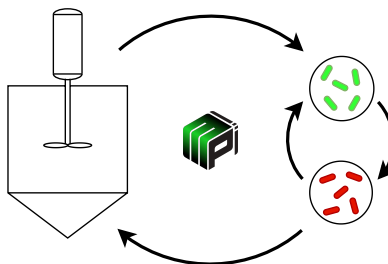


FITNESS COST RELATED TO NATURAL AND SYNTHETIC GENE CIRCUITS DRIVES COMPLEX BUT PREDICTABLE CELL POPULATION DYNAMICS

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Fitness cost related to natural and synthetic gene circuits drives complex but predictable cell population dynamics

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Abstract

Microbial populations are ubiquitous in our environment and are involved in a wide variety of applications, from food production to human health and bioproduction. However, they are often heterogeneous or unstable, with different phenotypes and genotypes coexisting in the same population and evolving over time.

The behaviour of these populations has been linked to the fitness of its constituent phenotypes and genotypes. This was observed in various systems, both natural, such as the sporulation system of *Bacillus subtilis*, and synthetic, such as *Escherichia coli* BL21 with a T7 production system. In this thesis, the links between fitness, heterogeneity and stability are explored and challenged with an additional synthetic toggle switch system, specifically designed to create a fitness cost without any other known biological interactions.

The various systems are studied in continuous culture with the help of automated flow cytometry, which allows for the measurement and control of the phenotypes of a microbial population in real time. In addition, several mathematical and computational tools are developed to analyse the data and study the underlying mechanisms of the population dynamics. The results show how the decreased fitness of some phenotypes can directly lead to both heterogeneity and instability, and why the result can be independent of the specific biological system, and caused instead by the interactions between the microbes and their environment.

Résumé

Les populations microbiennes sont omniprésentes dans notre environnement et interviennent dans une grande variété d'applications, de la production alimentaire à la santé humaine en passant par la bioproduction. Cependant, elles sont souvent hétérogènes ou instables, avec différents phénotypes et génotypes coexistant au sein d'une même population et évoluant au fil du temps.

Le comportement de ces populations a été associé à l'aptitude de leurs phénotypes et génotypes à survivre dans leur environnement. Ceci a été observé dans divers systèmes, tant naturels, comme le système de sporulation de *Bacillus subtilis*, que synthétiques, comme *E. coli* BL21 avec un système de production T7. Dans cette thèse, les liens entre aptitude de survie, hétérogénéité et stabilité sont explorés, notamment à l'aide d'un toggle switch génétique synthétique spécialement conçu pour créer une diminution d'aptitude sans aucune autre interaction biologique connue.

Les différents systèmes sont étudiés en culture continue à l'aide de cytométrie en flux automatisée, qui permet de mesurer et de contrôler les phénotypes d'une population microbienne en temps réel. En outre, plusieurs outils mathématiques et informatiques sont développés pour analyser les données et étudier les mécanismes menant aux dynamiques de population observées. Les résultats montrent comment la diminution de l'aptitude de survie de certains phénotypes peut directement conduire à l'hétérogénéité et à l'instabilité de la population indépendamment du système biologique étudié, et pourquoi les interactions entre les microbes et leur environnement sont déterminantes.

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A PhD is a path both walked alone and not. On the one hand, it was my project, my decisions and my work, but on the other hand, the project is inscribed within a team, with previous work established – standing on the shoulders of giants, as they say – and other projects advancing in parallel to mine, most of that work directly impacting my decisions. But the people are even more important than the work, both the ones I talked to every day, and the ones I didn't, random scientists in conferences, members of different departments I barely talked to, sometimes just books whose authors have shaped my views or professors from my master's degree. I will try my best to thank every group of people who helped me through this journey, but I did want to mention all the people I could not hope to acknowledge directly, because their help, indirect and often undirected as it was, was also important.

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Acronyms

σ^S RpoS, sigma factor for the general stress response in *E. coli* (also termed σ^{38}).

aTc anhydrotetracycline.

C3Bio Continuous Co-culture Bioprocesses.

CyOFP Cyan-activated Orange Fluorescent Protein. In this work, CyOFP1 is used.

$E\sigma^S$ RpoS-containing RNAP holoenzyme.

FC flow cytometry.

FEC Fitness-Entropy compensation, a population-level mechanism where loss of individual cellular fitness (for example due to burdensome gene expression that reduces growth) is offset by an increase in phenotypic diversity. This diversity, quantified by Shannon entropy, is caused by less-induced (faster-growing) cells which sustain overall population growth and prevent washout, effectively compensating for the fitness loss caused by the trade-off between growth and gene expression.

GFP Green Fluorescent Protein. In this work, GFPmut2 is primarily used.

GSR general stress response.

IPTG Isopropyl β -D-1-thiogalactopyranoside (lactose analogue).

mCherry A red fluorescent protein.

MSCC microfluidic single-cell cultivation.

PBS phosphate-buffered saline.

PDMS polydimethylsiloxane.

RNAP RNA polymerase.

RpoS sigma factor for the general stress response (also termed σ^S or σ^{38}).

Tc tetracycline.

YFP Yellow Fluorescent Protein. In this work, sYFP2 is used.

Glossary

-omics A suffix and by extension a collective term for high-throughput biological data acquisition and analysis techniques, such as genomics (acquire large quantities of genetic information), transcriptomics (acquire large quantities of RNA expression information), proteomics (acquire large quantities of protein expression information), and so on .

Accelerostat A continuous culture operated with a slowly increasing dilution rate, used to probe physiological responses as growth rate changes..

Auxotroph A microorganism that cannot synthesize a particular organic compound required for its growth, and must obtain it from the environment .

Batch culture A liquid microbial culture with a fixed volume of medium where no fresh medium is added and no culture is removed. Growth proceeds until a limiting nutrient is exhausted, glucose in this thesis. This encompasses classical flask experiments, and bioreactors where no fresh medium is added.

Bioprocess An industrial process that uses living cells. Bioprocesses can be used for the production of chemicals, pharmaceuticals, biofuels, and other products. They can also be used for environmental applications such as waste treatment and bioremediation .

Carbon catabolite repression A regulatory mechanism in microorganisms that prioritizes the utilization of preferred carbon sources (e.g., glucose, usually the most energy-efficient) over less preferred ones (e.g., lactose, which requires splitting before being useable) .

Carbon source see substrate.

Cell-machine interface A system that allows for the interaction between cultured cells and culture device, enabling real-time monitoring and control of cellular behavior. This includes measurement of the biological state (fluorescence, for example

through flow cytometry or microscopy), and control of the culture conditions (medium composition, dilution rate, etc.) based on the measured state .

Chemostat A continuous culture where the dilution rate is set to a constant value, which sets the growth rate of the culture and the residual limiting substrate concentration. The limiting substrate in this thesis is the carbon source. When the dilution rate is increased, cells have an increased growth rate. An increased growth rate can only be sustained if the limiting substrate concentration is increased in the culture, so the residual glucose concentration in the culture also increases with the dilution rate. If the dilution rate is increased too much, the cells cannot grow fast enough to keep up with the dilution and are washed out of the culture, the culture dies out.

Co-culture A culture containing two or more different microbial species or strains. Co-cultures contain a limited number of known organisms, by opposition to cultures of natural microbial communities, usually called mixed cultures. Co-cultures can combine the metabolic capabilities of different organisms to produce behaviours that are not possible with a single organism .

Continuous culture A liquid microbial culture where fresh medium is continuously added and culture liquid is continuously removed. This allows the culture to be maintained much longer than in a batch culture.

Cross-feeding A type of interaction between two species where one species produces a metabolite that is used by the other species, which can be used to stabilise co-cultures when strains are auxotroph to the metabolite produced by the other species .

Cybernetic model A mathematical modeling approach which considers that cells evolved to behave in the best possible way to maximize their fitness in a given environment. It assumes that cells have evolved regulatory mechanisms that allow them to optimally allocate resources and produce enzymes to maximize growth or survival under specific conditions, and bypasses the need for detailed knowledge of the underlying genetic regulation .

Decelerostat A continuous culture operated with a slowly decreasing dilution rate, used to observe cellular adaptation when growth is progressively limited..

Dilution rate (D) The rate at which fresh medium is added to a continuous culture, expressed in h^{-1} . It is defined as the flow rate of fresh medium Q divided by the culture volume V , $D = Q/V$. In most cases for continuous cultures, the volume is kept constant, which provides useful simplifications for steady-state calculations, and makes it possible to express all quantities in terms of concentrations and

dilution rates instead of absolute amounts and flow rates. In a chemostat, the dilution rate is set to a constant value, which sets the growth rate of the culture, as the defining equation for the biomass concentration X is $dX/dt = (\mu - D)X$, where μ is the growth rate .

Entropy A measure of variability or disorder within a probability distribution. In this thesis, entropy is used to quantify phenotypic heterogeneity.

Fed-batch culture A liquid microbial culture where fresh medium is added over time without removing culture liquid. This allows for longer culture times than in a batch culture, and can be used to control the growth rate of the culture by controlling the feeding rate. It is often used in industrial bioprocesses to maximize product formation while avoiding substrate inhibition (too much substrate causing a decrease in growth rate) or overflow metabolism (too much substrate causing the production of by-products such as acetate in *E. coli*) .

Fitness The reproductive or survival success in a given environment. It can be expressed for species, genotypes (selection of the fittest genotypes within a species), or phenotypes (elimination of unfit phenotypes from a culture). In chemostat contexts fitness can be expressed as the ratio μ/D , and a fitness >1 indicates a phenotype that will not be washed out of the system. See CHAPTER 1, section 1.3 and CHAPTER 3 for details .

Fitness cost The reduction in fitness caused by a response induced in the cell such as expression of heterologous genes (metabolic burden) or a stress responses. It manifests as a competitive disadvantage relative to the original phenotype, in a chemostat, that is a lower growth rate.

Gene circuit A set of genes and regulatory elements that interact to produce a specific behaviour. Natural gene circuits include: the stress response and sporulation, with the cell sensing stress in various ways and activating a set of genes in response; the lactose and arabinose operons, with the cell sensing the presence of these sugars and its own depleted energy reserve to cause the production of lactose/arabinose uptake and metabolism proteins. Synthetic gene circuits are designed to reproduce natural behaviours or create new ones. Two prominent example are the toggle switch circuit, acting like a switch between two states (see CHAPTER 3, section 2.5), and the repressilator, which produces temporal oscillations in gene expression .

Genomic integration Stable insertion of genetic material into the host genome, by opposition to plasmid-based expression.

Genotype The genetic composition of an organism, containing all the main genes of the species. For microorganisms, the genotype is often complemented with plasmids. The genotype partially determines potential phenotypes.

Genotypic heterogeneity Variation in genetic composition across individuals in a population (e.g., mutations, plasmid presence), which can produce different phenotypes and selection outcomes.

Growth rate (μ) The rate at which a microbial population grows, expressed in h^{-1} . Microbial populations grow exponentially, the more there are bacteria, the more new bacteria are produced, with a typical equation $X = X_0 \exp(\mu t)$, where X is the biomass, t is the time, and X_0 the initial biomass at $t = 0$. The growth rate is expressed in base e . The doubling time, which is the time required for the biomass to double, is given by $t_d = \ln(2)/\mu$.

Heaviside function A function that is 0 for negative values and 1 for positive values. It is often used to describe a switch-like response of a system to a stimulus. In the context of a biological response, it is 0 below a threshold K and $V_{\max}$ above, or vice-versa, similarly to Hill functions. A Heaviside function can be seen as a Hill function with an infinite Hill coefficient $n \rightarrow \infty$, a perfect threshold function, fully on, or fully off.

Hill function A function typically used to describe the sigmoidal response of a system to a stimulus. The typical Hill function $H(x)$ has a value between 0 and 1, and reaches the value of 0.5 at $x = K$, where K is the half-maximal value. It can be described by the equation $H(x) = x^n / (K^n + x^n)$ or $H(x) = K^n / (K^n + x^n)$, where n is the Hill coefficient, which describes the steepness of the function. The first version, $x^n / (K^n + x^n)$, worth 0 in $x = 0$ and 1 in $x \rightarrow \infty$, describes the activation of a system by a stimulus of intensity x , typically the activation of a gene based on an inducer concentration. The second version, $K^n / (K^n + x^n) = 1 - x^n / (K^n + x^n)$, worth 1 in $x = 0$ and 0 in $x \rightarrow \infty$, describes the inhibition a system instead. Often, the Hill function directly incorporates the maximal value of the system, and is then written as $H(x) = V_{\max} x^n / (K^n + x^n)$ or $H(x) = V_{\max} K^n / (K^n + x^n)$, where $V_{\max}$ is the maximal value.

Isogenic A population of cells that are genetically identical. While growing a population from a single cells should produce an isogenic population, mutations can occur, and an isogenic population is verified only for the specific genes of interest.

Metabolic burden The cost, for a microorganism, of maintaining and expressing a genetic constructs for industrial production, which diverts resources away from growth and other functions and can reduce fitness.

Microfluidic device A device which contains micrometer-scale channels and chambers, making it possible to manipulate and grow microorganisms in a controlled environment while observing them under a microscope. In the context of flow cytometry, a microfluidic device containing several channels is used to align the cells in a single file .

Phenotype The observable characteristics of an organism. The phenotype is determined by the genes of the cells, the environment in which they grow, and an element of random chance. In the context of this thesis, the phenotype is often the combination of the fluorescent proteins expressed by a cell and their growth rate. Phenotypic heterogeneity, where cells with identical genes still produce different phenotypes [1–3], is also contrasted with genotypic heterogeneity, where different genomes are the source .

Phenotypic heterogeneity Differences in observable traits among cells with identical genotypes arising from stochastic gene expression, environmental variation, or cellular history. Because this work focuses on chemostats at small scales, where the environment is perfectly mixed, phenotypic heterogeneity is primarily due to stochastic gene expression .

Plasmid A small piece of DNA, often found in bacteria, that replicates independently of the genome. Plasmids often naturally carry useful but not essential genes, such as antibiotic resistance genes. A lot of species can share plasmids between individuals. In biotechnology, for gene insertion, strains that cannot share plasmids can be injected with plasmids that carry the gene of interest. These plasmids can have different copy numbers, from a few copies per cell, comparable to the genomic DNA, (low copy number) to hundreds of copies per cell (high copy number). .

Quorum sensing A cell-to-cell communication mechanism in bacteria that allows them to sense and respond to changes in population density through the production and detection of signaling molecules called autoinducers. Each cell produces these autoinducers, and autoinducer concentration lets cell respond to population density. Quorum sensing can regulate various behaviors such as biofilm formation or motility .

Segregostat culture A chemostat equipped with an automated flow cytometry setup (see CHAPTER 3) which measures the phenotypic distribution of the population and can automatically activate pumps in consequence to change the culture conditions [4]. This produces oscillating culture conditions and oscillating phenotypes (fluorescent protein concentrations), usually with very low phenotypic heterogeneity, and is used in CHAPTER 3, section 3 to infer the fitness cost of a given phenotype from the oscillation period of the segregostat .

Substrate A molecule consumed by microorganisms to obtain biomass and/or energy. In the context of this thesis, all nutrients in the medium are in excess compared to the carbon source, and the carbon source is a sugar (e.g., glucose, arabinose). Therefore, this sugar is the limiting substrate, the one that will cause the culture to stop/reduce growth when it is exhausted.

Toxin-antitoxin system A genetic module found in many bacteria that consists of a toxin gene and an antitoxin gene. The toxin can inhibit cell growth or kill the cell, while the antitoxin neutralizes the toxin. These systems can play roles in plasmid maintenance, for example. They can be used to stabilize production in bioprocesses, for example, by linking antitoxin production to product concentration .

Washout The loss of cells from a continuous culture when the dilution rate exceeds the growth rate, leading to a decline in biomass concentration and potential culture collapse.

Yield The amount of biomass or product formed per unit of substrate consumed. For biomass, often expressed as biomass yield $Y_{X/S}$ (g biomass per g substrate). Usually, this value is around 0.5, half the carbon provided goes to energy production, and half to biomass production.

Notations

This section gathers several notations used throughout the thesis. Several of the concepts referenced are defined in the GLOSSARY or ACRONYMS sections.

Regarding molecular biology:

Species are italicised, with a capitalised Genus, for example, *Escherichia coli* or *E. coli*.

genes, the sequences of DNA, are italicised (e.g. *lacA*), except for fluorescent proteins (GFP, YFP, mCherry, CyOFP), which are also acronyms.

List of related, contiguous genes can be shortened. For example, *lacA*, *lacY*, and *lacZ* are three genes produced together and related to lactose consumption. They can be written *lacAYZ*.

Promoters, the DNA sequences controlling whether a gene is produced, are indicated as P_{tet} , where "P" indicates it is a promoter, and " $_{tet}$ " denotes which one.

Proteins are capitalised (LacA).

Gene deletions (Δ) are indicated with a Δ (an *E. coli* $\Delta serA$ is missing a functional *serA* gene).

Gene insertions ($::$), whether in the genome or in a plasmid, are indicated as $P_{tet}::lacI$, where P_{tet} is the promoter driving LacI production.

Regarding biological quantities and symbols:

D refers to the dilution rate in a chemostat.

μ is used to indicate the growth rate of the cells, in h^{-1} .

φ_i can be used to refer to the phenotype i from a strain.

H can refer to two different things based on context: the Shannon entropy (from information theory, NOT thermodynamics), or a threshold function, commonly modelled using a Hill or a Heaviside function.

K (**capital**) , the input value of the Hill/Heaviside function at which it reaches half of its maximum value. For a Hill function $H(S) = S^h / (S^h + K^h)$, $S = K$ means $H(S) = 0.5$. For a Heaviside function, K represents the threshold value. In biological systems, K is often the affinity constant of a given biochemical reaction.

k (**lowercase**) is used for constant values without specific meaning, often with $k \in [0, 1]$.

σ is either the standard deviation of a distribution, or the sigma factor for sigma factor for the general stress response in *E. coli* (RpoS), depending on context.

S is used to refer to a substrate concentration for the cells, in $\text{g}\cdot\text{L}^{-1}$. We are working with carbon limited media, so the substrate is a sugar, such as glucose, that the cells consume.

X is used to refer to the biomass (mass) concentration of the cells in liquid media, in $\text{g}\cdot\text{L}^{-1}$, with additional indices indicating the portion of biomass constituted of different phenotypes/genotypes/species.

$Y_{X/S}$ is the biomass yield on substrate, in $\text{g}_{\text{biomass}}/\text{g}_{\text{substrate}}$.

1

Introduction: Directing the fate of microbial populations

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Microbial populations are ubiquitous, inhabiting virtually every environment on Earth and living symbiotically with almost all animals and plants. Humans learned to harness their abilities first to ferment food, then to produce penicillin and other compounds through industrial fermentation processes. As a result, microbes play key roles in various research fields such as ecology, as microbes are a fundamental part of every ecosystem, human health, because of both helpful microbiota and harmful pathogens, or bioprocess engineering, to directly make use of microbes for the production of various products. All these fields are faced with a similar challenge: microbial populations are heterogeneous and their behaviour is difficult to predict accurately. Even a single species can present multiple genotypes as mutations accumulate over time [5, 6]. A single genotype can present multiple phenotypes (see GLOSSARY) due to noise in gene expression, both because each cell is subjected to a slightly different environment, and because low amounts of several molecules in a cell make the progression of their chemical reactions random [7–9]. A microbial culture can therefore present numerous sub-populations with distinct behaviours, some of which appearing with mutations, and all of them communicate with each other by modifying the composition of their shared growth medium. This evolving complex system may produce stable, oscillating, unstable or chaotic behaviours, and decyphering these behaviours is essential to understanding and directing microbial populations [10–12].

1. Understanding microbial populations through sub-population dynamics

Solving the problem of heterogeneity and predictability falls in the domain of systems biology, that is, "a systems approach to address the function and dysfunction of organisms by considering how function arises in dynamic interactions between the different components of a biological system" (paraphrasing [13]). In addition to biological components such as metabolic processes, physico-chemical conditions can also affect and be affected by microorganisms. The following sections summarise the

key biological and abiotic factors that can impact the fate of microbial populations, and how they interact with each other.

1.1. Key biological factors

The first biological component to consider is phenotypic heterogeneity [14, 15]. Even when environmental conditions are homogeneous and the cell population is isogenic, gene expression can be highly heterogeneous [1, 16–19]. This happens because the low amount of some molecules (DNA or mRNA for example) in a cell makes the occurrence of some biochemical reactions effectively random [1, 2], and phenotypic heterogeneity can actually help in creating thriving microbial populations [3, 20].

In addition to heterogeneity, there can be communication between cell sub-populations, either directly through quorum sensing or toxins, or indirectly through shared nutrient pools [10, 21–23]. This is more pronounced when the culture involves a co-culture of multiple microorganisms: using multiple microorganisms multiplies their potential capabilities, but it requires these microorganisms to survive in the same conditions, while competing for key resources. In that context, cell-to-cell interactions can be a blessing or a curse, either stabilising the co-culture [12, 24–26] or making it unpredictable [27, 28].

Even mono-cultures can effectively turn into co-cultures: in any microbial population, cells undergo mutations that can drastically alter their behaviour, creating genotypic heterogeneity [5, 6, 29, 30]. This evolutionary process is critical for microbial populations to thrive in the wild, but can be problematic. For example, bacteria can develop antibiotic resistances, or, production pathways of interest can be disabled in bioprocesses [29, 31].

1.2. The impact of abiotic factors

The behaviour of microbial populations depends on the microbes, but also on the environment they are exposed to. The availability of different nutrient sources, the presence of oxygen, the temperature, the pH, the presence of toxins, and more, all shape microbial population dynamics [32].

For research purposes, the environment is entirely defined by the type of culture and the culture medium, and several parameters such as the pH or temperature can be controlled directly. Within this work, the main culture device used is a chemostat, a continuous culture where fresh medium and culture broth are exchanged at a fixed rate (the dilution rate, see GLOSSARY).

The chemostat was invented in order to create a steady-state with constant conditions in the culture and a constant cell growth rate. The medium is prepared with a single limiting factor, often the carbon, nitrogen or phosphorus source, while all other components are in excess. Variations in that limiting factor and in the dilution rate are virtually the only determinants of the culture at steady-state, greatly simplifying research. [33]

However, in most cases outside the chemostat – and even in some cases in chemostat, as will be shown later – the environment is not constant. Most microbial populations are faced with heterogeneous conditions, over time (e.g. in a flask culture, nutrient concentrations decrease over time), over space (e.g. in a large scale bioreactor, parts of the reactor have higher concentrations than others [34]), or both (e.g. microbial populations outside of a laboratory).

This has two main consequences. First, there are phenomena that cannot be studied in constant, homogeneous conditions: for example, spatio-temporal heterogeneity can amplify existing phenotypic heterogeneity or cause stress, and can decrease the production level of a bioprocess [1, 34, 35]. Second, bacteria never evolved for a stable environment, because natural conditions are constantly changing. This implies that heterogeneous conditions would be a better choice for some applications. Such conditions were in fact used to stabilise cultures even as far back as 1986 [4, 36–40], and are used in CHAPTER 5 to manage a co-culture.

1.3. Interactions between biological and abiotic factors: the concept of fitness

Microbial populations are directly affected by their environment and can respond by adapting their metabolism. However, because of phenotypic and genotypic heterogeneity, some cells are more suited to survive in a given environment than others; this suitability is called the fitness. The concept can be defined over different time scales, for individuals or for genotypes [41–45], but it always has an impact on the population structure.

When considering genotypic heterogeneity, fitness causes evolution. Over time, the fitter organisms for a given environment out-compete the others and the best genotypes are selected. The characteristics that determine which organism is 'fitter' depend on the environment: the same trait can be beneficial in some circumstances and not others. In a chemostat, as will be used in this thesis, the selection is based on the growth rate of the organisms under nutrient limitations [46].

Fitness can also be applied to phenotypes instead of genotypes, although it is much less common. In a previous work [7], we classified different gene circuits (groups of genes regulated together, such as the arabinose consumption pathway or the stress response, see GLOSSARY) based on how much they reduced the fitness of the cell when activated. We subsequently discovered that this fitness cost of a gene circuit is directly linked to the phenotypic diversity observed when it is induced (FIGURE 1.1): if a gene has a low fitness cost, there is no heterogeneity, if it has an intermediate cost, the population becomes heterogeneous, and if the cost is too high, the gene expression spontaneously oscillates. How these patterns emerge is the subject of CHAPTER 4.

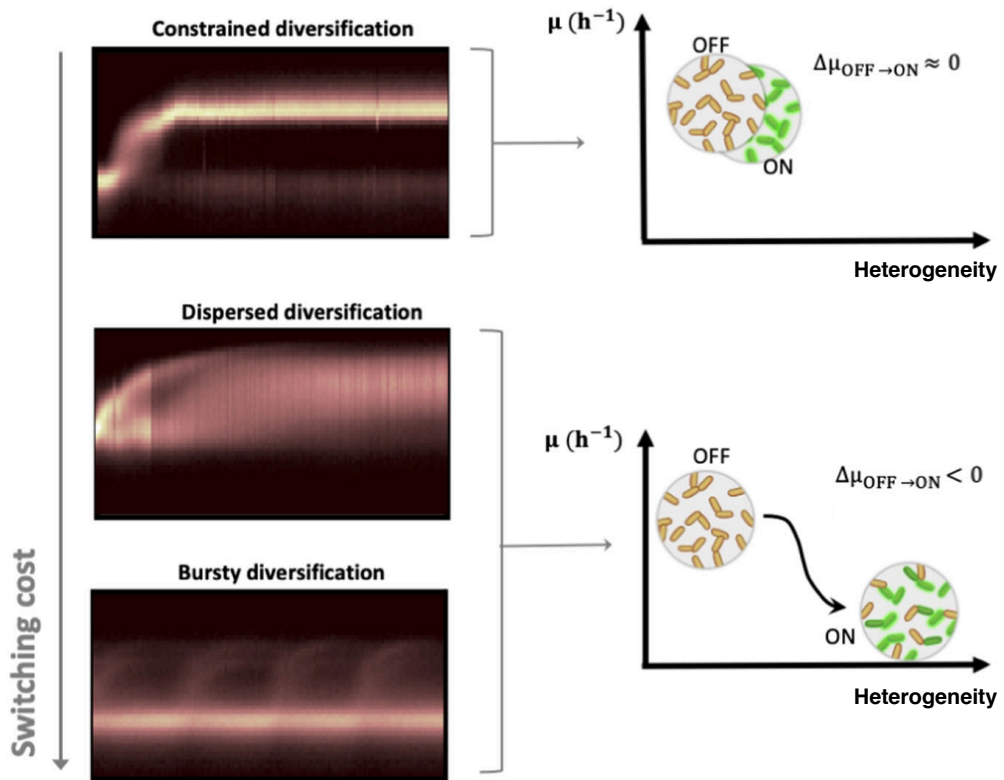


Figure 1.1: Diversification patterns associated to different fitness costs. In the constrained diversification regime, the growth rate μ is equal for the induced and uninduced phenotypes, and the population is uniformly induced. If the growth rate of the induced phenotype is lower than the uninduced one, there is a fitness cost, and the population becomes heterogeneous (dispersed diversification regime). When the fitness cost is high enough, waves of induction appear (bursty diversification regime). Figure taken from [7] (under CC4.0 license, <http://creativecommons.org/licenses/by/4.0>).

2. Controlling microbial populations

When using microbial populations, it becomes important to control their behaviour instead of only studying it. To that end, two sets of parameters can be affected: the strains themselves (the specific genetic variants of a microbe), and the abiotic factors.

Modifying the strains is done through synthetic biology, the field of study dedicated to modifying cells to produce complex behaviours, and, for bioprocesses, maintain production for as long as possible. The field started with the repressilator [47] and the toggle switch [48], carefully designed genetic architectures able to generate periodic and bistable protein production patterns, respectively. These pioneering papers led to numerous additional architectures and were eventually applied to strains designed for bioproduction, with several types of applications: one, dynamic metabolic engineering, redirects resources for growth or production based on the stage of the process, reducing metabolic burden (the fitness cost due to production) when more biomass is required and increasing productivity when the target compound is desired [49–51]. Another is self-selecting cultures, which have individual cells suiciding when they stop producing the target compound [52, 53].

