Anticipation of a serial transfer protocol through the evolution of fine-tuned growth dynamics

Keywords: microbial dynamics, eco-evolutionary dynamics, serial transfer protocol, anticipation, Virtual Microbes, in silico evolution, diversification

Bram van Dijk[1*], Jeroen Meijer[1], Thomas Cuypers[1], Paulien Hogeweg[1],

1 Theoretical Biology and Bioinformatics, Utrecht University, the Netherlands

* B.vanDijk@uu.nl

Abstract

Experimental evolution of microbes often involves a serial transfer protocol with repeated dilutions and transfers to fresh media to start a new growth cycle. Here we study how *in silico* evolved Virtual Microbe "wildtypes" (WTs) adapt to such a protocol, study the generic evolutionary features, and investigate how these features depend on prior evolution. All WTs adopt a balance of growth and survival, therewith anticipating the regularity of the serial transfer. We find that this anticipation can happen by means of a single lineage, or by coexisting lineages that specialise on either the growth phase or the stationary phase. Parallel experiments of the same WT show similar trajectories with respect to growth and yield, and similar biases towards diversification. In summary, all our *in silico* WTs show the same anticipation effects — fitting the periodicity of serial transfer protocol — but prior adaptations determines what solution is found by subsequent evolution.

Introduction

In order to see microbial evolution in action, we often rely on experimental evolution under controlled laboratory conditions. The Long-term Evolution Experiment (LTEE) [1] and similar shorter studies [2–4] have, for example, evolved many generations of microbes using a serial transfer protocol, in which microbes are repeatedly diluted and transferred to a fresh medium to start a new growth cycle. Conceptually, if we learn to understand how microbes adapt to such a simple protocol, we might one day be able to predict evolution in the lab and — ideally — also in nature. Indeed, a lot of evolution in the lab seems remarkably reproducible, where microbes show parallel adaptations both on the level of the phenotype as well as the genotype [4–11]. However, there also seems to be strong potential for divergent evolution, leading to diversity both between and within replicate populations [12–14]. Diversification events within populations often entail cross-feeding interactions [12, 13, 15–18], where species emerge that grow on metabolic by-products. These cross-feeding interactions are increasingly well understood with the help of metabolic modeling and digital evolution [19, 20]. A recent metagenomic study has revealed even more coexisting lineages in the LTEE than were previously reported [21]. It is however not yet clear whether all these polymorphisms are the result of uni-directional cross-feeding interactions, or if other mechanisms could drive coexistence in a simple experiment such as a serial transfer protocol.

Prior to being subjected to lab conditions, the microbes used in the aforementioned experimental studies all evolved for billions of years in natural environments, experiencing harshly fluctuating and — more often than not — unfavorable conditions. While a serial transfer protocol such as that of the LTEE at a first glance selects mostly for higher growth rates when resources are abundant (*i.e.* during the log phase), there is also selection to survive when resources are depleted and the population no longer grows (*i.e.* during the stationary phase). In fact, given the

unpredictable conditions found in nature, some of the ancestors of *Escherichia coli* might have survived precisely because they diverted resources *away* from growth. Indeed, *E. coli* does exactly this during the stationary phase by means of the stringent response, regulating up to one third of all genes during starvation [22]. This response lowers the growth rate, but promotes efficiency and survival (*i.e.* a higher yield). While most microbes have ways to deal with starvation, the physiology of growth arrest varies a lot across different microbes (for an excellent review, see [23]). Responses to starvation, as well as other features that organisms have acquired in their billions of years of evolution (such as gene clustering, gene regulatory network architecture, metabolic regulation), might strongly influence the adaptation and reproducibility we observe in the lab today.

Given that microbes are clearly adapted to more than just high growth rates, what do we expect when a complex microbe adapts to a serial transfer protocol? In order to obtain a fresh perspective we here use Virtual Microbes (see methods) in order to mimic natural evolution to acquire Virtual "wild types", which we then expose to a serial transfer protocol. We show that all wild types evolve the same anticipation towards the serial transfer protocol, and that this can happen by means of a single, or by multiple lineages.

Results

What is Virtual Microbes?

Virtual Microbes is a model of the eco-evolutionary dynamics of microbes. The Virtual Microbe model is unsupervised, meaning that it aims to combine relevant biological structures (genes, genomes, metabolism, mutations, ecology, etc.) without a preconceived notion of "fitness", which is instead an emergent phenomenon. By not explicitly defining what the model *should* do, it allows for a serendipitous approach to study microbial evolution.

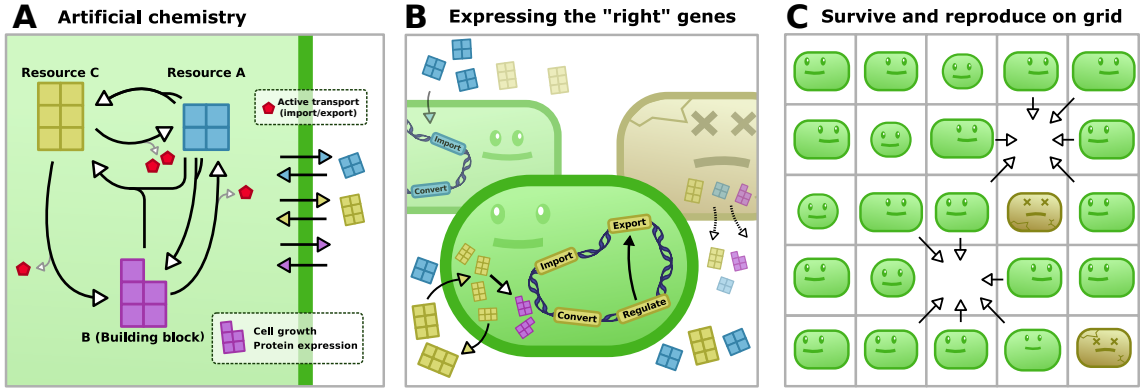

Fig 1. Virtual Microbes model overview
A) At the basis of the Virtual Microbe model is an artificial "metabolic universe", describing all the possible reactions that can be catalysed. Resources (yellow and blue) are fluxed in, but building blocks (purple) and energy (red) must be synthesized to express proteins and transport metabolites across the membrane, respectively.
B) A Virtual Microbe only needs to express a subset of all possible reactions to be viable, and that no metabolic strategy is necessarily the "right" one.
C) The individuals grow and reproduce on a spatial grid, and can only reproduce when there is an empty spot. Death happens stochastically or when a cell has accumulated toxicity by having excessively high concentrations of metabolites. Since only cells that have grown sufficiently are allowed to reproduce, we simulate evolution with no prior expectation.

Before we start evolving Virtual Microbes in a serial transfer protocol, we first evolved a set of Virtual "wild types" (WTs). Instead of optimizing these WTs solely for high growth rates, we here mimic natural circumstances by fluctuating resource conditions (Figure 2A). When too little resource is available, the Virtual Microbes cannot grow. When too much resource is available however, the Virtual Microbes run the risk of accumulating too high concentrations of metabolites, resulting in increased death rates due to toxicity. To avoid extinction, we divided the total grid (40x40) into four sub-grids (20x20). In these sub-grids, the two resource metabolites A and C (Figure 1A) change in their influx rates with probability 0.01. Both these resources can be converted into the purple building blocks required for growth. Maximally flourishing Virtual Microbes live on average 100 time steps. Thus, a healthy Virtual Microbe experiences on average one fluctuation in resource conditions in its lifetime (see full configuration in S1). As the rates of influx span four orders of magnitude, conditions will vary from very favourable to very poor. This in turn depends on which resources the evolved Virtual Microbes like to consume (and at which rate), whether or not there is too much or too little resource, and whether or not space for reproduction is available. All in all, this results in an unsupervised evolutionary process where there is no prior expectation of what metabolic strategy or gene regulatory networks might be best suited to survive. In other words: fitness is not a priori defined.

Using this protocol, we evolved the same initial clone in the exact same "random" resource fluctuations, *only* varying the mutations that happened across ~10.000 generations of evolution. This produces 16 distinct WTs with their own evolutionary history, which we then expose to the serial transfer protocol (Figure 2B). Despite experiencing precicely the same fluctuations, no two WTs evolved to be precisely the same. For example, we observe a great diversity in gene content, kinetic parameters of enzymes, gene regulatory networks and their complexity, and responses to environmental stimuli. The core metabolism is however strikingly similar across WTs, always consisting of a simple metabolic cycle. The rates of building block production and death rates are also very similar across all WTs (Figure S3). In other words, it appears that there are many different ways to be fit, and that no solution is

evidently better. The similarities and differences between our WTs are summarized in Figure 2C, but we discuss this in more detail in Supplementary Section 1.

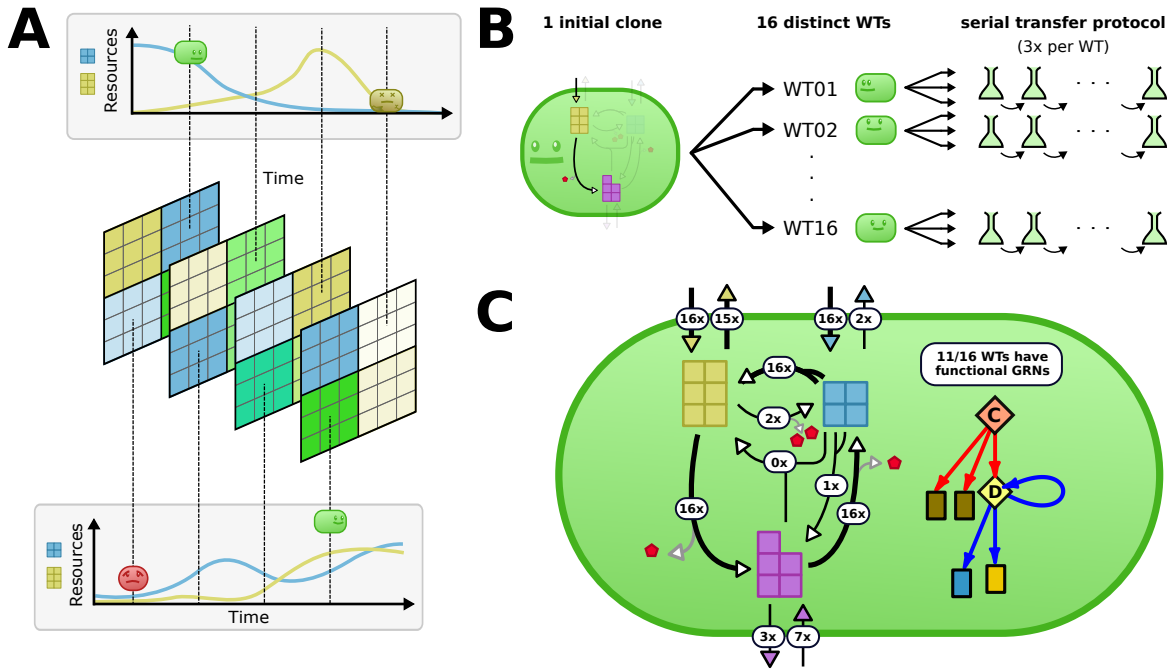

Fig 2. Evolution of Virtual "wild types" under naturally unpredictable and fluctuating resource conditions
A) Natural evolution is mimicked by (harsly) fluctuating resource conditions, resulting in a wide variety of resource conditions. The (actual) grid is 40x40, with four 20x20 subspaces where the rates of influx vary stochastically. These subspaces do not impede diffusion of metabolites or reproduction. The fluctuations of the A and C resource (blue and yellow respectively) are independent, resulting in a variety of different conditions. Building blocks (purple) must be synthesized for growth and protein expression.
B) We repeat the evolution in natural conditions 16 times starting from the same (minimally viable) initial clone (varying the mutations that happen) yielding 16 distinct WTs. These WTs are later transfered to a serial transfer protocol
C) In the white labels we how many of the evolved WTs evolved to use particular reactions. The thicker arrows represent the shared core genome which consists of two resource importers, a metabolic cycle, and a C-exporter. Transcription factors (diamonds) were always present across WTs, but only 11/16 WTs visibly display changes in gene expression correlated with changes in the environment.

