REVIEW ARTICLE

The functional basis of adaptive evolution in chemostats

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Abstract

Two of the central problems in biology are determining the molecular basis of adaptive evolution and understanding how cells regulate their growth. The chemostat is a device for culturing cells that provides great utility in tackling both of these problems: it enables precise control of the selective pressure under which organisms evolve and it facilitates experimental control of cell growth rate. The aim of this review is to synthesize results from studies of the functional basis of adaptive evolution in long-term chemostat selections using *Escherichia coli* and *Saccharomyces cerevisiae*. We describe the principle of the chemostat, provide a summary of studies of experimental evolution in chemostats, and use these studies to assess our current understanding of selection in the chemostat. Functional studies of adaptive evolution in chemostats provide a unique means of interrogating the genetic networks that control cell growth, which complements functional genomic approaches and quantitative trait loci (QTL) mapping in natural populations. An integrated approach to the study of adaptive evolution that accounts for both molecular function and evolutionary processes is critical to advancing our understanding of evolution. By renewing efforts to integrate these two research programs, experimental evolution in chemostats is ideally suited to extending the functional synthesis to the study of genetic networks.

Introduction

In 1950, Jacques Monod introduced a method for culturing microorganisms in which cells in a liquid medium of fixed volume grow continuously by constant replenishment of the medium. In the same year, Leo Szilard and Aaron Novick reported a device for culturing cells using the same approach, which they named the 'chemostat' (Novick & Szilard, 1950a, b) – a name that has since endured. The central principle of the chemostat is that through the continuous addition of medium containing a single growth-limiting nutrient and simultaneous removal of culture, a stable equilibrium is achieved. In this steady state, the rate at which the population of cells grows is equal to the rate at which the culture is diluted. The key advantages of the chemostat are that a steady state in a constant, defined environment is attained and the growth rate of the cells can be experimentally controlled by modulating the rate of culture dilution. The inventors of the chemostat argued that these unique properties had great utility for two areas of investigation: the study of cell growth and the study of evolution by mutation and selection. More than 60 years later, the advent of genome-scale experimental methods and a reinvigoration of a quantitative approach to biology (i.e. systems biology) have stimulated renewed interest in the use of chemostats (Hoskisson & Hobbs, 2005; Bull, 2010; Ziv *et al.*, 2013a). The primary research applications of chemostats remain the study of cell growth and adaptive evolution, which owing to their central importance in cell and evolutionary biology, remain vital areas of investigation.

Rather than treating the study of cell growth and adaptive evolution as separate research enterprises, much stands to be gained from their integrated study. Here, we argue that many of the important questions in evolution including the role of different types of genetic variation, the predictability and repeatability of evolution, the causes and consequences of antagonistic pleiotropy, the distribution of fitness effects, and the role of epistasis are best addressed on the foundation of an understanding of the genetic networks that are the targets of selection. This requires *a priori* knowledge, and precise experimental control, of the selective pressure experienced by the organism. The chemostat is ideally suited to fulfilling

FEMS MICROBIOLOGY REVIEWS

these criteria. Moreover, as a chemostat environment usually represents a novel environment for the organism, fitness increases in evolving lineages, and populations, are typically large providing increased statistical power when dissecting multigenic and epistatic effects. Finally, the chemostat provides unparalleled control for systematically varying important parameters in adaptive evolution including population size and the strength of selection.

At the same time, identifying the targets of selection in a chemostat provides insight into how a cell regulates its growth across a range of environmental conditions. Identifying the functional basis of increased fitness in a chemostat is informative about the regulatory mechanisms that control cell growth in different environments. In principle, there may be multiple ways in which fitness can increase in a particular environment. However, only a subset of these options may be evolutionarily viable, and determining how genetic networks that control growth evolve provides insight into their structure and plasticity. As many of the principles of cell growth control are conserved across orders of life and many of the proteins and processes required for cell growth are evolutionarily, conserved, insight into the evolution of the genetic networks controlling cell growth has the potential to be broadly informative.

Several reviews of experimental evolution in microorganisms have been published in recent years (Elena & Lenski, 2003; Zeyl, 2006; Bennett & Hughes, 2009; Buckling *et al.*, 2009; Conrad *et al.*, 2011; Dettman *et al.*, 2012; Desai, 2013). Reviews focused on experimental evolution in chemostats have appeared less frequently. Dykhuizen and Hartl (1983) published a comprehensive review of early microbial evolution studies using chemostats, and subsequent publications have provided updates on progress in the field (Dykhuizen, 1990; Adams, 2004; Ferenci, 2008). In addition, studies of selection in chemostats have been featured in collected volumes (Garland & Rose, 2009) and treatises on adaptive evolution (Bell, 2008). In this review, we introduce the concept of the chemostat and discuss the theoretical basis for adaptive evolution in chemostats. Although experimental evolution in chemostats has been performed in a range of microorganisms, we focus here on studies in *Escherichia coli* and *Saccharomyces cerevisiae*, which currently have the best functionally annotated genomes and therefore are ideally suited to enabling the integrated approach that we advocate. We discuss recent progress in understanding the molecular basis of adaptive evolution in chemostats in these two organisms and conclude by outlining some of the future goals that are now attainable. Our aim is to synthesize these studies to define the state of the field and to argue that selection in chemostats is an ideal system for extending the functional synthesis – the reunification of molecular biology and evolution (Dean & Thornton, 2007) – to the study of genetic networks.

The principle of the chemostat

The culturing of microorganisms in a chemostat differs in several ways from the method of batch culture growth typically employed by microbiologists. In a batch culture, a small number of individual cells are inoculated into fresh medium, and following a physiological and metabolic adjustment, cell growth and division commences. Initially, the rate at which the population grows is unconstrained by nutrient abundance, and population expansion proceeds at a maximal rate. Once a population reaches a sufficiently high density, nutrients become increasingly scarce, and exhaustion of essential nutrients ultimately leads to the cessation of cell growth and initiation of a quiescent state. In a chemostat, population growth is initially qualitatively similar to batch culture growth. However, as the population grows, the medium in the culture is continuously replenished by addition of fresh medium (Fig. 1). Simultaneously, an equal volume containing both cells and spent medium is removed from the culture vessel. Thus, a chemostat is said to be 'diluted' through continuous addition of fresh medium and removal of culture. A single essential nutrient is present at a growth-limiting concentration in the chemostat vessel (analogous to the nutrient that is first exhausted in batch cultures), while all other nutrients are present in excess. The growth-limiting nutrient is predetermined by the experimenter and can be any nutrient essential for cell growth. For example, if glucose is the limiting nutrient, a chemostat is said to be 'glucose-limited'.

In a chemostat, there are two important dynamic variables: the number of cells (x) and the concentration of the growth-limiting nutrient (s). The number of cells increases with time due to growth and division and decreases with time due to culture dilution. The concentration of the growth-limiting nutrient increases with time through addition of fresh medium and decreases through both dilution and consumption by the cells in the chemostat vessel. The temporal dynamics with which the number of cells (dx/dt) and the nutrient concentration (ds/dt) change can be modeled using the coupled ordinary differential equations:

$$\frac{dx}{dt} = \mu_{max}\frac{s}{K_s + s}x - Dx \quad (1)$$

$$\frac{ds}{dt} = DR - Ds - \frac{x}{Y}\mu_{max}\frac{s}{K_s + s} \quad (2)$$

In these equations, the growth rate of the cells (μ) is related to the concentration (s) of the limiting nutrient

