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Synthetic approaches to understanding biological constraints

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Microbes can be readily cultured and their genomes can be easily manipulated. For these reasons, laboratory systems of unicellular organisms are increasingly used to develop and test theories about biological constraints, which manifest themselves at different levels of biological organization, from optimal gene-expression levels to complex individual and social behaviors. The quantitative description of biological constraints has recently advanced in several areas, such as the formulation of global laws governing the entire economy of a cell, the direct experimental measurement of the trade-offs leading to optimal gene expression, the description of naturally occurring fitness landscapes, and the appreciation of the requirements for a stable bacterial ecosystem.

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Introduction

Living organisms are optimized by evolution to survive and reproduce. Optimization targets all levels of biological organization at once, from elementary biomolecules to large anatomical features, from the expression levels of single genes to complex individual and social behaviors. The complex interplay of physical, chemical, biological, and ecological constraints does not guarantee that absolute optima are achieved by the evolutionary process, and makes the design of optimal organisms hard to predict from first principles [1–3]. The evolutionary dynamics itself introduces further complications: for instance, finite populations can accumulate deleterious mutations [4], and species which are not the fittest may nevertheless prevail if they are more tolerant to mutations (survival of the flattest versus survival of the fittest) [5]. Moreover, since no organism can be optimal in every environment, trade-offs are imposed on the development and maintenance of any structure and function in living systems. Microbes are ideal experimental systems to

develop and test theories about optimality, trade-offs, and constraints in biology. The relative ease in manipulating microbial genomes and fast reproduction rates allows experimentalists to generate mutants with the desired features, to measure their costs and benefits, and to follow the evolutionary dynamics for thousands of generations [6].

Metabolic constraints

The common observation that the growth rate of a cell is inversely correlated with its resistance to stress [7] suggests the existence of a trade-off between growth and repair. More broadly, the growth rate may affect the entire economy of a cell through system-level constraints. An example of this effect is the up to 10-fold variations in the production rates of constitutively expressed genes during different growth phases [8]. Eventually, nonlinearities in the growth-rate/gene-expression feedback may even lead to bistable growth [9] and possibly cause some cells to enter a dormant state [8]. Growth-rate dependent global constraints have recently been expressed as simple linear relations involving the expression levels of different classes of proteins (ribosomal, growth-rate dependent, growth-rate independent, and unnecessary) [10^{••},11,12].

The macroscopic description of metabolism based on growth rates is complemented by the detailed study of metabolic constraints and the optimization of metabolic fluxes to maximize biomass production [13,14]. These methods have been formalized into flux balance models describing all the chemical reactions in a cell [15]. Even more comprehensive models account also for the costs and benefits of enzyme synthesis [16] to describe, for instance, the switch between efficient respiration in nutrient-poor environments and inefficient fermentation in nutrient-rich environments [17].

Trade-offs and gene regulation

One of the primary roles of gene regulation in microbes is to modulate metabolic fluxes and to ensure that high growth rates can be achieved in favorable environmental conditions. A common synthetic technique to characterize cellular responses is then to decouple a response in gene expression from its triggering signals. Poelwijk *et al.* [18^{••}] cleverly improved on previous experiments [19] measuring the costs and benefits of the lac system in *Escherichia coli* by decoupling induction and metabolic activity. The cost of the lac operon expression has been measured by tuning protein synthesis with an inducer which cannot be metabolized, whereas the benefits of expression have been measured with a metabolite which

