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Continuous directed evolution for strain and protein engineering

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Continuous directed evolution is the 'while loop' of synthetic biology, autonomous cycles of mutation, selection, and self-replication that can lead to the rapid development of industrially relevant organisms, pathways, or molecules. Although this engineering strategy requires particular mutagenesis methods and well-defined selections, recent advances have facilitated its implementation. Control over selection pressure has been augmented by novel cost-effective continuous culturing devices with open source designs. New *in vivo* targeted mutagenesis methods have enabled continuous directed protein evolution in various organisms. Furthermore, advances in automation have enabled rational, semi-continuous directed evolution strategies that may yield fewer artefacts or parasites. Overall, continuous directed evolution is persistently demonstrating its capacity to rapidly generate biotechnologically valuable strains and proteins.

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Introduction

Classically, many industrial microorganisms have been evolved by continuous propagation to achieve particular production phenotypes [1], but it was not until the advent of high-throughput sequencing technologies that reliable phenotype-to-genotype relationships could be made that better informed the course of such continuous evolution experiments [2]. Although the principles behind biocatalyst evolution via continuous dilution or serial culture have largely remained the same since their initial inception, a number of recent technological advances have enabled a higher level of control over selection parameters, mutation rate and scope, and have limited necessary manual intervention. This review will highlight recent developments in both automation technologies and genetic tools for the continuous evolution of strains and proteins, and how these distinct advances can synergize for biotechnology applications.

Advances in continuous culturing devices

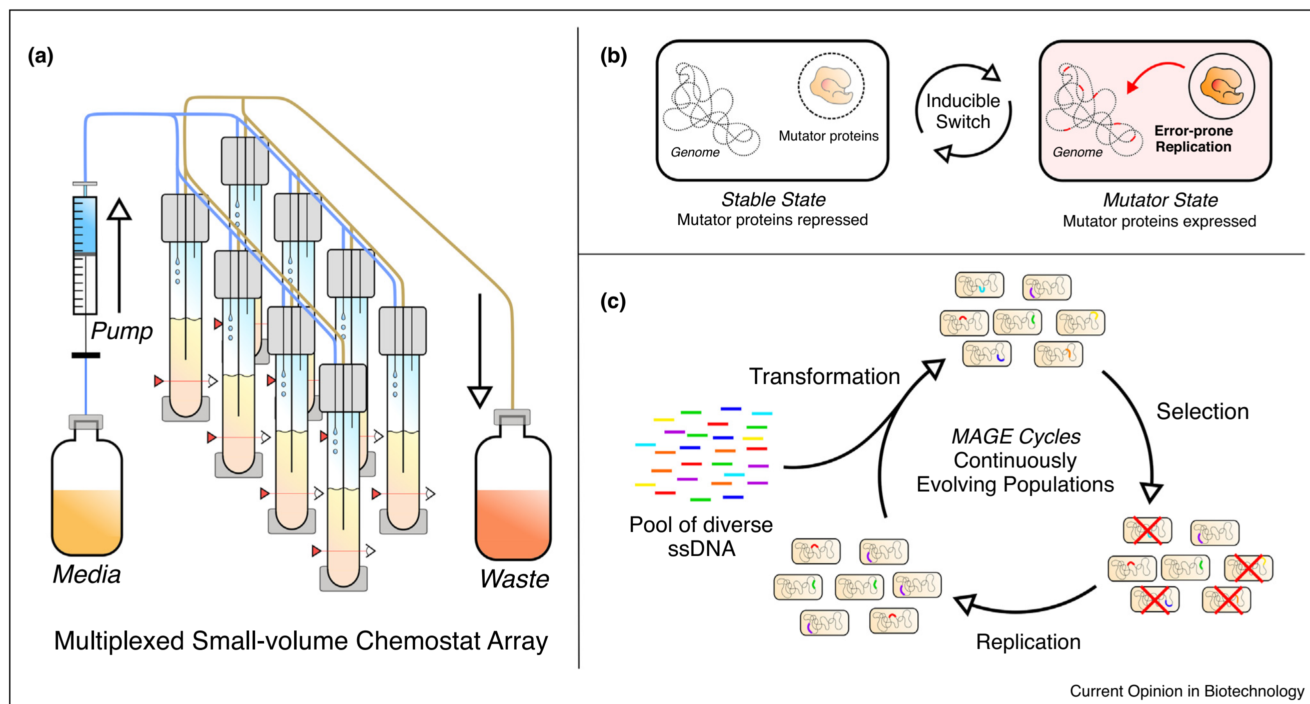
Culture propagation via the continuous dilution of cells is carried out either by an automated culturing device or by manual serial dilution. However, since the latter approach is more likely to suffer from population bottlenecking, dynamic fluctuations in culture density, and inconsistent selection pressure, improvements in continuous culture procedures have been necessitated. Issues such as high cost, biofilm accumulation, restricted diversity of culturing conditions, and limited multiplexing discourage the adoption of continuous culturing devices. Recent advances have helped mitigate some of these challenges: to address biofilm accumulation, the de Crecy group developed a continuous culturing device, termed the Evolugator, which limits wall growth by cycling cultures to different isolated partitions of the bioreactor [3,4]. The principle of culture cycling was also advanced in a device developed by Mutzel and coworkers that includes sterilization steps after culture transfers [5,6]. Taking a more biological tack, biofilm accumulation has also been limited by the discovery and subsequent elimination of genes involved in their formation [7,8].