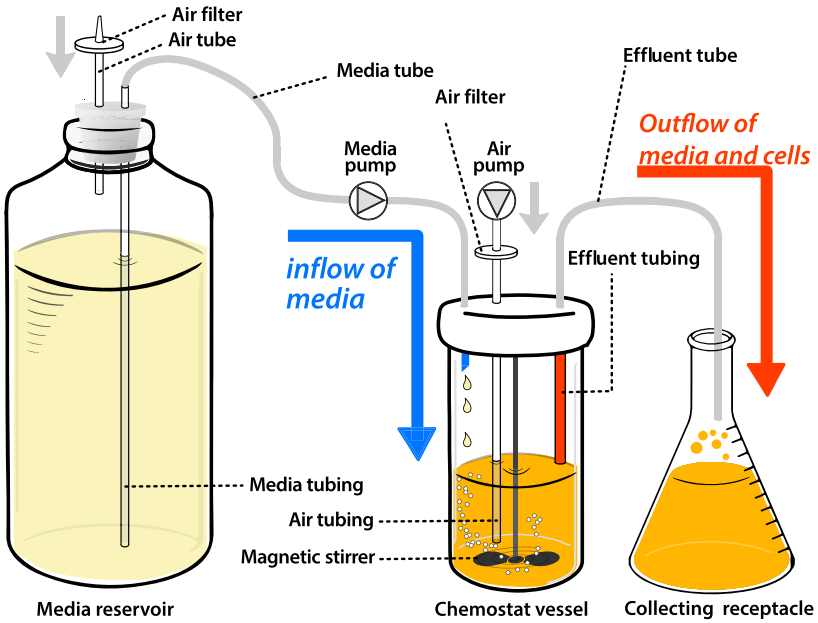

Fig. 1. Design of a chemostat. Typically, a chemostat comprises a culture vessel in which the population grows under continuous agitation and aeration. New media flows into the vessel at a defined rate. At the same rate, culture containing cells and medium is removed from the chemostat. The flow of media and culture is maintained using a pumping apparatus and holding the chemostat vessel under positive pressure by means of a constant air flow.

with saturating kinetics as described by the hyperbolic function, $\mu = \mu_{max} \cdot s/(K_s + s)$, where K_s is the substrate concentration at half-maximal μ. This relationship between growth rate and nutrient concentration was proposed by Monod on the basis of empirical measurements of *E. coli* growth rates in different glucose concentrations (Monod, 1949) and also observed by Novick and Szilard (1950b) when an *E. coli* strain auxotrophic for tryptophan was grown in the presence of different concentrations of tryptophan. Recently, this model has been shown to agree with sensitive measurements of *S. cerevisiae* cells growing across a range of glucose concentrations (Ziv *et al.*, 2013b).

The number of cells produced per mole of the limiting nutrient is defined by the parameter *Y*. Thus, μ_{max}, K_s, and *Y* are properties of the cell and therefore potentially modified by genetic variation. By contrast, the experimenter controls the parameters *R*, the concentration of the limiting nutrient in the feed medium and *D*, the culture dilution rate, by modulating the flow rate (*F*) of media addition, and removal for a given culture volume (*V*) according to the relationship, $D = F/V$.

This system of equations predicts the establishment of a single nonzero stable steady state in which the cell number and the concentration of the growth-limiting nutrient remain constant per unit time (i.e. $dx/dt = 0$ and $ds/dt = 0$) (Fig. 2). At steady state, growth rate (μ) is submaximal and exponential. The remarkable implication of this model is that in steady-state conditions, the exponential growth rate constant (i.e. the 'specific growth rate') is equal to the dilution rate (*D*). Thus, the doubling time of the exponentially growing population (i.e. the generation time) is simply $\ln(2)/D$. By varying the dilution rate, a variety of steady-state conditions can be established all of which have the property of growth rate being equal to the dilution rate so long as the dilution rate is less than the maximal growth rate of the cells. In principle, these steady states can be maintained indefinitely, enabling long-term selection in a constant environment. Although the experimental reality of some of the parameters of this model (Ferenci, 1999a) and the existence of a true steady state in the chemostat have been questioned (Ferenci, 2006), the model provides a useful conceptual framework for understanding selection in a chemostat. For further background on the principle of the chemostat, the reader is referred to the introductory book by Kubitschek (1970) and a comprehensive mathematical treatment of the chemostat (Smith & Waltman, 1995).

Experimental evolution in chemostats

The environment in a chemostat differs in several ways from that of a batch culture with important implications for understanding the relevant selective pressures. In a batch culture, cells initially experience nutrient-rich

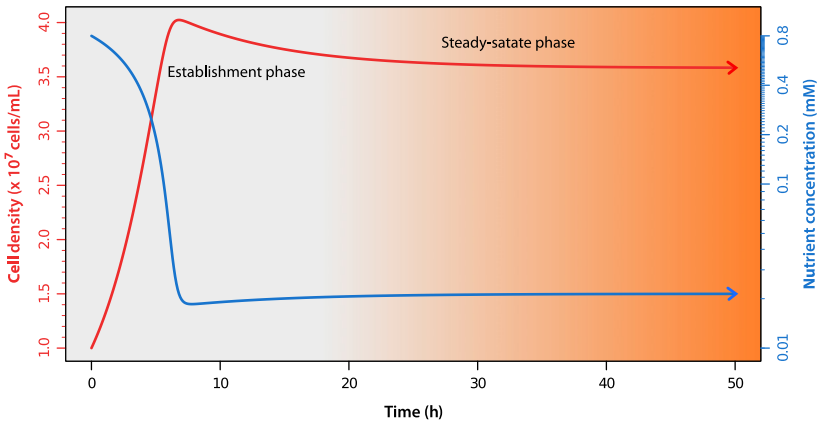

Fig. 2. Establishment of a steady state in the chemostat. Following inoculation and initiation of culture dilution, the chemostat is characterized by a period during which the population increases and nutrient abundance declines. Eventually, a steady state is established in which the cell population remains high and the concentration of the limiting nutrient remains low. The steady state is predicted by the fundamental equations of the chemostat and depends on the parameter values used in the simulation. In this simulation, $\mu_{max} = 0.4\ h^{-1}$, $K_s = 0.05$ mM, $Y = 4.6 \times 10^7$ cells mmol^{-1}, $R = 0.8$ mM, and $D = 0.12\ h^{-1}$. The simulation was initialized with $x = 1 \times 10^7$ cells mL^{-1} and $s = 0.8$ mM.

conditions, but as the population grows nutrients continuously decline, and are ultimately exhausted leading to arrest in cell growth and division. For the purposes of experimental evolution in batch cultures, these cycles of boom and bust are repeated using serial dilution (Elena & Lenski, 2003). During each cycle, population size change dramatically (typically, by several orders of magnitude), and in addition to a decline in nutrients, the environment is altered in other ways including the pH, oxygenation, and presence of waste products. As a consequence, this experimentally simple method of propagating microorganisms results in a complex and dynamic environment making it difficult to define the evolutionarily relevant selective force(s). By contrast, in a steady-state chemostat, cell growth is continuously constrained by the abundance of a single essential nutrient ensuring that cells are maintained in a 'hungry' (Ferenci, 2001) or 'poor, not starving' (Saldanha *et al.*, 2004) state. The population size in a steady-state chemostat remains constant, and all environmental factors are essentially invariant. Thus, despite the increased experimental complexity of chemostats, their use greatly simplifies the selection for the purposes of experimental evolution.

Additional factors of central importance to evolutionary processes also differ between the two methods of long-term selection. Serial dilution results in regular population bottleneck events when a subset of the population is used to found the subsequent cycle of growth. This results in random purging of genetic diversity increasing the role of genetic drift (random sampling of alleles between generations) in the evolution experiments. The extent of random genetic drift is a function of the effective population size, which in a serial dilution regime is determined by the number of cells passaged with each dilution. In a chemostat, the population size remains constant; however, the dilution process introduces an element of chance dictating which genotypes are maintained in the population. For example, at a dilution rate of 0.1 h^{-1}, there is a 10% chance that a cell will be removed from the population each hour regardless of its genotype. More generally, a cell containing a newly introduced mutation has a 50% chance of being removed by the dilution process before the population doubles.

As with most evolutionary scenarios, population size (N) and mutation rate (μ) are critical parameters that dictate the speed, outcome, and reproducibility of adaptive evolution in a chemostat. The product of population size and the mutation rate, $N\mu$, determines the mutation supply rate. In chemostats containing *E. coli* or *S. cerevisiae*, population sizes are typically on the order of 10^8–10^{10} individuals. Whole-genome sequencing of mutation accumulation lines results in estimates of 1×10^{-3} nucleotide substitutions per genome per generation in *E. coli* (Lee *et al.*, 2012) and 4×10^{-3} nucleotide substitutions per genome per generation in *S. cerevisiae* (Lynch *et al.*, 2008). Thus, the mutation supply rate in a chemostat containing 10^8–10^{10} cells is around 10^6–10^8 mutations per generation. As this is equal to, or exceeds, the number of nucleotides in *E. coli* (4.6×10^6 bases) and *S. cerevisiae* (1.2×10^7 bases), on average, every possible one-step mutation is introduced into the population each generation. Moreover, the rate at which other types of genetic variation occur such as copy number variants (CNVs) and transposition events may exceed the rate of nucleotide sub-

stitutions. As a result, depending on mutation rate and population size (Chao & Cox, 1983), adaptation in a chemostat is typically not limited by mutation supply, and multiple adaptive mutations are expected to coexist in the population. Depending on the fitness effect of a particular mutation, new adaptive mutations are expected to reach 50% frequency in the population in less than a few hundred generations (Fig. 3). If an adaptive mutation was already present at more than one copy in the population, this time will be further reduced.