Synthetic biology is powerful but can be time-consuming. Any cell modification needs to coexist with everything else the cell is doing and work consistently from lab to production scales. The alternative or sometimes complementary option is to adapt the process itself to the strains. This can be done not only by ensuring the pH and other process parameters stay in their optimal range, but also by actively measuring the biological process and dynamically adapting the process conditions accordingly. The idea of monitoring and controlling biological processes with on-line gas analysers [54–56], Raman spectroscopy [55–57], mass spectrometry [56, 58, 59] or fluorescence measurements [4, 40, 56], among others, has become more widespread in recent years.

More than just keeping every parameter constant, these techniques enable potential ‘cell-machine interfaces’ that automatically adjust culture conditions [4, 60, 61]. Since cell populations can be heterogeneous and/or unstable even in constant conditions, these interfaces dynamically adjust the culture based on what the cells are doing instead. There are at least two important arguments for these interfaces. First, characterising the optimal induction level of a strain could be much easier than optimising it with genetic modifications. Second, cells can be sensitive to very small changes in concentrations, and it is often very difficult to provide the exact concentration of all the chemicals they require. If the behaviour of the cells is monitored, the process can be adapted over time to correct for any error in the provided concentrations [40].

A dynamic adjustment requires knowledge of how changes in culture conditions will affect the cells. To this end, *in silico* models are emerging as a key element to develop these cell-machine interfaces. They can be used both to learn about biological behaviours before trying to control them, and to apply model predictive control [56, 61–65]. By definition, cell-machine interfaces need to consider the kinetics of biological

processes, excluding the traditional steady-state assumptions that underlie, for example, chemostat equations and most large-scale metabolic models [33, 66, 67]. Within the remaining models, the two dominant techniques are stochastic simulations such as the chemical master equation, which can distinguish single-cell phenotypes but are difficult to apply to macroscopic cell populations [68], and deterministic whole population models such as cybernetic models (see GLOSSARY), which consider large cell populations with a homogeneous behaviour [69]. Currently, there is no standardised way to model phenotypic diversity kinetics in large scale cultures.

3. Hypothesis and thesis outline

Biological systems are complex. There are a lot of different components, intervening at different scales, and strains are characterised in conditions that are completely different from their natural or intended environment.

In this thesis, I argue that a systemic approach integrating both the biology and the process conditions is necessary to understand and control a number of biological phenomena. In addition, new tools are required to apply this systemic approach as current tools cannot properly account for the interplay between phenotypic and genotypic heterogeneity and the culture conditions. In practice, the chapter will be focused on continuous cultures bioprocesses and how the fitness of different phenotypes and genotypes impact their stability, but many of the conclusions drawn can apply to different systems.

CHAPTER 2 is a mini-review of the literature on the use of synthetic and systems biology to understand and design bioprocesses, with a focus on continuous co-culture bioprocesses which represent the current frontier of research.

In CHAPTER 3, the focus is on automated flow cytometry, the key technique used in this thesis to measure bioprocesses. The chapter includes a general description of the technique, and highlights how its data was used to provide new insights into culture dynamics.

Automated flow cytometry results shed light on complex phenotypic diversification behaviours in continuous cultures, with similar trends observed irrespective of the biological system. This hinted at a non-biological cause to the phenomena, and CHAPTER 4 investigates this hypothesis by focusing on the interactions between the culture device and the biological system.

CHAPTER 5 is designed as a challenge to the current knowledge on biological systems. Past chapters already highlight that a large part of bioprocess complexity is still unknown. However, with what we know, is it possible to design a co-culture from the ground up, and predict its behaviour quantitatively? This chapter picks a reasonable approach to the problem, explores how far it can go, and what it can teach us.

Finally, CHAPTER 6 summarises the key insights from the thesis.

2

Enabling Continuous co-culture bioprocesses: A Systems and Synthetic biology review

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As synthetic and systems biology are the key topics involved in creating integrated bioprocesses, this first chapter reviews how they are currently used. In particular the focus is on continuous co-culture bioprocesses, continuous cultures of several microorganisms together. They gather all the difficulties of a mono-culture, and are further constrained by their continuous nature and the presence of multiple strains. As such, they represent an aspirational goal which pushes current technologies to their limit and which, if achieved, also enables traditional bioprocesses.

This chapter is adapted from the following mini-review, which remains unpublished:

[70] Vincent Vandenbroucke, Delphine Soyeurt, Christian Dusny, Frank Delvigne, and Juan Andres Martinez. “Enabling Continuous Co-Culture Bioprocesses (C3Bio): A Systems and Synthetic Biology Approach.” Unpublished manuscript. Nov. 28, 2022

1. Abstract

Next-generation bioprocesses will have to rely on new conceptual designs to expand the capabilities offered by industrial biotechnology. In this context, the continuous culture of multiple strains offers many advantages by enhancing metabolic potential, flexibility, and productivity. However, several challenges still need to be confronted for its satisfactory use in the industry. Critically, achieving functionality and microbial population stability at laboratory and industrial scales is especially troublesome for C3Bio. While many biomanufacturers desire continuous processes for their operation and production price reduction and their theoretical capacity for improved quality control, such processes suffer from two key drawbacks when devised for co-culture operations. First, it is challenging to maintain equilibrium between the

different microbial species with different fitness profiles. Second, when stressed by survival-demanding or competing environments, microorganisms often lose their functional capabilities in order to maximise their fitness over time. However, these challenges are now being tackled through systems and synthetic biology approaches via the introduction of new metabolic functions in microbes, enabling their assembly in synthetic communities with targeted capabilities and increasing the production of diverse biochemicals. Systems and synthetic biology approaches have proved promising towards stabilising co-cultures based on controlled microbial interactions and the directed activation of gene circuits and pathways. Here we present a review of these concepts, approaches, and examples of their integration that portray recent systems and synthetic biology advances in the context of C3Bio technologies.

2. Introduction

Microbial cultures have been extensively used in industrial processing, from traditional fermentation products to modern recombinant synthesis of an increasing amount of valuable chemicals and other bioproducts [49, 71]. From these processes, the view emerged of a cell as a "factory" which transforms simple sugars and nutrients into complex products. Through the advancement of genetic manipulation technologies, metabolic engineering, managing which chemical reaction a cell performs, and bioprocess engineering, this concept of microbial cell factories has readily evolved to produce ever more complex products with more robust, streamlined, and integrated industrial bioprocesses [71]. However, this paradigm also features severe limitations. For example, it is traditionally defined in mono-culture, which is by definition constrained to a single microorganism strain's metabolic functions and capabilities. While metabolic engineering provides several solutions to extend those capabilities, it is often difficult or impossible to introduce several degradation and synthesis capabilities while maintaining a reasonable growth rate and production balance [72–74]. To overcome these genetic and metabolic limitations, multiple strategies have been researched, such as multi-staged production processes coupled to dynamic metabolic control, where cells are first growing then producing instead of doing both [49, 75], or in vitro production technologies, where microbes are replaced with plant cells [76, 77]. While these strategies have resulted in several successful examples, they also require in-depth investigation of the careful balance between the host metabolism and the desired production. Typically, the integration of each new metabolic functional step requires the construction and tuning of new genetic parts, their assembly into functional regulated circuits, knowledge of their interplay with the host metabolism and ways to control the resulting population diversity [49, 75]. An alternative approach is the use of microbial co-cultures, either natural or synthetic, to allow different parts of the bioprocess to be performed by different organisms with complementary capabilities and divide their labour [74, 78, 79].

Microbial co-cultures can be divided into synthetic co-cultures, where the cultured microorganisms are known and assembled, given a particular interest in their functionality, and naturally occurring microbial communities, usually called mixed cultures instead of co-cultures. The latter commonly have an advantage in terms of resiliency to contamination and robustness while having the disadvantages of directing many resources to microbial fitness and biomass production, as they have evolved to survive, not to produce. Furthermore, they often portray complex metabolic and ecological interactions between the diverse members, most of which need to be better known or characterised. Thus, while they are useful in many cases, especially for degradation purposes (in which case productivity is closely related to the growth of the community) [80], it is difficult to understand whether a mixed culture can be optimised towards the production of a given compound. It could even be argued that it would be difficult to fully utilise natural microbial communities for several industrial production purposes without first being able to understand and manage synthetic co-cultures. In this chapter, their alternative, synthetic microbial co-cultures are the ones mainly discussed. Synthetic co-cultures are interesting in that, compared to natural communities, they are easier to study, and compared to mono-cultures, they have an expanded range of metabolic capabilities. In theory, they can be designed from sets of previously optimised microorganisms, often with the added efficiency of specialist strains with a naturally occurring function or a minimal trade-off between growth and production.

In this chapter, we would like to examine these microbial co-cultures through the lens of continuous bioprocesses. Continuous bioprocesses have the potential to make many processes much more economical, firstly through decreased downtime of the process and reduced sterilisation costs [81]. Secondly, continuous cultures provide an extended control capability compared to alternatives: unlike in batch and fed-batch, species are diluted, which can eliminate the accumulation of unwanted chemicals and makes it possible to correct abiotic parameters in the bioreactors over time, to reach the desired steady-state or dynamically control the production [82, 83]. These characteristics may not only impact productivity but also improve product quality through enhanced control in the microenvironment of the organisms [81]. It is difficult to classify which types of products or applications would be best performed in C3Bio processes and which would not, given that several challenges impede their adoption, whether be it consortium design, optimisation and control, process online measurement, or even the choice and analysis of such measurements to reach actionable conclusions on the process and efficiently control it. However, we can recognise certain design requirements that would make the C3Bio interesting for the desired application in combination. 1) when integration into a continuous upstream process or system is required, i.e. wastewater treatment, in which the main objective is the continuous degradation of a broad range of metabolites. 2) when the process requires the products or byproducts not to be accumulated to avoid either toxicity, production inhibition by the end-product or the product suffering further

modifications, which could reduce quality or shelf life (i.e., oxidation, glycosylation, degradation). 3) when the control of the fermenter's abiotic parameters is a must to achieve product quality or specifications. 4) when the process requires robust behaviours in the face of non-well-defined substrate inputs, i.e. biotransformation of mixed or complex substrates. 5) when the process is required to integrate more than one complex metabolic pathways, which could overwhelm a single strain, often due to energy or metabolite competition between pathways and cell growth. Continuous cultures also come with challenges in and of themselves and within their integration to upstream and, more importantly, downstream processes [81, 83, 84]. However, we will here restrict ourselves to the challenges they introduce specifically for a potential continuous co-cultures process. Finally, we would like to present the challenges, advances and perspectives of C3Bio within the perspective of Synthetic and Systems Biology, which are needed to address the microbial community's complexities and their control during culturing processes.

3. C3Bio: a balancing challenge

The more relevant characteristics of an industrially successful process are titer, productivity, yield and robustness [49]. For microbial co-cultures, these translate to achieving metabolic function and microbial population stability [14, 72, 85, 86]. This stability is one of the most demanding constraints for their industrial utilisation. That is because growth imbalances between organisms with different functions, added to phenotypic diversification processes, often result in unstable microbial communities favouring the extinction of a particular microorganism in the culture [25, 87–89]. Furthermore, when extinction is not the outcome for a microorganism, phenotypic diversification processes can reduce or inhibit the desired metabolic function within their population [14, 15]. These challenges become especially relevant for C3Bio, as dilution imposes stringent constraints on microorganisms' substrate consumption and growth rates, metabolite concentrations, stress and overall fitness distributions. The latter is crucial to industrial bioprocesses since the relative fitness of each microorganism's phenotypic state, defined as their growth or survival rate compared to the rest of the community, can dictate the fate of the co-culture [28, 90]. Limited knowledge and design considerations of these systemic phenomena commonly result in undesired stationary states in terms of population and metabolic function balance and stability, which directly impact the productivity and robustness of the bioprocess [15, 91]. A well-known example is the appearance of phenotypic heterogeneity, which impacts even mono-cultures [14], and in a co-culture, a simple predator-prey relationship can eliminate any steady state from the system, leading to unstable production over time [27, 28].

Accordingly, it is essential to control each organism's population composition and phenotypic and genotypic diversity to design an optimal co-culture process. To this

end, it is evident that tools to analyse, design and control these complex systems are required. Systems Biology and Synthetic Biology provide scientists with several such tools that have been developed to address different parts of the biological and ecological complexity (see FIGURE 2.1). However, integrating and implementing these various tools for a specific bioprocess design is often difficult, and their potential is not fully utilised. For this reason, this review encompasses a practical overview of some available bioprocess design and control tools provided by the systems and synthetic biology approaches, highlighting their potential integration into industrial applications and the associated challenges. In particular, we will focus on how those tools, which are now developed at the lab scale, could be integrated and further adapted within an engineering cycle (understanding, designing and controlling) of C3Bio industrial implementation.

4. Systems biology: understanding C3Bio

Systems biology studies biological parts within their context, whether an ecosystem and its food web or a gene and the associated metabolic fluxes and transcriptional network. For C3Bio, systems biology also implies considering both the complex behaviours of microorganisms and their interactions with each other and their environment [26, 92–94]. Those behaviours can be initially simplified into social descriptions such as commensalism or amensalism, that is, one organism benefiting or harming another without being affected, mutualism, two species benefiting each other, or competition [26, 92–94]. However, when studying a co-culture process, it may be more beneficial to approach the problem by considering the dynamics of multiple interactions types, as they could be simultaneously occurring between microorganisms, giving rise to different sets of phenotypes [26, 28, 95, 96]. Those interactions are usually regulated by chemicals that several microorganisms can sense or affect, like nutrient concentrations or quorum sensing systems molecules (produced by microbes to estimate their own abundance using the molecules' concentrations) [22, 28].

While these interactions are difficult to study, they can be used to boost production directly or can be employed indirectly to control cell populations [12, 94, 97, 98]. For example, competition has been found to induce the production of some desired compounds [94], and non-producing populations have been used to stabilise strain ratios in different co-cultures, minimising the accumulation of intermediates and byproducts while maximising the productivity of the desired compound [12, 97, 99, 100]. An example of this is the use of oxygen consumers to enhance anaerobic cultures, which have been in use for decades [98, 101]. Handling cell interactions, however, is not always straightforward, as a microbial community is a dynamic non-linear system in which a simple relationship could lead to the disruption, or even the absence, of a steady state, as in a predator-prey relationship [27].

In the context of C3Bio, the increased system complexity, which is precisely one

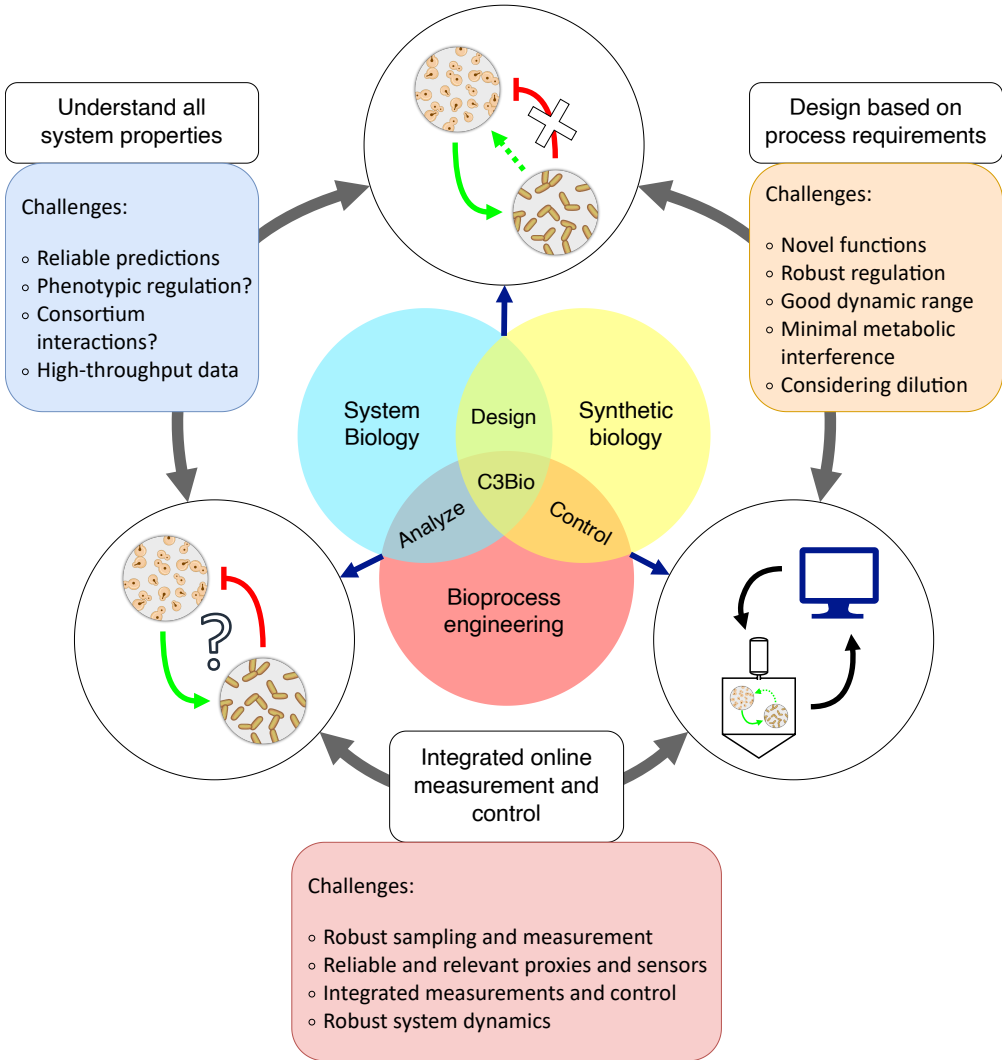


Figure 2.1: Systems biology makes it possible to analyse co-cultures, and understanding these systems can lead to the rational design of system dynamics for a co-culture process, usually with the help of Synthetic biology. The process, designed for control, can then be enhanced by online measurement to take into account the system's dynamics. However, several challenges need to be addressed to ensure a successful process implementation.

of the reasons they are relevant, is commonly in opposition to its robustness [73, 78, 79, 102]. However, the global perspective of systems biology helps to determine the emergent properties of such biological systems, required to understand and predict the behaviour and productivity of a co-culture. For example, Geng et al. [103] studied the metabolic interactions in a *Saccharomyces cerevisiae* and *Pichia stipites* co-culture through a custom genomic scale modelling workflow which allowed them to understand

metabolic interactions and complementation that could render the behaviour of their co-culture. Together with an experimental design to measure and quantify the expected metabolite pools in mono- and co-cultures, they identified and validated relevant metabolic exchanges that define their interactions. Interestingly, they found that the dynamics and behaviour of the organisms were different in mono- and co-culture due to metabolic network complementation. Such analysis would not be possible without the tools to measure and analyse biochemical, genetic, and genomic high throughput data [94, 104–106].

Furthermore, in a continuous culture, it is necessary to analyse both the interactions within the systems and their kinetics. Studying those kinetics can be supplemented by mathematical models to help predict the co-culture fate. For example, Dai et al. [107] studied the resilience of members in microbial communities by modelling the density of different populations through changes in environmental conditions. They used this to determine when environmental perturbation was close to the threshold beyond which populations would not recover stability. Mancuso et al. [106] also describe how a combination of experimental and modelling approaches provide better insight into how microbial communities are assembled, related to the environmental conditions, and regulate their diversity. Several other investigations have addressed many other characteristics of the interactions between populations (see TABLE A.1, ANNEX A), and the signal transmission across their different metabolic and cellular layers [108, 109].

Those advances make it possible to predict how a system will behave, and such understanding can be used for the rational design of stable systems [28, 110, 111]. This design needs to consider the cells themselves, but also how they are affected by physical and chemical perturbations [36, 88, 112–115]; this, in turn, combined with tools like online data measurement and treatment [116–118], and modelling [37, 114, 115, 119, 120], may allow to correct a biological process before deviations appear [4, 40, 99, 100, 121, 122]. Therefore, this integrated online measuring-modelling systems approach would improve the robustness of the process, a critical point in the context of a continuous culture, and optimise it for yield or productivity. However, it remains to be seen how the technologies can be adapted from the laboratory to larger-scale reactors. All types of data might not be available in a large-scale bioreactor due to practical reasons: fluorescence, for example, may produce an undesired burden on the cell and slow production down, the apparition of inhomogeneities in bioreactor parameters and the temporal transition of the organisms through them requires more complex and computationally demanding models, and the exact implementation of techniques like online flow cytometry at larger scales to provide meaningful and relevant data is still challenging. Thus, to be effective, this approach requires either obtaining more data from a large-scale industrial process to enhance models or producing strategies that require acquirable data to enhance control, whether online [117, 123] or simply at-line measurement for calibration and validation.

5. Synthetic biology: Designing C3Bio

Understanding the metabolism and interactions between the co-cultured strains allows the selection and design of tasks to be performed by each community member. However, metabolic networks in the would-be co-culture are rarely fully compatible. In this context integrating Systems Biology knowledge with Synthetic biology approaches can help steer the organism's behaviours towards a more desirable metabolic state. Synthetic biology development has led to the design of different gene circuits with tailored regulatory functions such as the toggle switch and the repressilator [47, 48]. Conversely to typical gene expression profiles with one steady-state behaviour, the toggle switch and the repressilator use promoters and genetic products to store information on multiple states of expression and generate bistable and periodic protein production patterns, respectively [124]. The invention of those synthetic circuits quickly led to a new molecular biology paradigm, as exemplified by Kobayashi et al. [125]'s three-layer description. Firstly, sensors detect physical or chemical stimuli in the environment; typically, light or the presence of small diffusible molecules directly triggers RNA synthesis [126–128]. Secondly, logical reasoning is applied to the resulting information, translating the input information into a decision thanks to synthetic gene circuits. Those can be memory circuits or biological oscillations, like the toggle switch and the repressilator, or in another broad category of circuits, the application of logic gates which combine multiple stimuli into a single decision [129]. Finally, the decision is translated into a phenotypic alteration, for example, producing a protein, redirecting carbon fluxes, or maintaining cell survival [49, 125, 130].

While those three components let one modify the behaviour of a single cell, one more layer can be added to the structure in microbial co-cultures: it is possible to recreate and repurpose cell interactions by coupling synthetic biology components between strains, leading to multicellular gene circuits [131, 132]. In natural communities, communication happens either through direct cell-cell contact or indirectly by affecting chemical concentrations in the medium. Synthetic biology approaches have reproduced the latter, integrating cross-feeding (sharing nutrients, see GLOSSARY), toxin-antitoxin, and quorum sensing (cell-cell communication) systems in cells [25, 89, 131, 132].

From this set of capabilities, research towards control of cell behaviour has mainly gone in two directions. On the one hand, it is possible to directly use synthetic biology to manage population stability and function, thus bridging the gap with systems biology. For example, Özkaya et al. [12] created artificial cheater populations to stabilise cooperation in a co-culture, Zargar et al. [133] cultured cells with designed quorum sensing inside alginate beads to manage phenotypic heterogeneity, and Kerner et al. [25] used the induced production of essential growth metabolites to control strain ratios in a culture. Controlling functions in cells is also possible, as shown by Gargatte et al. [134], who optimised the production pathway and maintained cell productivity thanks to internal tyrosine sensors, or by Gupta et al. [135], who used quorum sensing to

redirect metabolic fluxes dynamically. Another common example is the creation of substrate-selective strains, which allows for stable co-cultures where each organism consumes substrates independently of the others, eliminating competition [136–142]. Obtaining an optimal production, however, can then depend directly on the proportion of each substrate if the production pathway is split between the strains.

On the other hand, other research groups have regulated synthetic gene circuits externally by designing cell-machine interfaces where the cells' behaviour (e.g., in the laboratory, fluorescent proteins) is measured and external inputs such as inducer concentration or light exposure can affect them in response. Feedback control is then used to precisely manage phenotypic heterogeneity with pulsatile inputs Benzinger and Khammash [40], Nguyen et al. [116], and Henrion et al. [143], or interact with synthetic circuits, like Mondragón-Palomino et al. [144] who synchronised a genetic oscillator or Lugagne et al. [121] who reliably stabilised cells in the unstable state of a toggle switch circuit.

Several examples of synthetic biology applications are shown in TABLE A.1. Altogether, they paint a picture of synthetic biology as an enabling tool for systems biology. Whether it is used as a self-contained system or as a tool to interact with the existing system, synthetic biology expands the possibilities for system design by providing new biological bricks that react in predictable ways and let scientists build systems specifically to study their dynamics, as well as stabilise, improve and control their functions. Most of those studies, however, are performed at small scales or in microfluidics. Therefore, some approaches still require their robustness of behaviour to be tested in larger scale fermenters where environment and control can present temporal inhomogeneities.

6. Bio-process engineering: controlling C3Bio

As observed previously, systems and synthetic biology can provide the tools required to achieve function balance and stability, which are critical in designing and constructing microbial communities. Several reviews ([50, 99, 100, 145, 146]), and examples from the literature (see TABLE 2.1) show these different tools as a part of an integrated "analyse, design, and control" process (see FIGURE 2.1). Analysing the biological system can lead to its rational design by complementing and modifying the existing system properties. However, the process design also needs to consider the desired properties for the final C3Bio, as the co-culture must often be robust to non-well-defined feed or can be improved if it is controlled by external stimuli.

As discussed, two main factors define the success of using any microbial community in the industry. First is the balance between the different microbial species and metabolic functions involved, which is required for a highly efficient process. Second is process robustness, which means keeping this balance stable as long as production is desired and regardless of the biological noise introduced by scale changes.

Table 2.1: In this table, different examples where the various aspects of a co-culture design that have been identified in this review are shown. A more comprehensive list can be found in TABLE A.1 (ANNEX A).

Reference	What	Systems biology			Synthetic biology			Stability	Production
		Analyze	Design	Control	Analyze	Design	Control		
	Understand all system properties								
2014, Goers, Freemont, and Polizzi [102]	Review of techniques to design and control a stable co-culture, from natural interactions to external environment.	x	x	x	x	x		x	
2018, Rugbjerg et al. [29]	Study the interaction between the system, the metabolic load, and the appearance of cheaters, and use them to design more stable systems.	x	x		x	x		x	
	Design based on process requirements								
2017, Zargar et al. [133]	Combine quorum sensing and spatial segregation to control phenotypic response. The system and cells are designed to be controlled.		x	x		x	x	x	
2021, Hartline et al. [50]	Review of dynamic metabolic control, including a section on sensors and ways to apply that control, to redirect carbon fluxes or tackle phenotypic heterogeneity.					x	x	x	x

	Integrated online measurement and control							
2019, Rodríguez-Verdugo, Vulin, and Ackermann [88]	Known system kinetics can be used to stabilise a co-culture over time via dynamic environmental control.	x	x				x	
2022, Henrion et al. [143]	Review on how to analyse and control gene expression via control of the extracellular environment.	x	x	x		x	x	
	Optimization can be achieved both for stability and production							
2013, Minty et al. [87]	Systems & synthetic biology are used to design and optimise a co-culture system for production and stability.		x	x		x	x	x
2018, Bhatia et al. [111]	Improve production by choosing complementary strains both in terms of carbon source and different parts of a single production pathway.		x				x	x

In continuous culture, dilution rate can cause the washout of biomass and metabolites, ultimately leading to population and metabolic function instability [29, 107]. For example, quorum sensing, a vital tool in co-culture regulation, could be severely limited by continuous dilution. Population control in processes with co-utilised substrates may also be challenging when microorganisms have significant differences in growth and consumption rates, resulting in extensive fitness imbalances and, therefore, the eventual loss of a desired microbial species. Part of the bioprocess design, then, is to correctly determine the process conditions for which the production could be stable and optimal, a task that can be accomplished through online data measurement and analysis [116–118], dynamic feedback control [99, 100, 116] or oscillating conditions [36, 37, 88, 115, 119, 120, 143, 147]. As mentioned in the previous sections, such control strategies have helped regulate population dynamics [36, 88, 114, 115, 119] and synthetic gene expression [4, 40, 99, 100, 116, 121, 143, 144].

Regarding bioprocess control, periodic oscillations in metabolite concentrations and feedback control based on online data monitoring are only some of the ways to affect the process through the environment. Where the previous approach creates temporal niches for specific organisms, bioreactor and culture design can achieve similar spatio-temporal segregation and favour specific microorganisms or phenotypes [39, 79, 85, 91, 92, 133]. For example, biofilm-liquid bioreactors can have different strains filling the different niches, and changing the surface area or properties of the interface will affect the biofilm and thus the culture behaviour [79, 91, 92]. The cultures of different microbial species in communicating chambers or hydrogels can also be mentioned, as this limits their interactions and can improve population stability or manipulate strain ratios directly [38, 39, 79].

