [7–10]. For example, directed evolution can be used to optimise or even generate completely novel traits in proteins [11] or cells [12]. However, these methods rely on the ability of evolution to find solutions in a reasonable length of time. For most systems, the search space is so vast that the starting point in this process must have the potential to generate useful phenotypes relatively quickly.

Evolution may even be employed as a feature of the system during operation. For example, adaptive systems that evolve in response to environmental cues, or evolvable genetic circuits that can be designed with specific classes of phenotype that are reached as necessary through evolutionary change. To create such systems, it is critical that the biological design is *specifically evolvable*. This means it must have the potential to generate the types of phenotypes desired by the engineer from a single starting point in a reasonable time frame.

Even more critical is our moral obligation to develop a deeper understanding of how synthetic biosystems will continue to evolve if deployed into our bodies or the wider environment [13]. The field has rightly made efforts to develop tools to reduce and mitigate evolution [14], with fail-safes such as kill switches [15] or metabolic dependencies [16]. However, without a good theoretical understanding of how synthetic biosystems might continue to evolve once deployed, we risk these technologies developing unexpected faults with dire, but avoidable, consequences. Even breeding has at times had dire consequences. Notably, the inadvertent creation of the hyper-aggressive Africanised bee, which has had a severe impact

49 on humans and ecology [17]. As we develop technologies capable of even more rapid genetic

50 change, such as gene drives [18], these concerns will become even more salient.

51   Central to many of these issues is the view in traditional engineering disciplines that

52 the engineered artefact is a final destination in the design process. This view breaks for

53 biology. Instead, we believe that a new perspective is needed for a truly effective engineering

54 of biology; one that sees a designed biosystem as a starting point in a lineage of possibilities.

55 Whilst much of evolutionary biology has concerned itself with organisms' histories [19],

56 bioengineers must consider the future, and specifically how a biosystem will continue to evolve

57 when used [20]. Here, we describe a framework that enables this transition and offers a way to

58 specify, test and conceive the properties of biosystems in terms of their evolutionary potential,

59 and not just their phenotype (**Figure 1**). This provides a way to reimagine biological

60 engineering so it that works in harmony with life's ability to evolve.

61

The design type and the evotype

63 To better harness the capabilities of biology, we need a way of thinking about the evolutionary

64 properties of engineered biosystems. We must design for potential evolutionary change and

65 not just the immediate functionalities displayed by a system (i.e. its phenotypic traits). Though

66 these are properties of populations yet to exist, they can still be considered in the context of

67 an individual biosystem. We consider the *design type* as the system that has been engineered

68 consisting of a single genotype. The design type could be any biosystem capable of evolution:

69 a protein, genetic circuit, virus, cell, animal or plant, or even an ecosystem. We introduce the

70 concept of the *evotype* to capture the evolutionary properties of that system. The evotype is

71 the set of *evolutionary dispositions* of the design type, analogous to genotype and phenotype

72 being sets of genes and traits, respectively. Unlike a trait, a disposition is not a directly

73 observable property, rather it is a potential property of the system. For example, a protein may

74 have the evolutionary disposition of instability where its structure may change dramatically

75 when mutated. Designing the dispositions of the evotype is a challenge fundamental to

76 engineering biology.

77   For all but the very simplest biosystems, it is impractical to enumerate every potential

78 evolutionary disposition, just as it is impossible to consider every trait of the phenotype.

79 Instead, an appropriate sample of the evotype must be used for the purpose at hand, just as

80 samples of traits are used when describing the phenotype. How we take this sample, and thus

81 the scope of the evotype covered, should be determined by knowledge of the design type, its

82 intended function, and the context in which it will be used. This could include the size of

83 population, environment, and required number of generations over which the system must

84 operate reliably.

Broadly speaking, we may wish to seek one of two goals when designing the evotype: the first is that of *evolutionary stability*, where a system changes its function as little as possible as it evolves during use, and the second is *specific evolvability,* where the system can easily evolve new phenotypes of a specific class (i.e. the classes of function specified by the engineer), or adapt to changes in the environment (i.e. continuing to produce a desired chemical product). Specific evolvability requires an element of robustness: core functions of the phenotype must remain unperturbed throughout sequence space so that new phenotypes can be explored. This is analogous to natural evolvability [21] where the ability to generate novel phenotypes alone is insufficient, as they must also be adaptive. It also relates to the concept of plasticity, which is the ability to generate new features without total loss of function [22]. The relationship between robustness and evolvability in natural evolution has been explored in detail in prior literature[23]. For example, a genetic circuit may have been specified to produce an OR logic function in response to two inputs. That is, it expresses an output protein if either one of the two input chemicals are present. A population with an evolutionarily stable version of this circuit is likely to maintain an OR function during use. A specifically evolvable version of the circuit on the other hand might be designed to readily produce other logic functions when evolved (e.g. AND, NOR, NOT), without simply destroying existing functionality or causing lethality to the host cell.

Whether evolutionary stability or specific evolvability is the goal, it can be achieved through engineering genetic variation, the production of function from genotype, and both natural and artificial selection. How these processes interact to constrain and bias evolution can be understood by describing a landscape surrounding the design type in sequence space. This extends and generalises the fitness landscape concept as applied to natural systems[24] by accounting for the roles of variation, production of function, and selection in engineered biosystems. The bioengineer's goal is to sculpt this evotype landscape to their specification to ensure it has a structure in line with their requirements.

Engineering genetic variation

Not all point mutations are equally likely; transversions and transitions differ in their likelihoods [25], and methylation [26], genomic context [27], and species [28] all influence local and global mutation rates. Furthermore, *algorithmic mutations* [29] may occur. These are mutations that result in changes of several nucleotides in one event (thus an algorithm can describe the change) and can be thought of as shortcuts through sequence space (**Figure 1B**, left). The likelihood of an algorithmic mutation may be much greater than the summed likelihoods of the equivalent sequence of individual point mutations. For example, the chance of an insertion of the two-base motif 'AC' into a tandem repeat region due to slipped strand mispairing may be more

121 likely than two insertion events of 'A' and 'C' occurring independently [30]. Recombination [31] and

122 mobile genetic elements [32] are other examples of biological processes capable of producing

123 algorithmic mutations.

124   Sequence space is therefore not explored in a uniformly random way, even discounting

125 for the role of selection. Instead, the paths evolution can take are determined by the *variation*

126 *operator set*, which defines all the different point and algorithmic mutations that can occur in

127 the system. Each variation operator in this set has an associated probability distribution that

128 represents the likelihood of arriving at a given sequence from another (i.e. by this operator

129 acting on the design type). The distributions of the variation operator set combine to produce

130 the *variation probability distribution*. This describes the chance of arriving at any given

131 sequence from the design type due to all the biochemical and physical processes capable of

132 causing genetic variation that are present in the system (**Figure 1B**, right). The variation

133 operator set defines the rate and the likely directions in sequence space a design will explore

134 during evolution. As a design type evolves, the variation probability distribution changes as

135 further dispositions become available.

136   The variation operator set depends on the specifics of the biosystem being engineered,

137 and the set to be applied in practice is dependent on available knowledge of the system. For

138 example, the variation operator set of a design type biosystem may be said to include

139 transition mutations, transversion mutations, and recombination, each associated with a

140 unique probability that varies across the design type's sequence. A sample population can be

141 generated by applying the operator set to the design type. This population, with the design

142 type at its centre, may be named a *quasispecies*; as is used for the related concept in viral

143 evolution [33].

144   The variation probability distribution can be considered in all stages of the engineering

145 process. Global and local mutation rates could be specified in a design, and standardised

146 mutation rates could be listed in part datasheets [34] It is likely that improvements to the

147 prediction of mutation probabilities will be made with the increasing availability of sequence

148 data and associated computational methods. Design rules for influencing local genetic

149 variability are already known (e.g. avoiding the reusing of parts and repetitive sequences to

150 reduce homologous recombination and indel mutations) [35–37], and global mutation rates can

151 also be rationally engineered and manipulated [12,38].