The chemostat facilitates exploration of important factors that govern the outcome and dynamics of adaptive evolution. For example, the intensity of selection can be systematically varied, as increasing the dilution rate will increase the steady-state concentration of the limiting nutrient (Fig. 4a). The model of the chemostat predicts that as the dilution rate increases, the steady-state population size will decrease. However, it is possible to alter the population size in the chemostat independently of the growth rate by varying the concentration of the limiting nutrient (R) in the feed media. At an identical dilution rate, steady-state cell concentrations will increase linearly with increasing values of R, but the steady-state concentration of the limiting nutrient in the culture vessel will be identical (Fig. 4b). Thus, different population sizes can be maintained in otherwise identical selective conditions. The control of nutrient concentration and population size that is uniquely possible in a chemostat enables exploration of the effect of these parameters on adaptive evolution with unparalleled precision.

Theoretical routes to increased fitness in a chemostat

The mathematical model of the chemostat provides insight into the mechanisms by which fitness increases in the chemostat. Selection in the chemostat acts on those parameters that affect cell growth rate namely, the maximal growth rate (μ_{max}) and the nutrient concentration required for half-maximal growth rate (K_s). Changes in these parameters due to new mutations will effect the fitness of a lineage relative to the ancestral lineage (Fig. 5). Acquisition of a genetic variant that increases only maximal growth rate (μ_{max}) relative to the ancestral lineage results in increased fitness (mutation A in Fig. 5) as does a mutation that only decreases the nutrient concentration required for half-maximal growth rate (K_s) (mutation B in Fig. 5).

A single mutation may exert pleiotropic effects and result in changes in both μ_{max} and K_s. For example, a mutation that decreases K_s may also increase μ_{max} and therefore act synergistically to increase the fitness of a lineage in the chemostat (mutation C in Fig. 5). Alternatively, the effect of a particular mutation may be antagonistic with respect to these two parameters. For example, a mutation that decreases K_s may also result in decreased μ_{max}. Pleiotropic mutations that have antagonistic effects on both parameters can still result in a net increase in fitness so long as the magnitude of the effects is sufficiently different (mutation D in Fig. 5) and therefore may be selected.

Although yield (Y) is a property of the cell that is modeled in the fundamental equations of the chemostat,

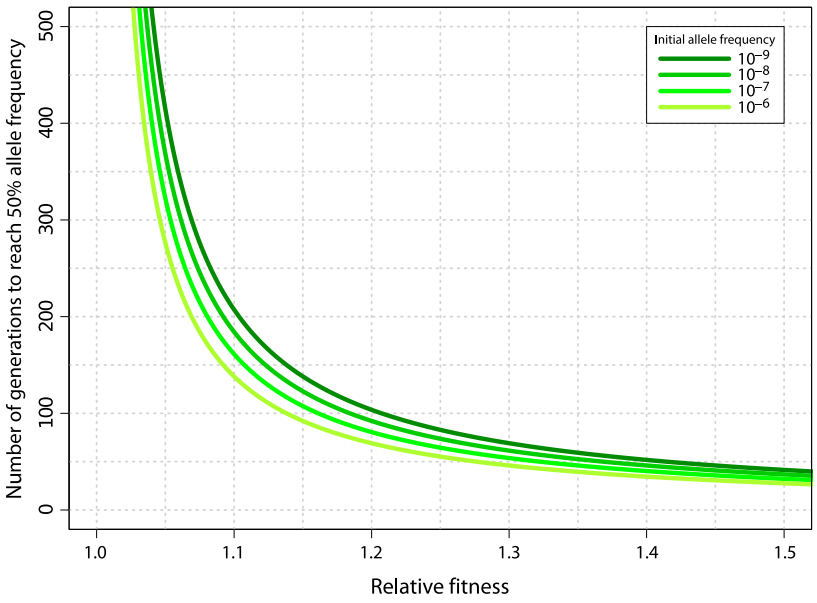

Fig. 3. Expected waiting time for beneficial mutations. The time until a beneficial mutations rises to a 50%, allele frequency in the population depends on the fitness effect of the mutation. Increased initial frequencies of a mutant genotype due to the occurrence of mutations during the establishment phase of the chemostat further reduce the waiting time.

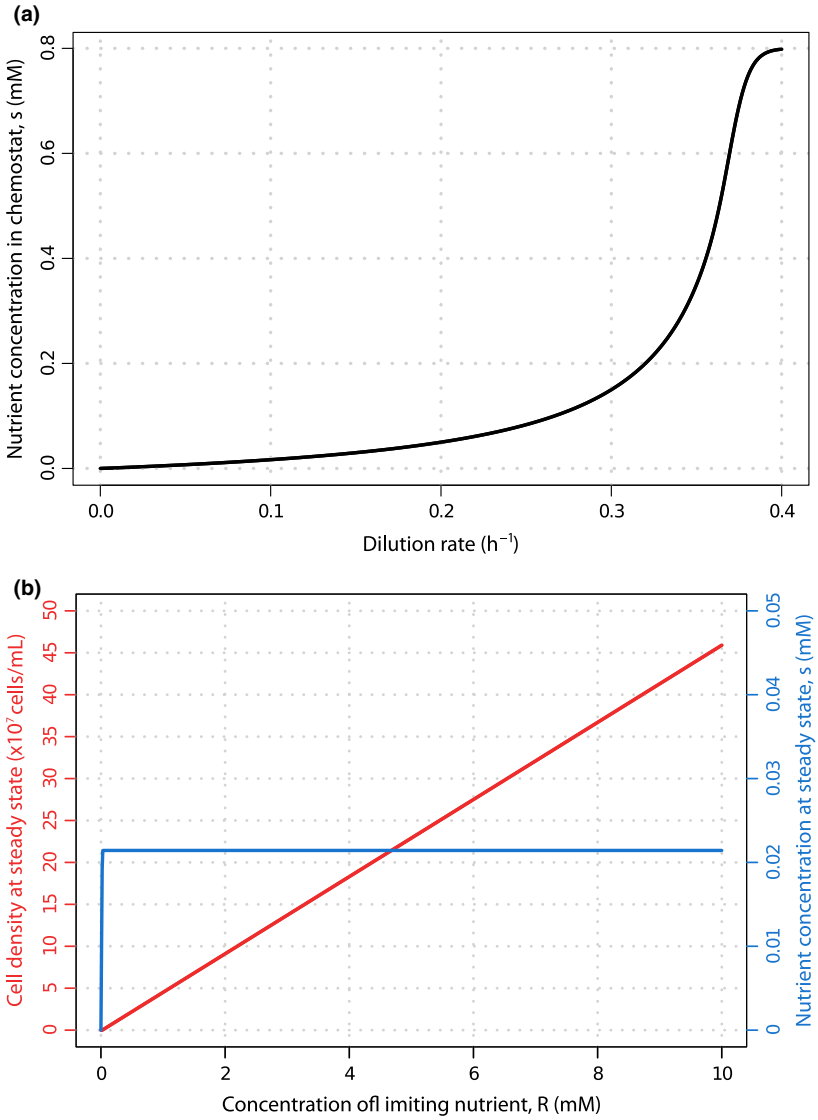

Fig. 4. Systematic variation of selection intensity and population size in a chemostat. (a) Steady-state nutrient concentration increases with increasing dilution rates. (b) Population size (red) in the chemostat can be controlled by varying the concentration of the limiting nutrient in the feed media (*R*) and maintaining a constant dilution rate. In this case, the steady-state concentration of the limiting nutrient in the chemostat (blue) remains constant.

alterations in yield do not directly affect fitness in the chemostat as a change in *Y* does not impact growth rate. However, an increase in *Y* could increase fitness indirectly by reducing the steady-state concentration of the limiting nutrient, *s*.

Long-term selection experiments in chemostats

Undertaking long-term selection experiments in chemostats is conceptually straightforward. Chemostat cultures can be established using a limiting concentration of any essential nutrient required for cell growth including carbon, nitrogen, phosphorous, and sulfur. The spectrum of possible limiting agents can be expanded by the use of auxotrophic strains. Indeed, early studies by Novick and Szilard (1950b) of mutation rates in chemostats made extensive use of *E. coli* auxotrophic for tryptophan. However, the fitness defects of auxotrophic strains (Baganz *et al.*, 1997; Pronk, 2002) and the potential for unanticipated effects (Boer *et al.*, 2008) associated with 'unnatural limitation' (Saldanha *et al.*, 2004) for the auxotrophic requirement suggest caution be employed with the use of auxotrophs in studies of cell growth.