Given the possibility of such spatio-temporal complexity, it becomes critical to understand the system's dynamics, in addition to its possible equilibrium states [28, 107, 120, 147–149]. Systems biology provides frameworks in which the microbial communities can be analysed and modelled, many of them have been used extensively for static interactions and characterised through several omics analyses, as stated in the systems biology section [28, 78, 102, 148–150]. However, in terms of system dynamics, analyses are often confined to relatively small systems and it is challenging to describe the full complexity of a co-culture [28, 102, 148]. This could be in part because intracellular processes are still challenging to address, although different strategies have been introduced to quantify evolution of strain ratios in co-culture, and phenotypic and genotypic diversification [28, 102, 107, 147, 148, 151, 152]. Among others, the use of the cybernetic modelling approach for understanding phenotypic and metabolic transitions in co-cultures allowed the study of fitness and consortium population changes across environmental oscillations [147]. This approach considers that biological function will be optimal for cell survival and allows rendering systemic regulation information without knowing the complete regulation architecture across the consortium microorganisms. However, constructing models that can readily confront

the metabolic behaviour of any microbial consortia with the transitions and inherent noisiness found in industrial-scale reactors still needs to be worked on.

Another common drawback of current models is that for co-cultures, a single phenotype is usually considered for each strain, and their interactions are usually simplified to the interaction through metabolite pools [37, 95, 114, 115, 119, 120, 147] (Matyjaszkiewicz et al. [96]’s work is one exception, though it does not pertain to bioreactors). Phenotypic and genotypic diversification have mainly been studied in single cultures, as they also negatively impact mono-culture bioprocesses. Multiple studies are shedding light on the dynamics of the processes and how they can be mitigated. For example using cell-machine interfaces [4, 29, 40, 99, 100, 116, 122, 130, 143].

Our understanding of system dynamics limits external control of a continuous co-culture, and it could be argued that to achieve their full potential, it is necessary to use models addressing better the complexity of the whole system, biotic and abiotic. These models must take into account phenotypic and genotypic heterogeneity, the effect of abiotic parameters and their spatio-temporal heterogeneity, the effect of synthetic gene circuits on cell metabolism, and the specific interactions that arise when multiple microorganisms are cultured together. All of the previous need to be integrated into a dynamic model if one is to understand the co-culture fully and explore all possibilities [28, 37, 95, 96, 107, 114, 115, 119, 120, 148, 149, 153, 154]. This is, however, extremely challenging. Therefore, developing new computational approaches that could integrate several models or address the complexity of all interactions efficiently is critical to understanding and then controlling the system with the current computational capacities.

It is worth mentioning that most of the previously mentioned studies still need to be adapted to industrial upscaling. Control is often qualified by proxies (i.e. fluorescent proteins) and rarely tested in actual production systems, and the study of cell interactions is usually performed with a theoretical outlook. While the study of production schemes in itself is essential, there is a vast, unexploited potential in combining them into single systems, with relevant data like yield, productivity and titer collected over time. However, this is especially difficult on large-scale where data profiles are often difficult to obtain. Such online large-scale data acquisition is another emergent challenge to address. Not only because such data could be used to more accurately understand and model the potential behaviour of the system in a real industrial bioprocess but because an efficient dynamic control is needed, and, therefore, it relies on the proper determination of the system’s instantaneous state.

7. Final considerations

Based on all the previously discussed tools and challenges concerning C3Bio, we can propose the following considerations as critical to designing a continuous co-culture. The primary constraint is that the analysis, design and control approach needs to be applied to obtain a stable co-culture over time and a stable, efficient expression of the desired metabolic function. This metabolic function is the purpose of the bioprocess and, therefore, its starting point. Multiple combinations of modified or unmodified microorganisms might be suitable to achieve the desired bioprocess, and the first step is to ensure that the organisms may grow and express their metabolic functions in the same conditions. Choosing the best initial consortia of specialists could reduce the number of modifications needed to achieve an efficient transformation. Once the microbial species of the co-culture have been identified, the main concern is to identify and quantify the effect the system's cell-to-cell interactions and environmental conditions that may impact both the stability of the co-culture and its performance. Typically, there are two interesting ways to manage these interactions. On the one hand, synthetic constructs with efficient and robust induction and repression patterns can become essential control levers in the system. When introducing new regulation constructs, it is essential to maintain the added interactions as simple as possible to minimise the added complexity. On the other hand, using different strains with clear different responses to certain stimuli may be a viable alternative, as the main differences in the natural growth conditions and metabolism of the strains can provide another set of control levers on the system. For example, available surface or agitation may affect biofilm-forming strains, differences in carbon source utilisation can impact the system, or two strains normally growing at different pH mean that pH could be used as leverage. It is relevant at this stage and along further design process to ensure that the model and data are as close as possible to each other and to the final desired system, and that relevant proxies or sensors can be used at the final operation scale to determine the state of the system. Once the dynamics of the culture and a set of critical parameters are known, it is possible to engineer the fermentation process, first to ensure that the co-culture can be maintained in continuous culture and then that it is as productive as possible. The former implies managing cell interactions, stress and growth, and thus may impact the productivity of the cells. In turn, managing cell productivity may imply, for example, the addition of controller cells or oscillating conditions that may affect the stability of the co-culture. In other words, the process is not limited by the existing control levers that can impact the synthetic constructs or the cells: synthetic gene circuits can be added or modified to add or remove critical parameters and make the culture manageable. These levers will require selecting or designing dynamic control rules and controllers that can follow the dynamic transitions of the microbial community. As a final note, we suggest that C3Bio design should be an iterative process where biological and bioprocess engineering must be considered and developed in parallel, rather than successively.

Real-time characterisation of microbial populations with automated flow cytometry

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The systemic approach described in the previous chapter is particularly important for continuous co-cultures due to their inherent complexity. It is, however, relevant for all bioprocesses or microorganism study in general. Currently, a number of individual biological components ranging from metabolic pathways to quorum sensing are reasonably well understood. There are ways to create whole genome metabolic models statically, at steady-state, and in some cases with ODE-based kinetics [66, 67, 155]. There are ways to simulate large-scale bioreactors or specific parts of the metabolism over time in silico [34, 68, 69]. But there is no simple way to predict the emergent properties of all these components that interact with each other and change over time.

Thankfully, investigating this gap in our knowledge is possible, thanks to innovative measuring techniques developed in the last decades. Microfluidics, studying individual cells in tightly controlled conditions, omics measurements, gathering data on large whole sets of biological molecules, and more, can provide a gold mine of information related to the dynamics of biological processes, if analysed appropriately.

In particular, in this work, an automated flow cytometry setup [4] is used to follow bioprocesses over time; this chapter describes this setup and how its data was analysed to provide key insights into bioprocesses.

1. Automated flow cytometry and segregostat

Flow cytometry is a technique that enables the analysis of tens of thousands of individual cells in a matter of minutes. Briefly, a microfluidic device aligns cells from a liquid sample in a single file, and each cell is analysed one by one as it flows past a laser beam. The scattered light and fluorescence emitted by each cell is recorded, providing information on cell size, granularity, and fluorescence intensity, that is, their phenotype (FIGURE 3.1). (several relevant definitions for this section are provided in the GLOSSARY)

In order to follow bioprocesses over time, an automated flow cytometry setup was developed (FIGURE 3.2, [4]). This setup allows for the regular sampling of a continuous culture bioreactor, the analysis of the samples by flow cytometry, and the automatic actuation of pumps to change the culture conditions based on the flow cytometry data.

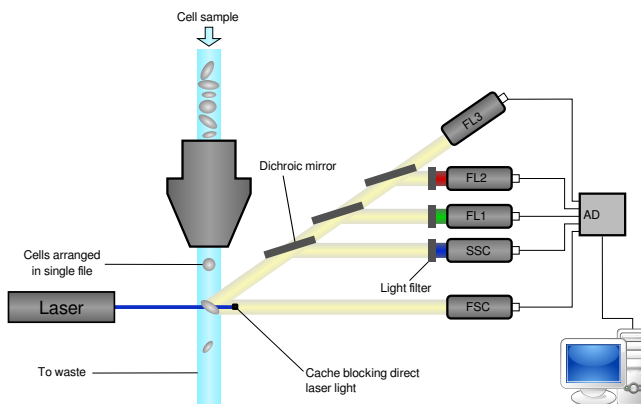


Figure 3.1: Schematic representation of a standard flow cytometer, showing the fluidic, optical, and electronic components.

FSC: Forward scatter measures how much cells deflected the laser, it is correlated with cell size.
SSC: Side scatter measures how much cells reflected the laser, it is linked with cell morphology.

FL1, FL2, FL3: Fluorescence channels measuring the fluorescence emitted by the cells at different wavelengths. The number depends on the cytometer, and the measured wavelengths depend on the mirrors and filters used.

AD: Analog to digital converter, transforms the electrical signals from the detectors into digital data that can be processed by a computer.

(Image adapted from <https://commons.wikimedia.org/wiki/File:サイトメーター.svg>)

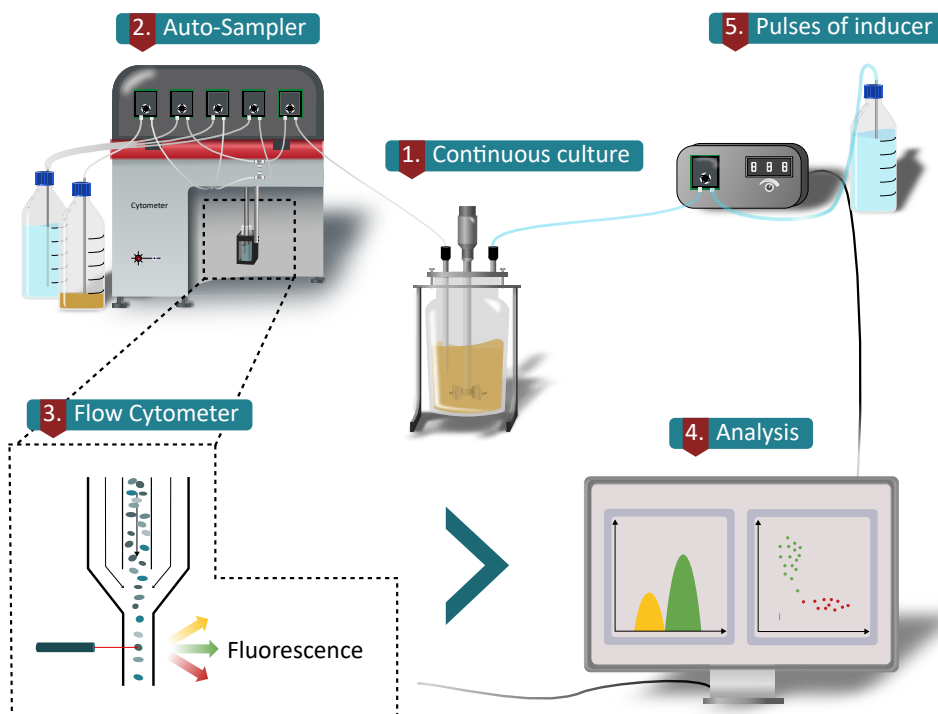


Figure 3.2: Automated flow cytometry setup

(Image credit: adapted from Maximilian Sehart's design)

1. A continuous bioreactor is used to culture the cells. Besides the flow cytometry, probe in the device can collect temperature, pH, oxygen density and biomass measurements, and the pH is regulated.
2. An autosampler, designed by Andrew Zicler, can collect samples from the bioreactor at regular intervals (every 15 to 45 min depending on the equipment) and dilute them for the flow cytometer.
3. The flow cytometer analyses the samples, providing a size and fluorescence distribution of the population.
4. The data is analysed in real-time and key statistics are gathered.
5. Arbitrary volumes of concentrated inducers can be added to the bioreactor, either with a pre-programmed schedule or in response to the flow cytometry data.

Depending on the equipment, the autosampler enables a flow cytometry measurement every 15 to 45 min, providing a detailed size and fluorescence profile of the microbial population in the bioreactor. This in turn makes it possible to automatically actuate various pumps and change the chemical parameters of the culture. These changes, for this work, are 'pulses' of inducer: a concentrated solution of an inducer is added to the culture in less than a minute, then the cells are left to respond to the new conditions. From the original publication, an experiment using these automated response pulses to enforce a specific level of fluorescence (segregate the cells into their target population)

is called a segregostat, by opposition to a chemostat which is a continuous culture with constant feeding conditions (see GLOSSARY).

Within a chemostat, the dilution rate D , expressed in h^{-1} , is equal to the flow rate of the feed divided by the culture volume. It indicates how fast the medium is renewed in the culture. If a steady-state is reached, the average growth rate μ of the cell population is equal to D . If any part of the cell population cannot grow at the rate D , because it exhibits a different phenotype, it is washed out of the bioreactor. The fitness of this sub-population can be defined as μ/D , so that a fitness ≥ 1 is required for the sub-population to grow in the given conditions. When a given phenotype is induced and its fitness decreases as a result, we call it a fitness cost. This can happen with the metabolic burden of heterologous protein expression or the stress response, for example.

One last important point regarding our continuous cultures is that they are made with defined medium and carbon limited: the medium does not contain variable sources of nutrient (no yeast extract, for example, which is rich in various nutrients and can grow a lot of microorganisms, but whose exact composition can vary), and all nutrient are in excess of the carbon source, that is glucose or another sugar. Therefore, the cells will run out of sugar well before anything else and its concentration can be used to characterise their growth.

2. Biological systems explored

Across this thesis, several biological systems are investigated using the automated flow cytometry setup described previously. Before presenting some of the techniques used to extract information from the data, this section summarises the biological systems studied, how they are induced, and what their effect on the cell is.

2.1. *E. coli* arabinose consumption pathway

The first system investigated is *Escherichia coli*'s arabinose consumption pathway. The arabinose regulon consists of seven genes, spread over three operons, *araBAD* (which produces the AraB, AraA and AraD enzymes, see NOTATIONS), responsible for the catabolism of arabinose, under the control of the P_{araB} promoter, and *araFGH* and *araE*, both responsible for the transport of the sugar into the cell. The expression of these genes is inhibited by the transcription factor AraC in the absence of arabinose. [156]

This consumption pathway is usually studied in the context of carbon catabolite repression, a phenomenon which lets *E. coli* preferentially consume higher quality carbon sources, such as glucose, before comparatively lower quality ones, such as arabinose. In this case, when glucose is present, the cells present a low level of cAMP. cAMP is a small molecule that indicates the energy status of the cell, and its presence is required for the expression of the arabinose consumption genes. Therefore, the arabinose consumption genes are only fully expressed when glucose is absent and the

cells are in a low energy state, and arabinose is present. [156, 157]

While this phenomenon is well known, it does not mean that several carbon sources cannot be consumed simultaneously, and it has been shown that glucose can be co-consumed with arabinose when in chemostat conditions (such as in our main experimental setup, where residual glucose concentrations are low. [158]

To study this system, we used a reporter from the Zaslaver collection [159], which consists of a low-copy plasmid (see GLOSSARY) containing the P_{araB} promoter driving the expression of GFP (GFPmut2 in this case). In a chemostat with glucose limitation, this reporter was activated either by adding arabinose to the feed, or by adding pulses of arabinose to the culture using our automated flow cytometry setup.

2.2. *E. coli* T7 expression system

E. coli is one of the most studied bacterial species, and is widely used both in the lab and in industrial biotechnology applications. For these industrial applications, various strains with enhanced production capabilities have been developed. In particular, for protein production, BL21(DE3) is a well-known host. The strain has a genomic integration (see GLOSSARY) of the T7 polymerase, a viral RNA polymerase that is both very active, and very specific to certain promoters, which the virus originally uses to hijack the cell's metabolism. This genomic integration is under the control of a lactose-dependent promoter to control the production. The strain can then be transformed with a plasmid containing a T7-specific and lactose-dependent promoter controlling the production of the protein of interest for industrial applications. [160]

This production is very effective, a significant portion of the cell's biomass under induction becomes the protein of interest. However, this effective production redirects most of the cell's biomass production capabilities away from growth, slowing it down dramatically (it creates metabolic burden). [161]

The T7 polymerase is induced with *E. coli*'s natural LacI protein and a variant of the natural lactose promoter, P_{lacUV5} [160]. Just like the arabinose operon mentioned previously, it is sensitive to carbon catabolite repression and glucose represses production [162].

In this chapter, we use this production system to produce GFP using the pET28:GFP plasmid [163], which lets us determine, through flow cytometry, the heterogeneity of the protein production in the population when it is induced with lactose. The induction itself could be, like for arabinose, either co-feeding of glucose and lactose, or pulses of lactose in a glucose-limited chemostat.

2.3. *E. coli* and *S. cerevisiae* stress response

In *E. coli*, the general stress response is a mechanism that allows adaptation to a wide range of environmental stresses, such as nutrient starvation or heat shock, and therefore appears in a wide range of contexts, from industrial metabolite production to antibiotic resistance. Different stressors trigger different genes, which all eventually converge towards activating the RpoS sigma factor. However, directing its resources towards the stress response comes at a cost for the cell, as it has to divert resources away from growth. This fundamental trade-off causes an observable decrease in growth rate when the stress response is activated, and is probably the reason why the stress response is not always active. [164, 165]

To study this system, unfortunately, the RpoS sigma factor cannot be used directly: even when stress is present, it is produced in very low quantity, which made an *rpoS* promoter based fluorescent reporter barely detectable under our microscope, and undetectable with our flow cytometer. Therefore, we tested a number of different genes that are under the control of RpoS (see ANNEX B, section 4) to find a suitable reporter. The reporter we settled on, $P_{ydcS}::GFP$ (from the Zaslaver collection, [159]), has a low baseline activation, is detectable in flow cytometry when the stress response is activated, and was correlated with both the intracellular RpoS sigma factor concentration and the growth rate of the cells under nutrient stress (the primary stress in a nutrient-limited chemostat such as our main experimental setup) in a microfluidic device (ANNEX B, section 5). The *ydcS* gene itself is associated with DNA transfer [166] and carbon flux distribution [167].

Just as *E. coli*, the common yeast *Saccharomyces cerevisiae* presents a trade-off between growth and stress response, and its stress response appears in various contexts.

Several stress response reporters exist for *S. cerevisiae*, and we chose $P_{glc3}::GFP$ [168], associated with the *glc3* gene, which is involved in glycogen metabolism. When under nutrient stress, the yeast stops growing, produces glycogen instead, to store energy while it is still available, and the *glc3* gene is up-regulated. [169]

Unlike the arabinose and T7 systems, the stress response was not induced by adding a chemical to the culture. Instead, in chemostat, a high dilution rate (0.6 h^{-1} for *E. coli* and 0.3 h^{-1} for *S. cerevisiae*) causes a high growth rate and low stress response, while a low dilution rate (0.1 h^{-1} for both) causes a low growth rate and high stress response. Notably, in nutrient stress condition, based on microfluidics measurements (ANNEX B, section 5), the stress response observed caused growth rates around 0.1 h^{-1} for *E. coli*, which is close to the stress dilution rate used, while *S. cerevisiae* showed near total growth arrest, which would cause a wash-out in a continuous culture. For segregostat experiments, the automated flow cytometry setup was used to temporarily inhibit the stress response by adding pulses of glucose to the culture.

2.4. *B. subtilis* sporulation

Bacillus subtilis is a bacterium relevant in soil microbiomes and industrial protein production, among others. Its stress response has multiple stages, going from a regular stress response, to cannibalism, where the cells produce toxins and kill part of their own population to consume their intracellular content, competence, to integrate foreign DNA into their own, or sporulation, for example. Sporulation is the species' last recourse against adverse environments: a sporulating cell will produce a spore, a non-growing capsule of proteins containing its genetic material and the resources required to re-start growing when external conditions are more favourable. [170]

The commitment to this sporulation process by the population is the one we study in this chapter, through a $P_{spoIIIE}::GFP$ reporter. *spoIIIE* is involved in the separation of the spore from the cell, after commitment to sporulation [171, 172], and is then released in the medium so that a $P_{spoIIIE}::GFP$ reporter lets us identify sporulating cells only but not spores.

Similarly to the stress response, the sporulation process appears in low dilution rate chemostats due to the lack of nutrients.

2.5. *E. coli* growth-limiting toggle switch

The previous systems all interact with growth by redirecting the cells' resource. To complete this list, we sought a way to arbitrarily control the growth rate of the cells and turned to synthetic biology. To achieve this goal, we combined two existing tools in an *E. coli* strain.

The first is a synthetic genetic toggle switch, providing a way to control the state of the cells. The toggle switch is a bistable genetic construct composed of two genes that repress each other (*tetR* and *lacI* in FIGURE 3.3). In effect, a cell equipped with a toggle switch can be in two states, both stable, with either of these two genes expressed. Additional genes can be added to each state so that the cell's state can control its behaviour independent of the rest of the rest of its metabolism. By choosing control genes that can be inhibited, it is possible to control the state of the cells, usually by adding inducers. In this case, we used the toggle-switch from Cameron and Collins [173], which has fluorescent proteins associated to each state and can be measured in flow cytometry.

The second is a dependency to a gene. Some genes are absolutely essential for cell survival, and, historically, moving one of these genes from the cell's genome to a production plasmid has allowed plasmid maintenance without antibiotic, effectively making sure the cells do not lose their plasmid by making them dependent on it [174]. We copied the original technique by creating a $\Delta serA$ strain and using the *serA* gene as a way to control the growth of the strain. The SerA protein is one of the proteins responsible for serine synthesis from, initially, glucose. When it is impaired, it makes

the strain auxotroph to serine, unable to grow in media without serine or other amino acids¹ [175–178].

serA was added to one side of the toggle switch (FIGURE 3.3) in a $\Delta serA$ strain. The result, in theory, is a strain whose growth can be turned off by flipping the switch with a chemical inducer, IPTG. In practice, the non-growing side of the switch only had a reduced growth rate (0.3 instead of 0.8 h⁻¹), most likely because the promoters are not perfectly repressed and some SerA is still produced in small quantities.

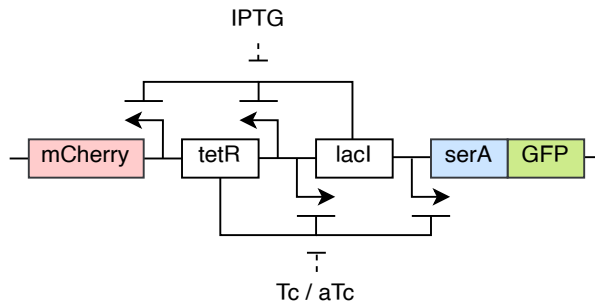


Figure 3.3: Growth-limiting toggle switch design. The switch itself, from Cameron and Collins [173] is composed of *tetR* and *lacI*; one stable state has LacI being produced and inhibiting TetR synthesis, while the other has TetR inhibiting LacI synthesis.

The states can be switched by adding IPTG, which inhibits LacI's effect and lets TetR be produced, or tetracycline (Tc) or anhydrotetracycline (aTc), which inhibit TetR's effect and let LacI be produced.

mCherry, *serA*, and *GFP* are genes put under the control of LacI and TetR so that the switch controls their state as well; *mCherry* is produced with TetR and *SerA* and *GFP* are produced with LacI.

mCherry and *GFP* are fluorescent proteins present so the state of the switch can be measured, while *serA* was added to control growth.

¹*E. coli* can produce serine from glucose (from 3-Phospho-D-glycerate issued from glucose) or from other amino acids, by transforming them back into glycine then serine. However, all amino acids are originally produced from serine, so that a culture medium without any amino acid provides no material for the cell to transform into serine. This was also verified with our $\Delta serA$ strain and our medium.

3. Inferring fitness cost from segregostat measurements

This section is adapted from the supplementary information for the following publication:

[179] Mathéo Delvenne, Vincent Vandenbroucke, Lucas Henrion, Maximilian Sehr, Juan A. Martínez, Alizée Sloodts, Samuel Telek, Andrew Zicler, and Frank Delvigne. *A Fitness–Entropy Compensation Effect Set the Trade-off between Growth and Gene Expression in Cell Populations*. July 9, 2025. DOI: 10.1101/2025.07.05.663304. Pre-published

The following resources are associated to this publication:

- doi.org/10.5281/zenodo.14273182
- gitlab.uliege.be/mipi/published-software/2024-fitnessentropycompensation

When working with continuous culture, the growth rate of the population is determined by the dilution rate. However, this growth rate is the average growth rate of a population at steady-state. When there are multiple phenotypes in a culture, or when the culture is not at steady state, it is entirely possible for different parts of the populations to have different growth rates.

This can be a problem when analysing the data: if different phenotypes become more or less prevalent in a culture, there is no way to tell whether that is because one or the other phenotype is growing slower (we say it has a fitness cost, as a lower growth rate compromises cell survival in a chemostat), or because cells are changing phenotype.

While it can be a problem, this specificity was used by Lucas Henrion in [180] to measure the growth rates of different phenotypes in a culture. By inducing a gene circuit with a fitness cost and a fluorescence tag, then stopping the induction (segregostats), a window of time is created where fluorescent proteins in the cells are not produced, only diluted as the cells grow. By measuring how fast this dilution happens in different sub-populations, it is possible to infer their growth rate.

In this section, we further refine that technique and apply it to all the *araB*, *T7*, *ydcS*, and *glc3* systems described in the previous section, in order to obtain growth rate data for chemostat experiments in the next chapter (detailed materials and methods for the experiment are described with the next chapter as well, code description is available in ANNEX B, section 8).

3.1. Basic principles of the method

The technique uses Segregostat measurements, where induction is periodic instead of constant. Thanks to the periodicity, there is a relaxation phase after each induction,

where no fluorescence is produced (see FIGURE 3.4A). We used this relaxation phase to estimate phenotype growth rates; this estimation relies on a few assumptions:

- The growth rate is constant during the portion of relaxation phase considered. Technically, the cells are changing phenotype (recovering from stress, for example), but we used data from only up to 1.5 h to have 5 measurements and decrease the influence of the change in phenotype.
- During relaxation, no fluorescence is produced, and the main source of decrease is dilution. In other words, the decrease in fluorescence is only due to growth, as the amount of fluorescent protein spreads through the cell's lineage, and calculating it is equivalent to calculating the growth rate. This is pertinent because protein degradation is negligible compared to growth in most cases [181].
- The function of growth rate with respect to fluorescence is monotonously decreasing. What this means effectively is that, when two phenotypes are measured at time t and $t + \Delta t$, a cell with a lower fluorescence than another at time t will also have a lower fluorescence at time $t + \Delta t$ because it is growing faster, hence diluting the fluorescence more, and there is no intersection between the trajectories of the cells. This assumption underlies the entire strategy we are using, as it lets us correlate the different portions of the population in different samples, and we will come back to its validity. When there is a trade-off between growth and gene expression, this is a reasonable assumption as a higher fluorescence means more protein production, hence less growth.

Using these assumptions, we can measure the growth rate of a population by following the fluorescence of different portions of the population over time: if the upper quartile of the population halves its fluorescence in 4 hours and the lower quartile halves it in 2, then the growth rate of the lower quartile is 0.35 h^{-1} , and twice that of the upper quartile (FIGURE 3.4B).

In practice, the population was split in the 90 percentiles between 5 and 95% of the fluorescence distribution in order to capture the full range of growth rates. The fluorescence was followed over five successive automated flow cytometry measurements, that is roughly an hour, to avoid too much phenotypic change over the measurement period. This was done for multiple relaxation phases and for multiple biological replicates, to determine a Hill function (see Glossary, here representing the inhibition of growth by fluorescence) that would correctly approximate the growth rate as a function of fluorescence (FIGURE 3.4C for a single biological replicate). This trade-off curve can then be used to determine the fitness of a population based on its fluorescence measurements.