152   A large toolkit for altering for controlling genetic variation has already been created by

153 bioengineers, which could be used to improve evolutionary stability or increase specific

154 evolvability. New tools will doubtless be developed from the diverse mechanisms that

155 generate genetic variation in nature. The variation probability distribution of the design type

156 can be modified by either adding or removing variation operators (e.g. by adding or removing

157 DNA modifying enzymes), or by modulating existing operators in the system across the

158   genotype. This may be through altering DNA sequence properties (e.g. avoiding simple
159   sequence repeats to reduce the chance of indels through slipped-strand mispairing[35]).
160   Variation operators can be highly targeted like the DNA methylation of specific bases to
161   increase likelihood of mutation through spontaneous deamination [26], or may have a global
162   effect such as the removal of error prone polymerases from a host organism [39]. Orthogonal
163   mutation systems that modulate genetic variation of a specific plasmid or region of DNA can
164   be used to overcome genomic error-thresholds, increasing the potential for directed evolution
165   [40].

166   Larger-scale genetic variation can be achieved through mechanisms such as site-
167   specific recombination, which can be used for inserting, removing, duplicating, inverting, or
168   shuffling large segments of DNA, exemplified by the SCRaMbLE system used in the synthetic
169   yeast Sc2.0 [41]. Finally, acquisition of foreign DNA either from other organisms in the population
170   through sex, horizontal gene transfer, or from free oligonucleotides in the environment[12] may
171   also be engineered. By combining these and other biochemical tools, it may eventually be
172   possible to precisely design the variation operator set to produce complex combinations of
173   genetic variation. For example, the variation operator set of a genetic circuit may be
174   engineered by avoiding repeat parts (removing the homologous recombination operator),
175   using a host with a high-fidelity DNA polymerase (globally reducing probability of point
176   mutations), and by incorporating DNA recombination sites (adding an operator for specific
177   DNA recombination, perhaps to be used for future directed evolution). **Table 1** provides
178   examples of methods for controlling variation operators that have been developed so far.

179

Engineering the production of function

181   Each genotype produces a phenotype via the processes of gene expression, growth, and
182   development. However, due to the constraints and biases of these processes [42] phenotypes
183   are not necessarily distributed evenly throughout sequence space, and not all conceivable
184   phenotypes may be possible. Many systems have shown similar properties in the structure of
185   their mapping from genotype to phenotype. Namely, redundancy (there are many more
186   genotypes than phenotypes), and bias (a small fraction of phenotypes are over-represented).
187   This has been shown both through simulation [43] and experimentally in RNA and protein
188   structures [43], and DNA binding sites [44]. How these principles apply to more complex
189   biosystems is a major challenge due to their vast genotype spaces. Nevertheless, there will
190   be a statistical structure in the mapping of genotype to phenotype. If this structure is sufficiently
191   well understood it could offer a powerful way of engineering the evotype.

192   Engineered biosystems have phenotypic traits that influence reproductive fitness, and
193   traits that influence *function* – the behaviour or properties specified by the designer (though

these are not mutually exclusive). The structure of the mapping from genotype space to function space is therefore a key part of the evotype. Function space may be discrete, or it may be continuous (**Figure 1C**). Designed functions could be literal mathematical functions, physical characteristics like colour or size, or combinations of several properties.

Any designed system has a degree of *utility* – the extent to which the system fulfils the specified function. The sole goal of a traditional engineering design process is to maximise the utility of the design type. However, the topology of the function landscape surrounding the design type is also important. It may be rugged and highly variable with the function rapidly changing across sequence space, or it may be smooth and have large neutral regions where function changes little or remains constant. These properties are a key design consideration: what is the functional range to be covered by the design type's evotype? Should the variation be large for increased evolvability, or limited, for evolutionary stability? Most likely the function landscape should be smooth and predictable, but how is this best achieved? Which regions of function space must be avoided, and which can be tolerated? For example, it may be necessary to reduce irrelevant or harmful functions as much as possible in a diagnostic application where regions of function space cause false negatives, whilst regions causing false positives can be tolerated.

Designs may have identical functionality but occupy regions of function space with very different topological properties. If a system is designed without considering its surrounding function landscape, a design with an undesirable evotype may be a likely outcome. Systems with identical phenotypes yet differing function landscapes were demonstrated by Schaerli *et al.*, who designed two genetic circuits, both with the same strip-generating function. It was found that each produced a different spectrum of new phenotypes when mutated due to differences in the regulatory mechanisms used [45]. We are only just beginning to understand what influences the structure of the mapping from genotype to phenotype. However, there are some general principles which seem to hold across scales and contexts. Fortunately, many of these principles are already familiar to engineering.

Prevalent phenotypes

Phenotypes that are more prevalent in sequence space can be both more robust (as they are more likely to be in genotype networks sharing the same phenotype), and more evolvable (as this allows a wider search of genotype space, increasing access to more novel phenotypes)[23]. Therefore, choosing prevalent components may aid both evolutionary stability and specific evolvability. For example, if designing a protein the codon chosen may influence evolvability: for a leucine residue, if UAA is chosen, its 1-mutant neighbourhood has a lower prevalence for leucine than any other codon (two versus four respectively). Therefore, UAA may have lower evolutionary stability, but higher specific evolvability than other codons (since it is able

231 to generate a wider range of non-polar amino acids). Remapping the genetic code itself has
232 been suggested as a way of altering its evolvability [46]. Other examples of applying phenotypic
233 prevalence include choosing RNA or protein structures that are highly represented in
234 sequence space [47,48]. An interesting question is how the phenotypic prevalence of a system's
235 parts relates to the overall phenotype compared to higher-order properties. Is the robustness
236 of a genetic circuit's parts or its network topology a greater determinant of its overall
237 robustness?

238

Redundancy

240 Redundancy is used in classical engineering and by evolution. It can add robustness by
241 allowing variation of parts of a system without overall loss of function and can aid in evolvability
242 by enabling redundant parts to mutate and thus explore new regions of function space. This
243 can be seen in serial homology, where repeated parts such as limbs or teeth enable evolution
244 of specialised functions [49], in gene duplications [50], and in the scale-free structure of genetic
245 networks where most nodes can be removed without altering overall function [51]. Note that
246 redundant parts may either be repeats of the same part, or different parts that can produce
247 the same function.

248

Modularity, regularity, and hierarchy

250 The organisational properties of a biosystem are a major influence on its evotype. These can
251 be summarised by modularity, regularity, and hierarchy [52]. Modularity is the division of a
252 system into subsystems (modules), where each has a high degree of internal connectivity, but
253 little interdependency between subsystems. Examples of this can be seen in the connectivity
254 of protein and regulatory networks, in RNA structures, and in limb development [53]. Regularity
255 is the use of patterns, repetitions, and symmetries (e.g. serial homology and animal body-
256 plans). Hierarchy is the recursive arrangement of a system into subsystems (that are
257 themselves composed of subsystems, etc.) [54] For example, an organism is composed of
258 organs, which is in turn composed of tissues, cells, etc. Hierarchy is also seen in gene
259 regulatory networks. For example, only nine proteins regulate half of all genes of *E. coli* [40].

260   These principles are distinct but often work together. For example, identical modules are
261 often repeated in regular patterns, and modules are arranged in a hierarchical structure. These
262 principles may each promote evolvability in different ways. Modularity allows parts of a system
263 to mutate and change function with a reduced negative impact on the rest of the system.
264 Regularity reduces the information required to describe the system (e.g. its genotype),
265 essentially reducing the size of the search space. Hierarchy allows the progressive increase
266 in the complexity of a system from the bottom up [54]. Though the widespread use of these
267 principles in both biology and technology clearly demonstrates their importance, how and

268 where these principles should be applied is context specific. This can be illustrated with an
269 imagined example.