Once a steady-state culture has been established, a chemostat can be maintained indefinitely with minimal maintenance aside from regular measurement of the dilution rate. The main practical challenges entail maintaining an adequate supply of fresh media, ensuring the integrity of the tubing and pump systems and preventing contamination (Fig. 1). These challenges are readily met with experience and care. For example, improved methods for

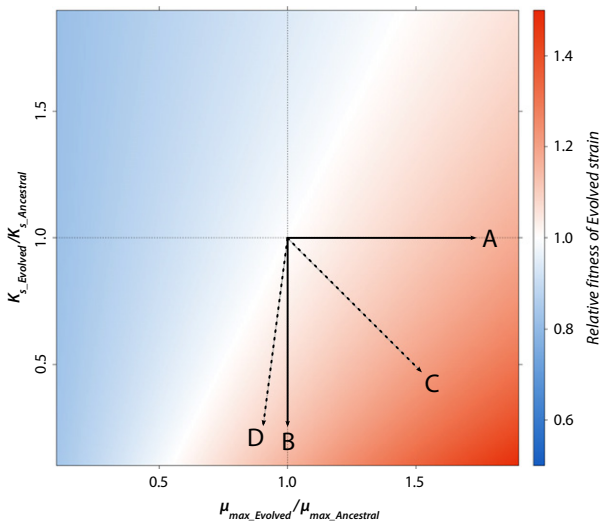

Fig. 5. The fitness landscape in a chemostat is determined by changes in K_s and μ_{max} due to adaptive mutations. A mutation that (A) increases μ_{max} or (B) decreases K_s will result in increased fitness. Pleiotropic mutations that (C) synergistically increase μ_{max} and decrease K_s will be adaptive. Pleiotropic mutations that act (D) antagonistically by decreasing μ_{max} and decreasing K_s can still be beneficial depending on the relative effects on the two parameters. Data were simulated using s = 10 mM, ancestral K_s = 10 μM, and ancestral μ_{max} = 0.4 h^{-1}.

sterile assembly of chemostats have largely overcome the problems of contamination that plagued earlier efforts of long-term selection in chemostats.

As with all experimental evolution studies, regular sampling from the chemostat allows curation of a 'fossil record' of the evolving population (Elena & Lenski, 2003). This record comprises unbiased samples of the population obtained periodically during the selection and archived at −80 °C. These samples can be used for reconstructing allele frequency dynamics during the populations' histories and for studying the emergence of adaptation-associated traits. In a chemostat, it is possible to obtain these samples without disturbing the population by temporarily diverting the outflow (Fig. 1) to a sample tube.

Phenotypic outcomes of long-term selection in chemostats

Typically, the expected (and desired) outcome of long-term selection in chemostats is the acquisition of improved growth capabilities in the low-nutrient environment of the chemostat. This is most rigorously quantified by performing competition assays in a chemostat in which the evolved strain and the ancestral strain are co-cultured. The relative abundance of the two strains is determined over a few generations (typically < 20) in steady-state chemostats. As both lineages grow exponentially, the growth advantage per unit time, which is defined as fitness, is determined by linear regression of the natural log of the ratio of two strains against time (Dykhuizen & Hartl, 1983). To distinguish the two strains and estimate their relative abundances, they are typically marked by neutral markers such as a drug (Paquin & Adams, 1983a, b; Gresham *et al.*, 2008) or phage resistance (Novick & Szilard, 1950b) allele, which facilitates genotype estimation by plating. The use of strains labeled with a constitutively expressed fluorescent protein facilitates genotype estimation using flow cytometry (Wenger *et al.*, 2011; Hong & Gresham, 2014), which greatly increases the precision of fitness measurements.

Typically, fitness increases of lineages selected within a few hundred generations in chemostats are large, often exceeding 10% (relative fitness, w = 1.1) per generation, and occasionally as much as 50% (w = 1.5) per generation. This is consistent with the notion that populations introduced into a chemostat start far from the fitness optima. Importantly, the increased fitness of chemostat-adapted lineages is specific to the low-nutrient environment of the chemostat. In fact, many lineages selected in chemostats appear to have reduced growth capabilities when growing in nutrient-rich conditions, suggesting that 'antagonistic pleiotropy' is frequently associated with beneficial mutations selected in the chemostat (Wenger *et al.*, 2011; Hong & Gresham, 2014).

As growth rate in a chemostat is constrained by nutrient abundance, one anticipated outcome of mutation and selection is enhanced nutrient uptake rates, which can be measured using radioactively labeled nutrients. *Escherichia coli* (Rosenzweig *et al.*, 1994; Manche *et al.*, 1999; Notley-McRobb & Ferenci, 1999b; Maharjan *et al.*, 2007) and *S. cerevisiae* (Brown *et al.*, 1998) lineages evolved in glucose-limited chemostats and *E. coli* lineages evolved in phosphate-limiting chemostats (Wang *et al.*, 2010) have greatly enhanced substrate transport velocities. Most likely, increased nutrient transport velocities have the effect of increasing growth rates by decreasing K_s. Enhanced transport capabilities have been reported to be associated with both increased (Wenger *et al.*, 2011) and decreased (Maharjan *et al.*, 2007) biomass yields.

Selection in chemostats also appears to result in altered metabolic strategies. A number of studies have accessed genome-wide changes in gene expression in yeast (Ferea *et al.*, 1999; Gresham *et al.*, 2008; Kao & Sherlock, 2008) and *E. coli* (Kinnersley *et al.*, 2009) lineages adapted to chemostats. These studies provide evidence for newly evolved metabolic strategies in chemostat-adapted lineages including increased oxidation of glucose in *S. cerevisiae* strains evolved in glucose-limited chemostats (Ferea *et al.*, 1999). Consistent with increased metabolic efficiency,

residual ethanol concentrations are typically decreased in populations evolved in glucose-limited chemostats (Gresham *et al.*, 2008).

In a chemostat, the organism lives on the edge of starvation. However, mounting a full scale starvation response, in which the cell enters a quiescent, nonproliferative state is an evolutionary dead end as these cells will be removed by dilution without contributing to subsequent generation. There is evidence that one outcome associated with increased fitness in chemostat is loss of the ability to appropriately mount a stress response. For example, strains of *E. coli* adapted to glucose-limited chemostats exhibit reduced survival when subjected to temperature or oxidative stress (Notley-McRobb *et al.*, 2002a). The loss of stress response pathways in *E. coli* can be qualitatively assessed by determining glycogen content in cells, using iodine staining, and catalase activity, using a 'bubbling assay' in hydrogen peroxide. Many *E. coli* lineages selected in glucose-limited chemostats show evidence for decreased glycogen levels and catalase activity, which has lead to the proposal of an antagonistic relationship between the capacity to mount a robust stress response and growth ability in nutrient-poor environments (Ferenci, 2003, 2005).

In addition to phenotypes that can be explained as a result of adaptive evolution in nutrient-limited environments, there are potential undesirable outcomes of long-term culturing in chemostats that may confound connecting genotype to phenotype. These include wall growth and flocculation, which are cases of niche specialization in the culture vessel that lead to heterogeneity within the population. However, selection for this class of mutants can be avoided by proper agitation of the culture to maintain all cells in suspension and the use of treated glass, which prevents microbial growth on vessel walls. In addition, an exception to the constant environment of a chemostat can occur if yeast cells synchronize cycles of oxidative and reductive metabolism in very slow-growing glucose-limited chemostats (Tu & McKnight, 2007). The phenotypic outcome to adaptation in this particular scenario is likely to be more complex.