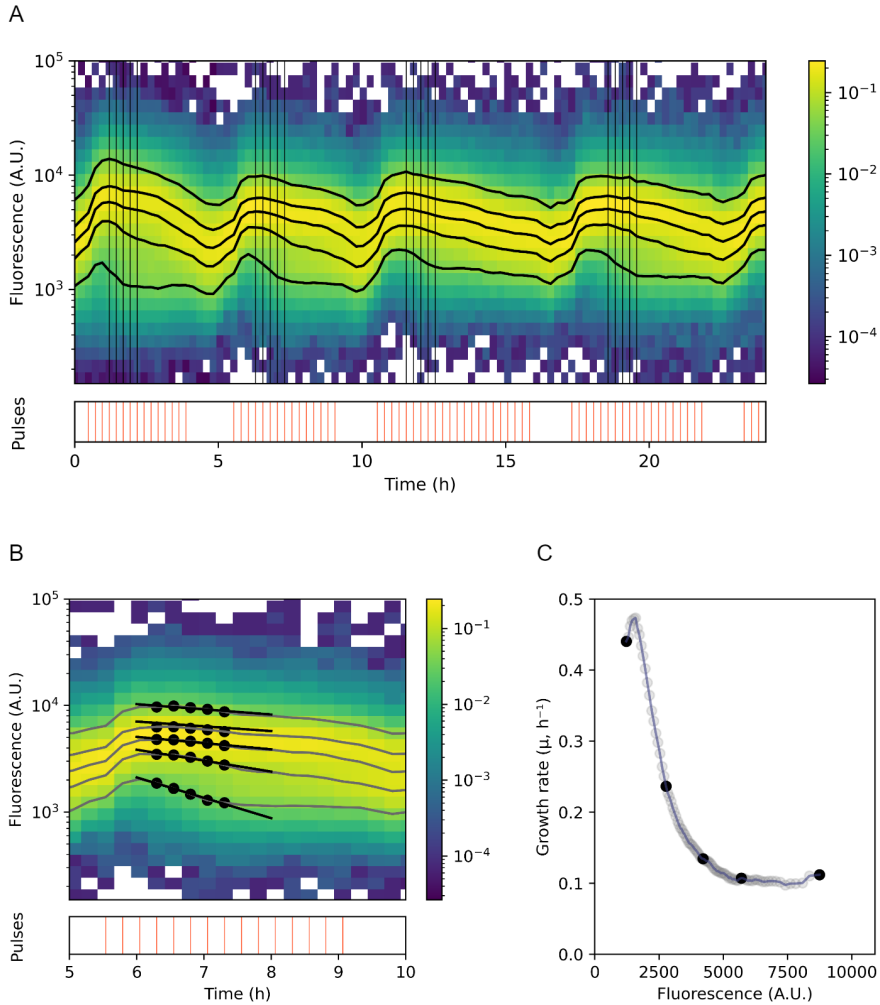


Figure 3.4: **A** The time scatter of a segregostat experiment for the yeast, showing the measured fluorescence over time, correlated with stress; lighter colours indicate more cells at that time and fluorescence. The lines in the 'pulses' rectangle indicate when glucose was pulsed to reduce the stress levels. The black vertical lines on the scatter plot indicate the sample times used to compute the trade-off curve. The black lines following the population indicate percentiles 5, 25, 50, 75 and 95. Periodic induction of stress and relaxation phases are produced. **B** For each relaxation phase, exponential curves (black lines) were fitted to the five selected time points (black dots, along the same percentiles (gray lines) shown in **A**). Note that these fits do not account for the mitigation of various sources of error described in the next section. **C** Trade-off between gene expression (fluorescence) and growth rate, as derived from the fitted exponential curves. Each round dot corresponds to a fluorescence percentile; black dots indicate the results for the specific percentiles illustrated in panels **A** and **B**.

3.2. Mitigation of various sources of error

While this basic method is useful, there are several sources of error that can impact the measurements significantly. Some of them were accounted for in our data treatment to improve the accuracy of the results, while some minor factors cannot be corrected for accurately.

- **Bigger cells have a higher fluorescence**, but do not necessarily grow slower because they also have more resources available. Instead, the measurement of interest is the fluorescence concentration in each cells. Therefore, instead of using the raw fluorescence, we used the fluorescence divided by the forward scatter (FSC) measurement, which is a proxy for the size of the cells. For yeast, FSC is linear with respect to cell volume [182], and this correction is valid. For bacteria, the relationship between FSC and cell volume is harder to establish, but at the very least, very stressed cells that filament and have a much higher FSC do not inflate the fluorescence excessively if this normalisation is applied.
- **The auto-fluorescence of the cells** implies that fluorescence measurements do not follow a simple decaying exponential (FIGURE 3.5). Rather, they follow a decaying exponential shifted up towards the auto-fluorescence ($Fluo = Fluo_0 \cdot e^{-\mu t} + Fluo_{auto}$, where $Fluo_0$ is the initial fluorescence, $Fluo_{auto}$ is the auto-fluorescence, $Fluo$ is the fluorescence at time t and μ is the growth rate of the portion of the population of interest) Without taking that into account, the growth rate would be systematically underestimated. To correct for this, we subtracted the mean auto-fluorescence of a blank sample without induction from the fluorescence measurements. Moreover, the auto-fluorescence is not the same for all cells. To avoid confusing fluorescence decay with auto-fluorescence, we excluded any fluorescence point that entered the range of auto-fluorescence from our measurements.
- **Because all portions of the populations do not grow at the same rate**, the abundance of each section of the population (in our case, each percentile at a given time) changes over time: the slower, high fluorescence cells are washed-out while the faster, low fluorescence cells grow and take over the reactor (FIGURE 3.6). This means, for example, that the 25th fluorescence percentile at a given time may correspond to 30% of the population one hour later instead of 25%. Therefore, with the basic method, we would over-estimate the growth rate of each sub-population. This was taken into account by iteratively estimating the growth rate of the population, using it to measure which percentiles at a given time correspond to which percentiles in the other samples, then re-computing the growth rate based on these new percentiles, until the change in growth rate was negligible.

We investigated the effect of this correction for the *glc3* system in *S. cerevisiae*

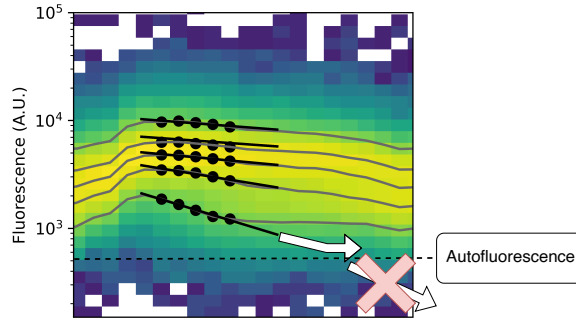


Figure 3.5: When fitting a simple decaying exponential, we assume the fluorescence is decaying towards zero. However, the fluorescence measurement is a sum of fluorescence due to GFP and auto-fluorescence. The decaying exponential should be going towards the autofluorescence (roughly 500 for yeast), so that going from 2000 to 1000 in fluorescence represents a 70% decrease in the fluorescence measurement due to GFP, instead of only 50%.

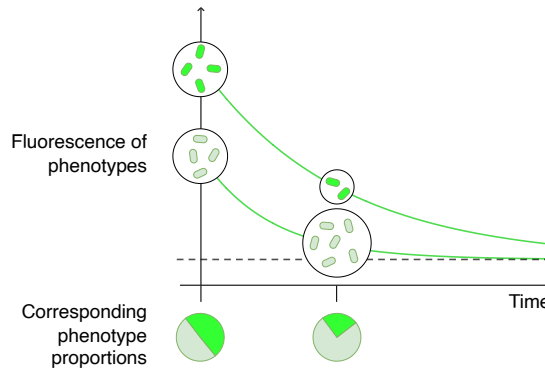


Figure 3.6: As the fluorescence decreases, faster-growing phenotypes also increase in proportion in the population. Based on the estimated growth rates, this change in proportion is estimated, and taken into account to correct the growth rates.

and found that it changed the estimated growth rate by up to 20%, showing that it is an essential step to obtain a good measurement.

Notably, in order to compute the change in abundance of each percentile of the population in the bioreactor, the dilution rate of the reactor is used. In our setup, the volume of the bioreactor may change by $\pm 5\%$ between experiments, and the tube used for feeding being autoclaved and wearing out may add error on top of that. To evaluate the impact of this error, we performed the calculations for the second replicate of the *glc3* system, which has the most relaxation phases, while setting dilution rates in the equations at 0.1, 0.125 (the actual dilution rate), and 0.15 h^{-1} . The resulting Hill function parameters changed by less than $10^{-5}\%$, showing that this error is negligible.

- **The assumption that the growth rate is monotonously decreasing with fluorescence** is reasonable, but not always measurable. In the range of fluorescence where the growth rate clearly decreases, everything works as intended. However, for low fluorescence values, the growth rate should be roughly constant with respect to fluorescence, and an increase in growth rate is often measured instead (as can be observed in FIGURE 3.4C for example). Similarly, for the highest fluorescence values, the growth rate reaches a minimum and, in some cases, we measure a small portion of the population with a small increase in growth rate. When correcting the percentiles, we replaced these aberrant values with the maxima or minima of the measured growth rates, and we did not use these points to fit the Hill function for the trade-off curve.
- **The growth rate of the cells may change over time**, as we are measuring it during the recovery from stress. To limit this effect, we only used about an hour of measurements, as early as possible when the fluorescence starts to decrease. The cells may be recovering anyway and it is not possible to accurately estimate this effect, we simply know that we may be over-estimating the growth rate of the stressed cells because they are recovering.
- **Our cytometers are not linear with respect to fluorescence for bacteria**, as mentioned in SECTION 1. Three different cytometers were used for the measurements, one BD Accuri C6 and two BD Accuri C6+, and the correlation between cytometers was not always linear. For yeast, this does not seem to be a problem, but for the T7 system in *E. coli* BL21, discrepancies were observed. The same discrepancies were not observed when measuring the *ycdS* system in both C6+ instead, suggesting the problem is specific to BL21. The relationship between our C6 and C6+ cytometers in particular is interesting as the correlation is of the shape $y = ax^b$, where y is the measurement of the C6+ cytometer, x is the measurement of the C6 cytometer, and a and b are parameters fitted to the data. Assuming that the relationship between actual fluorescence and any cytometer measurement follows a similar relationship, all measured growth rates would have an identical error factor of b . This was taken into account by measuring the average measured growth rate during the relaxation phase, and normalising the fitness-cost curve by the factor: 'average measured growth rate/dilution rate' (where the dilution rate, in a chemostat, should be equal to the actual average growth rate of the cells). We applied this correction for all bacterial systems. For the *ycdS* and *araB* systems, the resulting normalisation factors were 1.1 and 1.01, respectively, showing that the issue is quite small. For BL21, the normalisation factor was 1.7, showing large non-linearities between the reality and cytometer measurements.
- **The T7 system in *E. coli* BL21 stops being induced at different times based on fluorescence**. Unlike for other systems, where the onset of recovery from

induction was uniform across the population, for the T7 system, the lower fluorescence levels decreased in fluorescence while the higher levels were still increasing. Selecting samples too early therefore caused the apparition of negative growth rates for high fluorescence values in our measurements, due to the fluorescence increasing. We set these values to 0 h^{-1} when correcting the percentiles and did not use them to fit the Hill function, but if this part of the curve was negative, then the rest of it was underestimated. Similarly, when taking the samples too late, a high proportion (often 30 to 50%) of the population was already very low in fluorescence and couldn't be measured, and the rest of the population was likely already recovering from the stress, so taking that estimate would overestimate the growth rate.

To get a reasonable estimate, for each relaxation phase, multiple successive sets of samples were used (FIGURE 3.7). If the first are underestimated and the last are overestimated, then fitting the Hill function on all the points provides the best possible estimate.

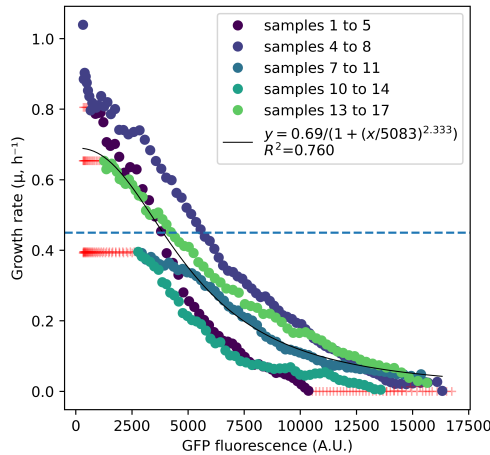


Figure 3.7: One relaxation phase of the T7 system in *E. coli* BL21. Each colour corresponds to one set of samples in the relaxation phase, from the earliest to the latest. The fitted Hill function was fitted on not all the relaxation phases of the replicate, though only one is shown here. The earlier measurements are under-estimated, the later ones are over-estimated, and the approximation is in between. Red '+' points were invalid and not used for fitting the Hill function. The dilution rate of 0.45 h^{-1} used in the Segregostat is shown in blue dashes.

3.3. Estimation of the trade-off curves for the four systems considered

3.3.1. The *ydcS* and *araB* systems in *E. coli*

The cases of *ydcS* and *araB* in *E. coli* are examples where the trade-off between growth and fluorescence should be small or nonexistent: in the first case, microfluidics shows only a small reduction in growth rate in stress conditions, while for *araB*, gene expression enables arabinose consumption and should be beneficial. The results are shown in FIGURE 3.9 (next page). Interestingly, for these two cases, correcting the final hill parameters by the dilution rate did not change the results much.

For the *ydcS* system, the measurements obtained with this technique in a segregostat at 0.325 h^{-1} do not reach the fluorescences observed in chemostat at dilution rate of 0.1 and 0.6 h^{-1} , which are the conditions used as control to reach a stressed or unstressed state. Therefore, and given the homogeneity of the population in chemostat, an approximation of the trade-off function was obtained using chemostat data acquired at various dilution rates (FIGURE 3.8). Only one replicate of chemostat at $D=0.3 \text{ h}^{-1}$ has been performed with an Accuri C6+. Nonetheless, the values at $D=0.3 \text{ h}^{-1}$ are close to the ones observed in segregostat with a C6+. Furthermore, cultivations at the 3 considered growth rates have also been performed with the C6 and provides similar results (FIGURE B.7, ANNEX B). In addition, microfluidics measurements show only a slight reduction in growth rate with fluorescence/stress. This is confirmed here, as we measure a growth rate that never gets below even 0.2 h^{-1} in segregostat.

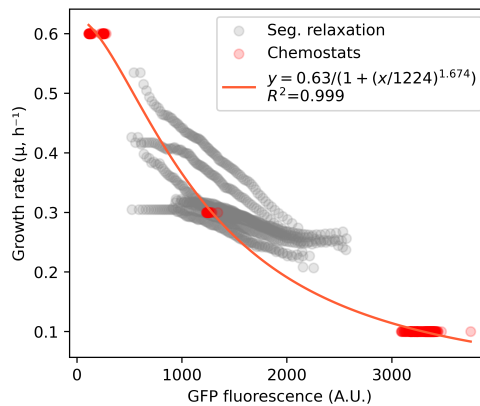


Figure 3.8: Trade-off function of the *ydcS* system fitted on median fluorescence values obtained during chemostat cultivations at different dilution rates. The points obtained with the method exploiting fluorescence relaxation in segregostat are indicated for comparison.

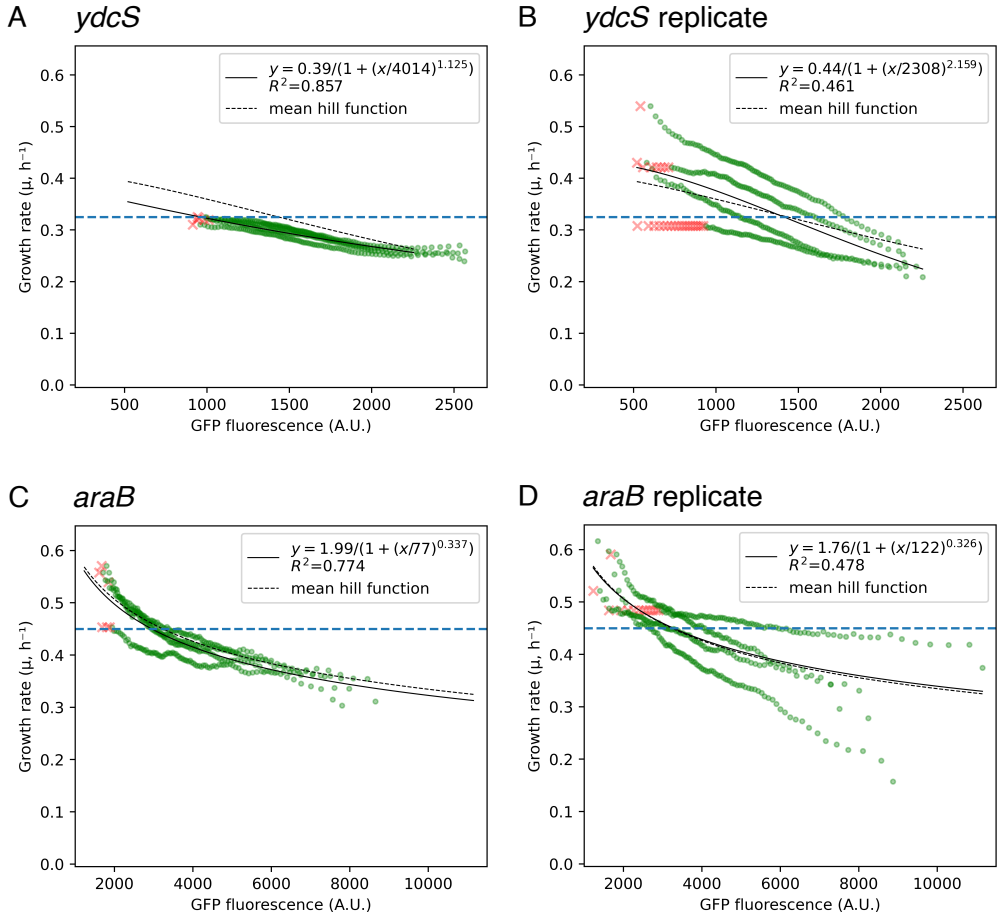


Figure 3.9: Growth rates measured for the *ydcS* and *araB* systems in *E. coli*. Each green point is a valid measurement of the growth rate as a function of fluorescence. No point was measured as <0 and replaced by 0 for the correction of percentiles (subpopulation proportions) based on the growth rate. Each red 'x' was before the maximum of the measured points, and none were after the minimum. This was inconsistent with the requirement that the curve be monotonic. Therefore, they were replaced by the maximum of the measured points for the correction of percentiles (subpopulation proportions) based on the growth rate. For the fitting, of the Hill function (full line), only the green points shown were used. The hill parameters were fitted independently for each replicate then averaged (dashed line). The horizontal dotted line is the dilution rates of $0.325 h^{-1}$ for *ydcS* and $0.45 h^{-1}$ for *araB* used in the Segregostat. The measured average growth rates before scaling, based on the individual Hill functions, for each replicate, were 0.30 and $0.35 h^{-1}$ for *ydcS* and 0.44 and $0.46 h^{-1}$ for *araB*. The scaling factor mentioned in the previous section is already applied in these figures.

For the *araB* system, there should not be any trade-off between growth and fluorescence, as the maximum growth rate on arabinose only of the strain is higher than 0.5 h^{-1} [7]. Similarly to *ycdS*, we show no growth rate below 0.3 h^{-1} .

For both systems, even though there should be very little trade-off between growth and gene expression, we still measure a difference in growth rate with fluorescence, although it is much smaller than the cases with high trade-offs shown later. This can be explained by two factors:

- On the one hand, while there might be little trade-off, we are not measuring the maximum growth rate, but only the growth rate in the culture for given fluorescences. Even with maximum growth rates above the dilution rates, some phenotypes may still be unable to compete with others for limited nutrients, and therefore be slightly disadvantaged. Considering the populations observed are unimodal, however, this effect should be minor.
- On the other hand, if we are expecting little or no trade-off, then the unimodal, homogeneous populations measured have their variance mainly produced by noise in the measurements and noise in GFP expression. The closer we are to measuring only noise, the more the assumption that cell trajectories are not crossing each other is violated.

Considering these facts, since we know there should be very little trade-off, we can consider the minimum growth rates obtained for these cases as a benchmark: they are the minima that need to be exceeded to ensure there is, actually, a trade-off between growth and gene expression, when measuring a system with this technique for which the result is a priori unknown. These minima are 30 to 40% lower than the dilution rate, and below that we can reasonably expect the growth curves calculated with this technique to reflect reality, as is the case for the *glc3* system in *S. cerevisiae* and the *T7* system in *E. coli* BL21, where we observe a reduction of at least 80% for the lowest growth rates.

3.3.2. The *glc3* system in *S. cerevisiae* and the *T7* system in *E. coli* BL21

First, for the *glc3* system in *S. cerevisiae*, a high trade-off is expected based on microfluidics cultivation. This trade-off is confirmed by the measurements in the Segregostat, as shown for three biological replicates in FIGURE 3.10. The growth rate as a function of fluorescence is well fitted by a Hill function, however the first replicate is slightly different compared to the others. Since it is the only one made with the C6+ cytometer, which was the model used for chemostat experiments, we use the parameters of the first replicate as the trade-off curve in the next chapter.

Based on the fitted Hill function, we computed the growth rate of these populations and measured slightly higher values than the dilution rate of 0.125 h^{-1} . This may have (and probably has) two main causes. It can indicate that we are slightly overestimating

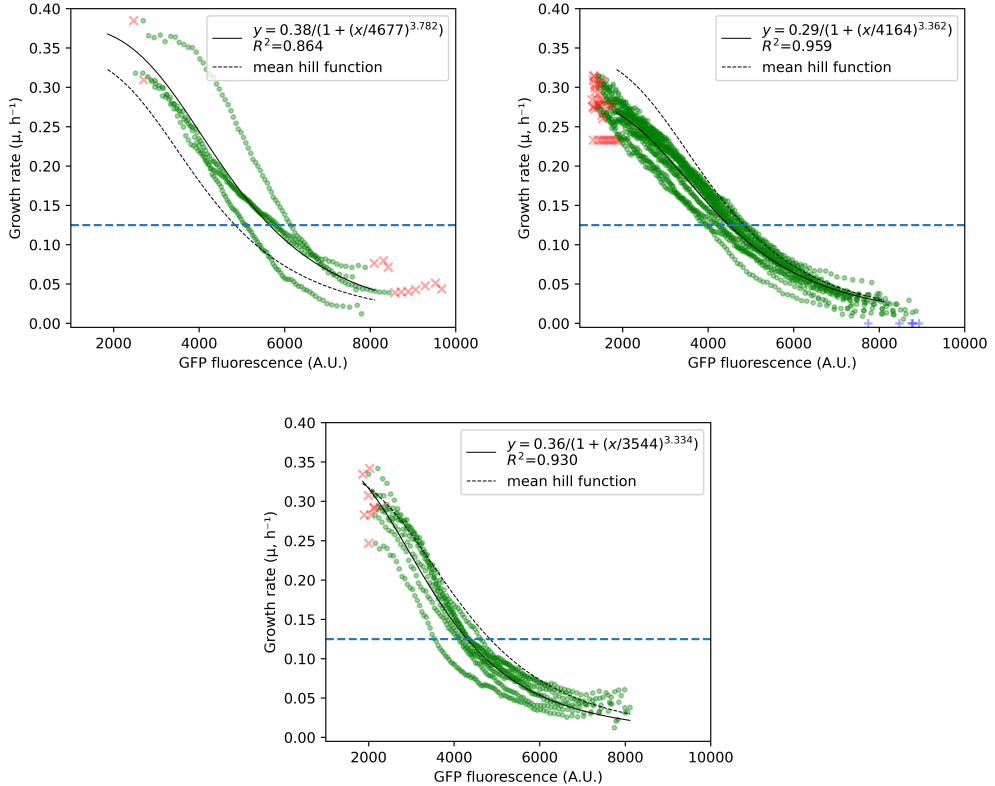


Figure 3.10: Fitness determination results for the *glc3* system in *S. cerevisiae*, in triplicate. Each green point is a valid measurement of the growth rate as a function of fluorescence. Each blue '+' was measured as <0 and replaced by 0 for the correction of percentiles (subpopulation proportions) based on the growth rate. Each red 'x' was either before the maximum of the measured points or after the minimum (and hence did not make sense since the curve needs to be monotonously). They were replaced by the maximum or minimum of the measured points, respectively, for the correction of percentiles (subpopulation proportions) based on the growth rate. For the fitting, of the Hill function (full line), only the green points shown were used. The hill parameters were fitted independently for each replicated then averaged (dashed line). The horizontal dotted line is the dilution rate of 0.125 h^{-1} used in the Segregostat, and the measured average growth rates based on the individual fitted lines were 0.18, 0.16 and 0.13 h^{-1} for the three replicates, respectively, in order of appearance.

the growth rate overall. However, for the *glc3* system, the relaxation phase happens when the culture is fed with additional glucose, which allows the biomass to increase. Therefore, another possibility is that the growth rate was actually higher than the dilution rate.

The *T7* system in *E. coli* BL21 is the system for which the measurements are the most imprecise due to the various caveats mentioned in the previous section, and we had to use a different measurement technique. Nevertheless, we obtained a reasonable estimate of the trade-off curve, as shown in FIGURE 3.7. The second replicate has more variance in the measurements but shows a similar resulting Hill function of $0.78 \cdot (4815^{1.8} / 4815^{1.8} + x^{1.8})$.

4. Entropy as a reliable indicator of population diversity

This section is adapted from the supplementary information for the following publication:

[179] Mathéo Delvenne, Vincent Vandenbroucke, Lucas Henrion, Maximilian Sehr, Juan A. Martínez, Alizée Sloodts, Samuel Telek, Andrew Zicler, and Frank Delvigne. *A Fitness–Entropy Compensation Effect Set the Trade-off between Growth and Gene Expression in Cell Populations*. July 9, 2025. DOI: 10.1101/2025.07.05.663304. Pre-published

The following resources are associated to this publication:

- doi.org/10.5281/zenodo.14273182
- gitlab.uliege.be/mipi/published-software/2024-fitnessentropycompensation

The automated flow cytometry device provides population distributions over time. Such distributions may not be directly useful for quantitative measurements, and different statistics can help summarise and compare these distributions. Most of these statistics have issues unfortunately, as, among other things, they often rely on the underlying distribution being normal (e.g. the t-test) or unimodal, they may be directly dependent on the average of the distribution (e.g. the Fano factor), making them unreliable when that average varies by orders of magnitude, or they may be sensitive to very large outliers (e.g. the mean) – which, when working with the tens of thousands of data points of a flow cytometry measurement, are common. One statistic that does not suffer from these drawbacks and has emerged as a reliable indicator of biological diversity in the literature is Shannon entropy [183–185].

In this section, we first introduce the concept of Shannon entropy and its application to biological measurements (this information is the foundation of information theory and can be found, for most of it, for example, in [186]). Then, starting in SECTION 4.3, we go into the details of what should happen to the statistic in a chemostat with different phenotypes that have different growth rates and introduce the Fitness-Entropy compensation effect for simplified, idealised scenarios. We conclude with a more detailed reasoning that can be applied to realistic cases such as the ones we observe in the next chapter (SECTION 4.4) and a short section on potential alternative behaviours (4.4.3).

4.1. Computing cell to cell variability in gene expression with Shannon entropy

The Shannon entropy (H , measured in bits) is a measure of how heterogeneous a probability distribution is in a given interval of measurements. FIGURE 3.11 illustrates how we can expect entropy to behave for different distributions.

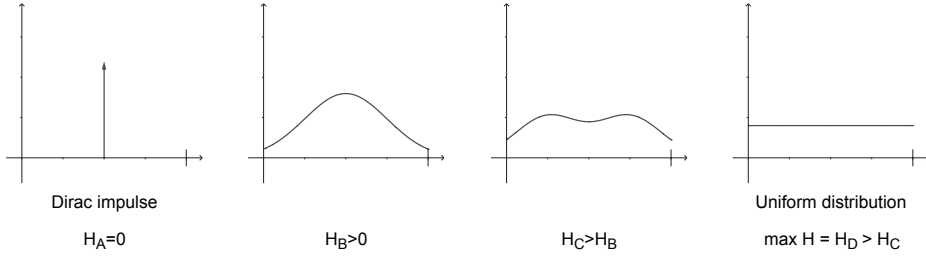


Figure 3.11: Illustration of Shannon entropy H for different distributions in the same interval. A Dirac impulse **A** has an entropy of 0, as all the probability is concentrated in a single point and there is no heterogeneity. A uniform distribution **D** is the most heterogeneous distribution possible within the range, and has the highest possible entropy for it. Between, these extremes, the more heterogeneous the distribution is, the higher the entropy.