270   Consider two biosensor circuits that each use red, green, and blue (RGB) fluorescent
271 proteins to produce a white output. In circuit A, the overall output of RGB should be as high
272 as possible when the input is positive (e.g. high sensitivity is required), the whiteness of the
273 signal is less critical. In circuit B, it is important that the positive signal remains precisely white
274 (e.g. other colours represent other input conditions), and overall output level is less critical.
275 Circuit A would benefit from a modular arrangement of red, green, and blue because a
276 mutation in any one of these genes does not affect the other two, thus reducing the impact on
277 overall output. However, for circuit B, a less modular design would be preferable: though a
278 mutation would have triple the effect on overall output, all colours would be impacted equally
279 conserving the overall hue. The nature of the design problem therefore relates to how
280 modularity should be used. In fact, Kashtan *et al.* showed that modular architectures evolve
281 in gene regulatory networks in response to modular environmental selection pressures, and
282 themselves prove to be more evolvable[55]. Similar relationships between how hierarchy and
283 regularity should relate to the design problem no doubt exist. However, we are far from having
284 design principles for their application, particularly for more complex problems.

285

Environmental robustness

287 Principles that improve robustness to environmental change or noise may also improve
288 robustness to genetic change. For example, if a genetic circuit is robust to noise in the
289 concentration of a regulatory protein, it may also be more robust to mutations that change the
290 promoter's expression level [56]. Similarly, proteins that are more thermodynamically stable may
291 also be more evolvable [57]. Systems could be buffered against environmental and genetic
292 perturbations through the use of negative feedback [58].

293

Designing parameter space

295 The structure of the parameter space of a system plays a large role in how function changes
296 under genetic variation. If the behaviour of a system can be modelled or inferred against the
297 variation of key parameters, this can provide information about which functions may be
298 accessible and most likely throughout sequence space. For example, by modelling the
299 regulatory mechanisms of two genetic circuits, Schaerli *et al.* explain why they produce
300 different distributions of functions when subjected to point mutations [45]. Similarly, with a simple
301 mathematical model of equilibrium binding, Mayo *et al.* showed that the cis-regulatory region
302 of the *lac*-operon in *E. coli* is incapable of accessing some input functions via point mutation
303 [22]. Parameter spaces are analogous to the morpho-spaces of evolutionary-developmental
304 biology, which provide constraints on organismal form [59].

305

Other principles

307 System specific principles may also provide design rules for the evotype. For example, RNA
308 gene regulation may be less evolvable than transcriptional regulation [60] so could determine
309 whether the regulation is applied at the transcriptional or translational level. Physical and
310 chemical processes of self-organisation may even be able to reduce a function's dependency
311 on the genotype. Perhaps ideas from developmental biology and morphogenesis could be
312 recast into engineering terms, such as concepts from the theory of facilitated variation [61],
313 particularly as bioengineering progresses to multicellular organisms. Metaheuristic design
314 approaches will also no doubt become an increasingly powerful tool: machine learning
315 approaches may be able to predict the evolvability of biological networks [62], and genetic
316 algorithms have been used to evolve more robust genetic networks *in silico* [63].

317

Engineering natural and artificial selection

319 Selection is the force that gives the otherwise random (but constrained) processes of genetic
320 variation a 'direction' by driving a population up the slopes of the adaptive landscape [64].
321 Uniquely, an engineered biosystem is a result of two forms of selection: natural selection and
322 the design process. Natural selection acts on reproductive fitness of the biosystem, and the
323 design process can be thought of as a sophisticated form of artificial selection acting on its
324 utility. Fitness and utility both form part of the evotype and understanding the interplay between
325 these two processes is critical for effective evotype design since there is often a tension
326 between the two (**Figure 1D**). If fitness and utility are uncorrelated, then natural selection is
327 likely to undo the work of the engineer. However, if fitness and utility are highly correlated,
328 then natural selection will also drive up utility. For example, one might design a cell in a
329 bioreactor, or a plant crop to produce a chemical product. Perhaps it uses control circuitry to
330 maintain optimal metabolic fluxes to maximise yield in fluctuating environmental conditions,
331 thus resulting in high utility. However, this will inevitably have a fitness effect on the organism
332 (e.g. due to metabolic burden of the circuit, or toxicity of the product), and thus natural
333 selection will favour mutants where this functionality is repressed. It should be noted that
334 natural selection here is meant as the process that acts on the reproductive ability of the
335 biosystem. Neither the environment nor biosystem need to be natural (e.g. the organisms
336 could be engineered to make use of non-canonical amino acids and grown within a bioreactor).
337 The critical distinction is that natural selection acts on survival of the biosystem without the
338 input of the engineer.

339 The aim of a bioengineer then, is to maximise *fitneity* – defined as a function that
340 combines the derivatives of utility and fitness (**Figure 1D**). Ultimately, evotype engineering is

341 controlling how fitneity changes throughout sequence space: it is the sculpting of fitneity

342 landscape. Exactly what form the fitneity function should take, and the best way to

343 mathematically describe the fitneity landscape to effectively capture the interaction between

344 these two forms of selection is not yet clear. Defining, modelling, and characterising the fitneity

345 landscapes of designed biological systems is a future avenue of research ripe with potential.

346 Nevertheless, the concept can already help in thinking about how to improve the fitneity of

347 designs on an intuitive level.

348   To design for evolutionary stability, it is sufficient to limit or neutralise the impact of

349 natural selection. This can be achieved one of three ways. Firstly, the fitness of the design

350 type and its immediate neighbours can be increased to create a local peak or plateau. This

351 could be done through adaptive evolution [65,66] after the design phase, reducing or dynamically

352 controlling burden [67], or by reducing toxicity of the associated function. Secondly, the fitness

353 of neighbouring genotypes can be decreased to flatten the surrounding fitness landscape, for

354 example by using organisms with a reduced genome that may be less fit than wild type

355 organisms [68], but with freed-up metabolic resources [69]. Thirdly, the utility landscape can be

356 flattened so that even if there is a natural selection pressure away from the design-type, it is

357 less likely to impact the design's function. Approaches for doing this have been outlined in the

358 previous section. To ensure a specifically evolvable evotype, fitness and utility must correlate:

359 both fitness and utility must slope in the same direction. An engineer could do this by coupling

360 function to survival, perhaps through a toxin-anti-toxin system [70], or by coupling function to

361 growth (e.g. by having the product of a system aid in metabolism of an energy source).

362 Alternatively, an artificial environmental pressure, such as repeated screening, could be used

363 to ensure utility and fitness correlate.

364

365 **Toward evotype engineering**

366 The evotype is a new way to think about the properties of engineered biosystems and how

367 they relate to each other (**Table 2**). It is a framework for thinking about an important but often

368 overlooked property: the role the biosystem itself plays in its future evolution. This is especially

369 critical due to the impact of an intervention (e.g. a new mutational method) being closely linked

370 to the composition of the system itself. For example, in a simple case, an identical protein

371 could be encoded by very different sequences and so be impacted by a targeted mutating

372 element in different ways. This is quite different to how engineers normally view systems. As

373 engineered biosystems are the result of both human creativity and natural adaptation, a

374 holistic consideration of both the roles of design and evolution is necessary. The evotype helps

375 us do this by explicitly considering the intertwined effects that genetic variation, production of

376 function, and multiple forms of selection will have on a design (**Figure 1**).

