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The omnistat: A flexible continuous-culture system for prolonged experimental evolution

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Abstract

1. Microbial evolution experiments provide a powerful tool to unravel the molecular basis of adaptive evolution but their outcomes can be difficult to interpret, unless the selective forces that are applied during the experiment are carefully controlled. In this respect, experimental evolution in continuous cultures provides advantages over commonly used sequential batch-culture protocols because continuous cultures allow for more accurate control over the induced selective environment. However, commercial continuous-culture systems are large and expensive, while available DIY continuous-culture systems are not versatile enough to allow for multiple sensors and rigorous stirring.
2. We present a modular continuous-culture system that adopts the commonly used *GL45* glass laboratory bottle as a bioreactor vessel. Our design offers three advantages: first, it is equipped with a large head plate, fitting two sensors and seven input/output ports, enabling the customization of the system for many running modes (chemostat, auxostat, etc.). Second, the bioreactor is small (25–250 ml), which makes it feasible to run many replicates in parallel. Third, bioreactor modules can be coupled by uni- or bi-directional flows to induce spatiotemporal variation in selection. These features result in a particularly flexible culturing platform that facilitates the investigation of a broad range of evolutionary and ecological questions.
3. To illustrate the versatility of our culturing system, we outline two evolution experiments that impose a temporally or spatially variable regime of selection. The first experiment illustrates how controlled temporal variation in resource availability can be utilized to select for anticipatory switching. The second experiment illustrates a spatially structured morbidostat setup that is designed to probe epistatic interactions between adaptive mutations. Furthermore, we demonstrate how sensor data can be used to stabilize selection pressures or track evolutionary adaptation.
4. Evolution experiments in which populations are exposed to controlled spatiotemporal variation, are essential to gain insight into the process of adaptation and

the mechanisms that constrain evolution. Continuous-culture systems, like the one presented here, offer control over key environmental parameters and establish a well-defined regime of selection. As such, they create the opportunity to expose evolutionary constraints in the form of phenotypic trade-offs, contributing to a mechanistic understanding of adaptive evolution.

KEYWORDS
auxostat, bioreactor, chemostat, continuous culture, experimental evolution, morbidostat, turbidostat

1 | INTRODUCTION

1.1 | The need for a systems-level approach in experimental evolution

In the last decades, experimental evolution has emerged as an important research approach to study evolutionary processes (Kawecki et al., 2012; Van den Bergh, Swings, Fauvart, & Michiels, 2018). This development has been accelerated by advances in molecular biology, especially in genetics. Experimental evolution is predominantly performed with micro-organisms because of their short generation time and relatively simple genetics and physiology. Moreover, many micro-organisms can be propagated for extended periods of time using a sequential batch-culture protocol. This straightforward approach to experimental evolution has yielded ground-breaking insights into adaptive evolution, including the processes of mutation, the spread of beneficial genes through populations and the emergence of biological innovations (Barrick & Lenski, 2013; Good, McDonald, Barrick, Lenski, & Desai, 2017; Herron & Doebeli, 2013; Lang & Desai, 2014; Winterbach, Mieghem, Reinders, Wang, & Ridder, 2013).

A recurring challenge in experimental evolution is to elucidate the molecular basis of adaptive evolution. In virtually all cases of interest, intricate developmental processes and molecular interaction networks create a genetic, regulatory and metabolic architecture that is so complex that it tends to obscure the relationship between genotype and phenotype. A promising way to address this problem is to investigate how trait architecture constrains evolutionary adaptation (Winterbach et al., 2013). In particular, by imposing divergent selection pressures, evolution experiments can reveal how constraints give rise to evolutionary trade-offs between fitness components, providing valuable insight into the underlying molecular mechanisms driving adaptive evolution in response to contrasting selective forces. Such experiments, however, require an ability to impose carefully controlled selective regimes.

In designing such selective regimes, it is important to consider that the outcome of evolution experiments is generally governed by selection for higher growth rate and/or growth yield. Both are constrained on multiple levels by trade-offs between biological functions such as substrate affinity, the synthesis of building blocks, replication, resistance to stress and the energetic expenditure of the cell (Bachmann, Molenaar, Branco dos Santos, & Teusink, 2017; Pfeiffer, Schuster, & Bonhoeffer, 2001; Schuetz, Zamboni, Zampieri, Heinemann, & Sauer, 2012). There is a need to more intimately understand how the molecular mechanisms related to the traits that are selected during evolution experiments (growth rate and yield), interact with other cellular functions. This will provide insights into when and how adaptations cause the phenotypic constraints that ultimately produce patterns of convergence and divergence between evolving populations. Evolution experiments have shown the importance of transcriptional regulation to overcome metabolic constraints related to growth optimization, by repressing or activating different catabolic pathways to suit specific environmental conditions (New et al., 2014; Price et al., 2019). Nevertheless, it still remains unclear under what conditions regulatory adaptations can or cannot overcome constraints and whether these constraints can be categorized and predicted. To start to answer these questions, a systems-level experimental-evolution approach is required, in which greater emphasis is put on obtaining a mechanistic understanding of adaptive evolution. Such a systems-level approach entails detailed knowledge on the molecular working of the selected function(s), tighter control over selection pressure and an integrated OMICS package of measuring tools to comprehensively quantify both positive and negative changes in the evolved mutants.