This entropy was first defined over discrete distributions as

$$H(\chi) = - \sum_{x \in \chi} p(x) \log_2 p(x), \quad (3.1)$$

where χ is the probability distribution for which the entropy is computed values and $p(x)$ is the probability of observing the value x according to χ , and any value for which $p(x) = 0$ is ignored in the sum.

While this definition can be generalised to continuous distributions, $-\int p(x) \log_2(p(x)) dx$ usually converges to ∞ as a continuous distribution has infinite information in most cases. Therefore, the simplest way to measure the information of a continuous distribution is to discretise it, by dividing the range of possible values into bins and computing the entropy over the resulting discrete distribution.

The choice of these bins will affect the resulting measured entropy. In order to correctly represent the entropy of a distribution, the bins should be smaller than the features of the distribution (i.e. it needs to be smaller than the peaks and troughs of the distribution for information on these peaks and troughs to be measured). However, there is no "convergence" of the entropy, where choosing smaller and smaller bins would lead to virtually identical results: to the limit, reducing the number of bins leads to an integral and, as stated above, increase towards infinity. In practice, when measuring the entropy of a measured distribution, individual points are available and binned. Decreasing the

size of the bins as much as possible would lead to having a single measurement per bin, in which case the entropy will be the maximum possible entropy for the number of measurements available, and that is not useful. Therefore, for continuous distributions, the bins are often arbitrarily chosen, and the entropy is comparative between different distributions with the same binning strategy. The binning strategy itself is a research topic on its own, but for this thesis we used a set of constant bins equally spaced in log space to compare different distributions.

For flow cytometry measurements, more than 10,000 cells are available, and in our case, only 2 major phenotypes are expected. To capture the heterogeneity, we used 50 bins covering the whole range of measurement values for each case study, as illustrated in FIGURE 3.12.

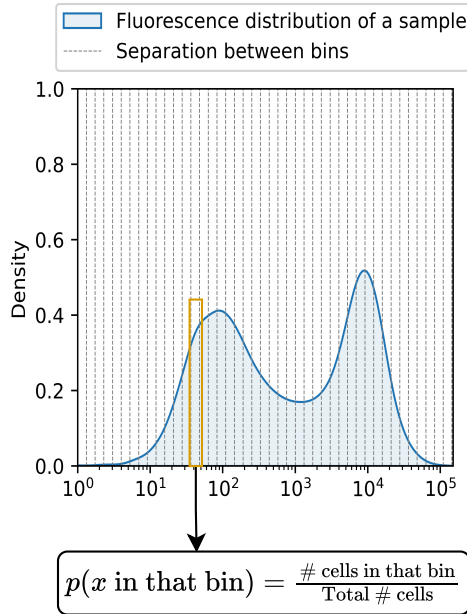


Figure 3.12: Example of the binning strategy used for the entropy measurements. The bins are equally spaced in log space, and cover the range of the measurements. The number of bins is much larger than the number of phenotypes expected (2), but much smaller than the number of cells measured (>10,000).

4.2. Using entropy to compare the level of heterogeneity between populations

In addition to the definition of entropy, there is one important property of Shannon entropy that is relevant here: mixing two distributions increases their entropy. Intuitively,

this can be understood as how mixing two distributions will make the result more heterogeneous. Mathematically, this is expressed as:

$$H(k \cdot \chi_1 + (1 - k) \cdot \chi_2) \geq k \cdot H(\chi_1) + (1 - k) \cdot H(\chi_2) \quad (3.2)$$

where k is a number between 0 and 1, χ_1 and χ_2 are two distributions, and the left-hand side is the entropy of the mixture of the two distributions.

In particular, when the ranges of $x \in \chi_1$ and $x \in \chi_2$ do not overlap, the entropy of the mixture can be directly calculated as:

$$H(k \cdot \chi_1 + (1 - k) \cdot \chi_2) = k \cdot H(\chi_1) + (1 - k) \cdot H(\chi_2) - k \cdot \log_2(k) - (1 - k) \cdot \log_2(1 - k) \quad (3.3)$$

where k being smaller than 1 means that this is indeed always larger than the mean entropy of the two individual distributions.

With this property, it is possible to determine what would happen to the entropy of a mix of two different cell populations, as depicted in FIGURE 3.13. On that figure, the x-axis represents the proportion of each population in the mixture, with 0 being 100% of the first population and 1 being 100% of the second one. The y-axis shows the entropy. Because of the aforementioned property, the lower limit of the entropy is the line joining the entropies of the two populations, and because there is a maximum entropy (the uniform distribution), any mix of two distributions must have an entropy somewhere within the depicted quadrilateral. Further, if the two populations do not overlap, for example when considering two independent phenotypes, then the entropy of the mixture can be calculated as in EQUATION 3.3, and the resulting entropy is shown with the blue curve.

The distributions of interest for this work are the distributions of fluorescence of cell populations. In particular, we are comparing the distributions obtained in continuous cultures to individual phenotypes. These phenotypes are measured either as the autofluorescence or fluorescence of the cells and have a baseline entropy. When contrasting them with a high entropy distribution from a continuous culture, we are measuring how much more heterogeneous the continuous culture is by comparison to the individual phenotypes. This measurement can be applied on multimodal distributions, is independent of the amplitude of the signal, and can be applied to multi-dimensional data if required. For these reasons, it can be more useful than standard statistics, such as the standard deviation or Fano factor, to determine the heterogeneity of a sample.

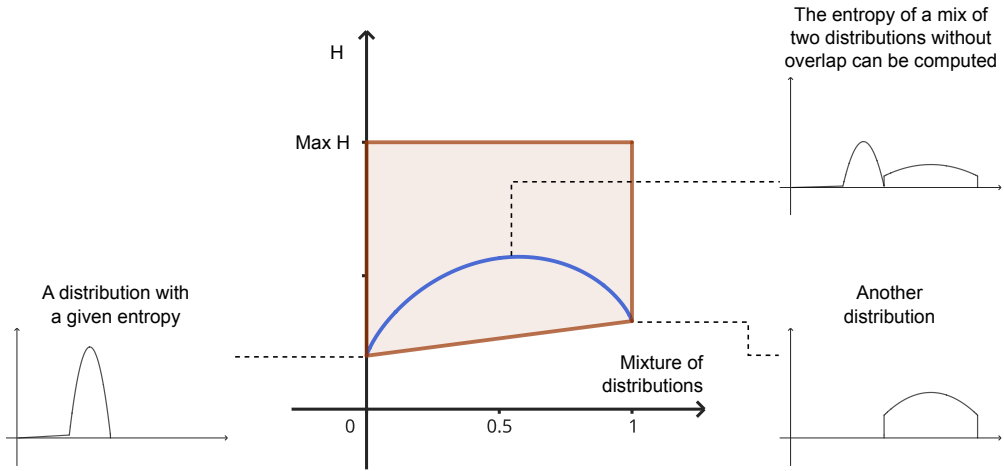


Figure 3.13: Entropy of the mixture of two different distributions; the x-axis represents the proportion of each population in the mixture, with 0 being 100% of the first population and 1 being 100% of the second one, and the y-axis shows the entropy. The quadrilateral on the entropy plot represents the area a mixture of any two distributions could have its entropy fall in. If those distributions do not overlap, like the ones depicted here, then the entropy of the mixture is the blue curve.

4.3. Differences in fitness: the root of a Fitness-Entropy compensation effect

As the previous sections showed, changes in phenotypes can lead to changes in fitness for cultured cells. This change leads to a change in the makeup of the population, which had to be taken into account in SECTION 3 to measure the growth rate of the cells. This change in population composition will also change its related statistics, including entropy. To understand the effect of the change in fitness, it is sufficient to consider two cases in a chemostat:

- A baseline case with two well-defined phenotypes, one induced and one uninduced, where all cells have the same growth rate.
- A case where we have the same phenotypes as previously, but the amount of induction affects the growth rate. Out of simplicity, we can consider that cells above a threshold expression level T have a reduced growth rate μ_{lim} , while cells below this threshold have the original growth rate μ_{max} .

In the baseline case, the two phenotypes φ_1 and φ_2 have the expected normal distribution as depicted in FIGURE 3.14A and B, respectively. By comparison, in the case of induction affecting growth, there is a selection effect: cells with a phenotype above the threshold T have a reduced growth rate μ_{lim} , and this decreases the relative

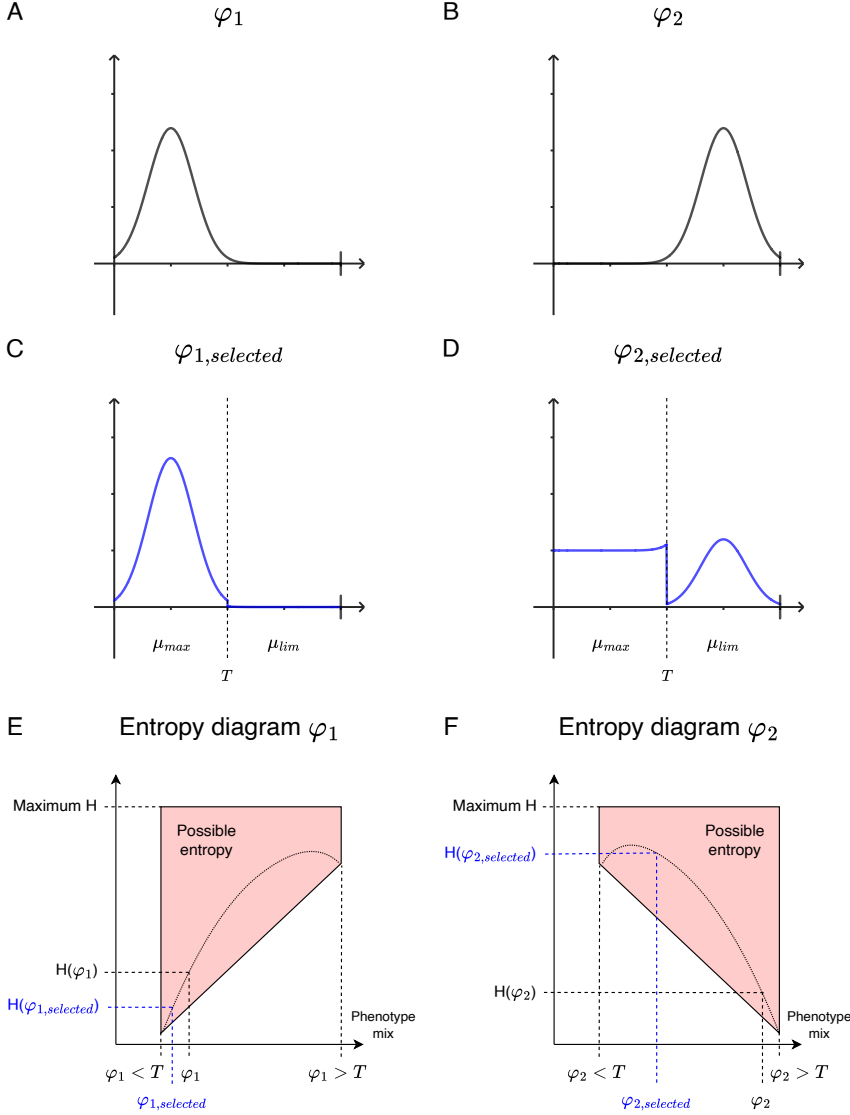


Figure 3.14: **A, B** Example probability distributions (not to scale) of fluorescence for two well-defined phenotypes, where the phenotype is not affecting fitness. **C, D** The same distributions, but where the phenotype is affecting fitness. Since they are well defined phenotype, we can expect most of the cells in φ_1 and φ_2 to be on the same side of the threshold T separating the two phenotypes. The other side is mainly the tail end of the distribution, and is eliminated (**C**) or selected (**D**) as $\mu_{lim} < \mu_{max}$.
(Caption continued on the next page)

Figure 3.14: E, F The entropy of the complete distributions expressed as a mixture of the entropies of the partial distributions, split between the uninduced (100% of cells are $< T$ to the left of the graph) and induced (100% of cells are $> T$ to the right of the graph) sub-distributions, and according to EQUATION 3.3. The entropy of the noisy tail of the distribution is much larger than the entropy of the main phenotype (by virtue of being noise). φ_1 sees nearly no selection since its main phenotype is already growing at μ_{max} , the mixture is shifted slightly towards the $\varphi_1 < T$ sub-distribution. φ_2 's distribution is strongly shifted by selection, which increases the importance of the noisy section of the distribution ($\varphi_2 < T$) in the mixture and correspondingly increase entropy.

abundance of the induced phenotype in the population, as shown in FIGURE 3.14C and D.

Regarding the uninduced phenotype, the distribution is nearly unaffected, as most cells are below the threshold T and therefore have the original growth rate μ_{max} . By contrast, the induced phenotype is selected against, and the selected distribution is a mix of the induced but unfit phenotype and a much larger amount of uninduced cells. These uninduced cells in FIGURE 3.14D would normally be the tail end of the distribution in FIGURE 3.14, and are the noise of the distribution.

This selection predictably affects the entropy of the resulting distributions. The entropy of each distribution can be split into the entropy of the uninduced region and the entropy of the induced region, according to EQUATION 3.3, since we are considering no overlap between phenotypes and a single threshold. In practice, there should be some overlap due to measurement errors and imperfect correlation between fluorescence and growth phenotype, but the principle holds. For each phenotype, the entropy of the region containing the bulk of the distribution ($H(\varphi_1 < T)$ and $H(\varphi_2 > T)$) will be quite small in comparison to their counterpart ($H(\varphi_1 > T)$ and $H(\varphi_2 < T)$), as the bulk of the distribution represents a defined phenotype, while the tails of the distribution are mainly noise and essentially flat, bringing their entropy close to the maximum possible entropy.

FIGURE 3.14E shows that selecting the main phenotype slightly reduces the entropy of the distribution as the portion of the distribution that is eliminated is the noisy one. However, because the noise for a well-defined phenotype is already a very small portion of the total distribution, this may not be measurable.

In FIGURE 3.14F, by contrast, selecting the noise instead of the induced phenotype naturally leads to an increase in entropy instead. This is a Fitness-Entropy compensation (FEC) mechanism, where the entropies of measured biological distributions will be directly shifted by the relative fitness of their phenotypes.

4.4. Applying the Fitness-Entropy compensation effect to our case studies

There are two additional points to consider when determining whether the concept of Fitness-Entropy compensation should apply to a real system. First, in the simplified case of the previous section, only a single transition between two phenotypes was considered. In practice, the growth rate probably changes continuously with the phenotype. Second, we only considered selection of phenotypes, but there was no mention of cells switching from one phenotype to another.

4.4.1. A fitness cost curve instead of a single transition between two phenotypes

In practice, we usually measured a continuous decrease in fitness with the measured phenotype, rather than two clear phenotypes with a clear threshold. To extend the previous reasoning, it is helpful to consider several discrete phenotypes rather than a smooth transition, knowing that if the principle holds for any number of phenotypes, then it holds to the limit if one considers the observed continuous decrease. Several discrete phenotypes could be depicted as in FIGURE 3.15.

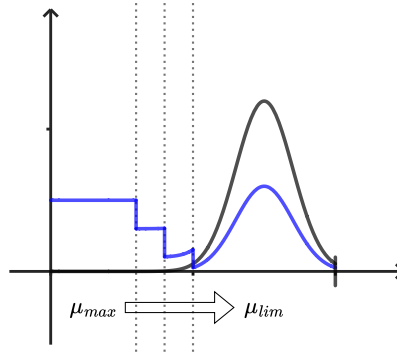


Figure 3.15: Entropy of a distribution with several phenotypes. In black the phenotype before selection. In blue, the phenotype after selection, where each successive region from left to right has a lower growth rate than the previous one.

To determine what is the entropy of such a distribution, EQUATION 3.3 can be generalised to any number of phenotypes:

$$\begin{aligned}
 H\left(\sum_{i=1}^n k_i \cdot \chi_i\right) &= \sum_{i=1}^n k_i \cdot H(\chi_i) - \sum_{i=1}^n k_i \cdot \log_2(k_i) \quad , \text{ where } \sum_{i=1}^n k_i = 1 \\
 &= \text{mean}(H(\chi_i)) - \sum_{i=1}^n k_i \cdot \log_2(k_i)
 \end{aligned} \tag{3.4}$$

This cannot be represented graphically as in FIGURE 3.14, but the same principle

applies: the entropy of the combined distribution will be slightly higher than the mean entropy of the individual parts of the distribution. Since the case of interest is the one where the main phenotype is selected against and phenotypes in the tail of the distribution are amplified, the entropy of the combined distribution will always be higher than the entropy of the phenotype without selection.

4.4.2. Switching between phenotypes

The second point to discuss is the switch of cells between the different phenotypes. Previously, the region selected against was always represented as having a smaller amount of cells. However, if cell lineages were to just switch once to a given phenotype then stop there, only the fastest growing phenotype would be present, over-growing any other. In practice, cells are likely travelling between phenotypes stochastically, and there should be a dynamic equilibrium between the faster growing phenotype that switches to the slower growing phenotype and the slower growing phenotype being washed out. A mathematical model of this equilibrium could be:

$$\frac{d\varphi_1}{dt} = \mu_1\varphi_1 - r_1\varphi_1 - D\varphi_1 + r_2\varphi_2 = 0 \text{ at steady state} \quad (3.5)$$

$$\frac{d\varphi_2}{dt} = \mu_{lim}\varphi_2 - r_2\varphi_2 - D\varphi_2 + r_1\varphi_1 = 0 \text{ at steady state} \quad (3.6)$$

where φ_1 and φ_2 are the concentrations of the two phenotypes, D is the dilution rate, μ_1 is the growth rate unlimited by the phenotype (lower than μ_{max} due to nutrient limitation), and r_1 and r_2 are the switching rates between the two phenotypes. If r_2 is nearly null (because the second phenotype is induced), then $r_1 + D > \mu_{max}$ leads to the first phenotype being washed out, and the second follows, making the FEC impossible when there is little noise and strong, fast induction.

4.4.3. Alternative fitness behaviours

The derivation presented previously describes Fitness-Entropy compensation for well-defined phenotypes. However, there is no reason it could not happen for naturally noisy induction instead. In other words, if the phenotypes are already spread-out and the entropy is already high before any selection is taken into account, what happens to the entropy when one phenotype is selected for?

As a thought experiment, we can consider a naturally bimodal induction where only part of the cells are induced, and the fluorescence is represented by the union of two similar normal distributions. The portion of cells naturally induced will then dictate the behaviour of the entropy in the system (FIGURE 3.16A & E are examples of possible distributions).

In that case, the diagram from FIGURE 3.13 becomes the one from FIGURE 3.16C instead. It demonstrates that, in a case where most cells are induced and have reduced

fitness, one would expect an entropy increase (FIGURE 3.16D & E). By contrast, if the proportion of induced cells is roughly equivalent to the ones that remain uninduced, it would be possible for the entropy to decrease as a response to selection (FIGURE 3.16A & B).

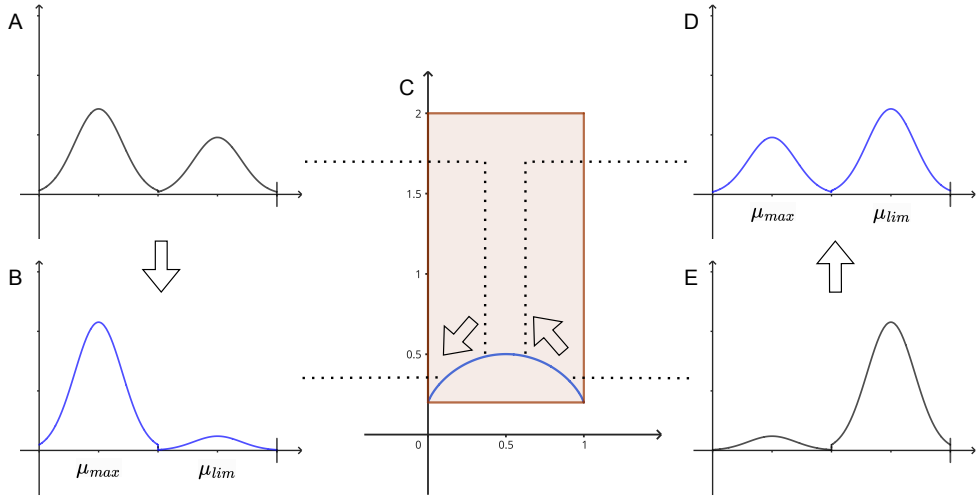


Figure 3.16: Entropy of a bimodal distribution with two phenotypes. If the distribution of has an initial low induction **A**, then the entropy of the selected distribution decreases instead of increasing through Fitness-Entropy compensation **B**. If the distribution has a high induction, then the entropy of the selected distribution increases as before (**E** to **D**). In **C**, the curve equivalent to FIGURE 3.13 but with two identical starting entropies.

4

**Elucidating complex population behaviours
By considering cell-system interactions**

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In the last chapter, various ways to analyse data from automated flow cytometry were introduced. Based on these techniques, this chapter investigates the results from Henrion et al. [7].

In that paper, we observed two phenomena appearing in continuous cultures with gene circuits (groups of genes regulated together, such as the arabinose consumption pathway or the stress response, see GLOSSARY) that cause a decrease in growth rate. That is a fitness cost, in a chemostat, as the induced, slow growing phenotype is washed out of the culture. When an induced gene circuit causes a fitness cost for the strain, the noise in gene expression increases in the cultures. When that decrease in growth rate is high enough, not only is the noise higher, but periodic induction bursts also became apparent.

These phenomena were not expected: the investigated systems are diverse, they are not inherently noisy, and they do not present the typical negative feedback loops present in oscillating gene network. We made an *in silico* model to investigate this behaviour, considering *Saccharomyces cerevisiae* in a chemostat with a stress response causing a fitness cost. When the stressed phenotype causes no fitness cost, gene expression is not noisy. When it is increased, all else staying equal, the apparent noise in gene expression increases as well, until at some point it becomes oscillating. While this demonstrated that the phenomena were intrinsically linked to the fitness cost and not to the properties of the induction system (which stayed identical throughout the simulations), it did not explain why the observations were what they were.

However, if these biological systems are investigated through their interactions with the culture system, which are accounted for in the model, then the key to understanding these phenomena may present itself.

In SECTION 1, we follow that reasoning and challenge the theoretical insights from CHAPTER 3, section 4 to deepen our understanding of Henrion et al.'s observed increasing entropy, showing that considering biological systems within their context can provide valuable insights. In SECTION 2, the reasoning is taken further by creating a minimal mathematical model of a system causing oscillating gene expression; it uncovers how this minimal model can reproduce and predict experimental observations.

1. A Fitness-Entropy compensation effect is set by the trade-off between growth and gene expression in cell populations

This section is adapted from the following publication:

[179] Mathéo Delvenne, Vincent Vandenbroucke, Lucas Henrion, Maximilian Sehr, Juan A. Martínez, Alizée Sloodts, Samuel Telek, Andrew Zicler, and Frank Delvigne. *A Fitness–Entropy Compensation Effect Set the Trade-off between Growth and Gene Expression in Cell Populations*. July 9, 2025. DOI: 10.1101/2025.07.05.663304. Pre-published

The following resources are associated to this publication:

- doi.org/10.5281/zenodo.14273182
- gitlab.uliege.be/mipi/published-software/2024-fitnessentropycompensation

1.1. Abstract

We present findings on a Fitness-Entropy compensation (FEC) mechanism which offsets the activation of gene circuits that compromise survival. It counteracts the resulting fitness reduction by increasing the diversity in gene expression among individual cells within the population. This diversity, quantified by the Shannon entropy, enables cells with lower expression levels to support the survival of the entire population. We investigated the presence of FEC in a range of synthetic and stress-related gene circuits in continuous culture. Our results reveal that it effectively stabilises cell populations by mitigating the detrimental trade-offs between growth and gene expression. This stabilisation is due to the reduced growth rate of the induced phenotype that leads to environmental changes, decreases induction strength, and promotes escape from unfit states. These findings suggest that the FEC mechanism may be a universal strategy for stabilisation in various cellular systems and set the basis for a quantitative description of the trade-off between growth and gene expression and its consequences at the population level.

1.2. Introduction

Individual cells within a population activate specific gene circuits in response to stress conditions, often at the expense of cellular growth [187–189]. Similarly, gene circuits employed in bioproduction can compromise cellular growth and both productivity and predictability [190, 191]. This trade-off between growth and gene expression is a crucial factor in understanding the physiological consequences of gene circuit activation, but its interpretation must be refined by accounting for the inherent variability in gene expression [14, 15, 190, 192].

Our recent findings have highlighted the importance of fitness cost, or the growth reduction associated with the activation of a given gene circuit, in driving cell population diversification dynamics [7]. We observed that low fitness costs result in relatively homogeneous cell population diversification profiles (FIGURE 4.1A), whereas high fitness costs lead to more heterogeneous diversification dynamics (FIGURE 4.1B) [7].

To quantify the degree of cell population dispersion in the activation of a given gene circuit, we utilised Shannon entropy from information theory. This metric was previously validated in various biological studies as a reliable estimator of signal transmission fidelity in gene expression [184, 185] and it offers a robust measure of population dispersion. Notably, entropy outperforms traditional metrics such as the Fano factor and coefficient of variation, as it is mean-independent and better suited to capturing heterogeneity in multimodal distributions [193, 194].

We hypothesised that a correlation exists between the increase in entropy observed in systems with high fitness costs and the overall fitness of the population. Previous studies have highlighted the crucial role of biological noise in enhancing the robustness of cell populations to perturbations by generating a diverse range of phenotypes adapted

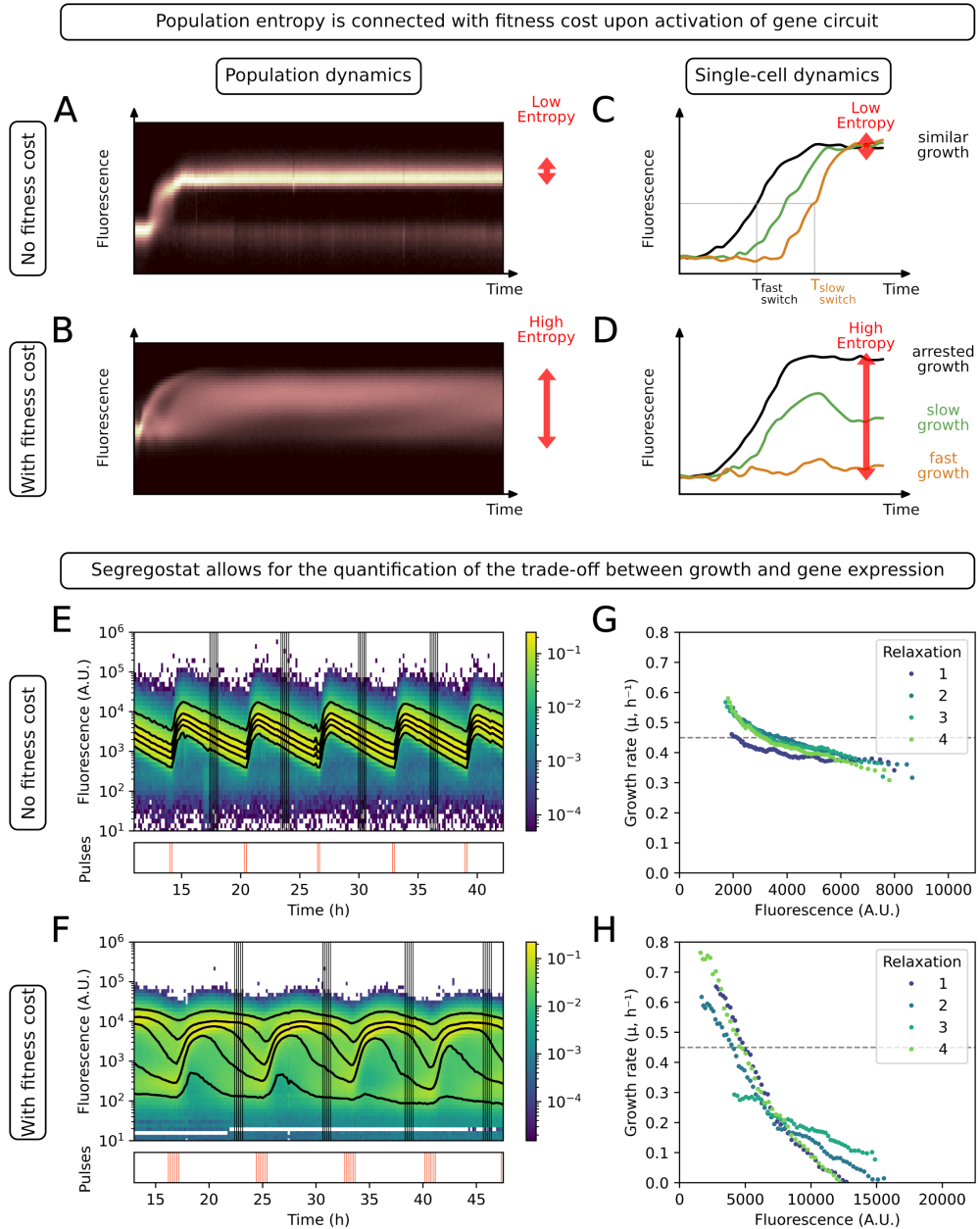


Figure 4.1: Relationship between gene expression, fitness cost, and population entropy.