Regulation of cell growth in nutrient-limited environments

The design, analysis and interpretation of adaptive evolution studies in chemostats is informed by an understanding of how cells regulate their growth. *Escherichia coli* and *S. cerevisiae* can modulate their rate of growth over a range of suboptimal nutrient concentrations. How cells regulate their growth in response to variation in environmental nutrient status and how this is coordinated with the myriad cellular processes required for cell growth is an enduring question of central importance in biology. Classic studies of cell growth in bacteria using chemostats showed that cell physiology changes with variation in growth rate (Kjeldgaard *et al.*, 1958; Schaechter *et al.*, 1958). These studies demonstrated that as cell growth rates decrease as a result of reduced environmental nutrients, the mass of cells, their RNA content, and their protein content decrease. More recent studies have revisited the question of how cellular processes vary with variation in cell growth. Using chemostats, it was found that a large fraction of *E. coli* (Ishii *et al.*, 2007) and *S. cerevisiae* (Regenberg *et al.*, 2006; Castrillo *et al.*, 2007; Brauer *et al.*, 2008) mRNAs vary in relative abundance as a function of growth rate as do many intracellular metabolites (Boer *et al.*, 2010). The gene expression and physiological changes associated with slowed growth rates in both *E. coli* and *S. cerevisiae* have important consequences for cells including increased resistance to stress (Elliott & Futcher, 1993; Notley & Ferenci, 1996; Ihssen & Egli, 2004; Lu *et al.*, 2009).

Conceptually, the regulation of cell growth in response to environmental nutrient status can be divided into distinct, but interconnected, modular processes (Fig. 6): (1) the sensing and transport of essential nutrients, (2) intracellular signaling pathways that converge on (3) transcriptional and (4) translational regulation, and (5) metabolic processes to coordinately regulate (6) the biogenesis of protein complexes and cellular organelles. In a chemostat, in which cell growth is constrained by the abundance of an essential nutrient, modification of any of these processes could potentially result in increased fitness and therefore may be targets of selection. The similarities in the principles underlying cell growth regulation in *E. coli* and *S. cerevisiae* present the possibility that common strategies may underlie adaptive evolution in chemostats.

In *E. coli*, many of the key components that contribute to regulation of cell growth are known. Specialized transporters import essential nutrients such as carbon, nitrogen, phosphorous, and sulfur, and many transporters are optimized for transporting specific forms of these nutrients. Many nutrient transporters are transcriptionally regulated, and as nutrient abundance declines, classes of higher affinity transporters are expressed. A key transcriptional regulator of the cellular response to decreased nutrient abundance is the sigma factor, σ^S, encoded by *RpoS* (Notley & Ferenci, 1996), the activity of which is regulated by decreased glucose, nitrogen, and phosphorous by different mechanisms (Peterson *et al.*, 2005). As nutrient abundance declines, *E. coli* cells increase expression of porins, which results in increased permeability of the outer membrane (Ferenci, 1999b). Interestingly, the environmental abundance of different essential nutrients appears to converge on the regulated increased produc-

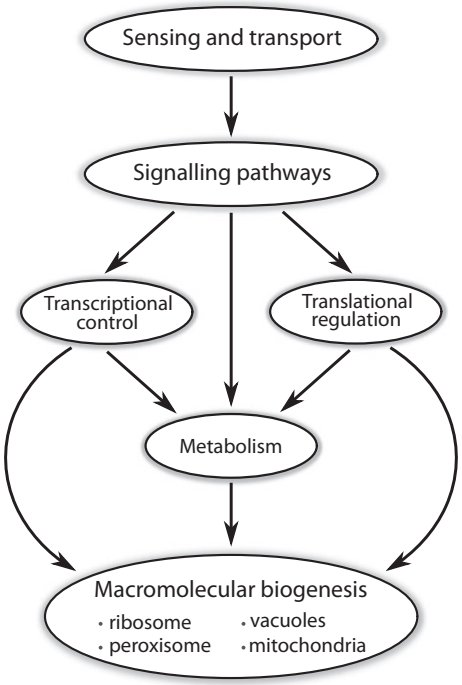

Fig. 6. Modularity of cell growth regulating processes. A hierarchy of cellular processes control cell growth and are potential targets of selection in the chemostat.

tion of important intracellular signaling molecules including cyclic adenosine monophosphate (cAMP) and guanosine tetraphosphate (ppGpp; Ferenci, 2001; Srivatsan & Wang, 2008). Production of ppGpp is controlled by the enzymes RelA and SpoT in response to distinct signals. ppGpp and the protein DksA exert growth inhibitory effects through a variety of mechanisms including inhibition of rRNA transcription (Potrykus & Cashel, 2008; Jin *et al.*, 2011).

In *S. cerevisiae*, the genetic networks that regulate cell growth have been intensively studied, in part due to their conservation in humans where the same networks regulate cell growth and are frequently dysregulated in disease such as cancer (Zaman *et al.*, 2008; Lippman & Broach, 2010; Loewith & Hall, 2011; Broach, 2012). The yeast genome contains an abundance of nutrient transporters with greatly varying affinities and specificities. The expression of specific nutrient transporters is controlled both transcriptionally and through a variety of post-transcriptional mechanisms. Environmental nutrient status is transmitted intracellularly by conserved signaling pathways including the TORC1 and Protein Kinase A/Ras pathways (Zaman *et al.*, 2008; Broach, 2012). Although the integration of diverse environmental signals that control cell growth is not well understood in yeast cells, there is evidence that signals from these pathways are integrated by converging on signaling molecules including the protein kinase RIM15 (Pedruzzi *et al.*, 2003). Consistent with the importance of coordinating protein translation with cell growth, the ultimate target of these signaling pathways are factors involved in ribosome biogenesis and protein translation (Jorgensen & Tyers, 2004; Loewith & Hall, 2011; Broach, 2012).

The mechanistic basis of increased fitness in nutrient-limited environments

Identifying the alleles that increase fitness in chemostats, and understanding the mechanistic basis by which the encoded products improve cell growth in nutrient-limited conditions, provides a path to understanding which of the myriad growth-related processes (Fig. 6) are limiting under conditions of suboptimal resources. However, until the advent of genome-scale methods, identifying the underlying genetic basis of increased fitness in chemostats was laborious and, with some exceptions, not typically amenable to standard methods for genetic cloning. Starting at the turn of the 21st century, the development of methods for comprehensive identification of genomic variation solved the long-standing problem of characterizing the genetic variation associated with adaptive evolution in chemostats. The aggregate of these studies provides insight into the types of mutations that contribute to adaptive evolution, the genes that are targeted by selection, and the mechanistic bases of increased fitness in chemostats.

The role of copy number variation in adaptation in chemostats

The adaptive role of gene amplification was an early finding from experimental evolution studies in chemostats. In some of the first studies of adaptive evolution of *E. coli* in lactose-limited chemostats, it was observed that evolved lineages produced unusually high amounts of β-galactosidase (Horiuchi *et al.*, 1962), which is the result of increased copies of the *lac* operon (Horiuchi *et al.*, 1963). In *S. cerevisiae*, amplification of the gene encoding acid phosphatase, which hydrolyzes organic phosphates, was identified in lineages selected in chemostats limited for phosphate (Hansche, 1975; Hansche *et al.*, 1978). A seminal study of *Salmonella typhimurium* lineages evolved in different carbon-limited chemostats identified amplification alleles containing transporters specific to the limiting carbon source (Sonti & Roth, 1989).

Subsequent studies have shown that CNVs are an important class of adaptive variation in chemostat-adapted lineages. Some of these CNVs are amplification alleles that contain specific nutrient transporter genes that

import the limiting nutrient (Table 1). Targeted analysis of the high-affinity glucose transporter genes, *HXT6* and *HXT7*, in an *S. cerevisiae* strain evolved in glucose-limiting conditions identified an amplification of the *HXT6-HXT7* locus (Brown *et al.*, 1998), which has subsequently been reported in multiple independent studies of adaptive evolution in glucose-limited chemostats (Gresham *et al.*, 2008; Kao & Sherlock, 2008). In repeated long-term selections in sulfur-limited chemostats using *S. cerevisiae*, multiple independent amplification alleles were identified that include *SUL1*, which encodes a high-affinity sulfur transporter (Gresham *et al.*, 2008). Similarly, long-term selections in diverse nitrogen-limiting environments identified CNV alleles containing transporter genes specific to the nitrogen source in the chemostat (Gresham *et al.*, 2010; Hong & Gresham, 2014). Although the role of CNVs has not been extensively studied in experimental evolution of *E. coli*, amplification of the *lac* operon, which includes the *lacY* permease gene, underlies adaptation of *E. coli* to lactulose (Zhong *et al.*, 2004). The role of CNVs in adaptive evolution of *E. coli* lineages warrants closer attention.