In addition to diminishing biofilm accumulation, the implementation of continuous culture is becoming increasingly easier, even for smaller labs, with the advent of low cost and user-friendly culturing devices made from 3D-printable or readily accessible parts, and supported by open source software [9,10,11••] (Figure 1a). Higher throughput parallelization has proven possible via liquid handling robots [12], miniature chemostat arrays [13,14•], and automated microdroplet systems [15]. While challenges remain, especially with respect to readily diversifying experimental conditions, the fact that there are both new avenues to low end devices [9,10,11••] and an increasing industrial emphasis on continuous culture [16] suggests that there will be a growing community of investigators to solve these problems.

Advances in strategies for mutating strains

Cellular adaptation during conventional continuous culture experiments is largely governed by strain mutation rates. Natural host mutagenesis can be insufficient [17,18] to achieve new functionalities, and methods which enhance basal mutation rates have been shown to accelerate strain evolution. Early demonstrations include the development of hypermutator *Escherichia coli* strains, such

Figure 1



Strategies for continuous strain evolution. **(a)** Example schematic of an open source design for a low cost, small-volume, multiplexable chemostat array. **(b)** Controllable mutator strains capable of switching between hypermutator and stable states upon induction via a metabolite-linked biosensor [21], pH-sensitive riboswitch [25•], or chemical inducer [23,26]. **(c)** Multiplexed Automated Genome Evolution (MAGE) [29]. A cell population is transformed with synthetic ssDNA, causing site-specific genome mutations, and subsequently grown in selective conditions thereby enriching certain variants. This process can be applied recursively using liquid handling robots to automate the semi-continuous evolution of strains.

as XL1-Red, which has a genomic mutation rate ~5000 times that of wild type strains [19], as well as the use of chemical mutagens [20].

Unfortunately, while a high basal mutation rate can expedite evolution, the accumulation of frequent, deleterious mutations often compromises overall strain stability. Thus, recent work has focused on developing strains capable of inducibly switching between high and low mutagenic states (Figure 1b). Such controlled mutator strains have been applied to evolving a range of phenotypes including butanol, acetate, and pH tolerance, as well as metabolite overproduction [21]. Inducible mutagenesis has been achieved through the use of plasmid expressed mutant error-prone polymerase subunit (dnaQ) introduction followed by plasmid curing [22], inducible expression of mutagenesis genes [23], or inducible expression of DNA repair genes in a hypermutator background [24]. Recently, Pham *et al.* described a similar system using an engineered pH-sensitive riboswitch that controlled an integrase which in turn controlled dnaQ production and could be used to evolve cellular tolerance to low pH [25•]. The riboswitch was off (and dnaQ was on) under acidic conditions (pH 5), thus allowing cells to mutate and adapt to the growth media by creating a neutral intracellular environment, at which point the riboswitch was turned on, leading to integrase production and flipping of the dnaQ cassette to an inactive conformation. The cells that best neutralized their acid environment were 'stuck' with the mutations that had been induced by dnaQ, and this method reliably generated a strain more tolerant to six organic acids compared to the starting strain. Additional technologies, including recently described inducible broad-spectrum mutagenesis plasmids [26], should further encourage adoption of switchable mutation across a wider variety of strains.

Recent advances in technologies for site-specific *in vivo* mutagenesis and automation [27,28] have also provided opportunities for the virtually continuous evolution of strains. One of the more high-profile methods has been called Multiplexed Automated Genome Engineering, or MAGE [29] (Figure 1c). In this method ssDNA oligonucleotides with specific mutations are transformed into *E. coli* and site-specifically incorporated within the host genome during lagging strand DNA replication. Efficient oligonucleotide initiation of replication relies on the expression of λ Red recombinase enzymes as well as

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