A, B Population diversification dynamics obtained based on automated flow cytometry (single-cell fluorescence distributions over time) of **(A)** *E. coli* W3110 $P_{araB}::GFP$, without fitness cost, and **(B)** *S. cerevisiae* $P_{glc3}::GFP$, with a fitness cost. Cells were cultivated in chemostats under specific induction conditions: glucose-limited medium supplemented with arabinose for *E. coli* **(A)**, and glucose-limited medium for *S. cerevisiae* **(B)**.

(Caption continued on the next page - Adapted from Henrion et al. [7])

Figure 4.1: C, D Schematic representation of single-cell traces for circuits without (C) or with (D) fitness cost. E, F Fluorescence distributions over time for (E) *E. coli* P_{araB}::GFP and (F) *E. coli* BL21(DE3) pET28:GFP under Segregostat control (continuous feed of glucose with pulses of arabinose or lactose, respectively). Black lines represent the 5th, 25th, 50th, 75th, and 95th percentiles. Under these conditions, the fluorescence relaxation phases can be used to estimate the growth rate as a function of the fluorescence intensity. The five samples used for growth rate approximation are indicated by vertical lines. G, H Trade-off between gene expression (fluorescence) and growth rate for (G) *E. coli* W3110 P_{araB}::GFP and (H) *E. coli* BL21(DE3) pET28:GFP based on Segregostat data. The gray dashed line indicates the dilution rate.

to various environmental conditions [3, 9, 20, 152, 195]. Specifically, in the context of heterogeneous expression of burdensome genes, cells with lower expression levels may compensate for the global loss of growth at the population level. This led us to propose the concept of a Fitness-Entropy compensation effect, a population-level phenomenon where the loss of individual cellular fitness (e.g., due to burdensome gene expression) is offset by an increase in phenotypic diversity, measured with entropy, thereby maintaining population fitness. To investigate this effect, we first examined the T7 expression system in *Escherichia coli*, which is known for its high fitness cost. We then explored whether Fitness-Entropy compensation (FEC) can also occur in natural gene circuits, focusing on genes involved in the general stress response (GSR) in *S. cerevisiae* and *E. coli*, as well as its potential sources.

1.3. Segregostat reveals the trade-off between growth and gene expression

The trade-off between growth and gene expression is a fundamental aspect of cellular biology [196, 197], yet a comprehensive framework for understanding its role at the population level remains elusive. In our previous work, we investigated this phenomenon across various cellular systems using chemostat experiments coupled with automated flow cytometry [7]. This approach allowed us to examine the relationship between the fitness cost associated with gene circuit activation and the resulting level of heterogeneity in gene expression, quantified by entropy. Our findings revealed that non-burdensome gene circuits exhibited low population entropy (FIGURE 4.1A), whereas burdensome gene circuits displayed significantly higher entropy (FIGURE 4.1B).

This observation led us to hypothesise that growth arrest or reduction may be the driving force behind cell-to-cell differences in gene circuit activation. For non-burdensome systems, cell-to-cell variation in accumulation of fluorescent protein could be observed, likely due to natural differences in gene expression timing (here using the arabinose system as an example (FIGURE 4.1A & C) [198]). However, these differences were quickly levelled out as the system reached steady state (FIGURE 4.1C). In contrast, burdensome gene circuits result in growth arrest and/or reduction, further amplifying the differences in fluorescent protein accumulation (FIGURE 4.1D). To

test these hypotheses, we conducted a detailed analysis of previously acquired data from the Segregostat, a cell-machine interface that enables automated monitoring and perturbation of gene circuits based on their natural dynamics [4, 7]. This system was used to generate successive induction and relaxation phases with *E. coli* cells containing either a non-burdensome arabinose utilisation reporter system (strain W3110 $P_{araB}::GFP$) or a burdensome T7 expression system (strain BL21(DE3) pET28:GFP) (FIGURE 4.1E, F). In these experiments, pulses of arabinose or lactose acted as inducers; their subsequent consumption by the respective cells naturally led to the transition toward relaxation. During the latter phase, where fluorescence levels decrease due to protein dilution caused by cell growth and division [181], we investigated the relationship between fluorescence intensity (GFP signal) and growth rate (FIGURE 4.1G, H, CHAPTER 3, section 3 ANNEX B, section 1. In accordance with our previous fitness cost analysis [7], our results pointed out that the arabinose system had no significant impact on growth (FIGURE 4.1G), whereas the T7 system displayed a pronounced fitness cost (FIGURE 4.1H).

1.4. Entropy compensates the loss of fitness upon the activation of burdensome gene circuits

The aim of this section is to determine whether entropy is linked to fitness at both the single-cell and population levels. The concept of 'fitness' is multifaceted and context-dependent, encompassing various levels of biological organisation and timescales. In the literature, fitness can refer to individual organisms, populations, or species, and can be measured over a range of timescales, from instantaneous propensities in a defined environment to evolutionary timescales where rare events influence population dynamics [41–45]. In this study, we consider fitness at two levels: (1) the phenotypic level, where gene induction can compromise cellular survival (e.g., growth rate in a continuous bioreactor); and (2) the population level, which comprises a diverse array of phenotypes. Furthermore, to investigate the mechanisms that enable a population to maintain its fitness over time, we consider population fitness as an instantaneous measure of the population's adaptation to its current environment.

To analyse the potential function of entropy as a population fitness stabiliser, we performed continuous cultivation of *E. coli* with the T7 expression system on a mix of glucose and lactose. This setup yielded a complex diversification profile, characterised by bursts of diversification, where cells periodically activated the T7 system, incurred a fitness cost, and were progressively washed out (see GLOSSARY) (FIGURE 4.2A, B). Despite this repeated washout, the cell population exhibited a robust behaviour in terms of cell density, with growth sustained by cells displaying lower induction levels (FIGURE 4.2C). Using automated flow cytometry (FC) data, we computed the time profiles of entropy (FIGURE 4.2D) and population fitness (FIGURE 4.2E), the latter being estimated as the mean fitness based on a trade-off curve generated from Segregostat data and fluorescence measurements (FIGURE 4.1H, CHAPTER 3, section 3).

These data point out that the generation of entropy during the successive bursts of diversification is correlated with a global gain in fitness, keeping the global growth rate of the cell population above the dilution rate and preventing washout. Ultimately, this FEC effect leads to a very stable cell density during the whole cultivation (FIGURE 4.2C).

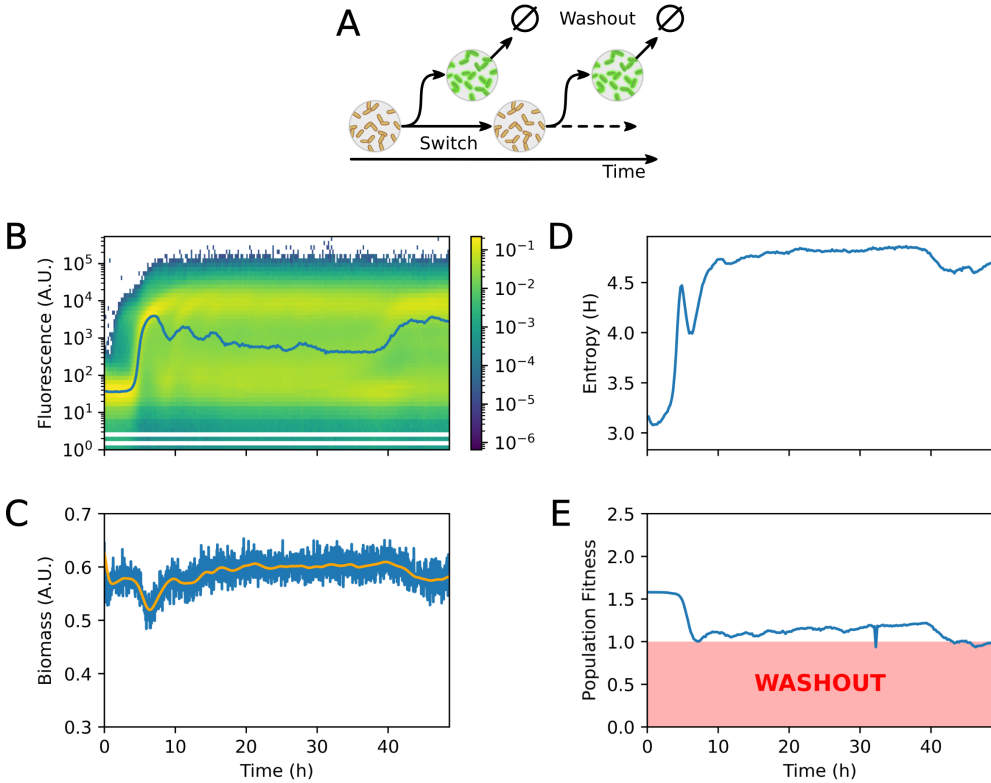


Figure 4.2: FEC for *E. coli* with a T7 expression system in continuous culture. **A** Schematic representation of the phenotypic diversification dynamics with bursts of diversification. **B** Fluorescence distributions over time determined based on automated FC (the blue line indicates the median fluorescence). The induction delay observed at the beginning of the chemostat results from the transition from glucose-only batch to glucose/lactose co-feeding. This lag integrates the time for lactose accumulation, the alleviation of carbon catabolite repression, and the sequential expression of the T7 system (T7 polymerase synthesis followed by GFP production). **C** Biomass over time, the blue line indicates raw data and the orange line indicates smoothed data. **D** Entropy computed based on the distribution of the fluorescence over time. **E** Population fitness computed as the mean fitness based on trade-off curve generated from Segregostat data and the fluorescence from automated FC.

A possible explanation about the origin of the FEC effect is the escape of cells from the unfit, slow-growing, states due to changes in environmental conditions (i.e., environmental escape). The bursty diversification pattern observed during chemostat cultivation suggests that the bursts of diversification may themselves trigger oscillations in the environmental conditions. Upon consumption of lactose, most of the cells are getting induced and exhibit reduced growth rate due to the fitness cost upon the activation of the T7 system, leaving out glucose in the supernatant [199, 200]. This temporary glucose increase, resulting from the feedback of cells on their environment, allows some cells to escape from the growth-limiting induction through carbon catabolite repression. Lactose and glucose concentrations are then expected to oscillate in phase opposition. This last feature will be addressed in the next section.

1.5. FEC involves two subpopulations exhibiting opposite trade-off

In the previous section, environmental escape was advanced as a potential driver for the generation of the FEC effect. Upon induction, the T7 system exhibits a bimodal behaviour with two subpopulations i.e., a slow-growing phenotype exhibiting a high induction level and a fast-growing one with a low expression level (FIGURE 4.3A). The co-occurrence of the two subpopulations is at the origin for the elevation of the entropy of the population, the lower subpopulations compensating for the loss of fitness of the high subpopulation. Since lactose and glucose are co-fed into the continuous cultivation device, these two subpopulations could be the result of the differential growth on a specific sugar. We validated this hypothesis by replacing glucose with xylose and observed a strong reduction in the proportion of the fast-growing, low GFP, subpopulation (FIGURE 4.3B). Xylose is not able to exhibit catabolite repression and is co-consumed with lactose [201], explaining the lack of environmental escape in this case. We then compared the measured biomass (FIGURE 4.3C) and entropies (FIGURE 4.3D) for the cultivations carried out on a mix of lactose and glucose and on a mix of lactose and xylose. When glucose is used, the entropy of the population increases during the first stage of the culture and then evolves to prevent the loss of fitness (FIGURE 4.2C – E and FIGURE 4.3C, D). By contrast, this is not observed when glucose is replaced by xylose. In that case, the entropy cannot be maintained, and the population collapses due to a lack of fitness (FIGURE 4.3C, D).

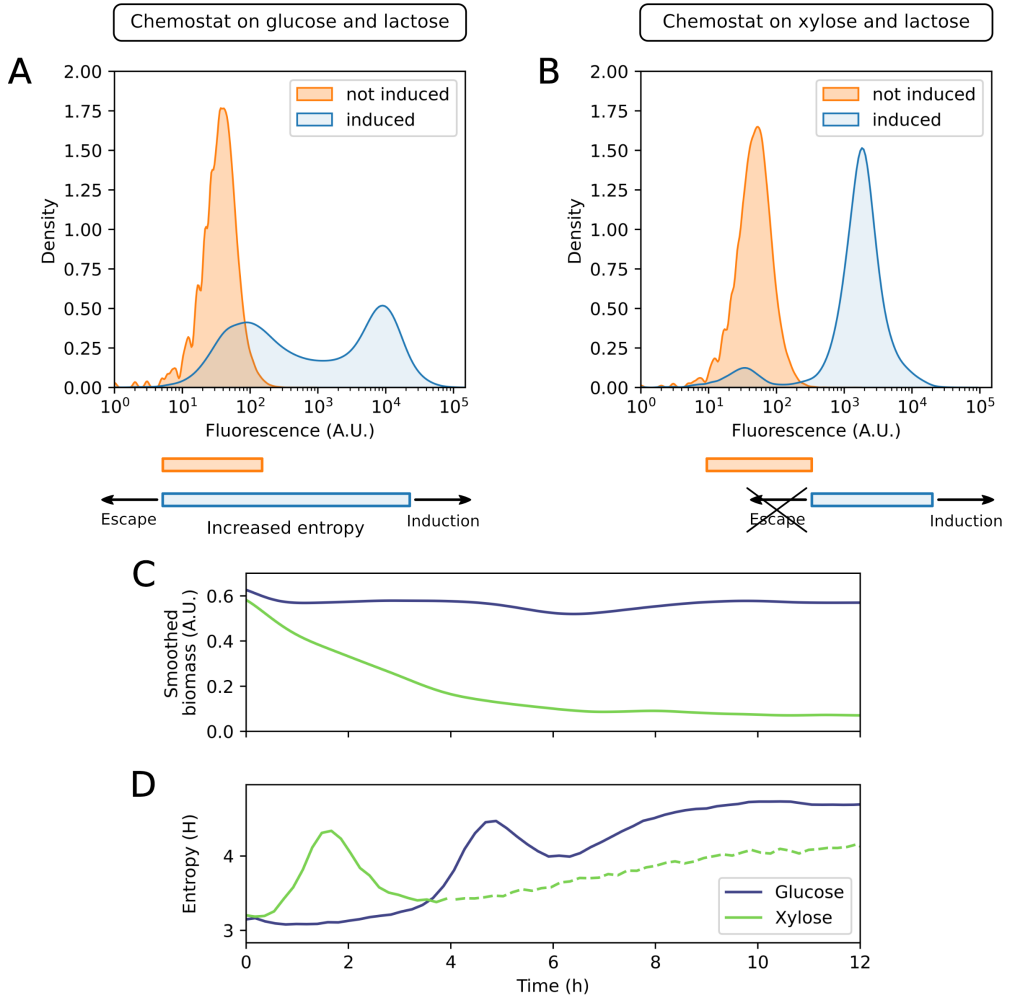


Figure 4.3: **A, B** Fluorescence distributions of *E. coli* cells with a T7 expression system cultivated in chemostat on **(A)** glucose and lactose, and **(B)** xylose and lactose. Orange: before induction, blue: after induction (20 h for glucose+lactose, and 2.5 h, i.e., just before the start of washout as measured by event numbers in flow cytometry, for xylose+lactose). The schematic below the fluorescence plots illustrates the presence **(A)** or absence **(B)** of environmental escape in these conditions. **C** Smoothed biomass over time. **D** Entropy computed based on the distribution of the fluorescence over time. The dashed line indicates an increase in entropy due to a higher proportion of noise resulting from the decrease in biomass, as fewer and fewer cells are measured. In this case, no subpopulation emerges to support growth. In **C** and **D** the purple lines refer to the glucose+lactose condition, while the green lines refer to the xylose+lactose condition.

1.6. Lack of environmental escape prevents FEC and leads to population collapse

To further explore the role of environmental escape in triggering the FEC effect, we engineered *E. coli* MG1655 strains where induction is decoupled from growth, thereby isolating the impact of phenotypic switching from environmental escape. To eliminate any potential environmental escape during our experiments, we employed IPTG as an inducer. As IPTG is not metabolised by the cells, its concentration remains constant, effectively precluding any cell escape due to changes in IPTG levels.

Our negative control was a genetic toggle switch without fitness cost (ANNEX B, section 3). This bistable circuit is controlled by two orthogonal inducers, IPTG and aTc; however, only the IPTG-input was exploited in this study. Upon addition of IPTG, the toggle switch is locked to a state preventing GFP production. As expected, automated FC analyses revealed that all cells exhibited a low and homogeneous GFP profile with a very low entropy and with no change in fitness at the population level.

To generate a fitness cost, the same toggle switch was used to control the *serA* gene in a serine auxotrophic *E. coli* mutant (depending on *serA* production to grow, FIGURE 4.4A). By placing the *serA* gene on the GFP side of the toggle switch, we created a system where IPTG induction represses GFP expression and thereby prevents serine synthesis. This resulted in a significant reduction in cellular growth rate (from 0.5 h⁻¹ to 0.3 h⁻¹ in microplate, ANNEX B, section 3), as the cells were unable to synthesise the essential amino acid serine. We subsequently analysed the population dynamics of this *E. coli* strain using automated FC (FIGURE 4.4) and observed no FEC effect, as well as the collapse of the population upon induction of the toggle switch.

In summary, our findings from two distinct gene circuits underscore the crucial role of environmental feedback in enabling cells to escape from growth-limited phenotypes through FEC.

1.7. Segregostat and time-lapse microscopy analyses indicate that FEC is unlikely for the GSR in E. coli but is expected for S. cerevisiae

In the previous sections, we demonstrated the occurrence of a FEC effect in synthetic gene circuits and wondered if natural gene circuits exhibited similar compensation effect. We then investigated natural stress responses in *E. coli* MG1655 and *S. cerevisiae*. For *E. coli*, we focused on the *rpoS* regulon, which is responsible for activating various stress response mechanisms [164, 187, 202, 203]. We conducted a comprehensive search for suitable transcriptional reporters to detect the activation of the GSR and ultimately selected the P_{ydcS}::GFP system for its dynamic range and sensitivity (ANNEX B, section 4). The strong correlation between *ydcS* and *rpoS* expression confirmed its relevance as a reporter of the GSR (FIGURE B.8, ANNEX B). For *S. cerevisiae*, we

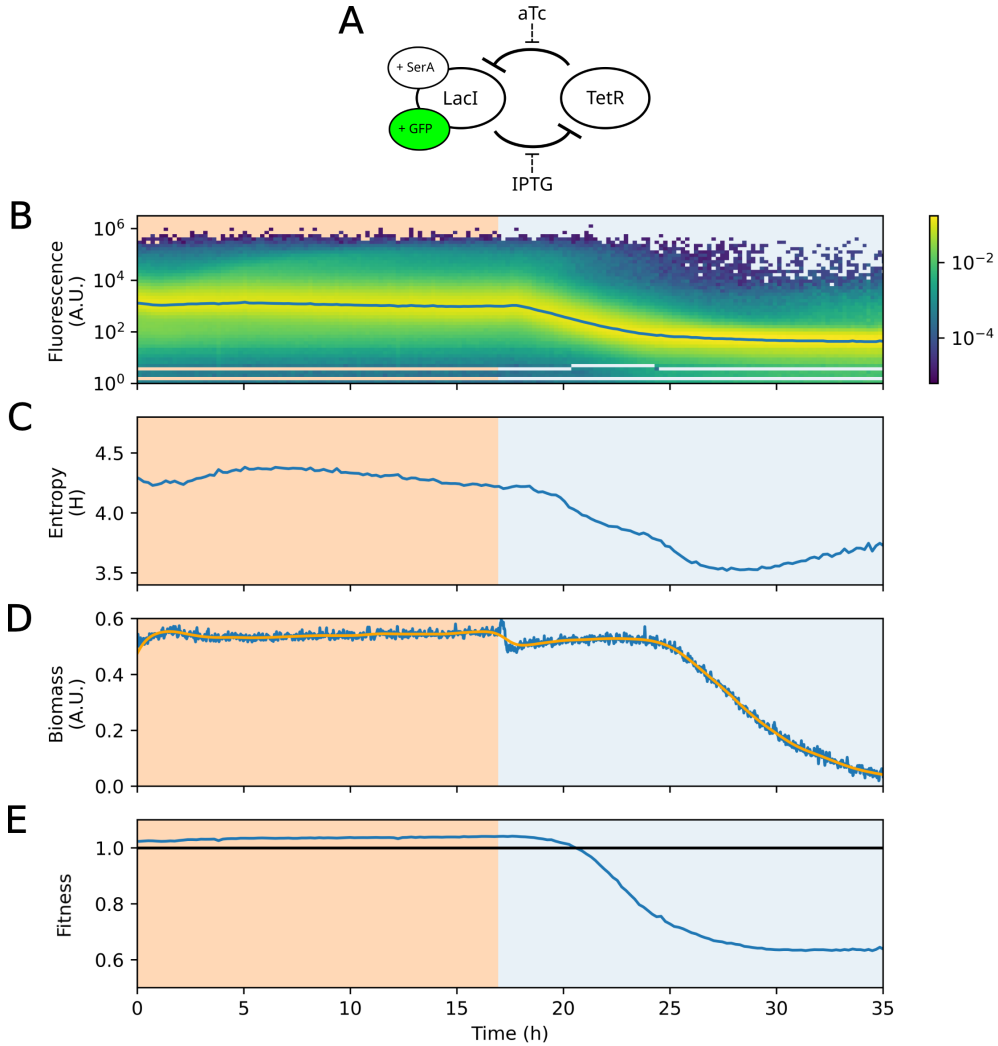


Figure 4.4: Continuous cultivation of a toggle switch controlling serine auxotrophy exhibits no environmental escape and no FEC. Upon addition of IPTG in the feed (lighter, blue background), the production of serine was inhibited. **A** Schematic representation of the toggle switch, **B** Fluorescence distributions over time as determined based on automated FC (the blue line indicates the median fluorescence). **C** Shannon entropy computed from the fluorescence distribution over time. **D** Biomass over time, the blue line indicates raw data and the orange line indicates smoothed data. **E** Population fitness computed as the mean fitness based on the fluorescence from automated FC and the independent measurement of the growth rate for each state of the switch.

employed a previously developed $P_{glc3}::GFP$ transcriptional reporter [7, 168, 204]. The *glc3* gene is associated with the GSR in yeast and has been reported to be involved in bet-hedging [169, 205, 206].

To visualise the trade-off between growth and gene expression of these systems, we monitored them by time-lapse microscopy in a microfluidic single-cell cultivation (MSCC) device. This system enables the cultivation of microcolonies as cell monolayers under precisely controlled environmental conditions to study cellular responses [15, 207, 208]. The activation of the *ycdS* gene in *E. coli* system did not induce growth arrest in cells exhibiting high expression levels (FIGURE 4.5A, B). Instead, we observed higher global gene expression in low-nutrient conditions, accompanied by continuous growth of microcolonies. These observations were confirmed by the results of the Segregostat experiment indicating a negligible trade-off for the GSR in *E. coli* (FIGURE 4.5C, D, CHAPTER 3, section 3). Given the significant impact of the stringent response on growth [209], one might expect a substantial fitness cost and corresponding increase in heterogeneity. However, *E. coli* exhibits mutual regulation between growth rate and stress response, allowing it to dynamically adjust its physiology [164, 187, 202, 203, 210, 211]. The GSR is regulated by the RpoS-containing RNAP holoenzyme ($E\sigma^S$). The concentration of RpoS and its competition with other sigma factors for the RNA polymerase (RNAP) core enzyme result from complex regulation at multiple levels [164, 189]. While $E\sigma^S$ represses processes associated with rapid cell proliferation, it does not completely inhibit growth. Instead, it facilitates the expression of stress-adaptive genes that support survival and limited metabolic activity. Notably, in certain contexts, RpoS is required for maintaining slow growth by sustaining the expression of central metabolism genes [164, 212].

We carried out similar analyses on the $P_{glc3}::GFP$ system in yeast at high and low nutrient conditions [7, 204]. In both cases, cells expressing the *glc3* reporter exhibited growth arrest, which ultimately halted the expansion of the entire microcolony in the case with low glucose concentrations (FIGURE 4.5E, F). This drastic growth arrest was confirmed based on Segregostat experiments (FIGURE 4.5G), with a trade-off curve pointing out a substantial reduction in growth rate upon full activation of the reporter system (FIGURE 4.5H, CHAPTER 3, section 3). This aligns with previous reports showing that, unlike *E. coli*'s, *S. cerevisiae*'s investment in stress resistance appears incompatible with proliferation, switching between growing and non-growing states [169, 188, 213].

The lack of a clear trade-off upon activation of the GSR in *E. coli* suggests that the population is homogeneous with individual cells growing at a similar rate during the relaxation phase. It implies that no FEC effect is anticipated for this system, in contrast to the GSR in yeast, where a significant trade-off was observed. This prediction will be further explored in the next section, based on experiments carried out in continuous cultures.

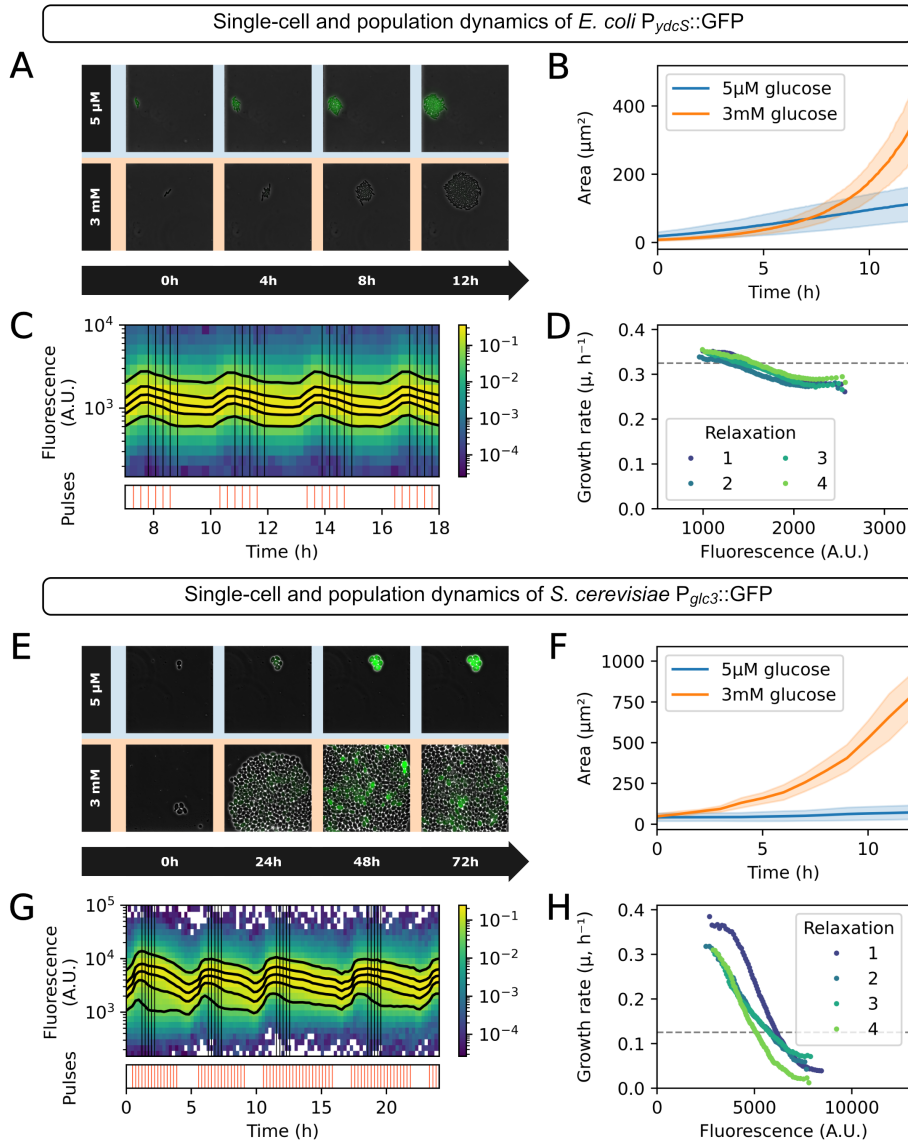


Figure 4.5: Single cell and population dynamics in MSCC and in Segregostat, for *E. coli* MG1655 $P_{ydcS}::GFP$ and *S. cerevisiae* $P_{glc3}::GFP$. **A & E** Time-lapse fluorescence microscopy images of colonies grown in mineral medium with 5 μM (top, blue) or 3 mM (bottom, orange) glucose concentration. **B & F** Colony growth in microfluidic chambers, quantified as the total cell area over time (for **(B)** *E. coli*, $n=3$ for both 5 μM and 3 mM glucose; for **(F)** *S. cerevisiae*, $n=4$ for 5 μM glucose and $n=3$ for 3 mM glucose). **C & G** Fluorescence distributions over time under Segregostat control with pulses of glucose. Black lines represent the 5th, 25th, 50th, 75th, and 95th percentiles. The five samples used for growth rate approximation are indicated by vertical black lines. **D & H** Trade-off between gene expression (fluorescence) and growth rate based on Segregostat data, the gray dashed line indicates the dilution rate.