The repeated selection of specific CNVs containing nutrient-specific transporter genes is consistent with enhanced nutrient transport capacity underlying a common adaptive strategy in chemostats. Measurement of fitness increases attributable to increased copy number of the *SUL1* locus suggests that relative fitness increases as large as 50% are associated with amplification of transporter genes (Gresham *et al.*, 2008). The critical role of CNVs in the rapid adaptive evolution observed in chemostats is consistent with their role in diverse adaptive evolution scenarios including animal domestication (Wright *et al.*, 2009) and human evolution in response to the advent of agriculture (Perry *et al.*, 2007). Although a subset of CNV alleles selected in chemostats contain transporter genes, many do not, and deletion alleles containing several contiguous genes are also detected (Gresham *et al.*, 2008; Hong & Gresham, 2014). Understanding the mechanistic basis by which the many CNVs that do not include transporter genes increase fitness in the chemostat remains to be determined.

Typically, amplification alleles are the result of tandem repeat expansions at their endogenous chromosomal locus. However, the mechanisms underlying CNV generation in chemostat-evolved lineages are not well understood. Although some CNV alleles in both *E. coli* (Zhong *et al.*, 2004)and *S. cerevisiae* (Hong & Gresham, 2014) are bounded by repetitive sequence elements, including transposon sequences, that likely facilitate intrachromosomal recombination (Mieczkowski *et al.*, 2006), many CNV junctions show no evidence of such repeats (Gresham *et al.*, 2008; Araya *et al.*, 2010; Hong & Gresham, 2014). Microhomology-mediated mechanisms (Payen *et al.*, 2008) may contribute to formation of these alleles. A notable exception to the chromosomal location of CNV alleles is the *GAP1* gene in *S. cerevisiae*, which is unusual in being one of only two isolated genes in the yeast genome flanked by tandem long-terminal repeats. These repeats facilitate the generation of a circular DNA element containing an autonomous replication sequence and *GAP1* (Gresham *et al.*, 2010), which transports glutamine and glutamate and is selected in glutamine- and glutamate-limited chemostats.

Determining the spontaneous rate at which CNVs are generated poses a considerable technical challenge. In *S. cerevisiae*, estimated rates of duplications range from as low as 10^{-10} amplifications per cell per division (Dorsey *et al.*, 1992; Peterson *et al.*, 2000) to 3.4×10^{-6} amplifications per cell per division (Lynch *et al.*, 2008). However, the rate at which amplification and deletion alleles are spontaneously generated is highly variable and dependent on local genome architecture (Romero & Palacios, 1997). Moreover, rates of CNV generation may be controlled in part by environmental factors, and the rate at which they are generated in the nutrient-poor conditions of a chemostat has not been determined. One considerable challenge to studying beneficial CNVs is that they appear to be unstable and revert at a high rate (Maharjan *et al.*, 2013b; Hong & Gresham, 2014); if the CNV is del-

Table 1. Transporter genes within copy number variants selected in nutrient-limited chemostats

Gene	Function	Nutrient limitation	References	Species
GAP1	General amino acid permease	Glutamine and glutamate	Gresham *et al.* (2010), Hong & Gresham (2014)	*Saccharomyces cerevisiae*
PUT4	High-affinity proline transporter	Proline		
DUR3	Plasma membrane transporter for both urea and polyamines	Urea		
DAL4	Allantoin permease	Allantoin		
HXT6/HXT7	High-affinity glucose transporters (99% identical)	Glucose	Brown *et al.* (1998), Kao & Sherlock (2008), Gresham *et al.* (2008)	
SUL1	High-affinity sulfate permease	Sulfur	Gresham *et al.* (2008)	
LacY	Lactose permease	Lactose	Zhong *et al.* (2004)	*Escherichia coli*

eterious outside of the chemostat environment, inadvertent selection for revertants may be difficult to avoid. Nonetheless, as the rate of occurrence of CNVs is likely to profoundly impact the evolutionary dynamics in chemostats, determining the rate at which these alleles are generated and the mechanistic basis by which they increase fitness is critical to understanding adaptive evolution in chemostats.

Chromosomal translocations and aneuploidies are frequently associated with increased fitness in chemostats

In addition to CNVs, large-scale chromosomal alterations associated with increased fitness in chemostats have been identified in *S. cerevisiae*. In lineages selected in chemostats limited for different organic phosphates, individual clones were found to contain variants in chromosome size as well as chromosomal rearrangements (Adams *et al.*, 1992). High resolution analysis of *S. cerevisiae* lineages evolved in glucose-limited chemostats using array comparative genomic hybridization (aCGH) identified repeated chromosomal translocations including reciprocal and nonreciprocal translocation events (Dunham *et al.*, 2002). In many cases, breakpoint mapping revealed that translocations are associated with transposon sequences as has been found in experimental studies of chromosomal rearrangements (Mieczkowski *et al.*, 2006).

Although the repeated selection of chromosomal translocations points to their adaptive benefit, the underlying basis by which they result in increased fitness is not clear. In the case of clones selected from glucose-limited chemostats, some translocation breakpoints were identified immediately adjacent to *CIT1*, which encodes citrate synthase (Dunham *et al.*, 2002). Although the translocation event is proposed to alter their transcriptional regulation of *CIT1*, this has not been formally demonstrated. More recently, a study of a diploid *S. cerevisiae*/*Saccharomyces bayanus* hybrid evolved in ammonium-limited chemostats identified reciprocal translocation events between species-specific homologous chromosomes that mapped within *MEP2* (Dunn *et al.*, 2013), which encodes a high-affinity ammonium permease. In this case, it seems likely (though untested) that the fusion protein product has enhanced transport properties compared with either of the species-specific gene products. Thus, chromosomal translocations may serve to both alter gene expression regulation and generate novel sequence variants that are beneficial.

In addition to partial chromosomal events, duplications of entire chromosomes (aneuploidies) are repeatedly associated with adaptive evolution in chemostats (Dunham *et al.*, 2002; Gresham *et al.*, 2008; Hong & Gresham, 2014). The adaptive benefit of chromosomal aneuploidies remains to be determined; however, their beneficial role in adaptive evolution in chemostats is consistent with their reported role in suppression of engineered genetic lesions (Hughes *et al.*, 2000; Rancati *et al.*, 2008) and adaptive evolution in other selective regimes such as high temperature growth (Yona *et al.*, 2012). The reason that aneuploidies are beneficial in particular circumstances is an open question with clear relevance to understanding their role in human tumors (Torres *et al.*, 2007, 2010; Pavelka *et al.*, 2010a, b; Tang *et al.*, 2011).

Transposons contribute to adaptive evolution in chemostats

Given their pervasive role in evolution (Biemont, 2010; Fedoroff, 2012), transposable elements – including 'cut and paste' DNA transposons and 'copy and paste' retrotransposons – are likely to play a role in adaptive evolution in chemostats. However, few studies have explicitly explored the contribution of transposition events to adaptive evolution in chemostats. In *E. coli*, both the Tn5 (Biel & Hartl, 1983) and Tn10 (Chao *et al.*, 1983) transposons have been shown to have beneficial effects in adaptive evolution in chemostats. In the case of Tn10, it was shown that this advantage is due to transposition events that presumably generate beneficial mutations (Chao *et al.*, 1983). Similarly in *S. cerevisiae*, the retrotransposon family, Ty1, has been shown to play an important role in generating adaptive-genetic variation in chemostats (Adams & Oeller, 1986; Adams *et al.*, 1992; Wilke & Adams, 1992).

However, retrotransposon sequences serve two distinct roles in generating genetic variation: first, their repetitive nature increases the frequency with which intra- and interchromosomal recombination events occur; second, they can generate novel alleles through retrotransposition events. The facilitation of genome rearrangements appears to be a particularly important role for transposon sequences as many rearrangements map to these repetitive elements (Dunham *et al.*, 2002; Zhong *et al.*, 2004; Gresham *et al.*, 2008; Hong & Gresham, 2014). In a small number of cases, novel transposition events in chemostat-evolved lines have been demonstrated to confer fitness benefits (Blanc & Adams, 2003; Gresham *et al.*, 2008; Gaffé *et al.*, 2011). However, the contribution of transposons to adaptive evolution in chemostats remains poorly understood. One of the potential perils of using next generation sequencing, with its current reliance on short read sequences, is that transposition events may be missed. Therefore, employing methods that specifically identify novel insertion sites of retrotransposon (Gabriel *et al.*, 2006; van Opijnen *et al.*, 2009; Mularoni *et al.*, 2012) remains an important aspect of analyzing genomes of evolved lineages.