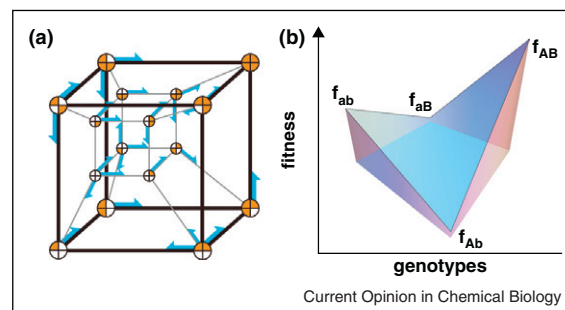
cannot induce. Evolutionary experiments in environments containing different concentrations of inducer and metabolite have confirmed how a theoretical costs/benefits analysis can predict the result of adaptation in each environment. Similarly, by decoupling glucose sensing and glucose uptake in yeast, it has been shown how the growth rate of yeast cells depends separately on these two variables. This is because sensing high concentrations of extracellular glucose triggers the synthesis of extra glucose-processing machinery and imposes a fitness burden if glucose import is less than expected [20]. When a regulatory system is not present, it can be engineered using synthetic techniques. As an example, in yeast, low levels of noise in the expression of a glutamate synthase provide a fitness advantage in limiting concentrations of ammonia, whereas noisy expression increases fitness at toxic levels of ammonia. A mutant with the ability to tune noise in response to a signaling molecule has been built, and its evolutionary advantage in fluctuating environments over its non-regulated wild type competitors has been established [21].

Fitness landscapes and evolutionary accessibility

Gene regulation confers a fitness advantage when the time scale of the environmental fluctuation is shorter than a few hundred generations [22,23]. If the environment changes much more slowly, however, regulatory functions are typically lost, according to a ‘use it or lose it’ principle [24,25] and the optimal expression level is fixed through genetic mutations. An example of this transition between regulation on cellular time-scales to adaptation on evolutionary time-scales has been repeatedly observed in laboratory populations of yeast cells: when a selective pressure favors fast growth, a trade-off between growth rate and mating efficiency causes quick silencing and an eventual loss of 23 genes in the mating pathway to achieve a 2% growth rate advantage [26]. Fitness landscapes assign a fitness value to each genotype or phenotype (in a specified environment) and they represent a geometric setting to study the evolutionary process connecting a low-fitness ancestral organism to its high-fitness descendants (Figure 1a). Whereas evolution can proceed rapidly along adaptive paths of increasingly fit organisms [27–29], real populations do cross moderately shallow-fitness valleys under a variety of conditions: if the fitness deficit of the intermediates is small enough [30], if the mutation rate is large enough, if the available time is long enough or by exploiting small effective population sizes (e.g. at the frontier of a range expansion [31]).

Synthetic approaches are increasingly applied to delineate naturally occurring landscapes by constructing all the possible genetic intermediates between ancestral organisms and their evolved counterparts [27,28,32,33,34,35,36,37,38]. Although such experimental studies are necessarily limited by the exponential number of possible

Figure 1



The geometry of fitness landscapes. (a) Example of an experimentally mapped four-dimensional phenotypic landscape describing the effectiveness (measured as MIC, Minimum Inhibitory Concentration) of a beta-lactamase enzyme in hydrolyzing the antibiotic cefotaxime [27,36]. Every circle represents a genotype for which each of the four mutations can be absent (white wedge) or present (orange wedge). The blue arrows indicate the selective pressure to acquire mutations which increase the MIC. The landscape in this example is rugged, meaning that some mutations can be beneficial or deleterious depending on the genetic background in which they appear. The landscape is nevertheless single-peaked. (b) An example of sign epistasis on a two-dimensional landscape. Whereas starting from a wild type genotype (ab) the combined fitness effect of two mutations (AB) is beneficial, the effect of only one of the two mutations (either A or B) is deleterious. For this reason the evolutionary dynamics is not guaranteed to find the global maximum of fitness (f_{AB}) and it might remain stuck for long times in a suboptimal local maximum (f_{ab}).

intermediates, various kinds of landscapes have been studied. Notably, the mapping involved in the definition of a landscape is not necessarily one-to-one: different genotypes can converge to the same phenotype [39] and, in case of multistable gene expression [40], the same genotype can produce different phenotypes. Moreover, even if genotype-to-phenotype landscapes may be approximately linear, the nonlinearity of the further mapping between phenotype and growth rate leads to a nonlinearity of the final genotype-to-fitness landscapes [32], which often display epistatic effects leading to diminishing returns [33,34,35].