1.8. FEC is also involved in natural stress response systems exhibiting a marked trade-off between gene expression and growth

To investigate the potential occurrence of FEC in natural gene circuits involved in the GSR, we cultured our reporter strains in chemostats and monitored them using automated FC. Cultivations were carried out at a high dilution rate (non-stressful control condition) and a low dilution rate (nutrient-limited, stress-inducing condition) for comparison.

For the *E. coli* $P_{ydcS}::GFP$ reporter, switching the chemostat from a high ($D = 0.6 \text{ h}^{-1}$) to a low dilution rate ($D = 0.1 \text{ h}^{-1}$) led to a strong and homogeneous activation of the reporter system (FIGURE 4.6A & E). The entire population transitioned from the non-stressed to the stressed phenotype, with the fluorescence distribution shifting uniformly from low to high GFP levels. The two corresponding distributions show minimal overlap, indicating that *E. coli* precisely modulates the expression of stress-related genes in proportion to stress intensity, functioning like a rheostat [214]. Furthermore, entropy did not increase upon switching to the stressed state; it may even be slightly reduced (FIGURE 4.6B & E). Notably, fitness remained unaffected (FIGURE 4.6D). Since the GSR in *E. coli* does not substantially impact growth under these conditions (even if it is reduced, the growth rate remains above the dilution rate), the FEC mechanism does not appear to be involved in this response.

For the *glc3* system in yeast, time-lapse microscopy and Segregostat experiments revealed that strong activation can reduce growth below the dilution rate, leading to growth arrest when fully activated (FIGURE 4.5E – H). Accordingly, we might expect population washout upon switching to the low dilution rate ($D = 0.1 \text{ h}^{-1}$), but this was not the case. In stark contrast to *E. coli*, *S. cerevisiae* displayed a stress response consistent with the “noisy switch model”, characterised by overlapping gene expression distributions across varying stress intensities [214](FIGURE 4.6F & J). At low dilution rates, the population split into two distinct subpopulations: one continued to elevate its stress response, while the other reverted toward pre-stress expression levels. This resulted in a bimodal fluorescence distribution and a significant increase in entropy (FIGURE 4.6G). Notably, population fitness did not decrease (FIGURE 4.6I), and biomass concentration remained stable (FIGURE 4.6H). The observed increase in entropy may therefore reflect a functional role at the population level, enabling partial escape from the slow- or non-growing stressed state and thereby preventing washout in continuous cultivation. This is consistent with the hypothesis that increased entropy compensates for the growth reduction associated with the activation of the stress reporter.

These observations confirm the fact that considerable reduction in growth rate upon activation of the target gene circuit is a crucial component of the FEC mechanism. The second condition previously reported for the generation of the FEC effect observed for the *glc3* system in yeast is the occurrence of an environmental escape mechanism. A possible explanation for the partial relaxation of cells from the stressed to the non-stressed state in low-dilution rate chemostats is that stressed cells exhibit reduced

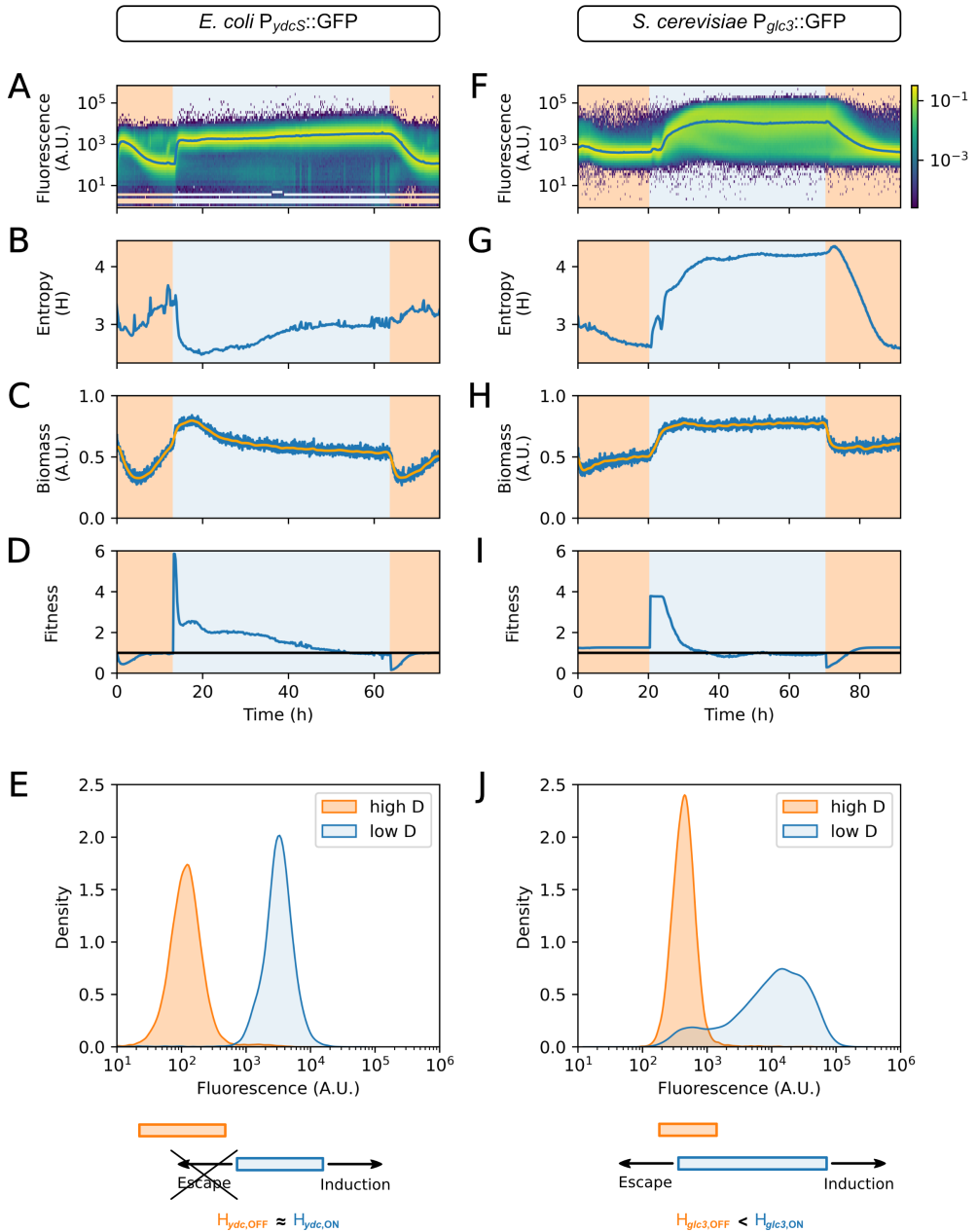


Figure 4.6: Single cell and biomass analysis of (A–E) *E. coli* MG1655 $P_{ydcS}::GFP$ and (F–J) *S. cerevisiae* $P_{glc3}::GFP$ cultivated in continuous cultures carried out at high (darker, orange background, $D = 0.6 \text{ h}^{-1}$ for *E. coli* and $D = 0.3 \text{ h}^{-1}$ for *S. cerevisiae*) and low (lighter, blue background, $D = 0.1 \text{ h}^{-1}$ for both strains) dilution rates. A & F Fluorescence distributions over time determined based on automated FC (the blue line indicates the median fluorescence).
(Caption continued on the next page)

Figure 4.6: **B & G** Shannon entropy computed from the corresponding fluorescence distribution over time. **C & H** Biomass over time (the blue line indicates raw data and the orange line indicates smoothed data). **D & I** Population fitness computed as the mean fitness based on trade-off curve generated from Segregostat data and the fluorescence from FC. **E & J** Fluorescence distributions of samples representative of cells cultivated in chemostat at high ($t = 75$ h for *E. coli* and $t = 90$ h for *S. cerevisiae*) and low ($t = 63$ h for *E. coli* and $t = 70$ h for *S. cerevisiae*) dilution rates. The schematic below the fluorescence plots illustrates the absence (**E**) or presence (**J**) of environmental escape.

metabolic activity and tend to shift toward a more quiescent state, thereby decreasing substrate consumption [169, 188, 213]. Fully stressed cells may even cease growth and nutrient uptake entirely. This reduced consumption enables cells with lower stress-related gene expression to benefit from a less depleted environment, allowing them to escape the stressed state via an environmental feedback mechanism.

Moreover, when cultivated in the MSCC device under low glucose concentrations, strongly activated cells remained locked in the stresses state, with no observable decrease in fluorescence (FIGURE 4.5E & F, FIGURE B.11 in ANNEX B). In microfluidics, cell physiology is decoupled from environmental conditions, thereby eliminating environmental feedback. Residual glucose concentrations ($[Glu_{res}]$) were measured in continuous cultures across various dilution rates and compared to those in the MSCC device (ANNEX B, section 6). At low dilution rates ($D < 0.3 \text{ h}^{-1}$), residual glucose concentrations $[Glu_{res}]$ remained very low around 0.1 mM, the threshold concentration at which the entire population exhibited growth arrest in the MSCC device (FIGURE B.11B in ANNEX B). This supports the hypothesis that transient environmental conditions enable cells to escape the stressed state.

1.9. Discussion

Gene expression noise and phenotypic switching enables microbial populations to adapt and thrive in dynamic environments [1–3, 8, 9, 20]. To date, most studies have treated phenotypic diversification both as a consequence of intrinsic gene expression noise and as an adaptive strategy for coping with fluctuating environments. However, with a few notable exceptions [20, 215, 216], the impact of phenotypic changes on population composition has received limited attention. This knowledge gap is critical, as fitness variation among phenotypes inevitably reshapes population structure through intra-population competition. Our study seeks to address it by laying the groundwork for a robust experimental framework to better understand these dynamics.

A well-documented functional consequence of noise in gene expression is bet hedging [3, 8, 9, 20], a strategy microbial populations use to enhance survival in unpredictable environments. This mechanism leverages noise to generate a small subset of phenotypes that, while suboptimal under current conditions, may prove

advantageous upon environmental shifts. These alternative phenotypes typically represent a minor fraction of the population, as they are usually outcompeted by the dominant, better-adapted phenotype [20]. But what happens when the dominant phenotype becomes maladapted to its environment? In such cases, the population would be expected to die out. However, several studies revealed a surprising resilience, with populations surviving conditions that would normally be lethal. Besides this work, a prominent example is bacterial persistence, where a resistant phenotype temporarily outcompetes sensitive cells even without mutations [217]. This unexpected survival can be explained by a FEC mechanism. According to this mechanism, when the dominant phenotype fails to persist, a minority of cells that stochastically escape this state — via variability in gene expression or repression — can proliferate and repopulate the culture. This competitive dynamic is essential, as it prevents any single phenotype from dominating the population. Instead, it promotes an equilibrium among phenotypes, resulting in increased heterogeneity (FIGURE 4.6). This heterogeneity is quantitatively captured by an increase in population entropy (as shown mathematically in CHAPTER 3, section 4, with additional numbers in ANNEX B, section 2).

The emergence of alternative phenotypes is facilitated by an environmental escape mechanism. Under our chemostat conditions, both *S. cerevisiae* under glucose starvation and *E. coli* BL21 grown on a mixture of glucose and lactose adopt phenotypes that are maladapted to survival. In *S. cerevisiae*, glucose limitation induces a non-growing, stress-related phenotype, while in *E. coli* BL21, lactose consumption triggers the activation of the burdensome T7 gene circuit, significantly reducing growth rate. In both cases, the decline of the induced, unfit subpopulation leads to a rise in environmental glucose levels. This, in turn, inhibits further induction of the maladapted phenotype — by relieving stress in yeast and via catabolite repression in BL21. As a result, the microbial population naturally adjusts its environment to a glucose concentration that permits sufficient gene expression noise for FEC to occur. This feedback mechanism is likely to arise in any context where the decline of the microbial population reduces induction strength.

The analysis of the FEC mechanism was enabled by quantifying the trade-off between growth rate and gene expression. In some systems, such as the growth-limiting toggle switch, it is possible to isolate and measure two clearly distinct phenotypes. However, biological systems are rarely so clear-cut. Microfluidics is the alternative option to map fitness to phenotype, but while it provides valuable temporal resolution, it is labour-intensive and does not accurately replicate the growth conditions of our systems of interest. By contrast, our approach using automated FC offers a direct and efficient means to assess growth rates within bioreactor environments. We propose that this technique represents a critical step towards a more robust understanding of bacterial population dynamics. Notably, we were only able to quantify a single trait at once, and an important question we did not address is whether additional heterogeneity, such as variations in transporter expression or metabolic regulation, could influence which cells

escape induction. This could be investigated through the use of multiple reporters [218, 219] or via omics approaches on subpopulation or even at the single cell level [220].

Overall, our findings seem to have far-reaching implications across biological systems. We measured FEC in chemostats, but the phenomenon is applicable to microbial population dynamics anywhere a fitness can be described, whether a bioprocess, an antibiotics resistance study, or a biofilm layer [217, 221]. In the laboratory, where FEC is relevant, applying results from one setting to another demands careful consideration because fitness and environmental escape are context dependent. When studying diversity itself, FEC alters how much gene expression noise is required for a species to effectively hedge its bets against a fluctuating environment and could affect our understanding of both the evolutionary path of microbes and the expression of recombinant protein genes in industrial settings.

1.10. Material and methods

1.10.1. Code and data availability

All relevant code is available at <https://gitlab.uliege.be/mipi/published-software/2024-fitnessentropycompensation>. All supplementary data are available at <https://doi.org/10.5281/zenodo.14273182>, besides the raw time-lapse microscopy files that can be made available on request due to their large size. See ANNEX B, section 8 for a description of the supplementary data and code.

1.10.2. Strains and plasmids

A strain of *E. coli* W3110 with a plasmid from the Zaslaver collection ($P_{arab}::GFPmut2$ with kanamycin resistance [159]) was used as an example of arabinose utilisation system with low fitness cost. The data acquired with this strain were already published [7].

The *E. coli* BL21(DE3) strain, one of the most important industrial production backbone, carrying the pET28:GFP plasmid (Addgene #60733)[163] was used to investigate the burdensome T7 expression system. Part of the data acquired with this strain were already published [7].

For the toggle switch study, the control strain without growth change is *E. coli* MG1655 $\Delta lacAYZ$ and $\Delta lacI$ with the toggle switch plasmid pECJ3 (Addgene #75465)[173]. To obtain the strain with auxotrophy to the plasmid, the $\Delta serA$ modification was added to the genome and the *serA* gene was added to pECJ3 between the promoter for GFP and the GFP (PLtetO-1::*serA*::GFP) with the same RBS as GFP, so that the latter is a direct reporter for SerA content. The genomic modifications were performed using the CRISPR-cas9 enhanced lambda red phage mediated homologous recombination method described by Jiang et al. [222]. *lacAYZ* and *lacI*, being contiguous, were removed in one step and *lacI* was used as target, which also eliminates any hypothetical copy of *lacI* in the episome. The primers used are available in TABLE B.2 (ANNEX B). The modified plasmid was produced through PCR and NEBuilder HiFi DNA Assembly (New England

Biolabs, Ipswich, MA, USA); the plasmid map is available in the supplementary data. Each phenotype of the strain with auxotrophy to its plasmid was measured in BioLector, a microfermentation system enabling online monitoring of biomass and fluorescence in microtiter plates. It showed that the growing side has a growth rate $>0.5 \text{ h}^{-1}$, while the non-growing side has a growth rate between 0.25 and 0.3 h^{-1} , slow enough to be washed out of the bioreactor in continuous culture, but not zero, likely due to leaky repression of serine ANNEX B, section 3 .

For the study of the general stress response of *E. coli* in chemostat, we utilised the *E. coli* MG1655 $P_{ydcS}::\text{GFPmut2}$ strain from the Zaslaver collection [159]. This strain carries a plasmid containing a transcriptional reporter for P_{ydcS} and confers kanamycin resistance. The same plasmid was introduced into the *E. coli* MG1655 $P_{rpoS}::\text{CyOFP1}$ strain to generate the double-reporter strain used in microfluidics. *E. coli* MG1655 $P_{rpoS}::\text{CyOFP1}$ was constructed using the same CRISP-cas9 system as above. The CyOFP1 gene was inserted downstream of the *rpoS* gene with a synthetic ribosomal binding site (RBS) 5'-AAAGAGGAGAAA-3' [47, 219]. The cassette containing the coding sequence of the CyOFP1 protein has been obtained by PCR overlap of the CyOFP1 sequence (obtained from the pNCS-CyOFP plasmid (Addgene #74278)) and two homologous regions of 400bp around the site of insertion. Tailed primers have been used to include the synthetic RBS in the cassette. The primers used are described in TABLE B.1 (ANNEX B).

For the study of the general stress response of *S. cerevisiae* in chemostat and in microfluidics, we utilised *S. cerevisiae* CEN.PK 113-7D strain with the chromosomal integration of a reporter cassette $P_{glc3}::\text{eGFP}$ [168, 223].

1.10.3. Growth media

Unless otherwise specified (e.g., regarding the carbon source and its concentration), the following media were used.

E. coli strains were cultivated in a mineral medium containing (in $\text{g}\cdot\text{L}^{-1}$): K_2HPO_4 : 14.6; $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$: 3.6; Na_2SO_4 : 2; $(\text{NH}_4)_2\text{SO}_4$: 2.47; NH_4Cl : 0.5; $(\text{NH}_4)_2\text{H-citrate}$: 1; glucose: 5; thiamine: 0.01. The medium was supplemented with $11 \text{ mL}\cdot\text{L}^{-1}$ of a trace element solution. This solution was assembled from the following solutions (in $\text{g}\cdot\text{L}^{-1}$): 3/11 of $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$: 16.7, 3/11 of EDTA: 20.1, 2/11 of MgSO_4 : 120 and 3/11 of a metallic trace element solution. The metallic trace element solution contains (in $\text{g}\cdot\text{L}^{-1}$): $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$: 0.74; $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$: 0.18; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$: 0.1; $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$: 0.1; $\text{CoSO}_4 \cdot 7 \text{H}_2\text{O}$: 0.21. For plasmid maintenance, it was supplemented with kanamycin ($50 \text{ mg}\cdot\text{L}^{-1}$). The trace element solution, the thiamine and the antibiotic were filter-sterilised ($0.22 \mu\text{m}$) before supplementation of the other component that were heat-sterilised (at 120°C).

S. cerevisiae was cultivated in a mineral medium based on the recipe of Verduyn et al. [224]. It contains, per liter, $(\text{NH}_4)_2\text{SO}_4$: 5 g; KH_2PO_4 : 3 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$: 0.5

g; EDTA: 95.55 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$: 22.5 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$: 5 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$: 1.5 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$: 1.5 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$: 2 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 22.5 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 15 mg; H_3BO_3 : 5 mg; KI: 0.5 mg and glucose: 7.5 g. After heat sterilisation (at 120°C) of those components, the filter-sterilised (0.22 µm) vitamins were added. The final medium contains per liter, D-biotin: 0.05 mg, calcium pantothenate: 1 mg, nicotinic acid: 1 mg, myo-inositol: 25 mg, thiamine HCl: 1 mg, pyridoxine HCl: 1 mg and para-aminobenzoic acid: 0.2 mg.

For bioreactor cultivations, 100 µL of TEGO Antifoam KS 911 were added per liter of medium.

1.10.4. Continuous cultivations

Continuous cultivations were performed in either Biostat B-Twin (Sartorius, Göttingen, Germany) or in Bionet F1 bioreactor (Bionet, Murcia, Spain), with a working volume of 1 l, at an agitation speed of 1000 rpm, with an aeration of 1 VVM. The temperature control was set either at 37°C (*E. coli*) or 30°C (*S. cerevisiae*) while the pH control was set either at 7 (*E. coli*) or 5 (*S. cerevisiae*). Bioreactor cultivations started with a batch phase lasting until full consumption of the carbon source (marked by an increase in oxygen concentrations) or after approximately 15 h for yeast. During the chemostat experiment, glucose was fed alone or with lactose for experiment with *E. coli* strains. During Segregostat experiments, glucose was the sole carbon source in the feed. The feedback control loop of the Segregostat (implemented via a custom MATLAB script exploiting flow cytometry data, the acquisition of which is detailed below) triggered a pump to deliver pulses of the activator. For inducible systems, the actuator was the corresponding inducer (i.e., arabinose for *E. coli* $P_{araB}::\text{GFP}$ and lactose for *E. coli* BL21). For systems with stress response reporters, glucose was pulsed instead. The control was based on a threshold of fluorescence, and the pulse was activated when more than 50% of the population was either below this threshold (for Ara and T7 systems) or above it (for systems with stress-related gene reporters). The specific operating conditions of the continuous cultures performed in lab-scale bioreactors are described below and summarised in TABLE B.3 (ANNEX B).

E. coli $P_{araB}::\text{GFP}$ and *E. coli* BL21 were precultured in 1 L baffled flask (with 100 ml of medium) overnight at 37°C at a shaking speed of 150 rpm. They were used to inoculate the reactor with an initial OD_{600} of ~ 0.5 . Continuous cultures were performed at a dilution rate of 0.45 h^{-1} . Concentrations of inducers (arabinose and lactose, respectively) were chosen to be high enough to ensure the induction of the whole population, avoiding the bimodal expression patterns typically observed at lower, intermediate concentrations [198].

The toggle switch strains were grown only in the same defined media as described above as a change in medium conditions would induce an unpredictable lag phase for the $\Delta serA$ strain. Glycerol stocks were prepared from a culture in shake flask with $10\text{ g}\cdot\text{L}^{-1}$ glucose medium and an OD_{600} above 5 (which is the maximal cell density

reached with $5 \text{ g}\cdot\text{L}^{-1}$ glucose). The precultures (40 ml of medium in 500 ml flasks) were then inoculated directly from glycerol stock solutions and incubated for 18 to 24 h at 37° at a shaking speed of 150 rpm. The continuous cultures were performed with a dilution rate of 0.5 h^{-1} . For both toggle switch strains, the continuous culture first ran for 12 h without inducer. The feed was then replaced by a feed supplemented with 0.1 mM IPTG to induce the culture. For the strains with the unmodified toggle switch, the continuous culture was run for 32 h with IPTG then stopped. For the toggle with growth control, the IPTG feed was kept for approximately 20 h.

Strains with stress-related genes reporters were precultured in two steps to avoid the induction of the reporter. Both steps were performed in 500 ml or 1 L flasks (with respectively 50 or 100 mL of the corresponding medium), either at 37°C (*E. coli*) or 30°C (*S. cerevisiae*) at a shaking speed of 150 rpm. The first preculture was inoculated either from glycerol stock or with one single colony from a LB (supplemented with $50 \text{ mg}\cdot\text{L}^{-1}$ kanamycin, for *E. coli*) or a YPD (*S. cerevisiae*) agar plate, and was incubated overnight. The second preculture was inoculated with cell suspension from the first preculture, to get an initial $\text{OD}_{600} \approx 0.5$, and was incubated until it reached enough biomass to inoculate the bioreactor ($\sim 3 \text{ h}$ for *E. coli*, $\sim 6 \text{ h}$ for *S. cerevisiae*). The batch phase was initiated with an $\text{OD}_{600} \approx 0.1$ (or 0.5 for experiments already published in previous paper [7]). The chemostat conditions alternated between high ($D = 0.6 \text{ h}^{-1}$ for *E. coli* or $D = 0.3 \text{ h}^{-1}$ for *S. cerevisiae*) and low ($D = 0.1 \text{ h}^{-1}$) dilution rates, with each condition maintained for a duration of at least 5 residence times. The Segregostat experiment of *E. coli* was performed at an intermediary dilution rate ($D = 0.3 \text{ h}^{-1}$). For Segregostat experiments with yeast, a low dilution rate ($D = 0.1 \text{ h}^{-1}$) was applied and, the glucose concentration of the feed was $5 \text{ g}\cdot\text{L}^{-1}$.

Dissolved oxygen and pH were monitored in all experiments. Excepted for experiment previously published [7], biomass was monitored with Hamilton Dencytte RS485 probes. All cultures were also monitored at the single-cell level using automated flow-cytometry. The automated flow-cytometry system is the combination of an in-house sampling device (part of the Segregostat [4, 7]) and a benchtop flow cytometer (BD Accuri C6 or BD Accuri C6+). Every $\sim 12 \text{ min}$, it takes a sample from the bioreactor and dilutes it with phosphate-buffered saline (PBS) before analysis. For data acquired with the BD Accuri C6 flow cytometer, the fluidics flow rate was set to custom ($24 \mu\text{L}\cdot\text{min}^{-1}$, $8 \mu\text{m}$ core) and a threshold was set either to 20 000 (*E. coli*) or 80 000 (*S. cerevisiae*) units of FSC-H. For the data acquired with the BD Accuri C6+ flow cytometer, the fluidics flow rate was set to medium ($35 \mu\text{L}/\text{min}$, $16 \mu\text{m}$ core) and a threshold was set either to 15 000 (*E. coli*) or 80 000 (*S. cerevisiae*) units of FSC-H. Under these conditions, about 40 000 cells were analysed per sample. The FL1-A channel (exc. 488 nm, em. 533/30 nm or em. 510/10 nm for the toggle switch) was used for GFP quantification.

1.10.5. Continuous cultivations: data treatment

To allow comparison of replicates acquired with different flow cytometers, we applied transformations on the data acquired with the BD Accuri C6 (ANNEX B, section 1). Flow cytometry data were cleaned by removing zeros and doublets. To compute the entropy, 50 fixed bins in log scale between 1 and 10^6 a.u. were used. Biomass data was smoothed using a Butterworth lowpass filter with cutoff frequency 0.4 h^{-1} .

The population fitness (or expected fitness) was computed based on flow-cytometry data as the mean growth rate of single cells in the population divided by the dilution rate. The single cell growth rate was inferred using functions linking fluorescence and growth rate. For most of our systems, this function was determined by exploiting the fluorescence relaxation phase in Segregostat (CHAPTER 3, section 3).