1.2 | Controlling selection in evolution experiments

The majority of experimental evolution studies rely on a sequential batch protocol to propagate cultures during the experiment. However, sequential batch propagation inadvertently generates periodic temporal variation in environmental conditions (e.g. nutrients, pH, overflow metabolites). Nutrient availability alternates between high and low as the culture is diluted (daily) and grown to stationary phase (Figure 1a). As a result, selection is fluctuating drastically during the experiment, which complicates the interpretation of the evolutionary outcome (Collot et al., 2018). In particular, it often remains unclear how different selective pressures (e.g. selection for rapid

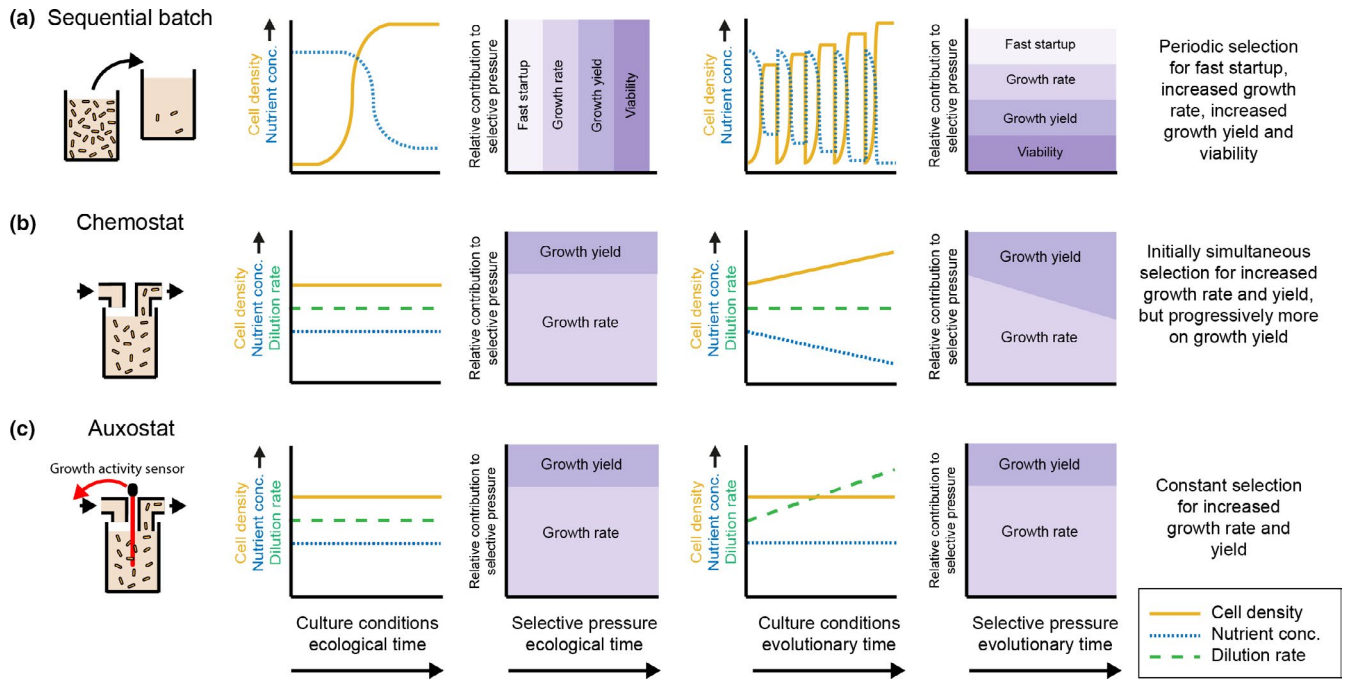


FIGURE 1 Changes in culture conditions (e.g. nutrient concentration, cell density and medium flux) and selective pressure (e.g. fast start-up, high growth rate, high growth yield and high viability) over ecological (first two columns) and evolutionary time (last two columns). For simplicity, evolutionary change is depicted to follow a linear trend. Culture conditions for (a) sequential batch culturing, (b) a chemostat and (c) an auxostat

growth start-up and fast growth at high and low nutrient availability) contribute to the observed outcomes (Figure 1a). To overcome this limitation, a more precise control of the selective pressures that are induced throughout the evolution experiment is required.

Continuous cultures such as chemostats solve the problem of unintended temporal variation in selection by inducing a physiological steady state via constant dilution of the culture (Figure 1b). This steady state is achieved by the continuous equal addition of fresh medium (nutrients) and removal of spent medium (waste products and cells). The steady state induced by a chemostat culture results in a culture with a constant growth rate that is equal to the dilution rate. Therefore, adjustment of the dilution rate allows for accurate control over the specific growth rate of the culture. Such a controllable steady-state selection regime also allows for representative and comparative sampling through evolutionary time, improving the quality of downstream analysis (e.g. transcriptomics, proteomics, metabolomics).

Chemostat continuous-culture systems are mostly used to induce constant growth conditions over ecological time, by keeping the culture dilution rate constant (Figure 1b). Often other important growth parameters are also kept constant, for example temperature, pH and dissolved oxygen (if the culture is grown aerobically). However, for the use of chemostats in experimental evolution, long running times (weeks or months) are required. During the evolution experiment, the adaptation towards increased growth will inadvertently lead to an increasingly higher steady-state density of the continuous culture (Figure 1b), which causes a shift in the selective regime over an evolutionary time scale. This shift is caused by the ecological feedback between the evolving population and its environment (the bioreactor). For example, the fixation of beneficial mutations may lead to an increase in culture density, generating a reduction of growth-limiting resource availability and, consequently, stronger selection towards the optimization of yield under nutrient-limited conditions. Similarly, an increase in culture density can reduce oxygen availability (if grown aerobically), increase concentration of overflow metabolites or change culture pH, all of which may feedback onto the selective regime experienced by the population. In fact, apart from selecting for higher cell yield, some of these changes may induce metabolic shifts or regulatory changes that trade-off against growth rate or yield.