Long-term evolution experiment *in silico*

After evolving a variety of different WTs, we mimic a serial transfer protocol like that of the LTEE. With regular intervals, all but 10 percent of the cells are removed, while at the same time refreshing the medium. Although time in Virtual Microbes has arbitrary units, we will refer to this process as the "daily" cycle from this point forward. Early in the day, during the log phase, high growth rates are very rewarding as there is a lot of opportunity to reproduce. However, once the population has reached stationary phase (having consumed all resources), it is favourable to survive and to not invest in growth any further. We will focus on how our WTs adapt to these alternating selection pressures. The results discussed here are found for a variety of different medium conditions (*e.g.* also see Table S2). We here present the 50 time step serial transfer protocol where the medium contained both the A- and C- resource, as this was a condition on which all WTs could be cultivated, ensuring equal treatment. We focus on the generic features of the adaptation towards this protocol, and how specific WTs and contingent factors from their evolutionary history shape these outcomes.

All wild types evolve to anticipate the serial transfer protocol After 800 days of evolving in a serial transfer protocol, we compare the ancestral WTs with the evolved populations. A first interesting observation is an increase in average cell volume during the log phase, which is also one of the first results from the LTEE [24]. We here further study the dynamics of average cell volumes. In Virtual Microbes, cell volume determines the ability to divide (minimal division volume) and survive (minimal viable volume). Interestingly, we find that the dynamics of evolved cell volumes fall into two possible strategies, which are illustrated in Figure 3A-B. Both outcomes show improvements in growth rates, but they clearly differ with respect to their daily yield. In the high yield scenario, cell volumes are maintained above the division volume until the very end of the day, whereas the low yield scenario leads to a transfer volume that is just above minimal. While the transfer volumes across (unevolved) ancestral WTs are mostly high, the evolved cells appear to be either ready to divide at transfer (high yield) or very small (low yield), with very few intermediates (Figure 3C). Despite the differences in daily yield, the cell volumes always show a pronounced decrease nearing the end of the day, suggesting deleterious effects on survival. Indeed, if we expose the populations to prolonged starvation by extending the day, the evolved populations die shortly after the anticipated serial transfer, while their WT ancestors survived for much longer (Figure 3A-B, right-hand side). When the extended yield (the total biomass that was generated after prolonged starvation) is measured, it shows a consistent decrease across all evolved populations (Figure 3D) relative to the WTs, as it is now masked from natural selection. In short, we have shown that all the different WTs learn to anticipate the regularity of the daily serial transfer protocol. We found the same anticipation effects when the transfers were done every 25 or 75 time steps (Figure S5, Table S2), suggesting that the key selection pressure in a serial transfer protocol is a trade-off between growing as fast as possible and remaining viable until the next day.

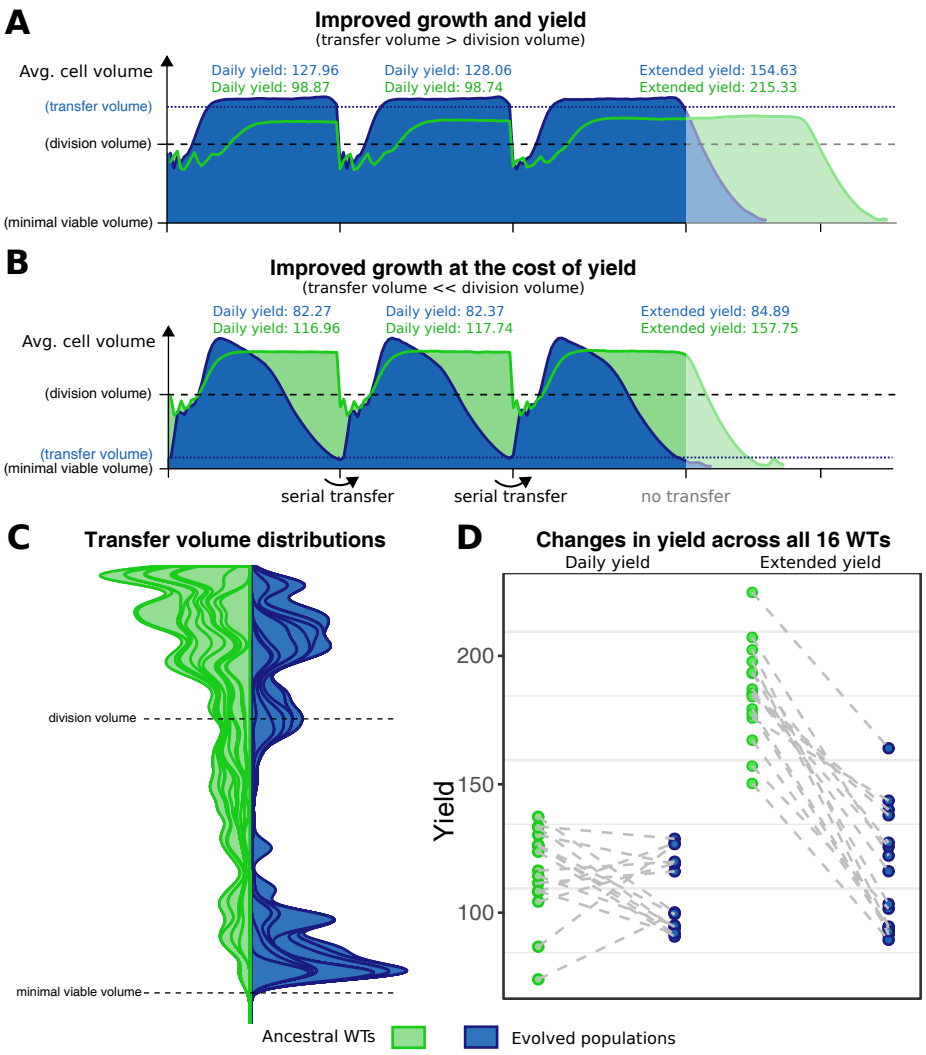

Fig 3. Virtual Microbes adapt to anticipate the regularity of a serial transfer protocol
A-B) Two WTs (green) and the population after prolonged evolution in the serial transfer protocol (blue) are shown as an illustration of the anticipation effects. Over the course of 3 cycles, the average cell volume is plotted against time for the ancestral WT (green) and for the evolved population (blue). The y-axis (cell volume) indicates the minimal viable volume and division volume (which are fixed for the model), and the evolved transfer volume (as measured at the end of the third cycle). Daily and extended yield are measured as defined in the method section. After the third cycle, serial transfer is stopped (transparent area).
C) Stacked density distributions are plotted for the transfer volumes both early (transfer 0-40, green) and late (transfer 760-800, blue).
D) The evolved changes in yield both "daily" (within one cycle of the protocol) and "extended" (after prolonged starvation) for all 16 WTs.

WTs have distinct trajectories toward a growth-yield trade-off The two extreme categories of cell volume dynamics from Figure 3 suggest a trade-off between growth and yield. We next investigate how our different WTs evolve towards this trade-off, and how reproducible these trajectories are. For this, we repeated the serial transfer protocol 3 times for each WT, and follow the trajectories over time. After ∼800 serial transfers, all populations have adapted along a trade-off between growth and yield (Figure 4A, $p << 10e\text{-}16$, $R^2 = 0.54$). This trade-off was not observed during the first cycle of the protocol, which instead shows a positive correlation between growth and yield (Figure 4B, $p << 10e\text{-}5$, $R^2 = 0.32$). Most WTs predictably evolve towards the trade-off by improving both growth and yield (*e.g.* by importing more resources, or producing more building blocks), but subsequent evolution is very WT-specific. Many WTs maintain high yield (*e.g.* Figure 4C), but others predictably trade off yield for a higher growth rate (Figure 4D). For instance, importing even more resources can improve growth even further, but leads to prolonged starvation and/or toxicity. Lastly, some WTs are showing variable trajectories after having arrived at the

trade-off (Figure 4E), which suggests that — for these populations — further evolution along the trade-off is neutral (All the 16x3 trajectories are shown in Figure S6). Finally, note how few WTs evolve intermediate growth rates and yield, which parallels the seperation between minimal transfer volumes and dividable transfer volumes depicted in Figure 3C. Taken together, these results illustrate how prior adaptations strongly shape the way subsequent evolution plays out. Evidently, specific WTs more readily give rise to certain solutions, having specific adaptations in their "mutational neighbourhood". This is also illustrated by two WTs that repeatedly gave rise to mutants with extremely high, but unsustainable growth rates, causing multiple populations to go extinct (black crosses in Figure 4). In summary, some WTs adapt predictably to the serial transfer protocol, while others have diverging evolutionary trajectories and can reach different solutions. The consistency of WTs in combination with the diversity of trajectories illustrates how prior evolution can bias — but not necessarily constrain — subsequent evolution.