We can now design and build genotypes with great precision, but we must account for the inevitable processes of genetic variation that will follow. The statistical structure of variation is unique to each biosystem and something we have control over. Yet understanding the details of genetic variation is insufficient if we do not also understand how this will manifest in changes of the designed function of the biosystem. Even a system with low mutation rates can be evolutionarily unstable if function changes wildly with small sequence alterations. Directed evolution will not be successful, despite the mutation strategy, if desired functions are simply not accessible from the starting point. If the biosystem's utility (i.e. its success as a design) and its fitness (i.e. its success as a biological replicator) are at odds, well designed dispositions for variation or function might not save the design from the pressure of natural selection. This must also be understood as conflict between utility and fitness landscapes across sequence space surrounding the original design type. It is clear then that all three of the aspects of the evotype must be considered together, and all offer significant scope for engineering. For instance, imagine a large genetic circuit that places an unavoidably high metabolic burden on the host cell. If it is crucial that the function of the circuit is maintained over long periods of time, then redundancy could be used to accommodate unavoidable mutations. However, if the dent to reproductive fitness is severe, this may still not be enough. Therefore, combining redundancy in the design with a hyper-stable host cell (e.g. one where all mobile genetic elements have been deleted and efficient DNA repair mechanisms are present [71]) might be the only way to achieve the desired goal for the system.

Designing biosystems with evolution in mind is a vital step towards a more complete engineering theory of biology. However, to be practical, supporting tools must exist that can provide key information regarding the genetic variation, genotype-function map, and selective pressures within a biosystem. Advances in sequencing offer a means to quantitatively measure millions of genotypes in parallel [72] and when combined with high-throughput techniques such as fluorescence activated cell sorting (FACS) make it possible to infer simplified genotype-function maps [73]. The local function landscapes of the green fluorescent protein[74], and transcription factor binding sites [44] have already been characterised experimentally with such methods. Detailed measurements of fitness in large populations of cells are also possible [75–77]. By combining sequencing with expression and growth measurements, genetic variation, function, and fitness could be characterised simultaneously to provide a complete picture of the evotype.

Even so, the vastness of evotype landscapes and the need for functions calculated from many outputs of a system mean that new methods with greater throughputs are also necessary. There is a particular need for methods able to measure many characteristics of each cell simultaneously (e.g. via automated high-content microscopy [78] or high-throughput Raman spectroscopy [79]). Parallel to these experimental methods, a promising direction to

bypass the need to directly measure these properties are the development of sufficiently comprehensive computational models (e.g. encompassing whole cells [80]) to allow for a mechanistic understanding of the biases in processes related to variation and reproductive rate. In these cases, if they are sufficiently accurate, the evotype could be predicted and used within computer aided-design workflows [81] to reduce the need to physically implement every possible design. Nevertheless, for systems of even moderate complexity, the evotype landscapes are much too vast to be exhaustively characterised or even modelled. It will therefore be of great importance to understand how they should be sampled [82], how large a region of the landscape needs to be characterised, and to what extent local landscape properties can be extrapolated. Use of machine learning is another method that holds great promise for increasing the ability to estimate the evotype landscape, albeit at the cost of mechanistic knowledge of the system.

In addition to characterising evotypes, tools for bioengineers to directly sculpt their landscapes must also be available (**Figure 2**). Here, we have touched upon the numerous repurposed biomolecular components that can alter the types of possible variation. However, there is a spectacular diversity of molecular machines dedicated to manipulating genetic information in the natural world, suggesting a need for an even larger toolkit to precisely modify genetic variation as needed. Likewise, principles for constraining and biasing the production of function and for controlling selection pressures have been suggested, but they are still poorly understood, and have barely been applied rigorously in an engineering setting.

An engineering theory of evolution is a new way of looking at evolutionary theory. It is also a new way of thinking about what it is that engineers do, and what the design process is in the context of bioengineering. The concept of the evotype, with some modifications, may also find use in evolutionary science, where it offers a framework for considering the mechanistic constraints of evolution and a way of talking about the evolutionary characteristics of organisms. It may also be applied beyond biological engineering fields to create new auto-adaptive technologies. Here the framework could be applied to ask how we design technologies to evolve, and not just how to engineer systems that already do.

Acknowledgements

S.D.C. was supported by the EPSRC/BBSRC Centre for Doctoral Training in Synthetic Biology grant EP/L016494/1. T.E.G. was supported by a Royal Society University Research Fellowship grant UF160357. T.E.G. and C.S.G. were supported by BrisSynBio, a BBSRC/EPSRC Synthetic Biology Research Centre grant BB/L01386X/1. This study did not involve any underlying data.

Author Contributions

Whilst T.E.G. and C.S.G. framed questions, suggested figures and tables and provided direction, the core concepts and new terminology here are mostly the work of S.D.C., who also wrote the first draft. S.D.C. and T.E.G. developed the figures with input from C.S.G. T.E.G. and C.S.G edited the manuscript and supervised the work.

Declaration of Interest

The authors declare no competing financial interests.

References

458

459   1.   Andrianantoandro, E. *et al.* Synthetic biology: New engineering rules for an emerging
460   discipline. *Molecular Systems Biology* **2**, 2006.0028 (2006).

461   2.   Grozinger, L. *et al.* Pathways to cellular supremacy in biocomputing. *Nature*
462   *Communications* 1–11 (2019) doi:10.1038/s41467-019-13232-z.

463   3.   Dobzhansky, T. Nothing in Biology Makes Sense except in the Light of Evolution. *The*
464   *American Biology Teacher* **35**, 125–129 (1973).

465   4.   Renda, B. A., Hammerling, M. J. & Barrick, J. E. Engineering reduced evolutionary
466   potential for synthetic biology. *Mol. BioSyst.* **10**, 1668–1678 (2014).

467   5.   Ellis, T. Predicting how evolution will beat us. *Microbial Biotechnology* **12**, 41–43 (2019).

468   6.   Fernandez-Rodriguez, J., Yang, L., Gorochowski, T. E., Gordon, D. B. & Voigt, C. A.
469   Memory and Combinatorial Logic Based on DNA Inversions: Dynamics and Evolutionary
470   Stability. *ACS Synthetic Biology* **4**, 1361–1372 (2015).

471   7.   Yokobayashi, Y., Weiss, R. & Arnold, F. H. Directed evolution of a genetic circuit.
472   *Proceedings of the National Academy of Sciences of the United States of America* **99**,
473   (2002).

474   8.   Giver, L., Gershenson, A., Freskgard, P.-O. & Arnold, F. H. Directed evolution of a
475   thermostable esterase. *PNAS* **95**, 12809–12813 (1998).

476   9.   Boder, E. T., Midelfort, K. S. & Wittrup, K. D. Directed evolution of antibody fragments
477   with monovalent femtomolar antigen-binding affinity. *Proceedings of the National*
478   *Academy of Sciences* **97**, 10701–10705 (2000).

479   10. Esvelt, K. M., Carlson, J. C. & Liu, D. R. A system for the continuous directed evolution
480   of biomolecules. *Nature* **472**, 499–503 (2011).

481   11. Guntas, G., Mansell, T. J., Kim, J. R. & Ostermeier, M. Directed evolution of protein
482   switches and their application to the creation of ligand-binding proteins. *Proc. Natl. Acad.*
483   *Sci. U.S.A.* **102**, 11224–11229 (2005).

484   12. Wang, H. H. *et al.* Programming cells by multiplex genome engineering and accelerated
485   evolution. *Nature* **460**, 894–898 (2009).

486   13. Anderson, J. *et al.* Engineering and ethical perspectives in synthetic biology. Rigorous,
487   robust and predictable designs, public engagement and a modern ethical framework are
488   vital to the continued success of synthetic biology. *EMBO Rep* **13**, 584–590 (2012).

489   14. Wright, O., Stan, G.-B. & Ellis, T. Building-in biosafety for synthetic biology. *Microbiology*
490   *(Reading, Engl.)* **159**, 1221–1235 (2013).

491   15. Chan, C., Lee, J., Cameron, E., Bashor, C. & Collins, J. 'Deadman' and 'Passcode'
492   microbial kill switches for bacterial containment. *Nat Chem Biol* **12**, 82–86 (2015).