To prevent changes in the selective regime over the course of prolonged evolution experiments, it is essential to monitor and control changes in culture density. Auxostats are bioreactors in which growth activity is measured to control the conditions inside the bioreactor to maintain a constant population density (Figure 1c). By doing so, auxostats provide the clear benefit of ensuring constant nutrient conditions for the cells not only throughout ecological but also evolutionary time. Turbidostats are the most common example of an auxostat. Here, population density is kept constant by measuring the cell density of the culture (turbidity) and subsequently adjusting the dilution rate (Gresham & Dunham, 2014). Alternative ways to control the culture conditions in auxostats are to regulate temperature (by heating or cooling), pH (by adding acid or base) or dissolved oxygen (by varying aeration). Logging the control parameters (e.g. dilution rate, activity of the pH-regulation pump and aeration flow) that are used to keep the growth conditions constant

can be helpful to identify the emergence of key adaptations during the evolutionary process. In particular, monitoring sudden shifts in the control parameters allows for a targeted sampling of biologically interesting time-points for subsequent phenotypic and genetic analysis (Dößelmann et al., 2017), facilitating the identification of causal major-effect mutations.

1.3 | Inducing variation in experimental evolution

Tightly controlling the selective regime in evolution experiments through the use of continuous cultures greatly facilitates the interpretation of evolutionary adaptations. However, microorganisms exhibit a tendency to hyper-specialize to a constant lab environment, at the cost of many functions that are essential under naturally varying conditions (Dillon, Rouillard, Van Dam, Gallet, & Cooper, 2016; Price et al., 2019). Mediating environmental variation is an integral part of the life cycle of most organisms. All organisms have evolved mechanisms to respond to selection that varies in space and/or time while being constrained by fundamental trade-offs between alternative strategies for maximizing fitness (Jaeger, Irons, & Monk, 2012). Therefore, continuous-culture evolution experiments benefit from applying controlled variation in selective conditions, forcing organisms to mediate essential trade-offs that constrain their performance under natural conditions. An accurate way to probe these trade-offs in a continuous-culture system is to induce temporal or spatial variation on top of a well-defined steady-state background condition. By minimizing the number of biologically relevant parameters that change, this design allows for systematic exploration of the effects of varying the impact or predictability of temporal heterogeneity. Alternatively, the possibility of coupling multiple bioreactors creates opportunities for realizing spatial variation in selection over an interconnected array of patches. Variation in the control parameters of each bioreactor (patch) can then be applied to induce gradients of nutrients, stressors, temperature or pH, or to create almost any arbitrary complex pattern of spatial variation.

1.4 | The need for the omnistat system

Motivated by these considerations, we propose here a dynamically controlled continuous-culture system that allows for (a) an accurate control and characterization of selective force(s); (b) the application of a wide range of patterns of spatial or temporal variation in selection to probe phenotypic trade-offs and (c) an integrated molecular characterization of evolved adaptations using genomics, transcriptomics, proteomics and metabolomics time series, to unravel the molecular mechanisms connecting the phenotype to the genotype.

Our system occupies an empty niche among the currently available alternatives. Many commercial bioreactor systems are developed for the biotechnology industry and feature an extensive array of monitoring and controlling devices. However, these biotech-derived bioreactors are usually very expensive and of relatively large sizes (0.25–10 L). Their large size presents a problem for running different treatments and many replicates in parallel because of the large amount of medium required (especially for extensive amounts of time) as well as the limited physical space available in most laboratories. Moreover, modifying these commercial bioreactors is costly because of their specialized parts. The other type of bioreactors are microtiter-plate-sized arrays of bioreactors or microfluidic bioreactors. These devices provide great potential to scale up replicates and the study of single cell dynamics. However, they are intended for short experiments (a couple of days) because they lack rigorous stirring and influx growth-back control, which results in fast biofilm formation/aggregation and clogging. DIY low-cost continuous-culturing devices of intermediate size (5–20 ml) come as an alternative to the industrial and microtiter/microfluidic bioreactors. Several recent DIY devices have been developed by researchers specifically for scientific experiments and address the problems related to price and scaling up the number of replicates (Miller, Befort, Kerr, & Dunham, 2013; Skelding, Hart, Vidyasagar, Pozhitkov, & Shou, 2018; Toprak et al., 2013; Wong, Mancuso, Kiriakov, Bashor, & Khalil, 2018). However, these DIY systems have three important limitations. First, they have reaction tube style bioreactors that are long but narrow in width. This limits the size of the head plate, making it impossible, in practice, to fit multiple different sensors to obtain precise control over culture conditions. Second, the high height/width ratio of these bioreactors limits the size of the magnetic stirrer and increases the distance of the stirring rod to the top of the culture. Both of these factors impair rigorous stirring. Rigorous stirring is particularly important for prolonged evolution experiments that last more than 2 weeks to avoid fouling. Third, to enable an integrated analysis of genetic and phenotypic data it must be possible to run multiple analyses on one culture sample (e.g. metagenome, metabolome and proteome analyses, and especially RNA isolation) in addition to retaining enough material for making glycerol stocks. These combined analyses can require a large amount of culture (20 ml) to be sampled from a single bioreactor, exceeding the capacity of many DIY systems.

The continuous-culture system we present here was designed to facilitate a next generation of experimental evolution studies. We named this system the omnistat because it can be configured to implement various alternative bioreactor modes (e.g. chemostat, retentostat, and various auxostat modes such as the turbidostat, pH-stat, oxistat and morbidostat). The omnistat is easily assembled, allows for precise control over culture conditions, and is highly flexible in terms of adding specific functions to the system and switching between intermediate to small culture volume. Lastly, the system is also particularly suitable for phenotypic characterization of evolved mutants in the analysis phase of an evolution experiment because of its adjustable modular structure, which allows for easy replication, and the ability to equip each bioreactor with various sensors.