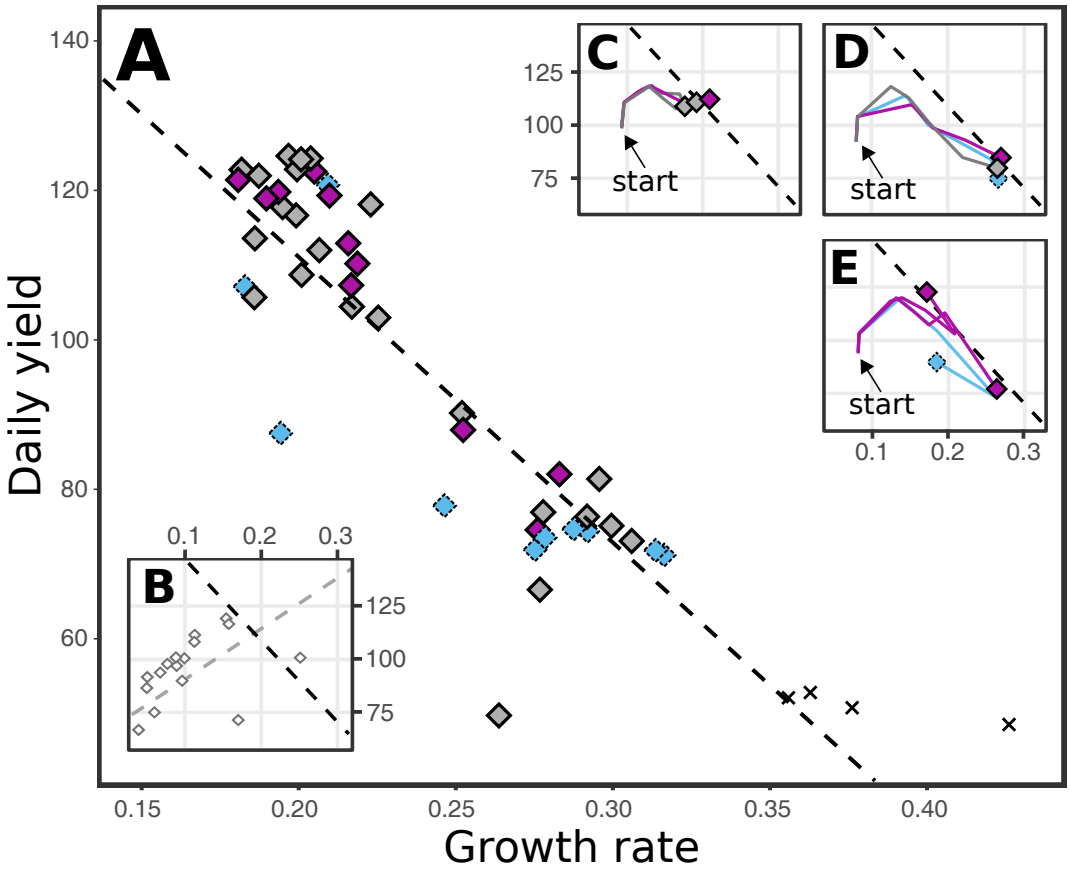

Fig 4. Prior evolution determines determines trajectories of adaptation to a serial transfer protocol
A) Growth rate (average building block production during log phase) is plotted against daily yield (average population biomass within a single cycle), for all the 48 experiments after adaptation to 800 serial transfers. The black dotted line is a linear regression model ($p << 10e\text{-}16$, $R^2 = 0.54$).
B) Shows the initial points for all 16 WTs, which actually have a positive correlation between growth and yield ($p << 10e\text{-}5$, $R^2 = 0.32$) instead of the negative correlation (black dotted line).
C-E) These insets display how the repeated evolution of most WTs produces similar trajectories towards the trade-off (time points are day 0, 20, 40, 100, 200 and 800), ending in either high daily yield (C) or low daily yield (D). A minority of WTs diverge after reaching the trade-off, and thus show more diverse trajectories when repeated (E). The colours of the end point symbols depict different modes of adaptation as discussed in the next paragraph (grey = no coexistence, blue = quasi-stable coexistence, purple = stable balanced polymorphisms, black cross = extinction).

Balanced polymorphisms repeatedly evolve in many different WTs So far we have only looked at population averages. Next, we study the dynamics of lineages and the evolved dynamics within cells. To track lineages we tag each individual in the population with a neutral lineage marker at the start of the experiment (analogous to DNA barcoding). When a single lineage reaches fixation, we reapply these neutral markers, allowing us

to quickly detect long-term coexistence. Moreover, these neutral markers also allow us to study which arising mutations are beneficial in different phases of the growth cycle. In Figure 5A we show dynamics where neutral lineage markers were frequently redistributed, indicating that there is no long-term coexistence of strains. Such coalescence events happen because a fitter strain takes over the entire population, or at a neutral rate due to genetic drift. In contrast, figure 5B displays a repeatedly observed quasi-stable coexistence, where two lineages coexist for some time, but coexistence was not stable in the long-term. Lastly, Figure 5C shows stable, long-term coexistence, where two lineages coexisted until the end of the experiment. Coexistence (either quasi-stable or stable) was observed in 21 out of 44 extant populations (Figure 5D).

By zooming in on the dynamics of coexisting lineage markers over a shorter time span (Figure 5B-C, right-hand side), we can better understand how these lineages stably coexist. Notably, one lineage is dominating during log phase, while the other lineage performs better during stationary phase. In other words, the lineages have specialized on their own temporal niche. We find that these dynamics can be the result of three mechanisms (or combinations thereof): 1) cross feeding on building blocks, 2) specialisation on resource A or C, 3) based on the growth vs. yield trade-off. While for cross feeding interactions the dependency is unidirectional, we found that lineages which diverged on the basis of the latter two mechanism often perform better together (Figure S8). This happens because these lineages have increased toxicity and/or experience prolonged starvation when only competing with their own kind, which is otherwise prevented by the presence of the other strain. The lineages thus appear to have adapted their fluxes to the presence of the other, which are now effectively a part of their environment. In accordance with these dynamics, invading mutants can have rapid increases early in the cycle, but decrease to much lower frequencies during the stationary phase (Figure S7A). Other mutants can however gradually increase in frequency all throughout the cycle Figure S7B), revealing that the nature of these dynamics depend on the specific type of mutation and the genetic background of the WT (*e.g.* cross feeding on building blocks is only possible if the ancestral WT had the necessary importer, which was true only for 7/16 WTs). In short, different types of coexistence emerge in a serial transfer protocol, based on a balancing effect where the abundance of one lineage favours the other and vice versa.

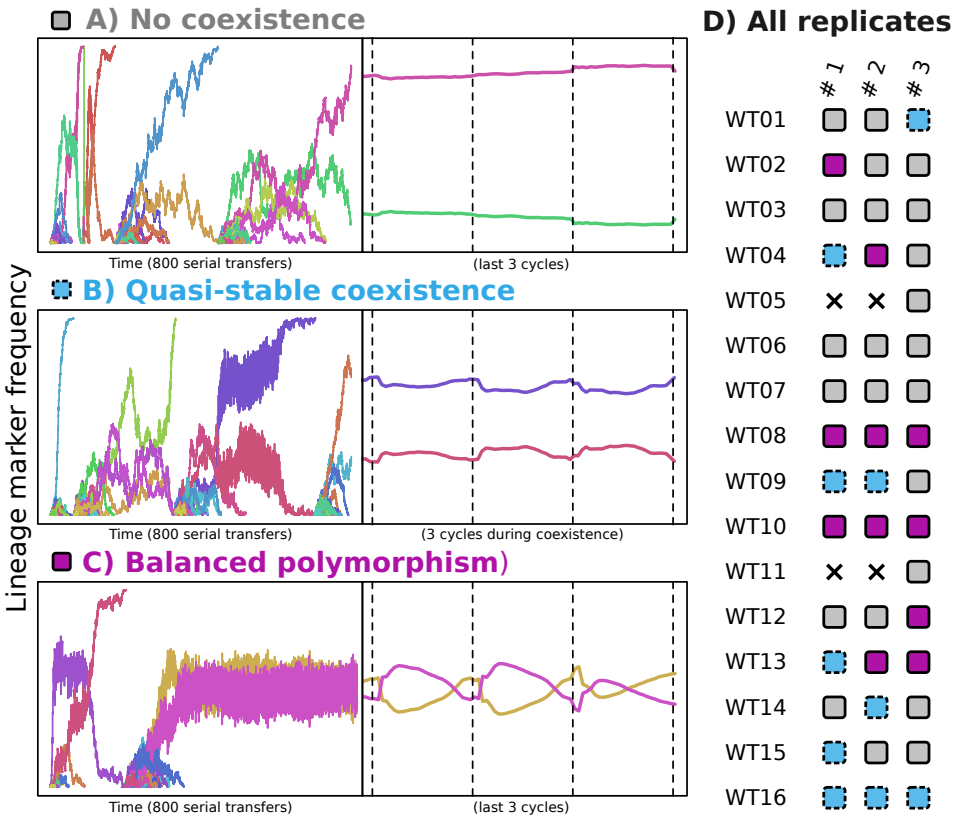

Fig 5. Dynamics of neutral lineage markers reveal balanced polymorphisms
A-C) Neutral lineage markers (random colours) frequencies are plotted along 800 serial transfers (left hand side) and along 3 cycles. Panel A shows an example with no coexistence which is found in 23 out of 44 replicates, and panel B and C show (quasi-)stable coexistence, found in the remaining 21 replicates.
D) shows these 3 possible outcomes for all 3 replicates of 16 WTs (grey = no coexistence, blue = quasi-stable coexistence, purple = stable balanced polymorphisms, black cross = extinction) Four replicates went extinct during the serial transfer experiment due to over-exploiting of the medium (black crosses).

Single lineage anticipation by tuning and trimming the gene regulatory network The previous section illustrates how multiple lineages can coexist because the predictable serial transfer protocol produces temporal niches. However, many of our WTs do not show any tendency to speciate like this, and instead always adapt to the serial transfer protocol as a single lineage (Figure 5D). In order to better understand this, we will now look at the intracellular dynamics of WT07, and how it changes when adapting to the protocol. WT07 is one of the more "clever" WTs with a relatively complex GRN, and displays strong responses in gene expression when exposed to "natural" fluctuations. In Figure 6 we show that WT07 consistently adapts to the protocol by switching between two modes of metabolism, where importer proteins are primed and ready at the beginning of the cycle, and exporter proteins and anabolic enzymes are suppressed during stationary phase. Despite some differences in the evolved GRNs, the evolved protein allocation patterns are virtually indistinguishable across the three replicates. Interestingly, although no parallel changes were observed in the kinetic parameters of proteins, we do observe the parallel loss of a energy-sensing transcription factor as well as increased sensitivity of the TF that senses the external resource C. In other words, evolution apparently happened mostly through loss, and tuning and trimming of the GRN. Modulation between two metabolic modes allows this single lineage to switch between log and stationary phase, occupying both temporal niches. Indeed, a second lineage never appeared for this WT (5 and Table S2).