Starting from the geometric properties of experimentally measured landscapes, the properties of the evolutionary dynamics can be predicted or, vice versa, some properties of naturally occurring fitness landscapes can be inferred by observing the evolutionary dynamics. Especially relevant properties of fitness landscapes are the ones which do not depend on how phenotypes or growth rates are scaled. Examples of these topological features are the number of accessible paths leading to a fitness maximum, the number of peaks in a landscape, and the presence of sign epistasis (Figure 1b), a necessary condition for the generation of multi-peaked landscapes [41]. Sign epistasis has been further shown to reduce the number of accessible evolutionary paths [42], to increase their length by imposing genetic detours [43], to increase

the repeatability of evolution [44], and to interfere with its reversibility [36,45].

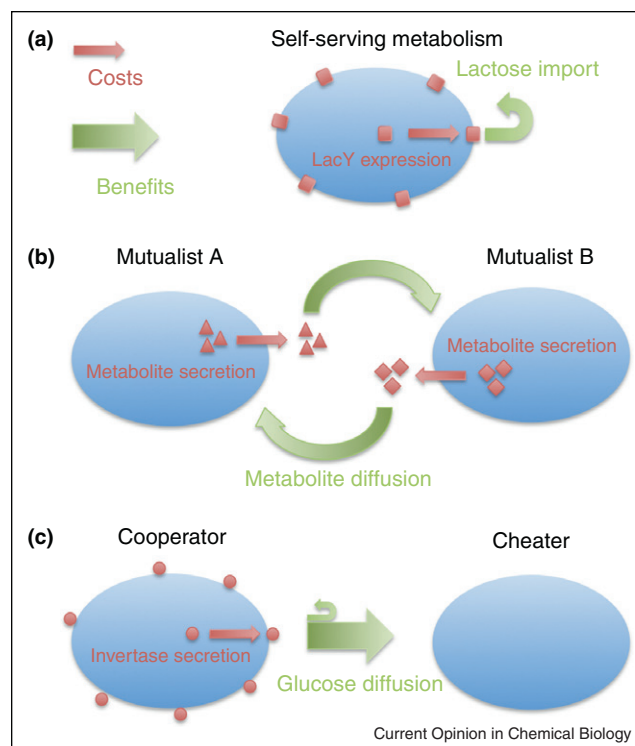
Notably, the biochemical constraints encoded by sign epistasis can also be exploited to encode biological functions. This is highlighted in a landscape describing the activity of a lac promoter as a function of mutations in the lac repressor and in its operator [37]. Each repressor/operator pair can be interpreted as a key-lock system and the fact that the landscape is multi-peaked (due to sign epistasis) indicates that a different key-lock system [46] can be generated with a few mutations in the repressor and a few compensatory mutations in the operator. Novel regulatory functions can indeed evolve with surprising rapidity [38,47]. In [38], a lac promoter controlled a synthetic operon for the simultaneous expression of two enzymes in a laboratory population of *E. coli*. High expression levels of the operon conferred higher fitness in environment A and low fitness in environment B. While alternating between the two environments, the experimenters added an inducer molecule only in environment B, so that the operon was highly expressed when low expression would have been optimal and repressed when high expression would have been optimal. After only three rounds of mutagenesis and competitive growth, the logic of regulation in the bacterial population was reversed, so that the inducer molecule had effectively become an inhibitor. Surprisingly, only three key mutations in the lac repressor protein were necessary to develop this new mode of regulation [38,48].

Synthetic ecosystems

Natural extensions of the formalism used to describe constraints and trade-offs at the single-cell level (Figure 2a) have recently been applied to small synthetic ecosystems, where each species can be considered as a module with specific metabolic inputs and outputs [49].

As a simple proof of principle, two variants of a yeast strain have been built, each overproducing an amino acid essential for the other strain (Figure 2b). In an environment without the two amino acids the two strains were obligate cooperators [50] and, in some conditions, they managed to coexist. In a similar experiment with *E. coli*, all possible pairing of 46 auxotrophic strains have been explored. In ~17% of the cases, the two strains were able to provide each others' essential nutrients and survive in environments which would have killed each of them separately [51]. Stable cooperation in synthetic ecosystems which did not coevolve is not guaranteed. In fact, in well-mixed conditions, asymmetries in growth rates, different tolerance to insults such as starvation or antibiotics and delays in the release of metabolites often result in the rapid rise of one strain, followed by the collapse of the whole population [52]. However, since these mutualistic interactions are based on the exchange of diffusible molecules, spatially structured environments