2 | MATERIALS AND METHODS

2.1 | An overview of the system

The omnistat system that we built consists of 24 individually digitally controlled and logged bioreactors. The system is operated via a real-time LabVIEW application that is run from a computer (*National Instruments compactRIO*). The bioreactors are grouped into six modules of four bioreactors that sit together in a stainless-steel frame (Figure 2). This frame allows for easy autoclaving and preparation of the bioreactors. Additionally, the frame provides support for the bioreactors, the tubing and bottles with additives. The bioreactor frame sits inside a computer-controlled water bath (*Julabo MB*) that controls the temperature of the culture. The culture is homogenized by a stirring rod powered by a submersible magnetic stirrer (*Cimarec micro*) that sits in the bioreactor frame underneath the bioreactor (Figure 2). Each individual bioreactor consists of a stainless-steel head plate and a glass reactor vessel (Duran *GL45 DIN*; Figure 3). A range of 25–200 ml culture volumes can be used for the bioreactors by changing the bottle size to 100 ml (shown in the figures), 150 or 250 ml and the height of the efflux port. To maximize the flexibility of the system, each head plate features nine ports (Figure 3): two sensor ports (pH, oxygen or cell density; *Applisens pH sensor for mini bioreactor 8 mm; Applisens 8 mm 15 cm polarographic DO_2 sensor for mini bioreactor; ABER futura pico 8 mm*), three static ports (chimney, nutrient, nitrogen gas and base additions) and four height-adjustable ports (efflux, sampling, migration and aeration; Figure 3). The potential to fit multiple sensors in the bioreactor greatly increases the ability to monitor and regulate the conditions within the bioreactor. Possible regulatory modes include maintaining constant pH, oxygen and/or viable cell density, or applying controlled fluctuations in these parameters through time or across bioreactors. Beyond their function in negative-feedback control, the sensors can also be used to follow phenotypic changes that affect these measurable conditions and occur as a result of ecological or evolutionary change throughout an experiment. The height-adjustable ports can be used for adding liquids and gas by raising the rods above the culture liquid. Alternatively, by sticking the rods inside the culture liquid, they can be used to remove spent culture, for aeration or for sampling. The three remaining static ports sit above the liquid and can be used as a chimney and/or input port. All the influxes and effluxes of liquids that take place inside the bioreactors are controlled via peristaltic pumps: *Ismatec IPCN24 (influx and efflux); Ismatec Reglo ICC (pH control pump)* and *Ismatec Reglo digital (migration pump*; Figures 4 and 5). The pumps can be used to control medium influx and efflux,

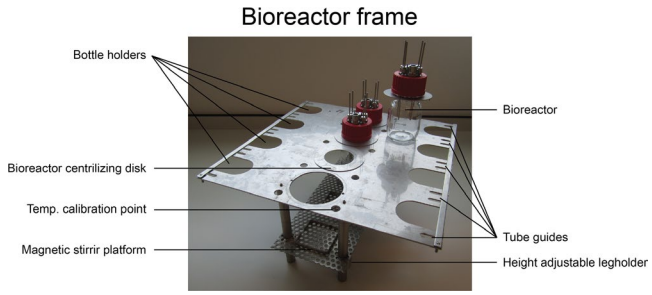


FIGURE 2 Overview of the features of the bioreactor frame

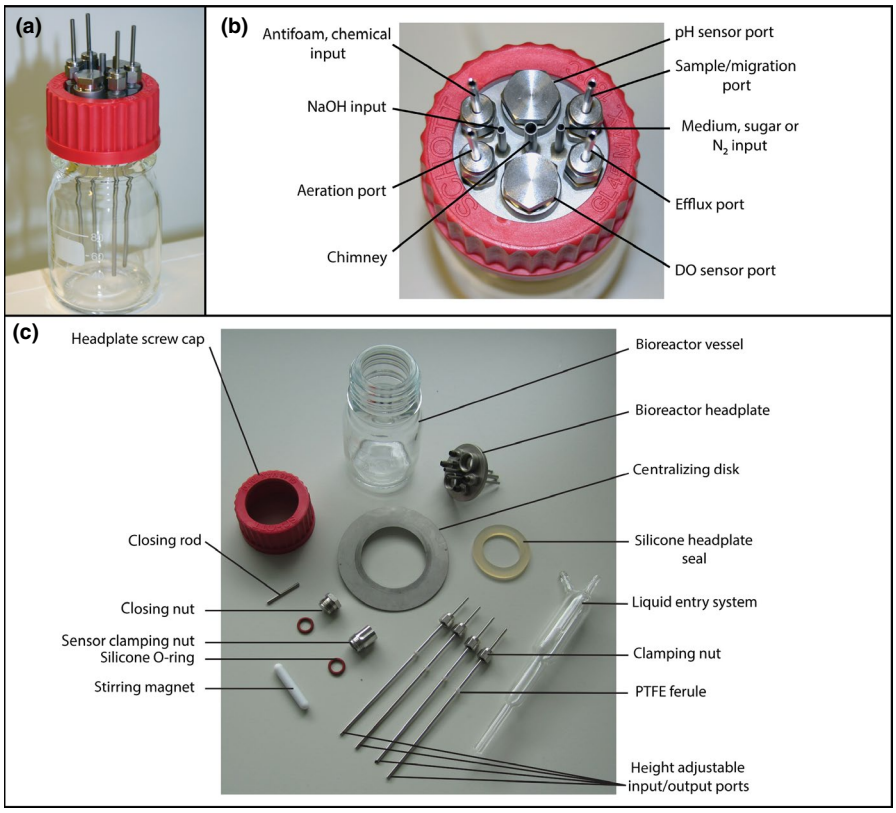


FIGURE 3 Overview of bioreactor components. (a) Bioreactor with headplate screwed on a 100 ml reactor vessel (duranGL45 DIN). (b) Inputs, outputs and sensor ports featured on the head-plate. (c) Separate components making up a single bioreactor