Strikingly, a GRN does not necessarily lead to a single lineage adaptation. For example, another regulating wild type (WT13) repeatedly evolved into multiple coexisting lineages, while maintaining the ability to regulate gene expression. *Vice versa*, non-regulating wild types (WT01 and WT15) also evolved single-lineage anticipation. Hence, even though the GRN of WT07 has a major impact on the repeatability of single-lineage adaptation (as illustrated in Figure 6), the presence of a functional GRN is neither sufficient nor necessary for single lineage adaptation.

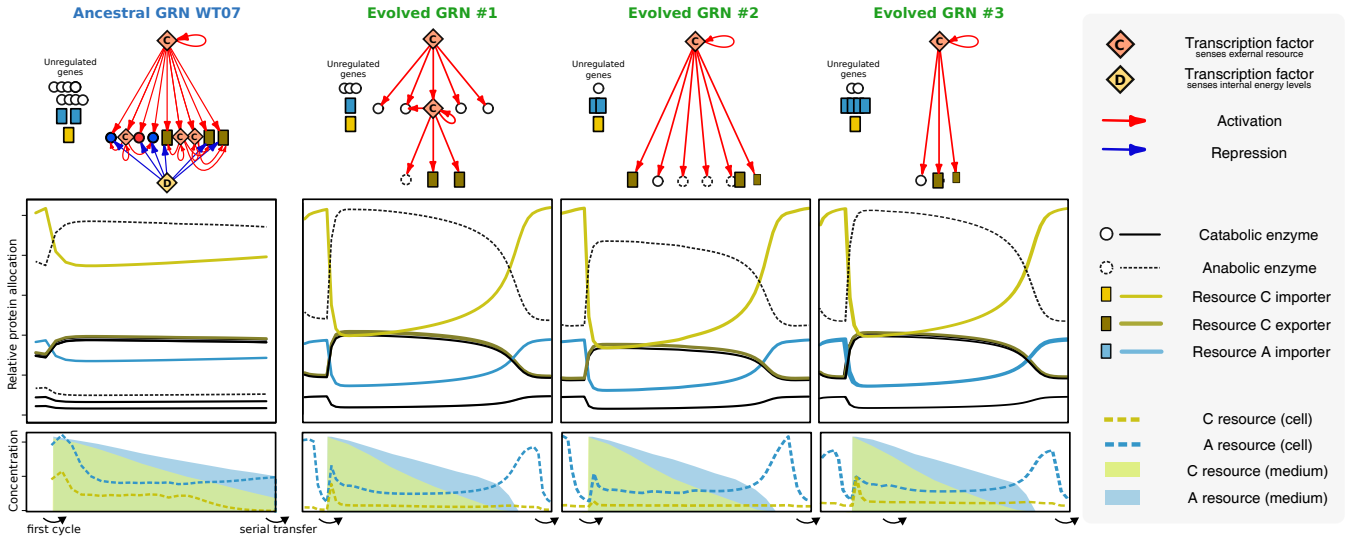

Fig 6. Consistent single-lineage adaptation through trimming and tuning of the gene regulatory network The ancestral gene regulatory network (GRN), protein allocation dynamics, and resource concentrations are displayed for WT07 during the first cycle of the serial transfer protocol, and for the three replicated experiments of 400 serial transfers.

Discussion

In this study we have taken a serendipitous approach to study how microbes adapt to a serial transfer protocol, and to what extent this is determined by their evolutionary history. The Virtual Microbe modelling framework serves this goal by not explicitly defining the concept of fitness. Instead, it builds up biology from the bottom up by implementing basic biological features and their interactions. We observe that regardless of their evolutionary history, all WTs learn to anticipate the regularity of the serial transfer protocol by evolving a fine-tuned balance between high growth rate and yield. Long-term survival, which is now masked from natural selection, always deteriorates after prolonged exposure to such a protocol. We next show that, if the same WT is repeatedly evolved in a serial transfer protocol, it has similar trajectories towards a growth versus yield trade-off, but may subsequently diverge along it. Polymorphisms within populations are frequently observed, which can happen by means of cross-feeding interactions, resource specialisation, or growth vs. yield specialisation. We furthermore find that coexisting lineages are dependent on each other, as they would perform better in the presence of the other. In general, our results are robust to details in the serial transfer protocol, such as using only a single resource, or varying the interval between transfers (see Table S2). The anticipation effects, as well as the balanced polymorphisms, therefore appear to be generic features of microbes exposed to prolonged evolution in a serial transfer protocol. Moreover, the concept of microbial populations anticipating predictable changes has also been observed in previous *in silico* [25] and experimental studies [26]. Combined with diversification and bet hedging strategies, anticipation might well play an important role in natural populations, the details of which are yet to be elucidated [27].

How do our results map onto experimental evolution in the lab? *E. coli* has been subjected to a daily serial transfer protocol for over 30 years (∼70.000 generations) in the LTEE. Many of our observations are remarkably similar to the LTEE, such as the improved growth rate and cell sizes during the log phase [24], the (quasi-)stable dynamics of coexisting lineages [21], and "leapfrogging" dynamics (*e.g.* Figure 5A-B) where an abundant lineage is overtaken by another lineage before rising to fixation [28, 29]. The comparison with respect to the growth versus yield dynamics and the anticipation effects discussed in this work is however less straightforward. We have observed how all our WTs quickly evolve to be maximally efficient given our artificial chemistry, and only subsequently diverge along the apparent growth versus yield trade-off (see Figure 6). For *E. coli*, growth and yield have continued to improve so far, and although a trade-off has been observed *within* the populations [30], no growth versus yield trade-off between the replicate populations has been observed yet. Likewise, our interesting results on the evolution of anticipation could not be corroborated as of yet (T. Hindré and D. Schneider, personal communication, November 2018). Nevertheless, we propose that anticipation of periodic environmental change, and a growth versus yield trade-off, provide a fruitful search image for the future of the LTEE, and similar experimental studies.

Cross-feeding interactions are commonly observed in the LTEE [11, 18], and modeling has shown that this adaptive diversification involves character displacement and strong niche construction [19], and can furthermore strongly depend on the regularity of a serial transfer protocol [20]. While similar cross-feeding interactions are observed in some of our *in silico* experiments, we found that balanced polymorphisms could also involve one lineage with high growth rates during log phase and a slower growing lineage which performs better in stationary phase. This can happen by means of resource specialisation, or purely on the basis of a growth versus yield specialisation which does not require cross-feeding or cannibalism. While the resource specialisation is only relevant to experimental studies that use more than one carbon source, the growth versus yield diversification also happens on a single resource (Table S2). Indeed, other studies have also suggested the importance of these dynamics, such as the coexistence of respiratory and fermenting strains in *Saccharomyces cerevisiae* [31] in a chemostat, and the presence of multiple selection pressures in a mathematical model of a serial transfer protocol [32]. Our findings show that these dynamics can emerge in a more complex eco-evolutionary setting. It however remains to be seen if such diversification happens in experiments such as the LTEE. Earlier work on the LTEE has shown that, although no significant negative correlation was found for an evolutionary trade-off of growth and yield, isolated clones from *within* a population do display a negative correlation [30], suggestive for the dynamics we observed in Virtual Microbes. With a great deal of polymorphisms in the LTEE left unexplained so far [21], we thus suggest this as a search image for future evolutionary experiments.

The dynamics of Virtual Microbes expose that even a simple serial transfer protocol entails much more than

sequentially evolving higher and higher growth rates. Instead, adaptation is an eco-evolutionary process that strongly depends on prior evolution, timescales, the presence of other competitors and mutants, and transient fitness effects. In accordance with these dynamics, temporal positive selection for certain alleles can be inferred from a metagenomics study on the LTEE [21]. Other recent studies have also discussed the important of eco-evolutionary dynamics [33], and how this can readily give rise to coexistence of multiple strains [34, 35]. In light of these shifting and emergent selection pressures, inferring "fitness" from competition experiments could be highly misleading. The fact that metagenomics have revealed much more diversity in the LTEE than previously anticipated [21], makes the subject more complex, and of course more intriguing, to study.

In conclusion, we have studied how *in silico* WTs of Virtual Microbes adapt to a serial transfer protocol. A fine-tuned balance between growth and yield causes an accurate anticipation of this protocol, which can happen in many ways. Furthermore, multiple lineages and dependencies can arise on the basis of the growth versus yield trade-off. Taken together, our results reveal important insights into the dynamics and relevant selection pressures in experimental evolution, advancing our understanding of the eco-evolutionary dynamics of microbes.

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Author contributions

B.D. performed simulations and provided the data. Results were analysed and interpreted by all authors alike. B.D. wrote the manuscript with input from P.H., J.M., and T.C. alike. P.H. supervised the project.

Code availability

The full python module of Virtual Microbes is publicly available via PyPi. The code is available online on `bitbucket.org/thocu/virtual-microbes`. Further help with installation, instructions on how to use Virtual Microbes, and full documentation of the methods, is available on `www.virtualmicrobes.com`

Data availability

The data that support this study are available from the author upon reasonable request.

Competing interests

The authors declares no competing financial interests.

Methods

Why Virtual Microbes?

Experimental evolution is, of course, done on organisms that have evolved for a long time under a wide variety of conditions. These modeling organisms all have their own evolutionary history, and differences in how they deal with starvation, stress, changes in resource etc. With Virtual Microbes we are able to evolve a *de novo* set of "wild types" (WTs), adapted to live in such severely fluctuating resource conditions. We can then explore how these WTs adapt to experimental evolution, and find generic patterns of evolution.

Model overview

Virtual Microbes metabolise, grow and divide on a spatial grid (Figure 1C). Here, we use two parallel 40x40 grids with wrapped boundary conditions. One grid contains the Virtual Microbes and empty grid-points, and the other describes the local environment in which the Virtual Microbes live. This environmental layer holds influxed metabolites, waste products of Virtual Microbes, and spilled metabolites from lysing cells (Figure 1B). In order to express proteins, grow, and maintain their cell size, Virtual Microbes must synthesize predefined metabolite(s), which we call building blocks. These building blocks are not directly provided, but must be synthesized by the Virtual Microbes by expressing the right proteins, allowing them to pump / convert metabolites into one another (Figure 1A). The expression of these proteins depends on genes on genomes that undergo a wide variety of possible mutations upon reproduction (Table 1). Genomes are circular lists of genes, each with their own unique properties (*e.g.* K_m, V_{max} for enzymes, K_{ligand} and binding motif for TFs). The level of expression is unique for each gene, and is determined by its evolvable basal transcription rate and how this rate is modulated by transcription factors. When an enzyme or transporter gene is expressed, that specific reaction will take place within the cell that carries that gene. Note however that in the complete metabolic universe, many more possible reactions exist. The genome of an evolved Virtual Microbes will typically only use a subset of all the possible reactions. Proteins to catalyse new reactions and novel TFs can be discovered through rare events.