16. Mandell, D. J. *et al.* Biocontainment of genetically modified organisms by synthetic protein design. *Nature* **518**, 55–60 (2015).

17. Winston, M. L. The Biology and Management of Africanized Honey Bees. *Annu. Rev. Entomol.* **37**, 173–193 (1992).

18. Oye, K. A. *et al.* Regulating gene drives. *Science* **345**, 626–628 (2014).

19. Pigliucci, M. Are ecology and evolutionary biology "soft" sciences? *Ann. Zool. Fennici* **39**, 87–98 (2002).

20. Bartley, B. A., Kim, K., Medley, J. K. & Sauro, H. M. Synthetic Biology: Engineering Living Systems from Biophysical Principles. *Biophys J* **112**, 1050–1058 (2017).

21. Kirschner, M. & Gerhart, J. Evolvability. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 8420–8427 (1998).

22. Mayo, A. E., Setty, Y., Shavit, S., Zaslaver, A. & Alon, U. Plasticity of the cis-regulatory input function of a gene. *PLoS Biology* **4**, 555–561 (2006).

23. Wagner, A. Robustness and evolvability: a paradox resolved. *Proceedings of the Royal Society B: Biological Sciences* **275**, 91–100 (2008).

24. Wright, S. *The roles of mutation, inbreeding, crossbreeding, and selection in evolution.* vol. 1 (na, 1932).

25. Stoltzfus, A. & Norris, R. W. On the Causes of Evolutionary Transition:Transversion Bias. *Mol Biol Evol* **33**, 595–602 (2016).

26. Jones, P. A., Rideout, W. M., Shen, J. C., Spruck, C. H. & Tsai, Y. C. Methylation, mutation and cancer. *Bioessays* **14**, 33–36 (1992).

27. Zhu, Y. O., Siegal, M. L., Hall, D. W. & Petrov, D. A. Precise estimates of mutation rate and spectrum in yeast. *Proc. Natl. Acad. Sci. U.S.A.* **111**, E2310-2318 (2014).

28. Drake, J., Charlseworth, B., Charlseworth, D. & Crow, J. Rates of Spontaneous Mutation. *Genetics* **148**, 1667–1686 (1998).

29. Chaitin, G. *Proving Darwin: Making Biology Mathematical.* (Vintage Books, 2013).

30. Levinson, G. & Gutman, G. A. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Mol. Biol. Evol.* **4**, 203–221 (1987).

31. Vos, M. Why do bacteria engage in homologous recombination? *Trends in microbiology* **17**, 226–32 (2009).

32. Frost, L. S., Leplae, R., Summers, A. O. & Toussaint, A. Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* **3**, 722–732 (2005).

33. Eigen, M. On the nature of virus quasispecies. *Trends Microbiol.* **4**, 216–218 (1996).

34. Canton, B., Labno, A. & Endy, D. Refinement and standardization of synthetic biological parts and devices. *Nature biotechnology* **26**, 787 (2008).

35. Jack, B. R. *et al.* Predicting the Genetic Stability of Engineered DNA Sequences with the EFM Calculator. *ACS Synth Biol* **4**, 939–943 (2015).

36. Sleight, S. C. & Sauro, H. M. Visualization of Evolutionary Stability Dynamics and Competitive Fitness of Escherichia coli Engineered with Randomized Multigene Circuits. *ACS Synth. Biol.* **2**, 519–528 (2013).

37. Hossain, A. *et al.* Automated design of thousands of nonrepetitive parts for engineering stable genetic systems. *Nature Biotechnology* 1–10 (2020) doi:10.1038/s41587-020-0584-2.

38. Geng, P., Leonard, S. P., Mishler, D. M. & Barrick, J. E. Synthetic Genome Defenses against Selfish DNA Elements Stabilize Engineered Bacteria against Evolutionary Failure. *ACS Synth. Biol.* **8**, 521–531 (2019).

39. Csörgő, B., Fehér, T., Tímár, E., Blattner, F. R. & Pósfai, G. Low-mutation-rate, reduced-genome Escherichia coli: an improved host for faithful maintenance of engineered genetic constructs. *Microbial Cell Factories* **11**, 11 (2012).

40. Ravikumar, A., Arzumanyan, G. A., Obadi, M. K. A., Javanpour, A. A. & Liu, C. C. Scalable, Continuous Evolution of Genes at Mutation Rates above Genomic Error Thresholds. *Cell* **175**, 1946-1957.e13 (2018).

41. Dymond, J. & Boeke, J. The Saccharomyces cerevisiae SCRaMbLE system and genome minimization. *Bioeng Bugs* **3**, 168–171 (2012).

42. Ahnert, S. E. Structural properties of genotype– phenotype maps. *Journal of The Royal Society Interface* **14**, 20170275 (2017).

43. Ferrada, E. & Wagner, A. A Comparison of Genotype-Phenotype Maps for RNA and Proteins. *Biophys J* **102**, 1916–1925 (2012).

44. Aguilar-Rodríguez, J., Payne, J. L. & Wagner, A. A thousand empirical adaptive landscapes and their navigability. *Nature Ecology & Evolution* **1**, 0045 (2017).

45. Schaerli, Y. *et al.* Synthetic circuits reveal how mechanisms of gene regulatory networks constrain evolution. *Molecular Systems Biology* **14**, 1–18 (2018).

46. Pines, G., Winkler, J. D., Pines, A. & Gill, R. T. Refactoring the Genetic Code for Increased Evolvability. *mBio* **8**, mBio.01654-17, e01654-17 (2017).

47. Schaper, S. & Louis, A. A. The Arrival of the Frequent: How Bias in Genotype-Phenotype Maps Can Steer Populations to Local Optima. *PLOS ONE* **9**, e86635 (2014).

48. Li, H., Helling, R., Tang, C. & Wingreen, N. Emergence of Preferred Structures in a Simple Model of Protein Folding. *Science* **273**, 666–669 (1996).

49. Carroll, S. Homology, Serial Homology, and Williston's Law. in *Endless Forms Most Beautiful* 29–36 (Weidenfeld & Nicolson, 2006).

50. Zhang, J. Evolution by gene duplication: an update. *Trends in Ecology & Evolution* **18**, 292–298 (2003).

51. Albert, R., Jeong, H. & Barabási, A.-L. Error and attack tolerance of complex networks | Nature. *Nature* **406**, 268–382 (2001).

52. Lipson, H. Principles of modularity, regularity, and hierarchy for scalable systems. *Journal of Biological Physics and Chemistry* **2007**, 125–128 (2007).

53. Wagner, G., Pavlicev, M. & Cheverud, J. The road to modularity. *Focus on Evo-Devo* **8**, 921–931 (2007).

54. Simon, H. A. The Architecture of Complexity. in *Facets of Systems Science* (ed. Klir, G. J.) 457–476 (Springer US, 1991).

55. Kashtan, N. & Alon, U. Spontaneous evolution of modularity and network motifs. *PNAS* **102**, 13773–13778 (2005).

56. Kaneko, K. Evolution of robustness to noise and mutation in gene expression dynamics. *PLoS ONE* **2**, (2007).

57. Bloom, J. D., Labthavikul, S. T., Otey, C. R. & Arnold, F. H. Protein stability promotes evolvability. *PNAS* **103**, 5869–5874 (2006).

58. Afroz, T. & Beisel, C. L. Understanding and exploiting feedback in synthetic biology. *Chemical Engineering Science* **103**, 79–90 (2013).

59. Brakefield, P. M. Evo-devo and constraints on selection. *Trends in Ecology & Evolution* **21**, 362–368 (2006).

60. Payne, J. L., Khalid, F. & Wagner, A. RNA-mediated gene regulation is less evolvable than transcriptional regulation. *PNAS* **115**, E3481–E3490 (2018).