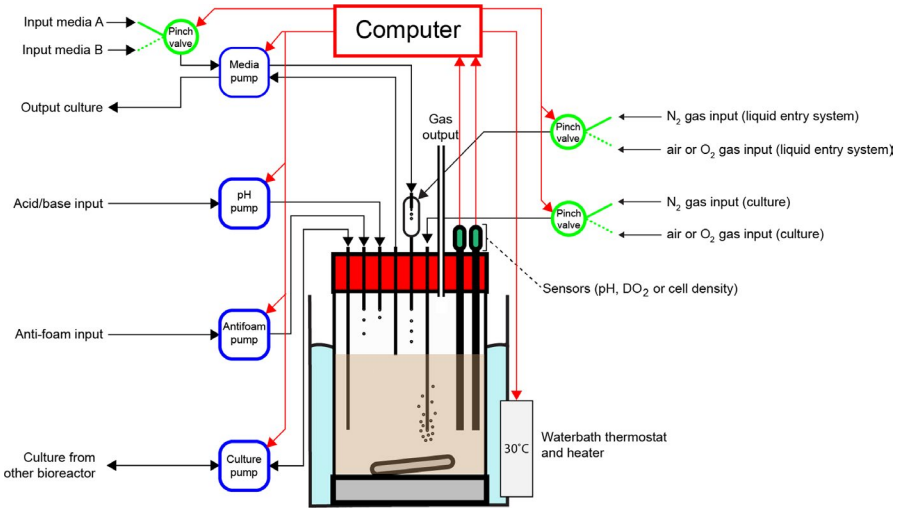


FIGURE 4 Schematic representation of the influxes and outfluxes of the bioreactors with their respective control loops. Pumps are indicated in blue. Computer-controlled pinch valves are indicated in green. Black arrows indicate fluxes of liquid or gas going in and out of the bioreactor. Red arrows represent input parameters flowing from the bioreactor sensors to the computer and the output parameters flowing from the computer to the controlling equipment (pumps, pinch valves and water baths)

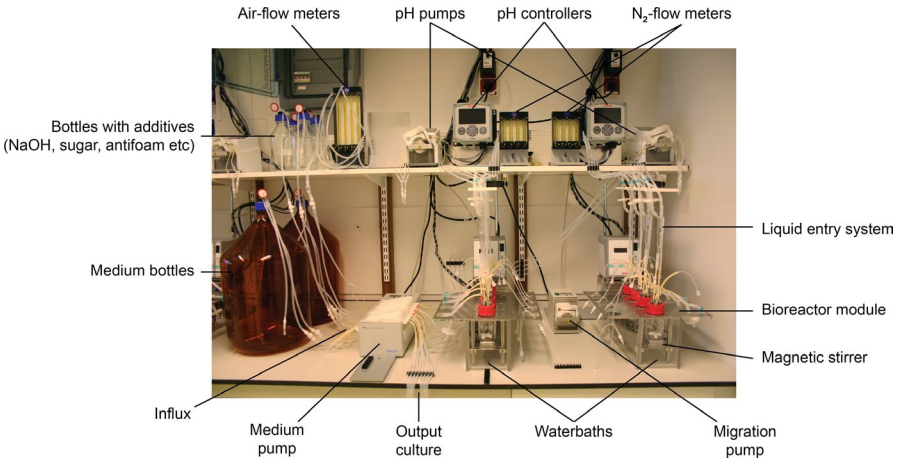


FIGURE 5 Overview of two 4-bioreactor modules, with water baths, pumps, media bottles and gas-flow meters

pH-control, migration or additions of other liquids such as signal molecules, antibiotics and nutrient components. The pumps are all controlled via a LabVIEW application to set their speed and direction of flow. The omnistat system additionally features three arrays of computer-controlled dual channel pinch valves (*Sirai 3/2 NC-NO solenoid pinch valve*; Figures 4 and 5). These valves enable liquid or gas additions to be varied in time. The behaviour of the pumps and pinch valves can either be pre-programmed or dynamically respond to output parameters from the bioreactor (e.g. pH, dissolved oxygen or cell density).

2.2 | Inducing spatial and temporal variation

To create spatial structure with the omnistat system, individual bioreactors can be setup as spatial patches that are connected via tubes and peristaltic pumps. Migration between patches can then be induced by continuously pumping culture back and forth between bioreactors at a controlled (migration) rate (Figures 4 and 5). A great variety of spatial selection regimes can be created by varying the conditions among bioreactors, the connectivity pattern between patches or the rate or directionality of migration. Examples are spatial gradients in nutrients, temperature or antibiotics. Alternatively, source–sink dynamics can be employed by inducing different migration rates or nutrients availabilities between patches. Temporal variation can be induced by changing the set values of the pump rates, pinch valves or the thermostats. Adjusting these control parameters enables the induction temporal variation in growth rate, nutrient availability, aeration (cellular respiration), pH, temperature exposure to stressors.

2.3 | Running modes that can be induced with the omnistat system

The omnistat system can be setup to enable different basic culturing modes. By installing different sensors, different aspects of growth activity can be monitored such as population density, density of catabolic waste products and cellular respiration. These measured variables can be used to control parameters such as pump rate, gas flow, temperature and pinch valves. A broad range of culturing modes can be induced by programming control loops between the measured variable and control parameters (Figure 6). In addition, a large variety of selective regimes can be induced by allowing the control loops to vary between connected modules or in time.