Note that unlike most evolutionary models, fitness is not explicitly defined. Both the rate of birth and death are dynamically defined, and evolvable properties for Virtual Microbes. Birth depends on the availability of empty space and resources to synthesize building blocks, whereas death depends on the ability to survive under a variety of different conditions and the potential accumulation (and avoidance) of toxicity. The resulting survival of the fittest (referred to as "competitive fitness" by Fragata *et al.*, 2018) is an emergent phenomenon of eco-evolutionary dynamics [36].

Experimental setup

Metabolic network and wild type evolution We use a very simple metabolic network with 2 resource metabolites, 1 building block metabolite, and an energy carrier (Figure 2A). We initialised 16 minimally viable Virtual Microbes, and evolved them for ∼10.000-15.000 generations in fluctuating resource conditions by applying random fluctuations of the influx rates for the A and the C resource. Because the rate of influx for the two resource metabolites fluctuates between very high (10^{-1}) and very low values (10^{-4}), conditions can be very poor, very rich, and/or potentially toxic. To avoid total extinction, we subdivided the 40x40 grid into four 20x20 subspaces, in which these fluctuations are independent (see Figure 2B). Note however that these subspaces do not impede diffusion and reproduction, but merely define the rate at which resources flux into different positions on the grid. In this study, the microbes do not migrate during their lifetime. These conditions aim to simulate natural resource fluctuations, evolving what we call "wild types" (WTs) of Virtual Microbes. (see supplement S1) The initial population consists of cells that have 3 enzymes, 3 pumps, and 5 transcription factors. All these proteins are randomly parameterized, meaning that these proteins are unlikely to have good binding affinities and catalytic rates. The amount of building block required to grow and produce protein is therefor very minimal in the early stages of evolution, and increases up to a fixed level as the Virtual Microbes improve.

***In silico* serial transfer protocol** We mimic a serial transfer protocol like that of the LTEE by taking our evolved WTs and – instead of fluctuating the resource conditions – periodically supplying a strong pulse of both the A- and the C-resource. While WTs are evolved in a spatial setting where resources flux in and out of the system, we here mix all cells and resources continuously and fully close the system, meaning no metabolites wash out. To apply strong bottlenecks while at the same time allowing for sufficient growth, we increased the size of the grid from 40x40 to 70x70. We dilute the approximately tenfold, transferring 500 cells to the next cycle.

While we used relatively high mutation rate for the evolution of WTs, we bring it back to a more reasonable number in the *in silico* LTEE. Furthermore, horizontal gene transfer was disabled to represent the asexual *Escherichia coli* B clone that is used in the LTEE [1].

Growth rate and yield measurements Yield was approximated by taking sum of all cell volumes, normalized by the maximum number of cells (i.o.w. the size of the grid). We measured yield both within a single serial transfer cycle ("daily yield"), and as the extended yield when we tested for long-term survival. As all WTs had slightly different log phases, we estimated the growth rates as the average building block production during the first half of the protocol.

Curating (quasi-)stable coexistence Coexistence of lineages was manually curated by looking at the dynamics of neutral lineage markers. When two neutral markers had relatively stable frequencies with a daily pattern as visualised in Figure 5C for at least 10.000 time steps (approximately 100 generations), it was scored as coexistence. When this persisted for a while, but later got lost, it was scored as quasi-stable. When two neutral markers had balanced frequencies for at least 10.000 time steps and this pattern lasted until the end of the 800 serial transfers, it was scored as stable. If neither happened, it was annotated at no coexistence.

Further configuration of Virtual Microbes Apart from the parameters within the confines of this article (Table 1), we have used the default settings for Virtual Microbes release 0.1.4, with the configuration files provided in Supplementary Section 2. Further details on the model and parametrisation are available online `https://bitbucket.org/thocu/virtual-microbes`

Table 1 - Types of mutations in Virtual Microbes and their probabilities
Mutation rates in WT evolution and serial transfer protocol (STP)

Mutation	**Description**	**Probability - WT**	**Probability - STP**
Duplication	A stretch of 1-n genes is duplicated	0.005	0.0015
Deletion	A stretch of 1-n or more genes is deleted	0.005	0.0015
Inversion	A stretch of 1-n or more genes is inverted	0.005	0.0015
Translocation	A stretch of 1-n or more genes is moved	0.005	0.0015
Stretch length (n)	Drawn from geometric distribution with p equals...	0.3	0.3
Gene discovery	Probability of discovering a new (randomly parameterised) gene.	0.0002	0.0000
Horizontal Gene Transfer	Probability of copying a gene from a cell closeby	0.002	0.000
Point mutation	Per gene probability of modifying a single parameter (prom strength, Michaelis Menten constants, etc.)	0.005	0.0015
Regulatory mutation	Per gene per generation probability of (partially) modifying the upstream binary operator sequence of a gene	0.005	0.0015
Other *in silico* serial transfer options	**Description**	**Value**	
Time steps of cycle	This represents, for example, the 24 hour serial transfer protocol of the LTEE	50 (AUT)	
[A] at beginning of cycle	Amount of resource A given at the beginning of the cycle	1.25	
[C] at beginning of cycle	Am mount of resource C given at the beginning of the cycle	1.25	
Extracellular metabolite outflux	Assuming metabolites cannot flux out of the experimental system	0.0	
Maximum population size	As defined by the size of the grid (70x70)	4900	
Number of cells serially transferred	A (near) tenfold dilution of cells	500	

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Supporting information

S1 - Evolution of Virtual Microbes wild types

Convergent and divergent evolution in Virtual Microbe wild types In the evolution of our WTs we observed strong convergence as well as divergence in the metabolic and gene regulatory networks that evolved. Because the evolved populations consist of a rich mix of different genotypes, we here describe the WTs by profiling the gene repertoires and GRNs at the end of the simulation (∼10.000 generations). For this, we took 20 (maximally unrelated) individuals from the evolved populations and determined the consensus metabolism (Figure S1A). While there is some diversity in the metabolic networks across WTs, the shared gene repertoire constitutes a metabolic network that forms a metabolic cycle complemented with resource importers and an exporter for the C metabolite (Figure S1B). We observed that the discovery of both the metabolic cycle as well as the exporter favour survival, as it coincides with an increase in population size and a decrease in the number of generations per time step (Figure S4). Note that in Virtual Microbes survival is improved by avoiding toxic effects of high metabolite concentrations and by only investing in growth when conditions are favourable for growth. The latter can be done via gene regulatory networks that respond to the quality of the environment, but we also found forms of metabolic regulation where microbes accurately fine-tuned kinetic parameters to automatically maintain homeostasis.

Although the shared gene repertoire from Figure S1B does not contain transcription factors (TFs), all of the 16 WTs have at least one type of TF. These TFs can constitutively repress or activate certain genes, or can respond to environmental conditions by binding to a ligand molecule. The latter response depends on the kinetic properties of the TFs and the properties of the genes which they regulate, all of which are evolvable (see Table S1). To get a better overview of how the WTs respond to environmental stimuli we therefore chose to directly measure the gene expression levels in a variety of different resource concentrations (displayed for 6 WTs in Figure S2). On the level of these GRNs, and their sensitivity the environment, we clearly see signs of strong divergent evolution. Note however, that the *effect* on the importer and exporter proteins seems very similar between WTs with different networks, showing that similar responses can be encoded by different GRNs. Finally, as seen in these graphs, some WTs have no response to environmental stimuli. We found that these non-regulating WTs are equally "fit", in that they have the same rates of building block production and death rates (see Figure S3). However, the majority (11/16) WTs evolved clear regulatory mechanisms.

In short, during the *de novo* evolution of Virtual Microbe WTs, some evolved features seem highly predictable. Namely, all have evolved the metabolic cycle, all express both resource importer proteins, and all but one WT have a C-exporter. On the other hand, regulatory mechanisms and some of the secondary reactions display considerable diversity. Note that this divergence cannot be explained by differences in initial conditions or fluctuations in resource concentrations, because the WTs only differ with respect to the mutations that have happened in their evolutionary history. However, as shown in the main text, these differences have a profound effect on further evolution.

Table S1 - Important parameters for TFs for an environmental response

Property TF	Description
Expression TF	The TF itself needs to be sufficiently expressed
Binding motif	The binary binding motif (10 bits) must have a sufficient match to the operator sequences of genes (50 bits) in order to affect their expression
K_{ligand}	If the binding constant to the ligand K_{ligand} is not in range of the observed concentration of metabolites, the TF will always have the same (up or down) regulatory effect, regardless of the environment or internal concentrations.
Effect of ligand	The ligand-bound and ligand-unbound regulatory effects of TFs need to be different to effectively change expression of genes given any environmental stimulus

Table S2 - Anticipation and polymorphisms are also observed when changing in the serial transfer protocol For different transfer times, dilutions, and resources concentrations, seven WTs (11-16, and WT07 from Figure 6 from the main text) have been tested for the anticipation effect and polymorphisms. Note that anticipation is not tested by prolonging the cycle (like in the main text), but by comparing the patterns in cell cycle dynamics with those from Figure 3 in the main text. If a clear decrease in cell volume was observed at the end of the cycle, it was scored as anticipation. Polymorphisms are scored as defined in the methods.