Figure 2



Cost/benefit analyses at multiple scales. (a) Single cells quickly adapt to balance the costs associated with the expression of a gene and the benefits deriving from the gene products in order to achieve optimal growth rates [18]. (b) Bacterial communities are often based on mutualistic interactions. A-cells might pay a cost so that B-cells can benefit from it. In turn, A-cells indirectly benefit from the success of B-cells. (c) Despite the existence of cells which do not cooperate, cooperative behaviors can still be maintained [57] if cooperator cells have preferential access to a public good (e.g. if a diffusive dynamics ensures that producers are also exposed to higher concentrations of the common good). Quantitative details of the costs and benefits, together with the spatial [52] and temporal [50,53,55] structures of the interactions are often crucial for the evolutionary stability of these social interactions.

(typically with length scales in the hundreds of microns) may enable the existence of synthetically assembled multispecies microbial communities [52].

Alternatively, even in spatially unstructured environments, the temporal structure of a population can play a relevant role in the coexistence of multiple species. Starting from the same ancestral *E. coli* strain, the seasonality induced by daily serial dilutions promoted the coevolution of two strains, each one occupying its own temporal niche: early in the day a fast-growing strain feasted on fresh medium; later in the day a scavenger strain thrived by cannibalizing the first strain [53].

Because of the complexity of even the smallest laboratory ecosystems discussed above, microbial communities are a standing challenge for synthetic and systems biology.

Notably, about 1/3 of the genes in *E. coli* still have no known function and a fraction of those genes might be related to the microbes' social life. Even more strikingly, the majority of microbial species cannot be cultured in isolation and much of the uncharted territory in the microbial world might remain unexplored until the networks of ties and constraints in microbial communities are understood [54].

The spread of cooperation

In microbial consortia each organism follows a selfish strategy, exploiting other organisms, preying on them [55] or synthesizing toxins for chemical warfare [56]. However, cooperative and altruistic behaviors are often observed in nature, in apparent contrast with the evolution of selfish genes. The emergence and, even more importantly, the persistence of cooperative behaviors can be investigated using synthetic techniques which allow scientists to manipulate microbial strategies. Communities of engineered microbes become then a tool to understand ecosystems beyond the microbiology laboratory.

Yeast cells grown on sucrose provide a model system to study cooperative behaviors [57,58]. Cooperator cells pay an individual cost to hydrolyze sucrose, producing glucose as a common good which diffuses in the medium (Figure 2c). Cheater cells, instead, are engineered to take advantage of the glucose in the medium without paying the cost for its production. Cheater cells can readily invade a population of cooperators and spread. Surprisingly, however, even in a well-mixed environment it has been shown that coexistence is possible [57]. Although cooperators are always exploited by cheaters, cooperators can survive by having preferential access to the common good they produce, and fare relatively well when the common good is scarce. Spatially structured populations further promote cooperation [59,60]: since a common good typically diffuses in the environment, local subcommunities which happen to be rich in cooperators also become privileged recipients of the common good and grow faster, so that the overall fraction of cooperators increases. This is the essence of the Simpson's paradox, which recent experiments on engineered *E. coli* have explicitly demonstrated [61]. To further emphasize the importance of laboratory models for a thorough understanding of natural ecosystems, Chuang *et al.* [62^{*}] used a simple bacterial system to demonstrate that costs and benefits can be nonlinear functions of the population structure, and that costs and benefits measured at the cell level are not simply related to the same parameters for the whole population.

Conclusion

We briefly reviewed recent experiments and theoretical work to understand the structure of metabolic, regulatory, evolutionary, and social constraints. Despite considerable

effort, these pioneering studies are partial or isolated results, providing mostly anecdotal evidence. Encouragingly, there is a trend of ever cheaper and more widespread use of synthetic biology tools to read and write genomes, and of biophysics techniques to quantify the behavior of living cells. This trend is enabling the acquisition of an unprecedented amount of data [63] and of aggressive hypothesis testing. Independent studies on a variety of systems are rapidly accumulating: the generic features of fitness landscapes [64] will reveal the constraints of evolution as a stochastic optimization process, whereas the emerging motifs in the networks of interactions within microbial communities will soon have a direct impact on biotechnology and human health.

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