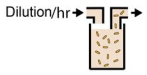
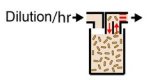
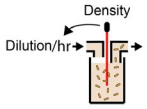
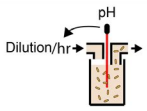
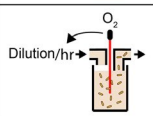
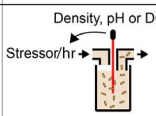
Culturing mode		Schematic visualization	Setup of omnistat device
Chemostat			**Sensor variable:** None **Feedback variable:** Medium flux pump rate (dilution rate) **Modification: none**
Retentostat			**Sensor variable:** None **Feedback variable:** Medium flux pump rate (dilution rate) **Modification:** Install crossflow filter on efflux
Auxostat	Turbidostat		**Sensor variable:** Optical density (total biomass), capacity (viable biomass), as proxy for population density **Feedback variable:** Medium flux pump rate (dilution rate) **Modification:** Install density-sensor with control loop to medium flux pump
	pH-stat		**Sensor variable:** Culture pH, as proxy for density catabolic end-products (catabolic activity) **Feedback variable:** Medium flux rate (dilution rate) of alkalinezed, acidified or buffered medium **Modification:** Install pH-sensor with control loop to medium flux pump
	Oxistat		**Sensor variable:** Dissolved oxygen in culture, as proxy for cellular respiration (aerobic catabolic activity) **Feedback variable:** Medium flux pump rate (dilution rate) **Modification:** Install DO-sensor with control loop to medium flux pump
	Morbidostat		**Sensor variable:** Density, pH or DO, as proxy for growth activity **Feedback variable:** Pump rate stressor (antibiotic, salt, toxin, acid etc.) or temprature **Modification:** Install density, pH or DO sensor with control loop to stressor input pump or heater

FIGURE 6 Table with a selection of culturing modes that can be adapted with the omnistat system, with their respective set-ups

In summary, the omnistat bioreactor system is a highly flexible, low-budget system that can be used in many bioreactor configurations. Both temporal and spatial variation can be pre-programmed to accurately induce a selective regime suited to the specific research question. The essential components of the omnistat are the bioreactor frame and head plate; the accessory equipment (pumps, sensors, bottles, flowmeters, etc.) can be easily changed, depending on the specific needs of the experimental setup. Technical drawings of the bioreactor frame (S1) and head plate (S2) are provided in the supplementary material, along with detailed protocols for constructing and operating the system (S3). Software for controlling the omnistat must be custom-developed to fit with the specific configuration of the instrument when it is initially built, but can be based on standard templates for control-loops available in software such as labVIEW. In our case, we opted for a labVIEW application that was run from a *National Instruments (compactRIO)* computer that connected to the hardware (pumps, sensors, waterbaths) and a standard office PC that displayed a graphical user interface for the omnistat. By means of this interface, users were able to monitor, log and adjust set-points and control algorithms (S3). The full labVIEW program code for our control application is available at https://doi.org/10.5281/zenodo.3731681.

3 | RESULTS

3.1 | Example experimental designs illustrating applications of the omnistat bioreactor system

To illustrate how the unique features of the omnistat can be exploited to address a variety of biological research questions, we outline two potential experimental designs for microbial evolution experiments. One of the two experiments has been completed and the other is in a preparatory phase, but here we present both experiments as project ideas (i.e. without [preliminary] data because these will be published elsewhere) to illustrate the range of possible applications of the omnistat system. The first example showcases the utilization and integration of pre-programmed pumps and pinch valves in a chemostat culture mode. This setup enables the induction of complex temporal variation in resource availability and environmental cues. In the second example, a morbidostat mode of culturing is combined with the induction of spatial variation in multiple stressors. This experimental design illustrates how sensor data of growth activity can be exploited to track multiple components of fitness and subsequently control a multivariate selection regime.

3.2 | Evolving anticipatory switching using temporal variation

The first experiment sets out to evolve the bacterium *Lactococcus lactis* to a temporally fluctuating resource environment in the presence or absence of informative cues about the future state of the environment. The aim of the experiment is to determine whether *L. lactis* can evolve anticipatory switching in response to predictive cues to prepare for upcoming changes in resource availability. Environmental variation is induced by switching between growth media containing either fructose or galactose as the sole carbon source. The resource influx randomly alternates between fructose and galactose media over a sequence of discrete time intervals (subject to the constraint that both sugars are supplied for equal amounts of time in total). The transition

from one time interval to the next always involves a short period in which we supply the bioreactors with water instead of a carbon source (Figure 7a,b), inducing a short period of starvation between periods of growth. During this starvation phase, we supply a pulse of either lactate or acetate to the bioreactor (Figure 7a,b). In one treatment, the lactate or acetate cues are predictive of the carbon source (galactose or fructose, respectively) that will be provided in the next time interval. In the other treatment, the lactate or acetate pulse do not contain information about the future state of the environment. In the treatment where informative cues are provided, *L. lactis* can, in principle, evolve anticipatory switching to prepare for the change in resource regime during the interval of starvation, allowing for a fast start-up when new resources become available. However, evolving such behaviour would require a rewiring of pre-existing metabolic regulation. This first experimental design illustrates that complex schemes of temporal variation can be realized by exploiting the flexibility of the omnistat's programmable pumps and pinch valves, without requiring major modifications to the system. Besides capitalizing on this flexibility, the first experiment also benefits from the ability to monitor the switching behaviour of the strains by tracking the acidification rate derived from the pH-sensor data (this can be interpreted as a proxy for metabolic activity). In particular, by quantifying the time it takes to reach stable acidification rate after switching from one carbon resource to another, we obtain valuable information about the evolution of the transcriptional/plastic response of the evolving mutants.