Shorter transfer time (25), 800 cycles		
WT	**Anticipation (Yes/No)**	**Polymorph (Yes,No,Quasi)**
11	Y	N
12	Y	N
13	Y	Q
14	Y	N
15	Y	Q
16	Y	Q
07	Y	N

Higher transfer time (75), ∼250 cycles		
WT	**Anticipation (Yes/No)**	**Polymorph (Yes,No,Quasi)**
11	Y	N
12	Y	N
13	Y	Y
14	Y	N
15	Y	N
16	Y	Q
07	Y	N

Single resource (A only), 800 cycles		
WT	**Anticipation (Yes/No)**	**Polymorph (Yes,No,Quasi)**
11	-	-
12	Y	N
13	Y	N
14	Y	Q
15	-	-
16	Y	Q
07	Y	N

Single resource (C only), 800 cycles		
WT	**Anticipation (Yes/No)**	**Polymorph (Yes,No,Quasi)**
11	-	-
12	Y	N
13	Y	Y
14	Y	N
15	Y	N
16	Y	Q
07	Y	N

S2 - Virtual Microbe configuration

The evolution of these WTs was done with Virtual Microbes version 0.1.4 which is publicly available as a Python package. Complete documentation on the methods is publicly available on `http://bitbucket.org/thocu/virtualmicrobes`. For this study, we used the configuration below. We removed options that are not relevant for reproducability (*e.g.* memory-limit, thread-count, data-storage-frequency etc.) or are default (*e.g.* universal-mutation-rate scaling=1.0) To reproduce these results with the newer versions of Virtual Microbes (0.2.4 as of January 2019), feel free to contact the corresponding author if help is required.

```
. virtualmicrobes.py @reggen.cfg - evo @reg-evo.cfg --name My_WT_Vmicrobe
```

reg-gen.cfg:

```
--base-death-rate 0.01
--mutation-rates
chrom_dup=0.0
chrom_del=0.0
chrom_fiss=0.0
chrom_fuse=0.0
point_mutation=0.005
tandem_dup=0.005
stretch_del=0.005
stretch_invert=0.005
stretch_translocate=0.005
stretch_exp_lambda=0.3
external_hgt=0.0002
internal_hgt=0.002
regulatory_mutation=0.005
reg_stretch_exp_lambda=0.1
--point-mutation-ratios
ligand_class=0.1
exporting=0.1
--rand-gene-params
base=10
lower=-1.0
upper=1.0
--mutation-param-space
base=10
lower=-0.5
upper=0.5
min=0.01
max=10.
--max-historic-max 0.1
--growth-rate-scaling 1
--competition-scaling 1
--selection-pressure historic_window_scaled
--historic-production-window 1000
--scale-prod-hist-to-pop
--small-mol-diff-const 0.02
--prot-degr-const 0.7
--transporter-membrane-occupancy .1
--influx-range
base=10
lower=-1.0
upper=-5.0
--fluctuate-frequencies 0,0.01
--init-external-conc 0.
--small-mol-ext-degr-const 1e-2
--bb-ext-degr-const 1e-1
--ene-ext-degr-const 5e-1
--transcription-cost 0.002
--energy-transcription-scaling 0.01
--spill-conc-factor 1.
--v-max-growth 1
--min-bind-score 0.85
--per-grid-cell-volume 8
--enzyme-volume-occupancy 3
--grid-sub-div row=2,col=2
--sub-env-part-influx 1.0
--grid-cols 40
--grid-rows 40
```

reg-evo.cfg:

```
--duration 1000000
--env-rand-seed 87
--reproduce-size-proportional
--cell-init-volume 1.5
--cell-growth-rate 2.0
--cell-shrink-rate 0.6
--cell-growth-cost 0.2
--cell-division-volume 2.
--init-prot-mol-conc .1
--max-cell-volume 5
--nr-resource-classes 3
--nr-energy-classes 1
--ene-energy-range 1,1
--res-energy-range 2,10
--nr-building-blocks 1
--building-block-stois 1,1
--nr-cell-building-blocks 1
--mol-per-ene-class 1
--mol-per-res-class 1
--nr-cat-reactions 3
--nr-ana-reactions 3
--max-nr-cat-products 2
--min-cat-energy 1,3
--max-nr-ana-products 1
--nr-ana-reactants 2
--chromosome-compositions tf=0,enz=1,pump=1
--binding-seq-len 10
--operator-seq-len 50
--prioritize-influxed-metabolism
--init-prot-mol-conc 0.01
--degradation-variance-shape 100
--no-toxicity-variance-shape
--toxicity 0.2
--toxic-building-blocks
--toxicity-scaling 1000
--tf-binding-cooperativity 2
--homeostatic-bb-scaling 1
--high-energy-bbs
--prioritize-energy-rich-influx
```

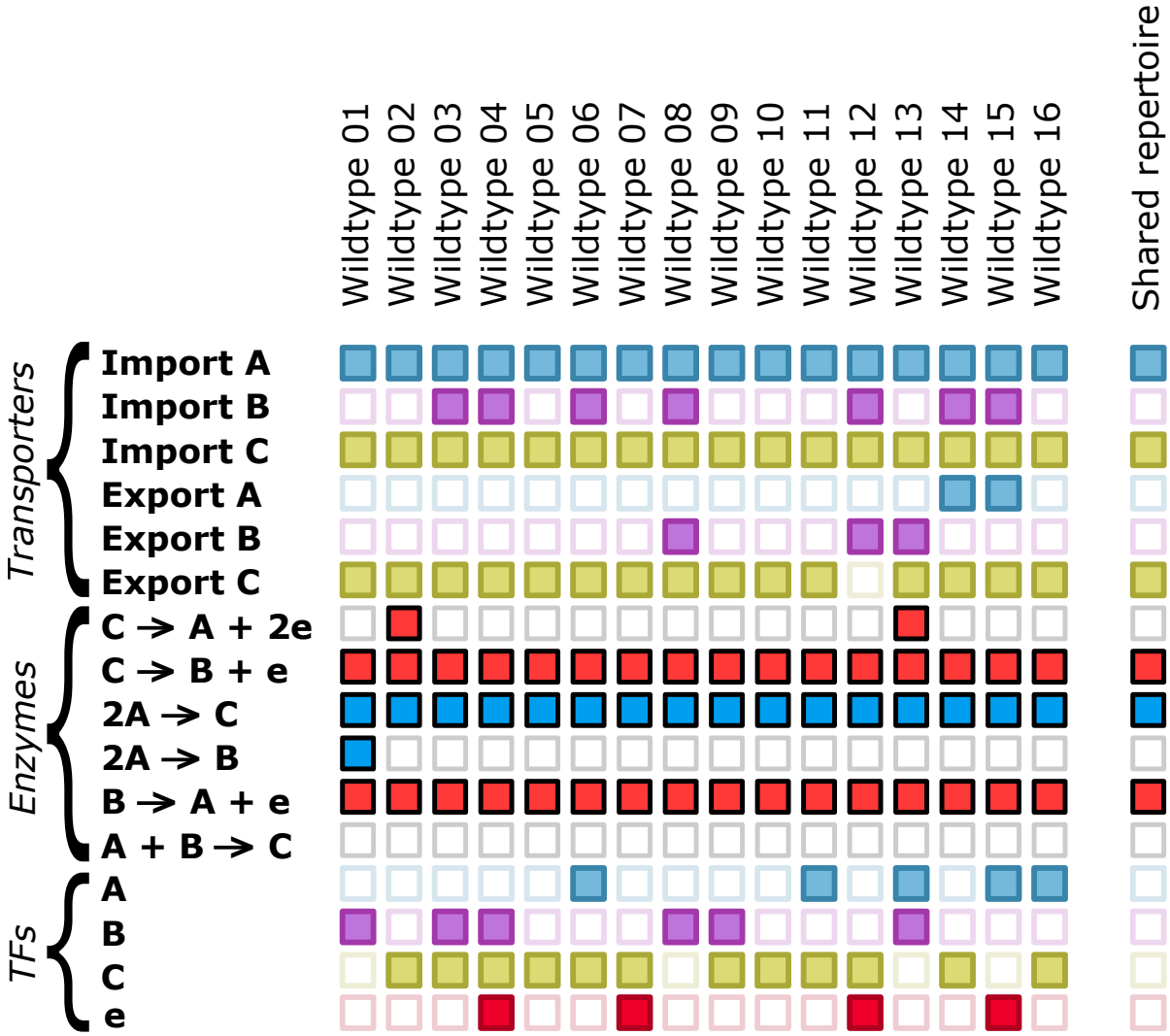

Fig. S1. The evolved gene repertoires for all 16 WTs The gene repertoires of WTs (20 maximally unrelated individuals) is displayed for all 16 replicate simulations after 1.000.000 time steps. Rows represent the different types of proteins (transporters, enzymes and TFs), and the columns the gene repertoires. Note that the presence of a gene does not imply it is functional, since properties such as K_s and V_max might be poorly parameterised. Genes found in less than 4 cells or genes with low concentrations (*i.e.* low expression) were omitted. The seperate column depicts the genes found in at least 90% of WTs.

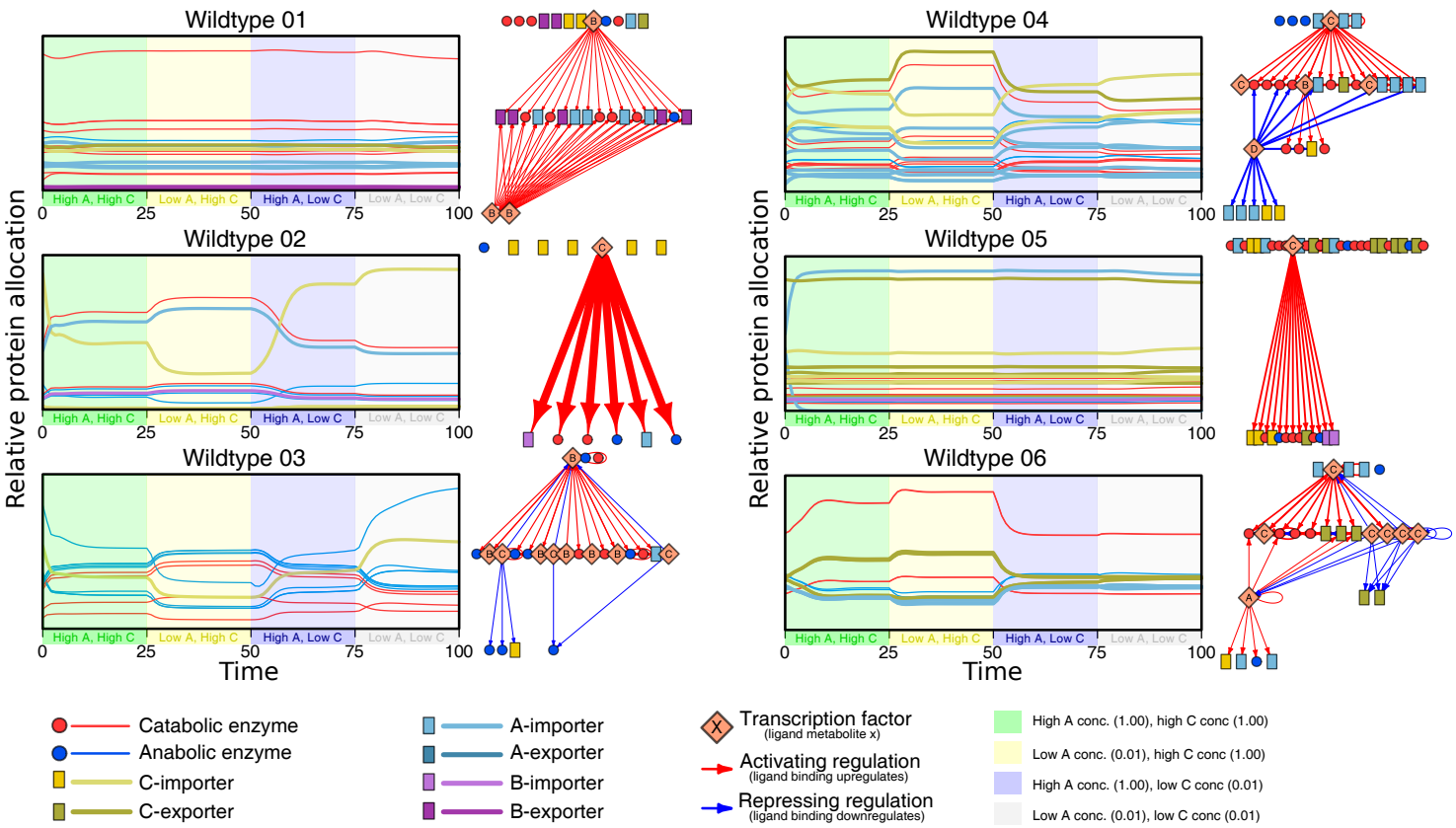

Fig. S2. WTs have great diversity of transcriptional regulation, and not all respond to changing resource concentrations The graphs shows how the protein allocation shift to imposed changes in resource concentration of the environment. Single clones from the WTs were taken and resource concentrations of the A- and C-resource were varied from low (0.01) to high (1.0). The gene regulatory network responsible for these changes is displayed next to each graph. The colours for different enzymes are as displayed in the legend. Thicker arrows in the gene regulatory network represent higher expression levels of the transcription factors. Genes with very low expression levels were omitted for clarity.