61. Gerthart, J. & Kirschner, M. The theory of facilitated variation | PNAS. *Proceedings of the National Academy of Sciences* **104**, 8582–8589 (2007).

62. Kim, H., Muñoz, S., Osuna, P. & Gershenson, C. Antifragility Predicts the Robustness and Evolvability of Biological Networks through Multi-Class Classification with a Convolutional Neural Network. *Entropy* **22**, 986 (2020).

63. Noman, N., Monjo, T., Moscato, P. & Iba, H. Evolving Robust Gene Regulatory Networks. *PLoS One* **10**, e0116258 (2015).

64. Kauffman, S. A. *The Origins of Order: Self-Organization and Selection in Evolution.* (Oxford University Press, USA, 1993).

65. Wannier, T. M. *et al.* Adaptive evolution of genomically recoded *Escherichia coli. Proc Natl Acad Sci USA* **115**, 3090–3095 (2018).

66. Springman, R., Molineux, I. J., Duong, C., Bull, R. J. & Bull, J. J. Evolutionary Stability of a Refactored Phage Genome. *ACS Synth. Biol.* **1**, 425–430 (2012).

67. Ceroni, F. *et al.* Burden-driven feedback control of gene expression. *Nature Methods* **15**, 387–393 (2018).

68. Kurokawa, M., Seno, S., Matsuda, H. & Ying, B.-W. Correlation between genome reduction and bacterial growth. *DNA Res* **23**, 517–525 (2016).

69. Martínez-García, E., Nikel, P. I., Aparicio, T. & de Lorenzo, V. Pseudomonas 2.0: genetic upgrading of P. putida KT2440 as an enhanced host for heterologous gene expression. *Microb Cell Fact* **13**, 159 (2014).

70. Stieber, D., Gabant, P. & Szpirer, C. Y. The art of selective killing: plasmid toxin/antitoxin systems and their technological applications. *BioTechniques* **45**, 344–346 (2008).

71. Umenhoffer, K. *et al.* Genome-Wide Abolishment of Mobile Genetic Elements Using Genome Shuffling and CRISPR/Cas-Assisted MAGE Allows the Efficient Stabilization of a Bacterial Chassis. *ACS Synth. Biol.* **6**, 1471–1483 (2017).

72. Reuter, J. A., Spacek, D. V. & Snyder, M. P. High-throughput sequencing technologies. *Mol. Cell* **58**, 586–597 (2015).

73. Sharon, E. *et al.* Inferring gene regulatory logic from high-throughput measurements of thousands of systematically designed promoters. *Nature Biotechnology* **30**, 521–530 (2012).

74. Sarkisyan, K. S. *et al.* Local fitness landscape of the green fluorescent protein. *Nature* **533**, 397–401 (2016).

75. Nevozhay, D., Adams, R. M., Itallie, E. V., Bennett, M. R. & Balázsi, G. Mapping the Environmental Fitness Landscape of a Synthetic Gene Circuit. *PLOS Computational Biology* **8**, e1002480 (2012).

76. Cira, N. J., Pearce, M. T. & Quake, S. R. Neutral and selective dynamics in a synthetic microbial community. *Proc Natl Acad Sci USA* **115**, E9842–E9848 (2018).

77. van Opijnen, T., Bodi, K. L. & Camilli, A. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. *Nature Methods* **6**, 767–772 (2009).

78. Pepperkok, R. & Ellenberg, J. High-throughput fluorescence microscopy for systems biology. *Nat. Rev. Mol. Cell Biol.* **7**, 690–696 (2006).

79. Goda, K. *et al.* High-throughput single-microparticle imaging flow analyzer. *PNAS* **109**, 11630–11635 (2012).

80. Marucci, L. *et al.* Computer-Aided Whole-Cell Design: Taking a Holistic Approach by Integrating Synthetic With Systems Biology. *Front. Bioeng. Biotechnol.* **8**, 942 (2020).

81. Nielsen, A. A. K. *et al.* Genetic circuit design automation. *Science* **352**, aac7341–aac7341 (2016).

82. du Plessis, L., Leventhal, G. E. & Bonhoeffer, S. How Good Are Statistical Models at Approximating Complex Fitness Landscapes? *Molecular Biology and Evolution* **33**, 2454–2468 (2016).

83. McDonald, J. I. *et al.* Reprogrammable CRISPR/Cas9-based system for inducing site-specific DNA methylation. *Biology open* **5**, 866–74 (2016).

84. Nivina, A. *et al.* Structure-specific DNA recombination sites: Design, validation, and machine learning–based refinement. *Science Advances* **6**, eaay2922 (2020).

85. Romanini, D. W., Peralta-Yahya, P., Mondol, V. & Cornish, V. W. A Heritable Recombination System for Synthetic Darwinian Evolution in Yeast. *ACS Synth. Biol.* **1**, 602–609 (2012).

86. Reis, A. C. *et al.* Simultaneous repression of multiple bacterial genes using nonrepetitive extra-long sgRNA arrays. *Nature Biotechnology* **37**, 1294–1301 (2019).

87. Umenhoffer, K. *et al.* Reduced evolvability of Escherichia coli MDS42, an IS-less cellular chassis for molecular and synthetic biology applications. *Microbial Cell Factories* **9**, 38 (2010).

88. Nyerges, Á. *et al.* CRISPR-interference-based modulation of mobile genetic elements in bacteria. *Synthetic Biology (Oxford, England* **4**, (2019).

89. Le Breton, Y., Mohapatra, N. P. & Haldenwang, W. G. In Vivo Random Mutagenesis of Bacillus subtilis by Use of TnYLB-1, a mariner-Based Transposon. *Appl Environ Microbiol* **72**, 327–333 (2006).

90. Greener, A., Callahan, M. & Jerpseth, B. An efficient random mutagenesis technique using an E. coli mutator strain. *Methods Mol Biol* **57**, 375–385 (1996).

91. Badran, A. H. & Liu, D. R. Development of potent in vivo mutagenesis plasmids with broad mutational spectra. *Nature Communications* **6**, 8425 (2015).

92. Halperin, S. O. *et al.* CRISPR-guided DNA polymerases enable diversification of all nucleotides in a tunable window. *Nature* **560**, 248–252 (2018).

93. Camps, M., Naukkarinen, J., Johnson, B. P. & Loeb, L. A. Targeted gene evolution in Escherichia coli using a highly error-prone DNA polymerase I. *PNAS* **100**, 9727–9732 (2003).

94. Vojta, A. *et al.* Repurposing the CRISPR-Cas9 system for targeted DNA methylation. *Nucleic Acids Research* **44**, 5615–5628 (2016).

95. Hess, G., Frésard, L., Han, K., Lee, C. & Bassik, M. Directed evolution using dCas9-targeted somatic hypermutation in mammalian cells | Nature Methods. *Nature Methods* **13**, 1036–1042 (2016).

96. Tyo, K. E. J., Ajikumar, P. K. & Stephanopoulos, G. Stabilized gene duplication enables long-term selection-free heterologous pathway expression. *Nature Biotechnology* **27**, 760–765 (2009).

97. Albert, R. Scale-free networks in cell biology. *J Cell Sci* **118**, 4947–4957 (2005).

98. Park, Y., Espah Borujeni, A., Gorochowski, T. E., Shin, J. & Voigt, C. A. Precision design of stable genetic circuits carried in highly-insulated E. coli genomic landing pads. *Molecular Systems Biology* **16**, e9584 (2020).

99. Meyer, A. J., Ellefson, J. W. & Ellington, A. D. Directed Evolution of a Panel of Orthogonal T7 RNA Polymerase Variants for in Vivo or in Vitro Synthetic Circuitry. *ACS Synthetic Biology* **4**, 1070–1076 (2015).