3.3 | Probing epistatic interactions in a spatially varying morbidostat setup

The second suggested experiment investigates the role of epistatic interactions between mutations conferring resistance to different types of antibiotics. Epistatic interactions between different resistance mechanisms are prevalent and can impact the acquisition of multi-drug resistance (Durão, Balbontín, & Gordo, 2018), a problem that increasingly undermines the effectiveness of antibiotic treatments. To study how epistatic interactions influence the evolution of multi-drug resistance, four bioreactors can be coupled together in a source sink configuration, with one source bioreactor connected to three sink bioreactors (Figure 8a). The connectivity between the bioreactors is achieved via a constant exchange of a small volume of culture from a central bioreactor (source population) to three peripheral bioreactors (sink populations) by pumping a small volume of culture continuously back and forth between the bioreactors. Each sink population is exposed to a specific antibiotic that is pumped into the bioreactor via a computer controlled peristaltic pump. At the beginning of the experiment, the concentration of the three antibiotics in the central bioreactor is set at the highest possible level that still allows the population to persist (i.e. the growth rate in the presence of antibiotics should exceed the dilution rate at low population density). In the peripheral bioreactors, antibiotic concentrations for two of three antibiotics are set to equal those in the central bioreactor, while the concentration of the remaining antibiotic is set slightly higher such that the population cannot persist initially. As a result, the bioreactor is continuously inoculated by the source bioreactor but cells are flushed out a higher rate than they are able to divide (Figure 8b). Hence, at the start of the experiment, cell density is high in the central bioreactor but very low in the peripheral bioreactors. However, when a mutant strain evolves that has a greater resistance to one of the three antibiotics, it will likely colonize the corresponding peripheral bioreactor (Figure 8b) and will rapidly increase in frequency, given its advantage of being able to occupy a nearly empty niche. If such an event occurs, it can be detected in the bioreactor because of changes in pH or turbidity in the given bioreactor. The

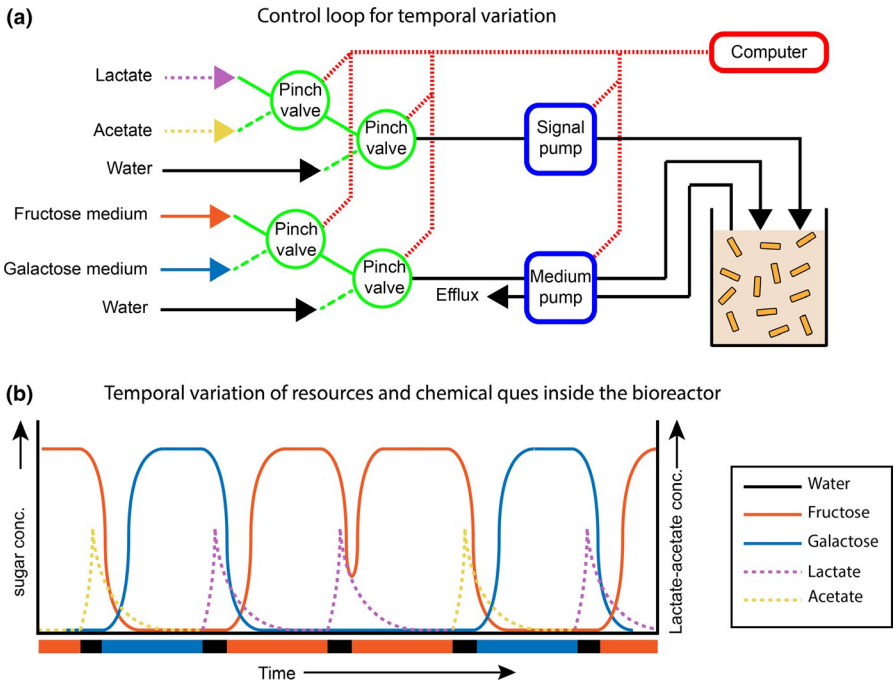


FIGURE 7 Schematic overview of the experimental set-up of the anticipatory switching experiment. (a) Pinch-valve-controlled inputs of media (fructose medium, galactose medium or water) as well as the pinch-valve-controlled signal chemicals (lactate, acetate and water). (b) Schematic example of concentrations of sugars and signal chemicals induced through time in the bioreactor

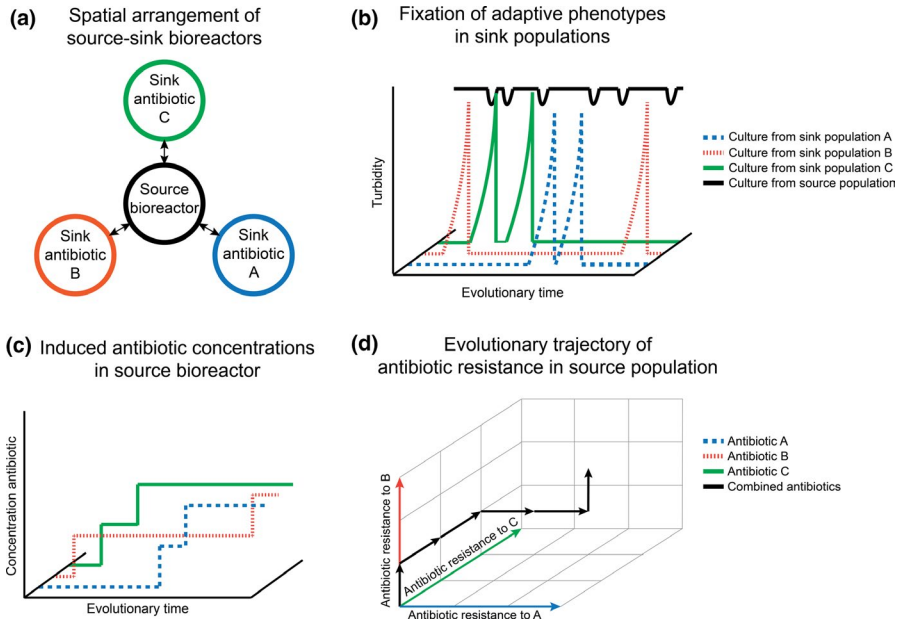


FIGURE 8 Epistatic interactions during the evolution of multi-drug-resistant bacterial strains. (a) Spatial connectivity of source and sink bioreactors (populations) via migration pumps. (b) Cell density in the source and sink populations throughout the evolution experiment. The steep increase in turbidity for the sink populations indicates moments when a newly acquired mutation related to higher resistance fixates in one of the sink populations resulting in a net increase in cell density. The subsequent steep decrease in turbidity marks the moment that the antibiotic concentration is increased in the specific sink bioreactor inducing a decrease or cease in growth resulting in the cells being washed out of the sink bioreactor. (c) Concentrations of different antibiotics in the source bioreactor as the population builds up multi-drug resistance through time. (d) The resulting evolutionary multi-resistance trajectory of the source population through time