Identification of genetic networks under selection by whole-genome sequencing

Prior to the advent of genome-scale methods for interrogating DNA sequence variation, the identification of nucleotide substitutions associated with adaptive evolution in chemostats largely relied on the identification of adaptation-associated phenotypes with known, or readily testable, genetic bases. In *E. coli*, the careful analysis of phenotypes in chemostat-evolved lines enabled significant progress in identifying the genetic bases of adaptation in chemostats. Loss of function mutations in *rpoS* results in decreased glycogen production and catalase activity in selected lineages (Notley-McRobb *et al.*, 2002a), facilitating identification of recurrent selection for loss of *rpoS* in glucose-limited chemostats (Ferenci, 2003; Kinnersley *et al.*, 2009). Similarly, on the basis of known regulation of the high-affinity glucose transporter encoded by *LamB*, mutations in the transcriptional repressor *mlc* and the transcriptional activator *malT* were identified in lineages adapted to glucose-limited chemostats (Notley-McRobb & Ferenci, 1999b; Kinnersley *et al.*, 2009). The *mglBAC* regulon, which transports galactose, is highly expressed in glucose-limited chemostats (Hua *et al.*, 2004) in which it also functions as a high-affinity glucose transporter. The repressor of the *mglBAC* genes, *mglD* (or *galS*), is repeatedly mutated in clones adapted to glucose limitation (Notley-McRobb & Ferenci, 1999a). Selection for *mgl* mutations appears to be specific to growth in aerobic glucose-limited chemostats: in anaerobic glucose-limited chemostat mutations in *ptsG*, a regulator of the PEP:glucose phosphotransferase system is repeatedly selected (Manche *et al.*, 1999).

The advent of next generation sequencing methods has enabled complete characterization of the spectrum of mutations in *E. coli* strains selected in chemostats. These unbiased characterizations have confirmed the importance of some of the loci identified in earlier studies including *rpoS*, *mglD*, and *malT* in glucose-limited clones (Wang *et al.*, 2010; Maharjan *et al.*, 2012). Whole-genome sequencing of clones selected in phosphate-limited chemostats identified mutations in *rpoS* (Wang *et al.*, 2010), providing evidence that loss of *rpoS* is not a specific adaptive response to glucose limitation.

Other stress responsive pathways may be general targets of selection. Sequencing of clones evolved in phosphate-limited chemostats also identified mutations in *hfq*, which encodes an RNA chaperone important for post-transcriptional regulation of many transcripts by small RNAs. Reduction of ppGpp production could potentially confer benefits in the chemostat as it inhibits cell growth. However, although ppGpp levels are high in *E. coli* cells growing in chemostats, whole-genome sequencing of lineages adapted to glucose limitation has not yet identified mutations in *spoT* (Maharjan *et al.*, 2012), a regulator of ppGpp production, whereas mutations in *spoT* have been identified in lineages adapted to phosphate limitation (Wang *et al.*, 2010). Interestingly, mutations in *spoT* are also frequently selected in experimental evolution using serial dilution (Herron & Doebeli, 2013).

In contrast to studies in *E. coli*, the absence of characteristic phenotypes of known genetic bases in *S. cerevisiae* strains evolved in chemostats impeded the identification of point mutations using candidate gene approaches in early studies. This was initially solved by the use of tiling DNA microarray-based methods to comprehensively identify genomic variation throughout the genome (Gresham *et al.*, 2006; Kao & Sherlock, 2008). A notable finding from early studies of accumulated sequence variation in *S. cerevisiae* lineages selected in chemostat was the relatively small number of mutations (Gresham *et al.*, 2006, 2008; Kao & Sherlock, 2008). Thus, mutator strains, which have been reported in experimental evolution studies of *E. coli* using chemostats (Cox & Gibson, 1974; Notley-McRobb *et al.*, 2002b,c) and serial dilution (Arjan *et al.*, 1999), do not seem to contribute to adaptive evolution of *S. cerevisiae* in chemostat environments.

More recently, next generation sequencing has been used to define the complete spectrum of mutations in many *S. cerevisiae* lineages selected in chemostats. Among repeated studies in glucose-limited chemostats, loci that regulate transcription of glucose-responsive genes are frequently mutated. In particular, a negative regulator of the glucose-sensing signal transduction pathway, *MTH1*, has been found to contain loss of function mutations in several lineages in different labs (Gresham *et al.*, 2008; Kvitek & Sherlock, 2011). In lineages selected in ammonium-limited chemostats, missense mutations in *GAT1*, which encodes a positive transcriptional regulator of the high-affinity ammonium permease *MEP2*, are recurrently selected (Hong & Gresham, 2014). Selection in ammonium-limited chemostats also provides a rare example in which a transporter gene has been found to acquire coding sequence variants as multiple independent mutations were found in *MEP2* (Hong & Gresham, 2014). A comparative analysis of loci selected in chemostats limited for different nitrogen sources identified repeated selection for variation in *VAC14* (Hong & Gresham, 2014). VAC14 is a regulator of phosphatidylinositol-3,5-bisphosphate production with roles in protein trafficking and vacuole biogenesis. This study also found that loss of function mutations in nutrient transporters that are expressed but futile in a particular environment are frequent including

GAP1 in urea and allantoin limitation and the proline permease gene *PUT4* in arginine limitation (Gresham *et al.*, 2010; Hong & Gresham, 2014). To date, the small number of genomes that have been characterized for *S. cerevisiae* strains evolved in phosphate- or sulfur-limited chemostats (Gresham *et al.*, 2008; Araya *et al.*, 2010) preclude the identification of common targets of selection in these lineages.

Comparison of loci that acquire adaptive variation in *S. cerevisiae* lineages evolving in glucose- and nitrogen-limited environments provides evidence for common themes underlying adaptive evolution in chemostats (Fig. 7). Although the repeated selection of the identical gene appears to occur infrequently, the function of genes that are mutated and selected in chemostats shows considerable coherence. Mutated loci are enriched for nutrient transport functions that are specific to the molecular form of the limiting nutrient consistent with the importance of nutrient transport for fitness in the chemostat. Genetic loci underlying adaptive evolution that are common to nitrogen- and glucose-limited environments are enriched for signaling pathways that control cell growth in response to nutrient status. Some of the same specific targets are shared and repeatedly observed to be mutated and selected in different chemostat selections (Hong & Gresham, 2014). In particular, loss of function mutations in *RIM15*, which encodes a protein kinase downstream of the TORC1 and PKA pathways, is selected in a variety of glucose and nitrogen-limited chemostat selections (Gresham *et al.*, 2008; Kao & Sherlock, 2008; Kvitek & Sherlock, 2011; Hong & Gresham, 2014). RIM15 regulates initiation of a quiescent G_0 state in response to different nutrient starvations (Reinders *et al.*, 1998; Pedruzzi *et al.*, 2003), and its loss presumably reduces the probability of a cell entering this state in response to the poor nutrient conditions in the chemostat. Thus, selection for loss of *RIM15* in *S. cerevisiae* lineages evolving in chemostats may be analogous to loss of *rpoS* in *E. coli* lineages evolving in similar conditions. It is likely that with deeper sampling and increased replication of selection experiments, additional mechanisms of adaptation in chemostats will be identified. The integration of orthogonal methods for identifying the genetic basis of fitness increases in chemostats, such as the use of pools of gene deletion mutants (Delneri *et al.*, 2007), with the outcomes of long-term selection is a potential way of anticipating and interpreting novel pathways that may be identified as targets of selection.