100. Kylilis, N., Tuza, Z. A., Stan, G.-B. & Polizzi, K. M. Tools for engineering coordinated system behaviour in synthetic microbial consortia. *Nature Communications* **9**, 2677 (2018).

101. Wei, S.-P. *et al.* Formation and functionalization of membraneless compartments in Escherichia coli. *Nature Chemical Biology* **16**, 1143–1148 (2020).

102. Xiang, N. *et al.* Using synthetic biology to overcome barriers to stable expression of nitrogenase in eukaryotic organelles. *PNAS* **117**, 16537–16545 (2020).

103. Richardson, S. M. *et al.* Design of a synthetic yeast genome. *Science* **355**, 1040–1044 (2017).

104. Steel, H. & Papachristodoulou, A. Low-Burden Biological Feedback Controllers for Near-Perfect Adaptation. *ACS Synth. Biol.* **8**, 2212–2219 (2019).

105. Gorochowski, T. E., Avcilar-Kucukgoze, I., Bovenberg, R. A. L., Roubos, J. A. & Ignatova, Z. A Minimal Model of Ribosome Allocation Dynamics Captures Trade-offs in Expression between Endogenous and Synthetic Genes. *ACS Synth. Biol.* **5**, 710–720 (2016).

106. Gorochowski, T. E., Van Den Berg, E., Kerkman, R., Roubos, J. A. & Bovenberg, R. A. L. Using synthetic biological parts and microbioreactors to explore the protein expression characteristics of escherichia coli. *ACS Synthetic Biology* (2014) doi:10.1021/sb4001245.

107. Mittal, P., Brindle, J., Stephen, J., Plotkin, J. B. & Kudla, G. Codon usage influences fitness through RNA toxicity. *Proc Natl Acad Sci USA* **115**, 8639–8644 (2018).

108. Abil, Z., Ellefson, J. W., Gollihar, J. D., Watkins, E. & Ellington, A. D. Compartmentalized partnered replication for the directed evolution of genetic parts and circuits. *Nature Protocols* **12**, 2493–2512 (2017).

109. Yang, G. & Withers, S. G. Ultrahigh-Throughput FACS-Based Screening for Directed Enzyme Evolution. *ChemBioChem* **10**, 2704–2715 (2009).

110. Smith, G. P. & Petrenko, V. A. Phage Display. *Chem. Rev.* **97**, 391–410 (1997).

111. Mozhaev, V. V. & Martinek, K. Structure-stability relationships in proteins: new approaches to stabilizing enzymes. *Enzyme and Microbial Technology* **6**, 50–59 (1984).

112. Archetti, M. Genetic robustness and selection at the protein level for synonymous codons. *Journal of Evolutionary Biology* **19**, 353–365 (2006).

709   **Tables**

710   **Table 1: Methods for engineering the evotype.**

Engineering Genetic Variation				
Mechanism	**Type of Variation**	**Stability**	**Evolvability**	**Examples**
Site specific recombination	Inversions Deletions Duplications Rearrangement	Remove host recombinases	Targeted recombination systems	CRISPR-recombinase[83], SCRAMBLE[41], De-novo recombination sites[84]
Homologous recombination	Rearrangement Deletions Duplications	Remove host recombinases	Inducible recombination	Heritable recombination system[85]
		Avoid repeated sequences	Design in repeated sequences	EFM calculator[35], Automated design of non-repetitive parts[37,86]
Mobile genetic elements	Rearrangement	Remove Transposons	Transposon mutagenesis	Abolishment of Mobile Genetic Elements[71,87], CRISPR-controlled Insertion sequences[88], *In vivo tr*ansposon mutagenesis[89]
DNA polymerase fidelity	Global point mutation rate	Eliminate error-prone polymerases	Mutator strains	Low mutation *E. coli*[39], XI1-Red Mutator strain[90], Mutagenesis plasmids[91]
	Local point mutation rate	–	Targeted DNA polymerase	CRISPR-DNA polymerase[92]
	Plasmid-specific mutation rate	–	Orthogonal mutation plasmids	Orthorep[40], Pol 1 mutagenesis system[93]
Slipped-strand Mispairing	Targeted small indels	Avoid simple sequence repeats	Employ simple sequence repeats	EFM calculator[35]
DNA Methylation	Point mutation	Remove host DNA methyltransferase	Targeted methylation	CRISPR-methylation[94]
Cytosine Deamination	Point mutation	–	Targeted deamination	CRISPR-deamination[95]
Engineering the Production of Function [a]				
Principle	**Stability**		**Evolvability**	**Examples**
Prevalence	Use prevalent phenotypes		Use prevalent phenotypes	Designable protein structures[48]
Redundancy	Use multiple copies of genes/constructs		Use multiple copies of genes/constructs	High gene-copy system for *E. coli*[96].
	Scale free networks[97]		Scale free networks	–
Modularity	Insulate genetic parts		Insulate function	Ribozyme insulators[81], Insulated genetic landing pads[98]
	Use Orthogonal systems		Use Orthogonal systems	T7 RNA polymerase[99]
	Spatial/temporal separation		Spatial/temporal separation	Microbial consortia[100], Targeting to a cell cycle stage, specific cell type or organelle[101,102]
Regularity	Standardise parts across system		Standardise parts across system	Standardised architecture of the Yeast 2.0 genome[103]

Hierarchy	Co-control related functions	Co-control related functions	–
	Use networks with wide, shallow hierarchies	Use networks with wide, shallow hierarchies	–
Environmental Robustness	Feedback control	Feedback control	Feedback control of transcription and translation[104]
	Use larger tolerances for parts	Use larger tolerances for parts	Maximise dynamic range of genetic logic gates[81]
Designing Parameter Space	Design a constrained parameter space	Design a diverse parameter space	Modelling phenotype distributions of genetic circuits[45]
Engineering Natural and Artificial Selection			
Principle	**Stability**	**Evolvability**	**Examples**
Increase design type fitness	Evolution for fitness after design phase	–	Adaptive evolution of recoded *E. coli*[65], Adaptive evolution of refactored phage genome [66]
	Reduce metabolic burden	–	Modelling ribosome allocation to reduce burden[105], Characterising burden of genetic parts[106]
	Dynamic control of burden	–	Burden-driven feedback control in *E. coli*[67]
	Reduce toxicity	–	mRNA toxicity[107]
Decrease neighbouring fitness	Minimised chassis organism	–	*Pseudomonas* 2.0[69]
Correlate fitness and utility	Couple function to reproduction	Couple function to reproduction	Phage assisted continuous evolution[10], Compartmentalized partnered replication[108]
	Couple function to survival	Couple function to reproduction	Toxin/antitoxin systems[70]
	Artificial selection pressure	Artificial selection pressure	FACS screening[109], Ribosome Display[89], Phage Display[110]

711 a. Methods for production of function are suggestions; the details of how to apply these principles to influence the
712   evotype in design type systems are still in the process of being understood.