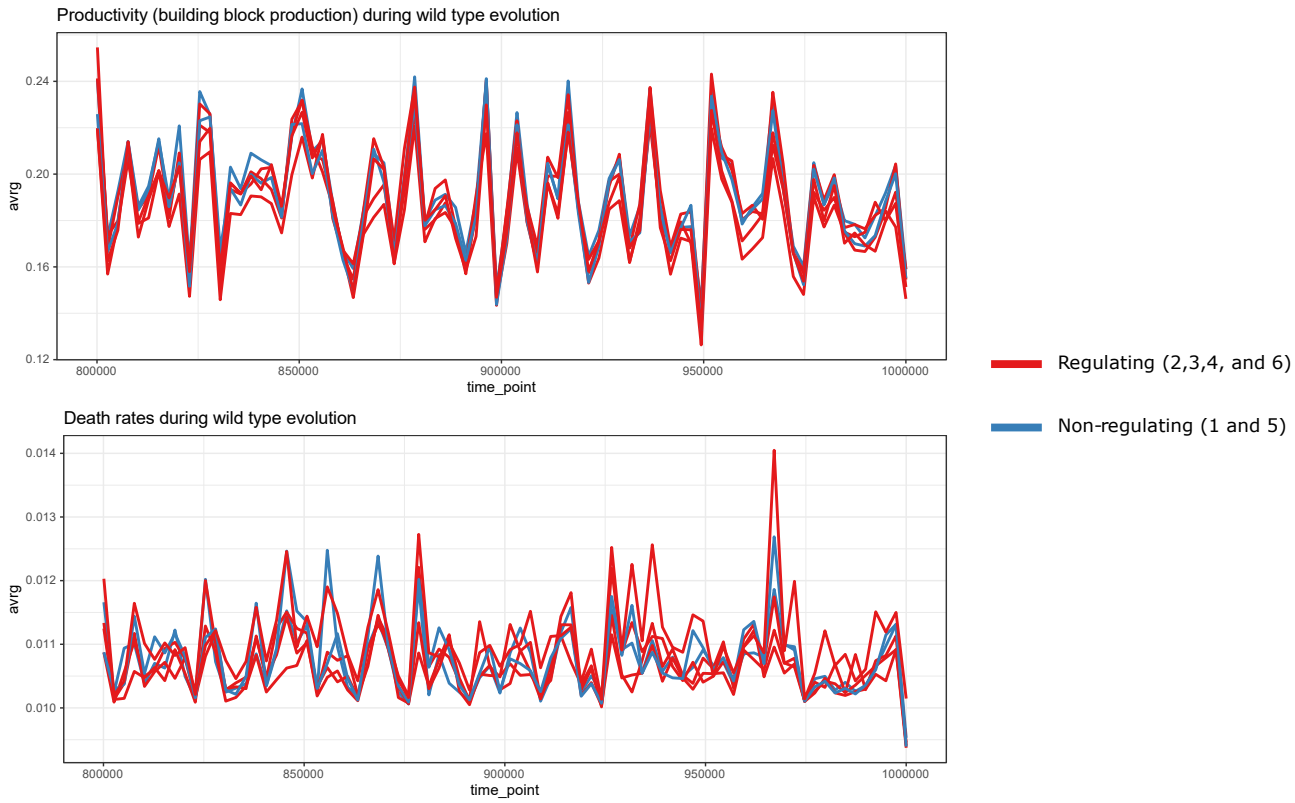

Fig. S3. Different WTs have similar building block production rates and death rates

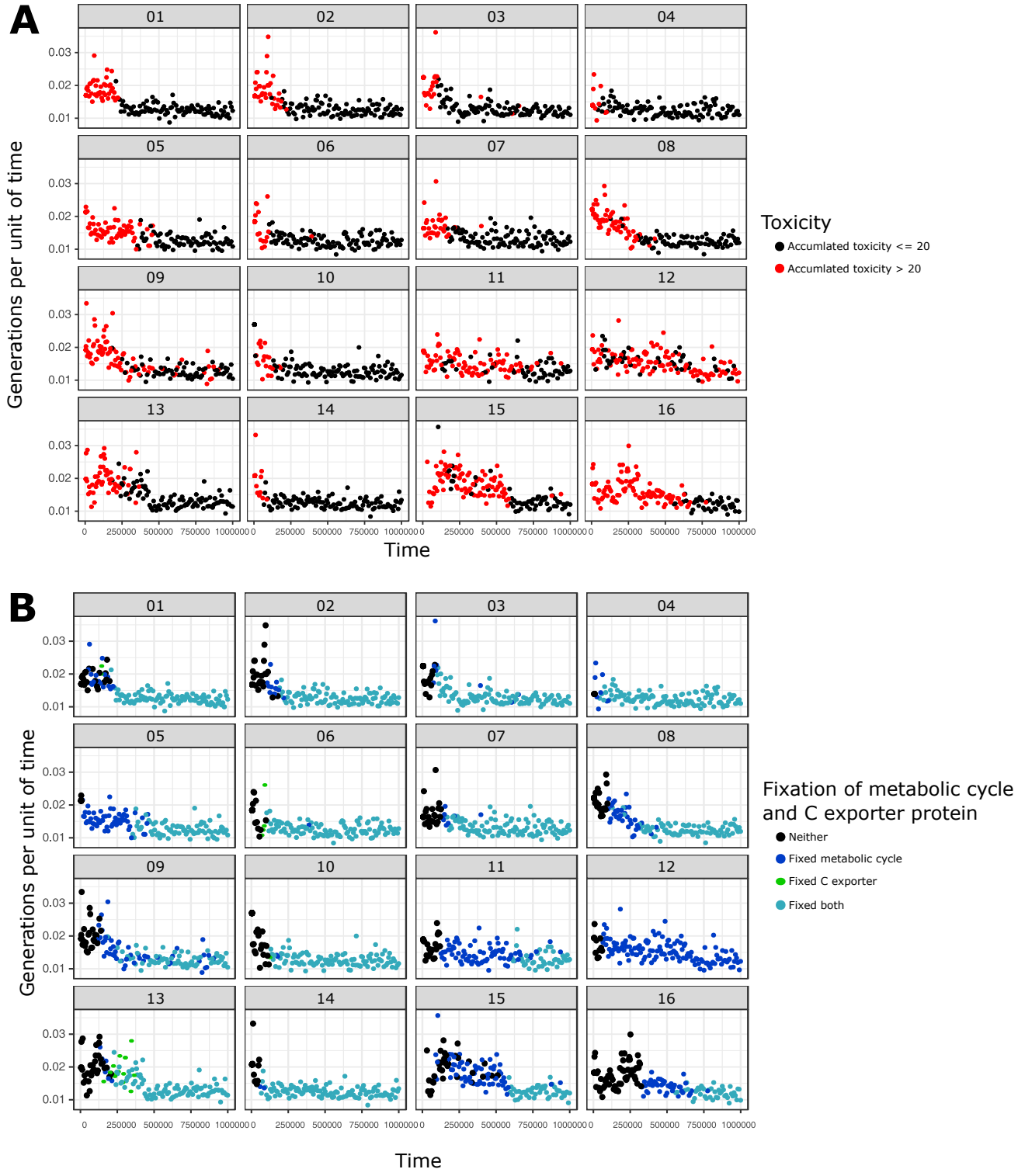

Fig. S4. Decrease in number of generations per time step for WTs coincides with a decrease in toxicity and/or fixation of a metabolic cycle Decrease in number of generations per time step coincides with a decrease in toxicity accumulation and/or fixation of a metabolic cycle. Every dot represents an average over a 100 generations of simulation. For panel A, red dots represent a toxicity level above which death rate is increased at least threefold (toxicity > 20), and black dots represent the lower toxic levels (toxicity $<= 20$). In panel B, the cyan dots represent the fixation of both the C-exporter and the metabolic cycle, green dots the fixation of only the C-exporter, blue dots the fixation of the metabolic cycle, and black dots the fixation of neither of these features.