Dynamics and constraints of selection in chemostats

Identification of the targets of selection in the chemostat facilitates the analysis of many questions important for our understanding of the dynamics and constraints of adaptive evolution. Early studies in the chemostat found evidence supporting periodic selection as the dominant mode of adaptation dynamics (Novick & Szilard, 1950b; Paquin & Adams, 1983a). In this regime, genotypes of increasing fitness are sequentially replaced by filter genotypes leading to selective sweeps, which were monitored using a neutral genetic marker. However, the role of periodic selection in the chemostat was questioned on the basis of the expected mutation supply rate (Dykhuizen, 1990), and more recent studies have shown that clonal interference – competition between multiple lineages carrying beneficial mutations – characterizes the evolutionary dynamics in a chemostat (Maharjan *et al.*, 2006; Kao & Sherlock, 2008). Clonal interference prevents genotypes from sweeping to fixation, and as a result, lineages are rarely found at fixation in chemostats (Gresham *et al.*, 2008; Kao & Sherlock, 2008). However, population level sequencing has shown that the extent of genetic heterogeneity is limited, and evolving populations contain only a

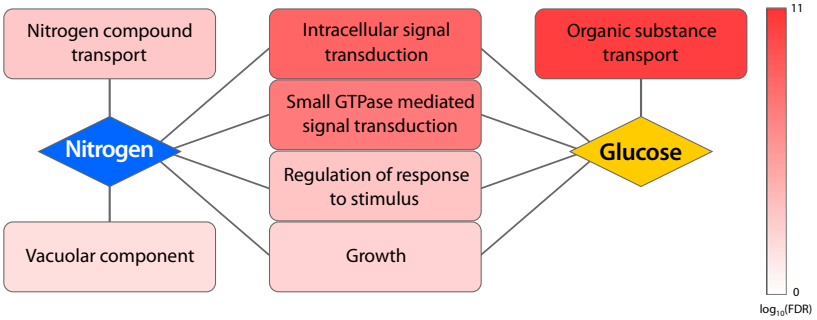

Fig. 7. Convergence of cellular processes that are targets of adaptive evolution in chemostats. We performed GO term enrichment of networks generated using GENEMANIA (Warde-Farley *et al.*, 2010) seeded with loci containing sequence variants identified in lineages and whole populations evolved in glucose-limited and nitrogen-limited chemostats from Gresham *et al.* (2008), Kvitek and Sherlock (2011) and Hong and Gresham (2014).

small number of dominant lineages even when more complex limitations are used (Hong & Gresham, 2014). This points to an important role for chance and luck in the evolutionary dynamics in the chemostat. Once a lineage containing adaptive mutations has risen to an appreciable frequency, it will limit the probability of a similarly fit lineage increasing in frequency even in large populations. New adaptive mutations are then likely to occur in the background of the dominant lineages, which results in the sequential accumulation of adaptive mutations within lineages (Hong & Gresham, 2014). Studies in *E. coli* suggest a more complex scenario in which multiple lineages with different genotypes, likely corresponding to distinct adaptive strategies, coexist within a population (Maharjan *et al.*, 2012).

The order in which adaptive mutations accumulate is likely to be constrained by epistatic interactions, which impacts both the pace of adaptive evolution and the topology of adaptive landscapes. Analysis of the fitness effect of individual mutations and their combinations from *S. cerevisiae* lineages selected in glucose-limited chemostats showed that combinations of adaptive mutations are less than the sum of their individuals effects (Kvitek & Sherlock, 2011). This phenomenon, termed 'diminishing returns epistasis', has also been observed in *E. coli* lineages selected in glucose-limited chemostats (Maharjan & Ferenci, 2013) as well as long-term serial dilution selections (Chou *et al.*, 2011; Khan *et al.*, 2011) and may be a general phenomenon of adaptive evolution (Kryazhimskiy *et al.*, 2014).

Although negative epistasis between beneficial mutations may be a general phenomenon, other types of nonadditive genetic interactions may depend more specifically on the functional relationship between the encoded gene products. Sign epistatic interactions occur when individually beneficial mutations are deleterious in the same genetic background (Weinreich *et al.*, 2005). One such example was found for loss of function mutations in *MTH1* and amplification of the *HXT6/7* locus, which are both adaptive in glucose-limited chemostats. It was observed that both mutations are never found in the same lineage and consistent with a sign epistatic relationship; when both mutations were engineered into the same strain, the strain was less fit than the ancestral lineage (Kvitek & Sherlock, 2011). Analysis of a recurrently selected three-locus genotype comprised of variation in *MEP2*, *GAT1*, and *LST4* found that variation in *LST4* is only beneficial in the background of a mutation in *MEP2* or *GAT1* consistent with positive epistasis (Hong & Gresham, 2014). Sign epistatic relationships have also been found between *rpoS* and *hfq* and between *mglD* and *malT* in *E. coli* (Maharjan & Ferenci, 2013). Interestingly, the negative interaction between *mglD* and *malT* mutations is alleviated by the presence of an *rpoS* loss of function mutation, suggesting that higher order epistasis may be important in chemostat evolution experiments.

The reproducibility of adaptive evolution outcomes in the chemostat has been addressed by performing parallel selection experiments in the same environment. It is clear that there is a finite number of solutions to the selection imposed by a chemostat. However, the extent to which convergent solutions are observed depends on the precise selection. For example, the diversity of outcomes at both the genetic and phenotypic level is far greater for selection in glucose- and phosphate-limited chemostats than sulfur-limited chemostats (Gresham *et al.*, 2008). Similar comparative studies have not yet been performed in *E. coli*; however, it seems likely that the reproducibility of evolution depends on the distribution of fitness effects associated with adaptive mutations, which will likely differ for different environments.

The beneficial effect of mutations selected in chemostats is uniquely specific to the environment. In fact, many mutations that are beneficial in the chemostat exhibit antagonistic pleiotropy in that they are deleterious in nutrient-rich conditions (Wenger *et al.*, 2011; Hong & Gresham, 2014). Mutations in *hfq* are beneficial at low dilution rates in which the mutations result in increased expression of glucose transporters; however, the same mutations are deleterious when cells are grown at a higher dilution rate for reasons that remain unclear (Maharjan *et al.*, 2013a). In general, the basis of antagonistic pleiotropy is not well understood and could result from specific functional effects that are deleterious in alternative environments or from the increased load imposed by increased production of particular proteins that are not required in the alternative environment. Understanding the 'cost' of protein production requires careful experimentation and interpretation (Stoebel *et al.*, 2008).

Concluding remarks and open questions

Several general themes emerge from the accumulated studies of adaptive evolution in chemostats. First, it is clear that selection for improved nutrient transport capabilities in both *E. coli* and *S. cerevisiae* underlies adaptive evolution in chemostats. The specificity with which CNVs containing nutrient transporters are selected is consistent with increased nutrient transport production underlying increased growth rates in nutrient-limited environments. Whereas amplification alleles result in increased production of nutrient transporter mRNAs (Hong & Gresham, 2014), adaptive point mutations preferentially target regulators of these same genes. This occurs through a variety of mechanisms including loss of function mutations in negative regulators (e.g. *galS* and *MTH1* mutations) and

possible gain of function mutations in positive regulators (e.g. *malT* and *GAT1*), which are expected to have the net effect of increasing transporter abundance. Second, signaling pathways, and their downstream targets, that regulate cellular responses to environmental nutritional status are targets of selection in the chemostat in both *E. coli* and *S. cerevisiae*. The repeated loss of function mutations in *rpoS* in *E. coli* lineages and *RIM15* in *S. cerevisiae* lineages in different nutrient limitations indicates that loss of the capacity to fully induce a stress response in nutrient-limited conditions is beneficial in the chemostat. Interestingly, there is little evidence in either species for selection of enhanced or new enzymatic functions in metabolic genes despite the centrality of metabolic processes for optimized growth in nutrient-limited environments. In addition, there is little evidence that translational regulation of nutrient transporters is a target of selection.

On the foundation of the precise control provided by the chemostat and our accumulated understanding of adaptive evolution in the chemostat from over 60 years of investigation, there are several evolutionary questions that are now readily tackled. It will be important to continue to identify the pathways that are targets of selection constraint and to determine which of these are nutrient-specific responses as opposed to generic solutions to growth by any nutrient. This will allow us to determine how many routes to increased fitness exist within a single selection. These studies will provide insight into the distribution of fitness effects attributable to acquired mutations and how this differs in different selective environments and different genetic backgrounds. The chemostat is uniquely suited to asking how the adaptive response changes with the intensity of selection and variation in population size. Furthermore, the maintenance of constant populations sizes provides the ideal scenario for characterizing the allele frequency dynamics of evolving populations. Finally, precise modulation of the environment enables analysis of the mechanistic basis of antagonistic pleiotropy.

Addressing these questions using chemostats is empowered by an understanding of the structure and function of the genetic networks and cellular processes that control cell growth. The study of protein evolution is making great strides by embracing the 'functional synthesis' (Dean & Thornton, 2007) in which molecular biology, biochemistry, and evolutionary biology are integrated. Experimental evolution in chemostats is an ideally suited to extending the functional synthesis to the study of gene networks. The convergence of DNA sequencing methods, a renewed appreciation for the critical importance of cell growth control, and detailed functional annotation of the genomes of *E. coli* and *S. cerevisiae* mean that experimental evolution in chemostats is entering an exciting new phase in which the study of adaptive evolution will benefit from renewing the close relationship with cell and molecular biology embraced by the founders of the field (Monod, 1950; Novick & Szilard, 1950a).

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