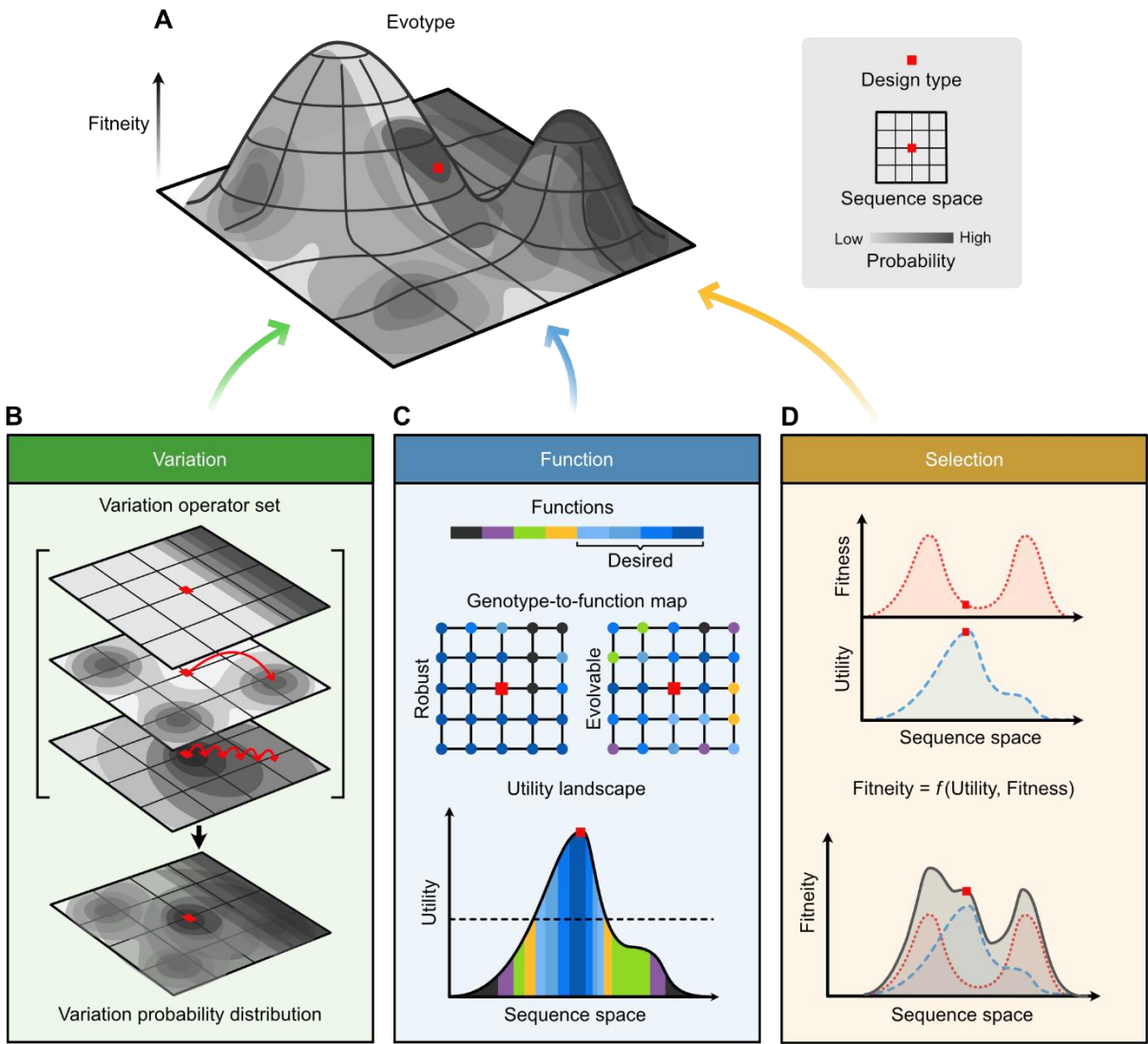
713 **Table 2: Role of genotype, phenotype and evotype when describing biological systems.**

	Genotype	Phenotype	Evotype
Describes	Informational/ Hereditary properties	Physical/Environmental properties	Evolutionary properties
Is a set of...	Genes/Sequences	Traits	Dispositions
Pig example	Hox gene	Four legs	Chance of evolving wings
Protein example	Codon	Structural stability under temperature [a]	Structural stability under mutation [b]
Biosensor circuit example	Genetic circuit sequence	Sensor sensitivity/specificity, Input-output function, Fluorescent output, etc.	Probability of: total failure, sensitivity loss, I/O function change, etc.

714 a. For example, strengthened hydrophobic interactions in the interiors of thermostable proteins [111].

715 b. For example, robustness of the genetic code to amino acid mutations due to synonymous codons [112].

716 **Figures and captions**

717

718 **Figure 1: The evotype and its key properties.** (**A**) The evotype visualised as a landscape

719 surrounding the design type (red square), where fitneity (the combined function of fitness and

720 utility) is plotted as a vertical axis against a 2D plane of sequence space with the probability

721 of evolution exploring regions of sequence space overlaid in grey. The properties of this

722 landscape are determined by the interaction of three components: variation, function, and

723 selection. (**B**) A variation probability distribution can be projected onto sequence space, which

724 represents the likelihood of exploring a given sequence through genetic variation. Darker

725 regions represent regions of higher probability. This is the sum of the distributions of the

726 individual variation operators present in the system (variation operator set). For example, point

727 mutation (bottom layer in set), recombination of homologous regions (middle layer in set), and

728 slip-strand mutation (top layer in set). Red arrows in middle and bottom layers represent

729 algorithmic and point mutations respectively. (**C**) How phenotypic functions are distributed in

730 sequence space surrounding the design type is critical. Function space may be considered as

731   discrete (top), where the space may have high genotypic robustness (left grid) or high

732   variability (right grid). A continuous utility space (bottom) plotted against a 1D projection of

733   sequence space. The colour under the curve represents the discrete function associated with

734   that region of sequence space and the utility that each has as a continuous value. For

735   example, if the goal is to produce blue-like functions, dark blue may have the highest utility,

736   followed by lighter variants in the spectrum. The bioengineer must define a minimal threshold

737   (dashed line), below which the design is deemed to be a failure (e.g. non-desired function is

738   exhibited). (**D**) Sequences differ in their reproductive fitness. This is the driver of natural

739   selection and can be plotted across sequence space as an adaptive landscape (red dotted

740   line). Utility (blue dashed line) may or may not correlate with reproductive fitness across

741   sequence space. The fitneity (grey solid line) is the combination of the fitness and utility.

742   Bioengineers must optimise fitneity both for the design type and throughout the landscape.

Figure 2: Engineering evotypes by sculpting their landscapes. Different biosystem designs may share the same phenotype but have very different evotypes (top row). Rational engineering approaches could be used to transform a naive design (middle column), where evotype has not been considered, into either evolutionarily stable (left column) or specifically evolvable (right column) evotypes, which are characterised by their fitneity landscapes. Bioengineers can sculpt the evotype by modifying three major factors: genetic variation, production of function and selection. Genetic variation (green row): In a naive design, a mixture of variation operators may be in play. This might create a system that can reach many different regions of sequence space. It could be made more stable by reducing global mutation rates (e.g. host strain engineering), or by removing homologous regions to reduce the chance of recombination. Conversely, a naive design might be made more evolvable by increasing mutation rates in focused areas of sequence space (e.g. via methylation) and incorporating site-specific recombination or gene-shuffling (e.g. the SCRaMbLE system). Function (blue row): A naive design may have high utility, however, if its function changes rapidly and chaotically across sequence space, it may be inherently

unstable. A robust evotype has large neutral regions in function space. Conversely a design can be made more evolvable if it can access a large range of new phenotypes, of a specific class (e.g. produce a colour), and the landscape may be smoothed (e.g. through removing crosstalk between features) and thus made amenable to evolutionary search. Production of function may be engineered by using prevalent phenotypes, designing in redundancy, modularity, regularity and hierarchy, increasing environmental robustness, or by designing a system's parameter space. Selection (orange row): If, as in the naive design, reproductive fitness (red dotted line) and utility (blue dashed line) are highly uncorrelated, then the design type may have a strong selection pressure acting against it, and regions where both fitness and utility are maximised may be rare or non-existent, so high fitneity (grey solid line), may not be achievable. For a stable design, one might act to reduce the effects of natural selection through global increases in fitness (e.g. through reducing metabolic burden of a genetic circuit), by reducing toxicity of gene products, or by reducing the fitness of neighbouring sequences (e.g. using minimised chassis organisms). A naive design can be made more evolvable by closely correlating fitness and utility (e.g. through coupling function to reproduction). This means natural selection will act to drive up the utility of the design: the precise goal of a directed evolution experiment.