computer system controlling the bioreactor will then copy the antibiotic concentrations of the newly occupied peripheral niche to the central bioreactor as well as increasing the specific antibiotic level in the other peripheral bioreactors by increasing the speed of the antibiotic pumps (Figure 8c). The stress level in the previously occupied peripheral bioreactor is then increased to a level in which the culture is again sufficiently hampered so that it turns into a sink population again (i.e. growth rate < flux rate). By repeating this process multiple times, we are able to track the phenotypic adaptations to each of the different antibiotics, that is, phenotypic innovations are detected while the experiment is running. In each evolutionary step, the direction of adaptation can be inferred from the pattern of population growth in the peripheral bioreactors. Moreover, system control parameters that are automatically logged provide a readout of the evolutionary trajectory followed by the adapting population (Figure 8d). Culture samples taken during different phenotypic improvement events can then be sequenced to specifically link genetic mutations to new phenotypic traits (acquisition of increased resistance). It will be very interesting to see whether replicate treatments follow identical evolutionary trajectories (parallel evolution) or alternative evolutionary paths due to epistasis and mutation order or other stochastic effects. Furthermore, it may be possible to track specific resistance patterns to specific starting points in phenotype space. Such mapping of evolutionary trajectories to specific preadapted states could facilitate the development of novel antimicrobial strategies guided by epistatic interactions between different antibiotic mechanisms. This second suggested experiment illustrates how the omnistat continuous-culture setup allows for the exploitation of real-time information on fitness proxies to dynamically alter the selective regime experienced by the population. In this way, it becomes feasible to set-up controlled, frequency-dependent selection regimes and manipulate the feedback between ecology and evolution, opening up an exciting new range of questions for experimental evolution.

4 | DISCUSSION

The omnistat fills a unique niche among the range of currently available continuous-culture devices. It provides a culturing system that supports the monitoring capabilities of a commercial device combined with a small (20–200 ml) culturing volume that allows for sufficient replication of evolution experiments. In terms of budget, the system's capital and operational costs are intermediate between those of commercially available bioreactors and (small volume) budget DIY systems: the bioreactor frame and head plates can be made relatively inexpensively, at around €700 (materials and labour) per four-chemostat module, but substantial additional investment is needed for the peristaltic pumps, the different types of sensors, controllers and other equipment. However, users are able to control the overall costs by limiting the number of bioreactor modules, or by (initially) equipping them with a subset of the functionality outlined here. In other words, owing to its modular design, the omnistat platform can be customized for specific

needs and budgets. It is this flexibility of the omnistat which allows the device to be used for many purposes and be continually modified or updated to explore current and future research questions.

The above-mentioned applications of the omnistat system illustrate how it can be used to address a variety of evolutionary questions. The ambition to understand the molecular basis of adaptation, which is motivated by the increased ability to dissect the mechanistic underpinning of biological functions, requires a new generation of experimental evolution approaches. Systems such as the omnistat allow for tight control over selection pressures and spatiotemporal heterogeneity, making it feasible to probe key trade-offs and constraints rooted in the genetic architecture or the organization of regulatory and metabolic networks of organisms. In this way, evolution experiments can further unravel the complex relationship between genotype and phenotype and clarify how selection and constraints interact to shape the outcome of evolution.

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AUTHORS' CONTRIBUTIONS

D.M.E. and G.S.v.D. wrote the manuscript and all authors contributed by providing comments on draft versions; F.B.d.S. conceived, built and tested a predecessor version of the omnistat and provided expertise for the further refinement of the system; D.M.E. modularized the bioreactor system, optimized the head plate design, developed an integrated operating system and the spatiotemporal variation systems; D.M.E. built final system; D.M.E. and C.A.M. tested the omnistat and used the omnistat in experiments; F.B. and G.S.v.D. facilitated the construction and development of the bioreactor system and provided expert input on its conception and design.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/2041-210X.13403

DATA AVAILABILITY STATEMENT

We have made the full LabVIEW code of omnistat available at https://doi.org/10.5281/zenodo.3731681.

GLOSSARY

Growth rate	The amount of biomass formed per unit of time
Growth yield	The amount of biomass formed per unit of resource
Influx	The incoming flow of a liquid (generally fresh medium)
Efflux	The outgoing flow of liquid (spent culture)
Flow rate	The rate at which the culture medium is fluxed through the bioreactor, measured in volume per unit of time
Dilution rate	The flow rate normalized by the volume of the culture (reflects the fraction of the culture that is refreshed per unit of time)
Resource limitation	Growth conditions under which culture density is controlled by nutrient supply
Stress limitation	Growth conditions under which culture density is controlled by the level of a stressor
Flux limitation	Growth conditions under which culture density is controlled by the dilution rate while resources are abundant
Sequential batch	A culturing method in which cells are grown in batch until all resources are consumed. Then, the batch culture is transferred to a new batch of fresh medium. This method can be repeated to propagate cultures for extended amount of time
Chemostat	A continuous-culturing device in which influx and efflux of medium are equal, thereby maintaining a constant culture volume that is continuously diluted and mixed. Optionally, pH and aeration can be stabilized with the use of sensors and pumps. This mode of culturing results in a physiological steady state
Auxostat	A chemostat with additional feedback mechanisms that control cell density
Turbidostat	An auxostat continuous-culturing device in which cell density (measured by turbidity) is subject to feedback control mediated by variation in dilution rate
Morbidostat	An auxostat continuous-culturing device in which cell density (measured by turbidity) is subject to feedback control mediated by variation in the level of a stressor (e.g. antibiotics, temperature, salinity, pH, etc.)

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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