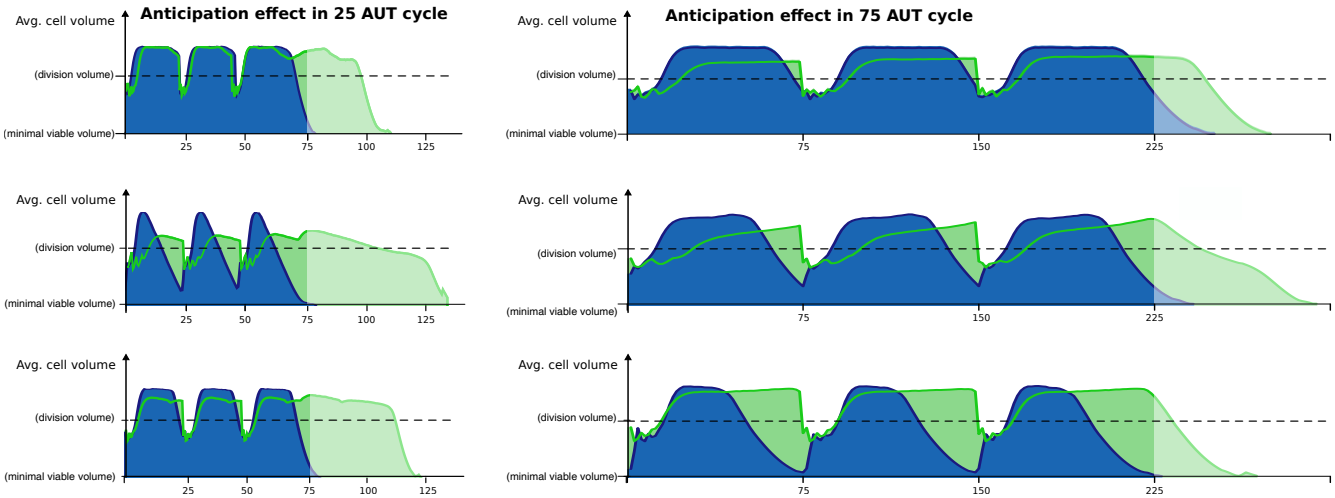

Fig. S5. The anticipation effect is present after evolving in shorter (left) and longer (right) time intervals

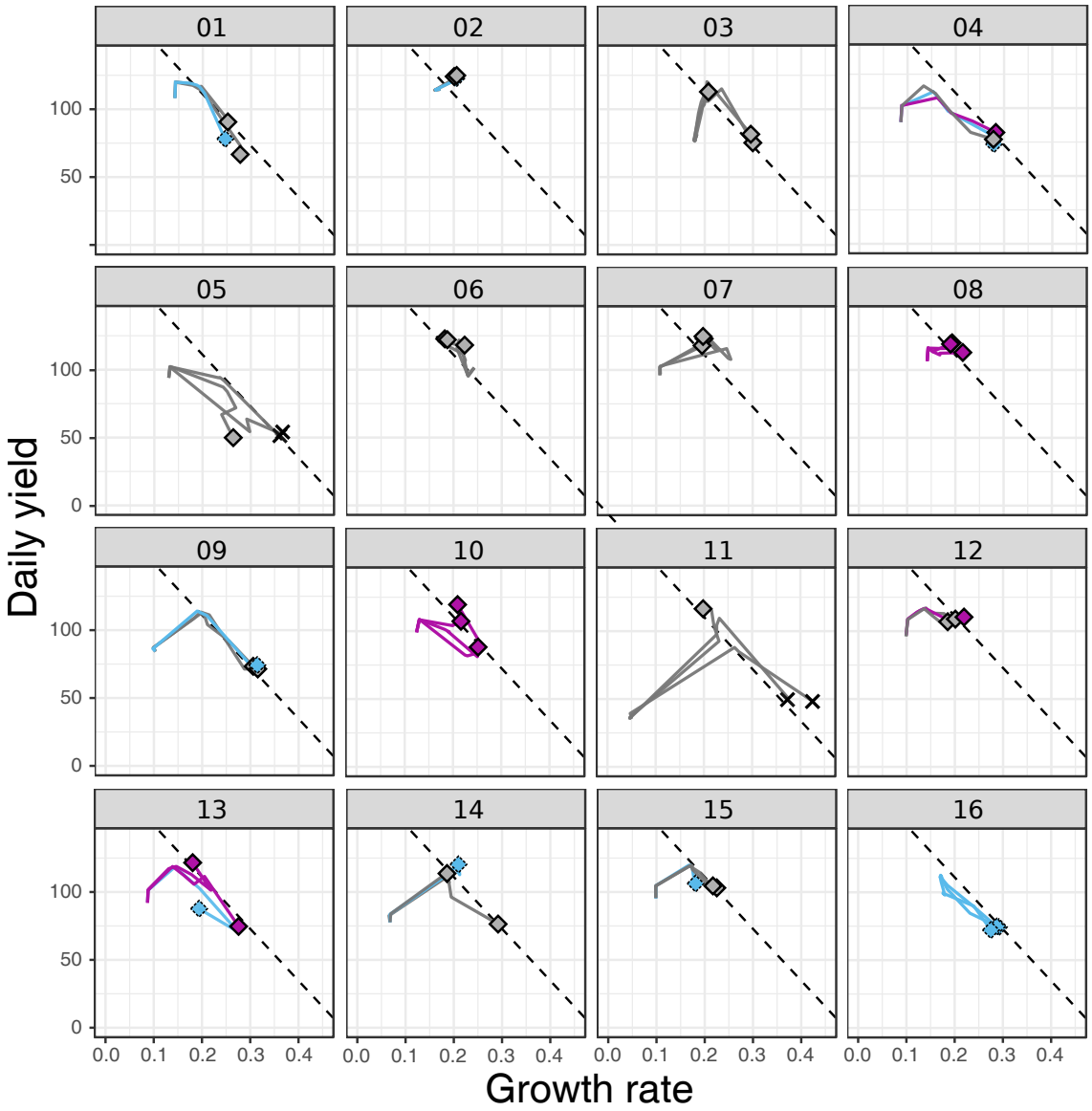

Fig. S6. Three replicate serial transfer experiments for all 16 WTs. This image is shows all the data from the examples from Figure 4.

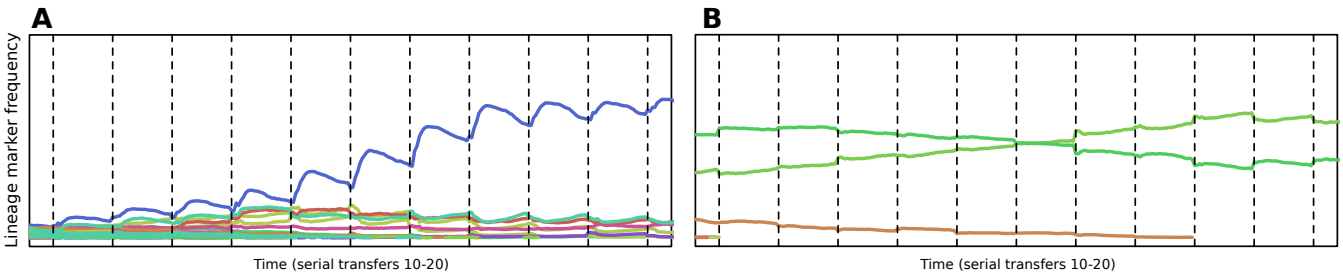

Fig. S7. Dynamics of different invading mutants A) Neutral lineage markers linked to mutations which cause high growth rates but poor survival increase in frequency early, but are losing in frequency during stationary phase. B) Neutral lineage markers linked to mutations that have higher growth rates without trading off against survival show no such temporal fitness effect.

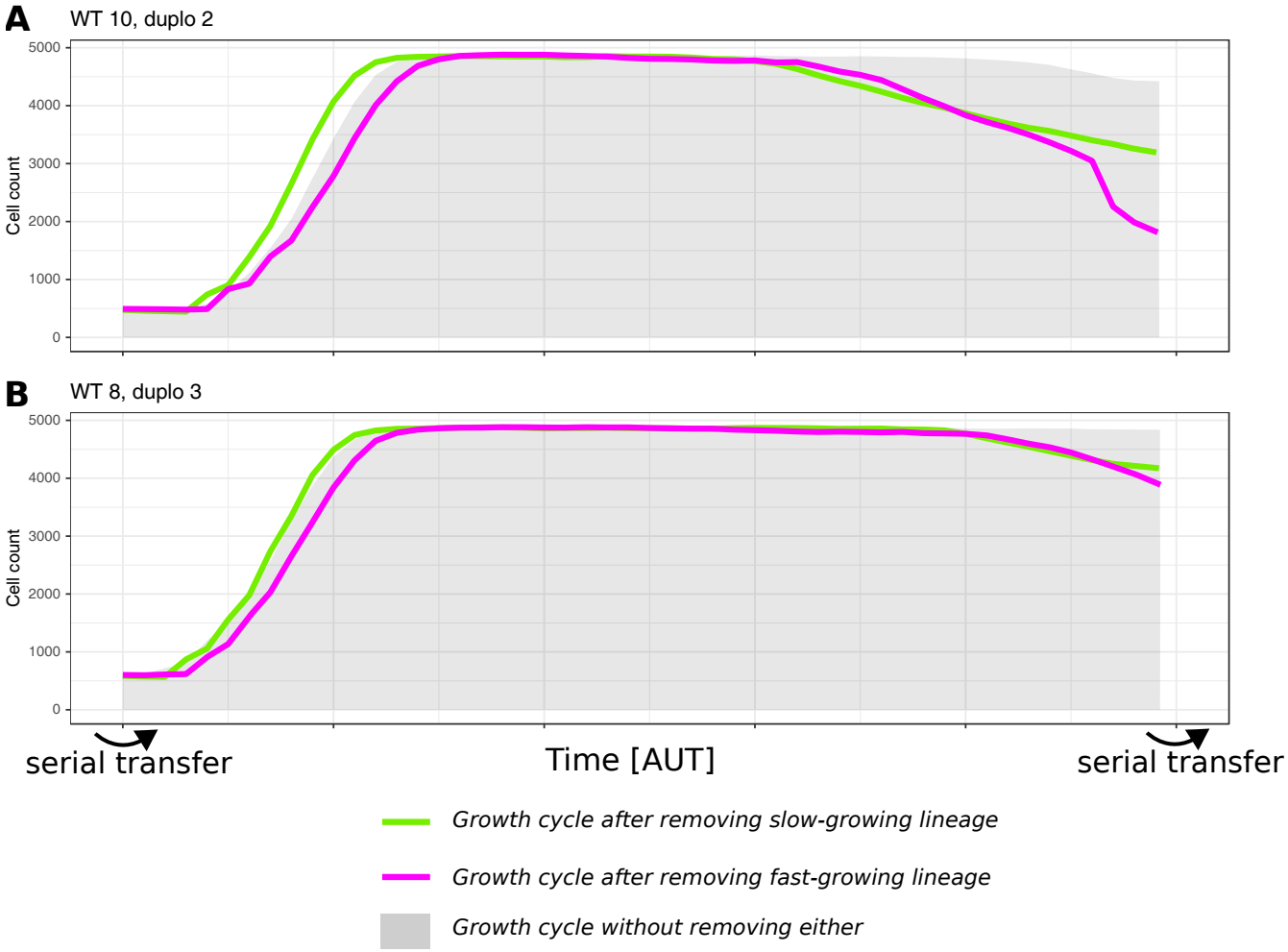

Fig. S8. Lineages based on resource specialisation (top) and growth vs. yield specialisation (bottom) perform better in the presence of the other In the upper panel, two resource specialists over-exploit their resource in the absence of the other. In the bottom panel, without evident resource specialisation, both lineages have increased toxicity (given that this happens through enzymatic changes, this is likely due to changes in fluxes), which accumulates to significant levels near the end of the cycle. This figure thus shows that lineages can grow dependent on one another, as they are part of each-others environment.