Regarding *E. coli* $P_{ydcS}::\text{GFP}$, no trade-off was observed between general stress expression and growth rate, making this computation less relevant. However, given the homogeneity of the population, an approximation of the function linking single-cell fluorescence and growth rate was obtained using chemostat data acquired at various dilution rates (FIGURE 3.8 in ANNEX B). For the toggle switch system, we considered two phenotypes: GFP^+ (able to produce serine) and GFP^- (without serine production) separated by a threshold of 100 fluorescence a.u.. In this case, the population fitness was calculated as a weighted average of the growth rates of the two subpopulations based on their relative abundances divided by the dilution rate:

$$\text{Population Fitness}_{\text{Toggle}} = \frac{p(\text{GFP}^+) \cdot \mu_{\text{GFP}^+} + p(\text{GFP}^-) \cdot \mu_{\text{GFP}^-}}{D} \quad (4.1)$$

Where D is the dilution rate and $p(\text{GFP}^+)$ and $p(\text{GFP}^-)$ represent the proportions of GFP positive and negative cells in the population. Their respective growth rates $\mu_{\text{GFP}^+} = 0.531 \text{ h}^{-1} \pm 0.014$ (std) and $\mu_{\text{GFP}^-} = 0.295 \text{ h}^{-1} \pm 0.010$ (std) were determined during the toggle switch phenotypic characterisation (ANNEX B, section 3).

1.10.6. Microfluidic cultivations and time lapse microscopy

Cells precultures were performed in the same way as those for the lab-scale bioreactor, but in smaller flasks (100 ml with 10 ml of the corresponding medium).

The media used are the same as described above, but with modified glucose concentrations ($5 \text{ }\mu\text{M}$ for stressful conditions and 3 mM for non-stressful conditions).

Cells have been cultivated in microfluidic chips provided by Alexander Grünberger's lab. They are polydimethylsiloxane (PDMS)-glass based MSCC device featuring mono-layer growth (chambers sizes: $80 \text{ }\mu\text{m} \times 90 \text{ }\mu\text{m} \times \sim 850 \text{ nm}$ for *S. cerevisiae* and $80 \text{ }\mu\text{m} \times 90 \text{ }\mu\text{m} \times \sim 700 \text{ nm}$ for *E. coli*) [208, 225, 226]. The experiments on *S. cerevisiae*, were already published [7, 204]. They were reprocessed to calculate colonies areas using the same method as for *E. coli* (see below). At least 3 cultivation chambers were selected manually for each glucose concentration condition. The settings of the

experiments carried out with *E. coli* are slightly different. The temperature was set at 37°C. The chambers were inoculated with a few cells by flushing the device with a cell suspension ($OD_{600} \approx 0.1$). Three biological replicates have been performed for each glucose concentration condition. For two of them, one chamber was analysed while 3 chambers (technical replicate) were analysed in the last biological replicate (for a total of 5 chambers for each glucose concentration condition).

Microscopy images of *E. coli* were acquired using a Nikon Eclipse Ti2-E inverted automated epifluorescence microscope (Nikon Eclipse Ti2-E, Nikon France, France) equipped with a DS-Qi2 camera (Nikon camera DSQI2, Nikon France, France), a 100x oil objective (CFI P-Apo DM Lambda 100x Oil (Ph3), Nikon France, France). The GFP-3035D cube (excitation filter: 472/30 nm, dichroic mirror: 495 nm, emission filter: 520/35 nm, Nikon France, Nikon) and the TxRed-4040C cube (excitation filter: 562/40 nm, dichroic mirror: 593 nm, emission filter: 624/40 nm, Nikon France, Nikon) were used to measure GFP and CyOFP1, respectively. The phase contrast images were recorded with an exposure time of 300 ms and an illuminator's intensity of 30 %. The GFP images were recorded with an exposure time of 500 ms and an illuminator's intensity of 2 % (SOLA SE II, Lumencor, USA). The CyOFP1 images were recorded with an exposure time of 800 ms and an illuminator's intensity of 50 % (SOLA SE II, Lumencor, USA). Phase contrast images were acquired every 5 min while both GFP and CyOFP1 fluorescence were measured every 20 min. The optical parameters and the timelapse were managed with the NIS-Elements Imaging Software (Nikon NIS Elements AR software package, Nikon France, France).

The cell segmentation of the images of *S. cerevisiae* was performed using the Matlab algorithm of Wood and Doncic [227]. Microscopy images of *E. coli* were analysed as follows. First, the images were exported in '.tiff' files using the NIS-Elements Imaging Software (Nikon NIS Elements AR software package, Nikon France, France). Then, they were pre-processed (normalisation, stabilisation, cropping, etc.) using a custom-made Python pipeline and Fiji/ImageJ. The cell segmentation was performed on phase contrast images using a custom-made Python code based on cellpose library (version 0.7.1) [228, 229] with the trained model of Dimalis (<https://github.com/Helena-todd/Dimalis/tree/96ac2c8>, commit 96ac2c8, 26 Jul 2023). Masks images of segmented cells were corrected manually using Fiji/ImageJ before tracking using the Fiji/ImageJ plugin TrackMate (version 7.11.1) [230, 231] with the "Label image detector" and the "Overlap tracker" options (precise IuO calculation, min. IuO = 0.1 and scale factor = 1.1). Singles-cells data and lineages were then exported as tables and further analysed. For instance, different approximations of the growth rates have been performed. The one used in FIGURE B.9 (ANNEX B) is based on the increased area during the life of single cells (until division) considering an exponential growth. This approximation is computed based on filtered area (to decrease the noise due to segmentation artefacts between time frames).

2. Biological oscillations without genetic oscillator or external forcing

Based on our previous paper, Henrion et al. [7], there were two different behaviours to explain. First, the increase in entropy with the increased fitness cost, which was the subject of the last section, and second, the appearance of bursts of gene expression for the high fitness cost systems tested, *Bacillus subtilis* sporulation and T7 based production. This section explores the emergence of these oscillations for *B. subtilis*, with a method that can be generalised to the T7 system (see ANNEX C)

This section is adapted from the following publication:

[232] Vincent Vandenbroucke, Lucas Henrion, and Frank Delvigne. “Biological Oscillations without Intracellular Oscillator or External Forcing”. In: *npj Systems Biology and Applications* (Aug. 5, 2026). DOI: 10.1038/s41540-026-00801-4

The following resources are associated to this publication:

- doi.org/10.5281/zenodo.13803913

2.1. Abstract

Oscillators are fundamental to biological systems, underpinning essential processes such as cell division, circadian rhythms, and developmental cycles. While both natural and synthetic genetic oscillators have been extensively studied, oscillatory behaviours in cells can also emerge without dedicated gene circuits. In earlier work, we uncovered sustained oscillations in phenotypic switching across diverse cellular systems and gene circuits, occurring spontaneously, without external forcing and linked them to the induction of slow-growing phenotypes. In this study, we identify the conditions that give rise to such intrinsic phenotypic oscillations, leading to population-level oscillations. We develop and analytically solve a simplified mathematical model of a stress-induced phenotype, mapping the range of continuous culture conditions that trigger oscillatory gene expression. This oscillation range, predicted by the model, was experimentally validated in *B. subtilis* cultures. Our findings reveal that oscillations can arise in the complete absence of genetic oscillators or external perturbations. Although demonstrated here for a stress response in continuous culture, this phenomenon may occur in any long-term cultivation where environmental feedback links an inducer to the cellular system, broadening the landscape of possible oscillatory behaviours in microbiology and synthetic biology.

2.2. Introduction

Oscillations in gene expression are traditionally attributed to dedicated gene circuits, metabolic pathways or external forcing [233, 234]. Here, we report sustained oscillations emerging in the complete absence of either, arising instead from the interplay between fitness cost and nutrient availability in a constant environment. Oscillatory behaviours are pervasive in biology, spanning multiple scales, from single cells to entire organisms, and regulating substantial portions of cellular metabolism [235, 236]. Their study has revealed natural gene circuits acting as biological oscillators, such as the circadian clock in cyanobacteria [237] and gut microbiota [238, 239], as well as the cell cycle in *E. coli* [240]. These discoveries have been complemented by synthetic designs [241, 242], including the iconic repressilator, which generates spontaneous oscillations in gene expression through a triad of repressor proteins [47, 243]. In general, biological oscillations arise from dedicated gene circuits [241, 243], external periodic forcing [121], or a combination of both [144]. In all reported cases, oscillations under constant culture conditions were linked to an underlying genetic oscillator e.g., yeast respiratory cycles [244], bacterial cell cycle [245] and plankton synchronisation [10].

Surprisingly, we previously observed that highly burdensome gene circuits can exhibit sustained oscillations in a simple chemostat [7], a continuous cultivation device initially designed to maintain constant growth conditions [33, 246]. Strikingly, the two systems that displayed this behaviour, the T7 expression system in *E. coli* BL21 and the sporulation program of *B. subtilis*, lack network architectures typically associated with oscillatory dynamics. To uncover the origin of these oscillations, we focused on the *B. subtilis* system, which can be distilled into: i) Two discrete phenotypes linked to growth capacity, vegetative, non-fluorescent cells that grow, and sporulating, fluorescent cells that do not; ii) A single trigger, glucose, whose depletion both induces sporulation [247] and serves as the primary carbon source.

To uncover the origin of these unexpected oscillations, we developed a minimal mathematical model of the stress response in a chemostat, a system with constant nutrient influx, where stress is triggered when nutrient levels fall below a critical threshold. The model predicts that, even under the classically “stable” operating conditions of a chemostat, there exists a specific range of dilution rates in which no steady-state population-level stress response can be sustained. This challenges the long-standing assumption that chemostats inherently stabilise gene expression across microbial populations [33]. We experimentally tested this prediction using *B. subtilis* 168 carrying a chromosomal *GFP* reporter under the control of the *spoIIE* promoter, SpoIIE being a key regulator of asymmetric cell division during sporulation [171, 172]. Cultures were grown at different dilution rates, and sporulation activation was tracked at the single-cell level using a custom flow cytometry–bioreactor interface [4, 248]. Consistent with model predictions, we observed oscillatory sporulation dynamics within the predicted operating range, manifesting as successive bursts of sporulating cells

sweeping through the population.

2.3. Results

2.3.1. A mathematical model predicts oscillations in gene expression for a specific range of dilution rate.

To probe how oscillations can arise in the absence of a genetic oscillator, we constructed a minimal ODE model capturing stress-induced dynamics in a cell population. The framework tracks two phenotypic states: non-stressed cells (X_1) and stressed cells (X_2) (EQUATIONS 4.2 to 4.6). The stressed phenotype grows more slowly, with a maximum growth rate $\mu_{\max,2} = k\mu_{\max}$ where $k \in [0, 1]$ and $\mu_{\max}$ is the maximal growth rate of the non-stressed biomass. Switching from X_1 to X_2 occurs at a constant rate H when the substrate concentration S drops below a threshold K_I and is implemented via a step function $H_1(S)$ (EQUATION 4.6). Because stressed cells grow slowly and stress-related proteins are primarily diluted through cell growth, the reverse transition from X_2 to X_1 is assumed negligible.

$$\frac{dS}{dt} = (S_{in} - S)D - \frac{\mu X_1}{Y_{X1}} - \frac{k\mu X_2}{Y_{X2}} \quad (4.2)$$

$$\frac{dX_1}{dt} = (\mu - D)X_1 - H_1(S)X_1 \quad (4.3)$$

$$\frac{dX_2}{dt} = (k\mu - D)X_2 + H_1(S)X_1 \quad (4.4)$$

$$\mu = \mu_{\max} \frac{S}{K_S + S} \quad (4.5)$$

$$H_1(S) = \begin{cases} H & \text{if } S \leq K_I \\ 0 & \text{if } S > K_I \end{cases} \quad (4.6)$$

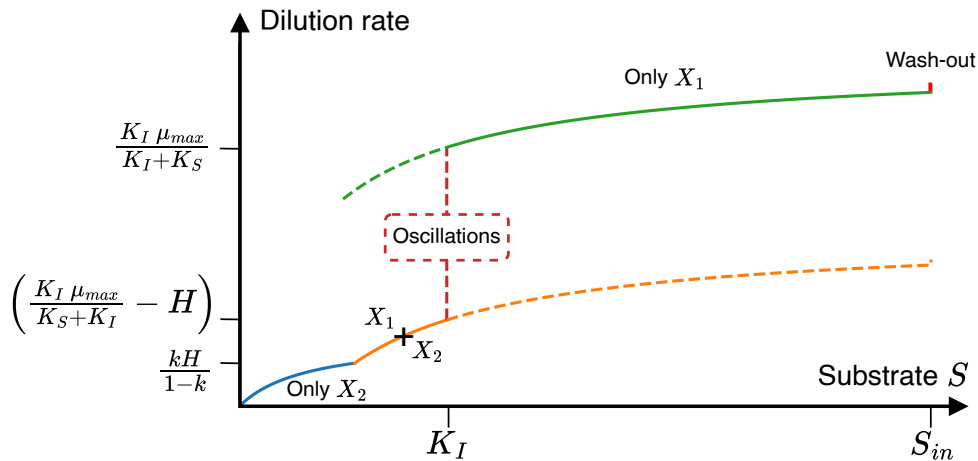
Here, S_{in} denotes the sugar concentration in the feed, D the dilution rate, $Y_{X,i}$ the biomass yield of each phenotype, and K_S the sugar concentration at half-maximum growth rate (see GLOSSARY and NOTATIONS). Analytical solutions of the model (see ANNEX C) reveal a critical range of dilution rates in which no steady-state equilibrium can be achieved among the state variables, making the system oscillate (FIGURE 4.7A).

Within this oscillation window, two mutually exclusive conditions prevent convergence to equilibrium. First, the classical chemostat formulation, omitting the stress phenotype, fails because the residual substrate concentration S stabilises below the threshold K_I , triggering the stress response. Second, the alternative scenario of continuous switching into the stressed state is also invalid, as the associated reduction in substrate uptake drives S above K_I , thereby suppressing the stress response.

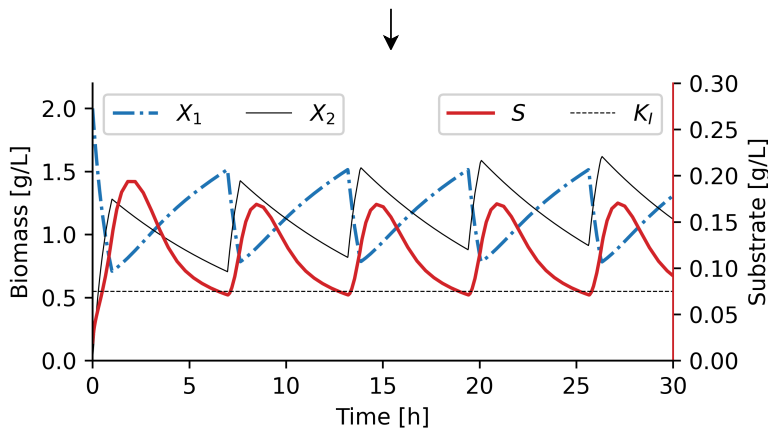
Rather than resolving into either case, the system hovers near the switching threshold K_I , forcing cells to continually adjust to environmental fluctuations. This creates a

negative feedback loop on population growth which, in combination with delayed phenotypic transitions, produces sustained oscillations analogous to those of the synthetic repressilator [47, 131, 243] (FIGURE 4.7B, C). The appearance and persistence of these oscillations are expected to depend on both the delay magnitude and the fidelity of the step function in capturing the switching dynamics, while the dilution-rate range for their occurrence can be experimentally validated.

A Solution space for S with respect to D and oscillation region



B Simulation of X and S over time in the oscillation region



C The cell population motif is analogous to a repressilator

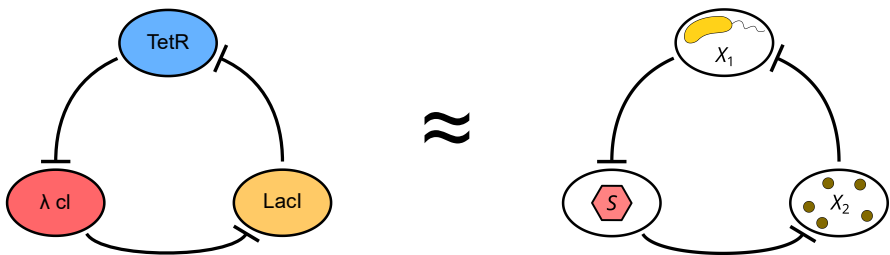


Figure 4.7: Representation of the solution space of the stress-response model at steady state. **A** Model predictions reveal three distinct regimes. At high dilution rates, only the non-stressed phenotype is sustained, as the sugar concentration remains above the threshold K_I . At low dilution rates, the stressed phenotype dominates, with sugar concentrations falling below K_I . Between these extremes lies a window where no steady state exists, and oscillations can emerge. **B** Introducing a delay in the stress response generates sustained oscillations *in silico* within the oscillation region. The example shown corresponds to *B. subtilis* sporulation dynamics with $D = 0.1 \text{ h}^{-1}$ and a 0.5 h delay, although small parameter changes can markedly alter the oscillatory behaviour.

C Schematic analogy between the sporulation system in *B. subtilis* (right) and the synthetic repressilator [47] (left). Here, the stressed and non-stressed subpopulations, together with the extracellular sugar concentration, form a triple negative feedback loop. In the chemostat, oscillations arise at the level of reactor concentrations rather than intracellular protein levels: sporulation (or stress induction) reduces the number of unstressed cells, decreasing sugar uptake, raising sugar concentration above K_I , inhibiting sporulation, and allowing unstressed cells to expand again.

2.3.2. The oscillation region can be experimentally observed in chemostat for a given range of dilution rates

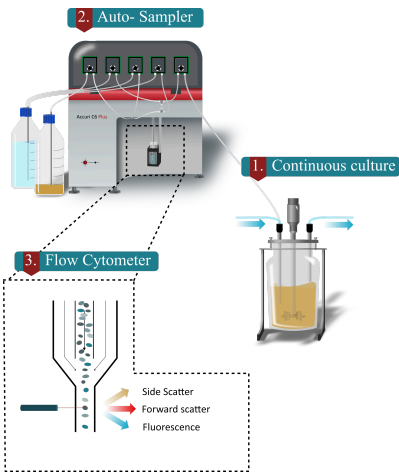
Model predictions were tested under continuous cultivation, a regime designed to maintain stable growth conditions through substrate (S) limitation, which can be tuned via the dilution rate (D). As a case study, we examined *B. subtilis* sporulation, a stress response triggered when residual glucose concentration falls below a threshold K_I . Here, X_1 denotes vegetative (non-stressed) biomass and X_2 denotes stressed biomass (spores). Sporulation was monitored at the single-cell level using automated flow cytometry and a $P_{spoIIIE}::GFP$ transcriptional reporter, where *spoIIIE* encodes a key regulator of asymmetric division during sporulation.

Using H , K_I , $\mu_{\max}$, and K_S values determined from batch cultures (ANNEX C), and assuming no growth for the stressed phenotype, the model predicts that at high D , with residual glucose above K_I , only X_1 persists. At low D , with residual glucose at or below K_I , both X_1 and X_2 are present, with X_2 (spores) periodically increasing in abundance. Due to the absence of growth in X_2 and a high switching rate from X_1 , the model anticipates no regime of stable spore concentration.

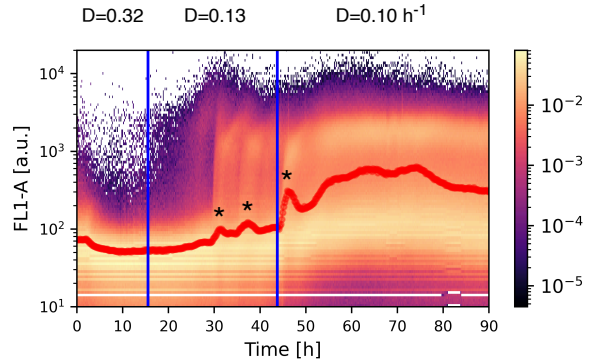
Experimental observations aligned with these predictions: at high dilution rates, residual glucose remained above the switching threshold and sporulation was not activated, yielding exclusively X_1 biomass (FIGURE 4.8). Reducing D caused residual glucose to fall below K_I , initiating bursts of sporulation from X_1 to X_2 . These oscillatory transitions persisted for at least five residence times before gradually decreasing in amplitude.

Fitness cost drives complex but predictable cell population dynamics

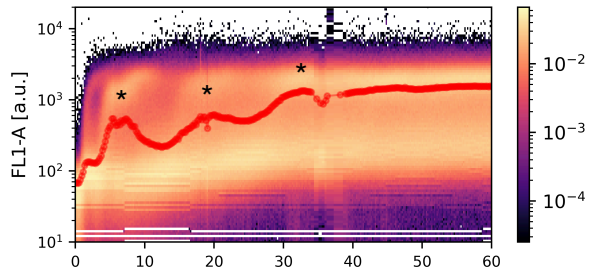
A Automated Flow Cytometry setup



B Dilution rate sweep reveals the oscillation region



C Confirmation for the presence of an oscillation region



D

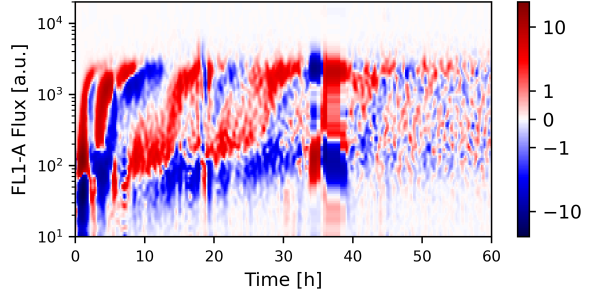


Figure 4.8: Mapping of cell population dynamics using automated flow cytometry reveals spontaneous oscillations in the activation of the *spoIIE* system during continuous cultivation of *B. subtilis*. **A** Schematic of the automated flow cytometry setup. Samples are withdrawn directly from the bioreactor and analysed at single-cell resolution every 15 min.

B Time-fluorescence density plots, where brighter colours indicate higher cell counts. The black line marks the 75th percentile of fluorescence; large oscillations are indicated by asterisks. In agreement with model predictions, gene expression dynamics vary with the dilution rate (D): at $D = 0.32 \text{ h}^{-1}$, cells remain vegetative, whereas lowering D to 0.13 h^{-1} triggers sporulation bursts detected by activation of the $P_{spoIIE}::\text{GFP}$ reporter. These bursts diminish in amplitude upon further reduction to $D = 0.10 \text{ h}^{-1}$. **C** Prolonged chemostat cultivation at $D = 0.11 \text{ h}^{-1}$ confirmed the persistence of oscillatory sporulation. **D** Differential fluorescence maps showing the increase (red) or decrease (blue) in cell counts per fluorescence bin between successive time points, smoothed for clarity. Until $\sim 40 \text{ h}$ of continuous culture, clear waves of sporulation are observed. Color units are arbitrary.

2.4. Discussion

Oscillatory behaviours in biology have long been associated with dedicated genetic oscillators, external forcing, or a combination of both [233, 249, 250]. From natural systems such as circadian clocks and cell cycles to synthetic designs like the repressilator [47], the prevailing assumption has been that sustained oscillations require an explicit genetic architecture to generate them. Our work challenges this paradigm by demonstrating that oscillations can arise spontaneously in constant environments, purely from the interplay between cellular physiology and environmental feedback.

Our results demonstrate that even gene networks lacking intrinsic oscillatory architecture can generate sustained oscillations in the absence of external forcing. These oscillations emerge from the interplay of two independent but complementary mechanisms. The first is the impact of gene expression on cellular growth. This phenomenon is widespread: many natural circuits, such as sporulation in *B. subtilis*, and synthetic systems, such as burdensome protein overproduction [251], can impose substantial growth penalties [252]. Similar effects are conceivable for genes affecting persistence traits, such as biofilm formation, where the “cost” to growth is offset by improved survival in specific environments [216].

The second requirement is environmental feedback, in which cells not only sense but also modify the concentration of their inducers. This feedback occurs when cells consume, degrade, or produce the relevant inducer, thereby reshaping their own stimulus landscape. In our case study, sporulation reduced glucose uptake, allowing residual glucose to rise above the sporulation threshold and thereby suppressing further induction in non-stressed cells. A similar mechanism was reported for a T7 expression system under lactose control [216], where growth inhibition from burdensome protein production increased residual glucose levels and suppressed induction via carbon catabolite repression.

Fundamentally, the oscillations observed here represent system-level circuits: emergent behaviours arising from population–environment interactions rather than from the regulatory topology of an isolated genetic network. Whereas genetic oscillators are defined by well-mapped gene–protein interactions [144, 241], system-level oscillators depend on how those interactions couple to resource flows and environmental feedback [216, 253, 254]. The example we present is relatively simple, but more complex interactions, especially where multiple gene networks intersect through shared environmental variables, could produce rich and unexpected dynamical patterns.

The emergence of oscillations in the absence of an explicit genetic oscillator has several important implications. First, it challenges the assumption, fundamental to chemostat theory [33, 255], that constant environmental conditions necessarily yield steady-state population structures and gene expression profiles. Second, it suggests that similar oscillations may occur in other long-term cultures whenever environmental feedback is tightly coupled to a phenotypic switch that significantly alters resource

consumption. This principle could extend to diverse microbial systems, from industrial bioprocesses [256] to natural microbial communities [88, 106], where environmental cues and physiological states are dynamically linked.

2.5. Materials and Methods

2.5.1. Mathematical Model Solution

The mathematical model was solved analytically (see ANNEX C) by considering independently the cases where there is or is not switching to the stressed phenotype, then assembling the solution. The reasoning was further expanded to exponential fed-batch cultures and additional considerations regarding the validity of the model are given.

Based on the mathematical solutions, there are values of dilution rate for which no solution exists, depending on the biological parameters.

The model was then verified by simulating a continuous culture using Matlab's delay differential equations (dde23) with parameters similar to the ones observed with our *B. subtilis* strain and with a delay of 30 min between the substrate concentration crossing the threshold and the cells activating or repressing the switch to the stressed phenotypes.

2.5.2. Experimental Design

This work aims to explain how gene oscillations can arise without a specific gene motif nor external feedback. To do so, we focused on the sporulation dynamics in *B. subtilis*. First, the growth and switch characteristics were experimentally determined as described in ANNEX C, section 4.

Based on these parameters and the analytical resolution of the model detailed in ANNEX C, section 1, we identified dilution rate regimes that should either prevent sporulation or lead to the predicted oscillations. We then performed chemostats under these dilution rates and confirmed the behaviour with our custom-built automated flow cytometry platform and a strain we engineered to produce GFP upon sporulation onset.

2.5.3. Strain and Growth media

All experiments were performed in minimal mineral media containing (in $\text{g}\cdot\text{L}^{-1}$): K_2HPO_4 : 14.6; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$: 3.6; Na_2SO_4 : 2; $(\text{NH}_4)_2\text{SO}_4$: 2.47; NH_4Cl : 0.5; $(\text{NH}_4)_2\text{H-citrate}$: 1; glucose: 5; thiamine: 0.01, tryptophan: 0.05. The medium is supplemented with a trace element solution totalling $11\text{ mL}\cdot\text{L}^{-1}$ assembled from the following solutions (in $\text{g}\cdot\text{L}^{-1}$): 3/11 of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$: 16.7, 3/11 of EDTA: 20.1, 2/11 of MgSO_4 : 120 and 3/11 of a metallic trace element solution. The metallic trace element solution contains (in $\text{g}\cdot\text{L}^{-1}$): $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 0.74; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$: 0.18; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$: 0.1; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$: 0.1; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$: 0.21. Both the trace element solution and the amino acids were filter sterilised ($0.22\text{ }\mu\text{m}$). To monitor the switching to the sporulating phenotype, *Bacillus subtilis* 168 was modified to express *gfpmut2* under the control

of the SpoIIE promoter. The strain, *B. subtilis* 168 P_{spoIIE}::gfpmut2, was built by AmyE chromosomal integration of a cassette containing the promoter, gfpmut2 and the selection maker, kanamycin.

2.5.4. Cultivation Conditions

Precultures were started from single colonies picked from a LB plate and grown overnight. To avoid starting bioreactor cultivations with sporulating populations, the precultures were done in overfilled shake flasks (20 % of filled volume) without baffles to reach oxygen limitation before glucose. All bioreactor cultivations were performed at 37°C, pH 7 and aeration at 1 VVM. For growth and switching parameters determination, experiments were performed in DASbox mini-bioreactors (Eppendorf) with a stirring speed of 400 rpm and a cultivation volume of 160 ml. Continuous cultivations were performed in Bionet F1 bioreactor with a cultivation volume of 1 L at an agitation speed of 1000 rpm. The transition from batch to continuous was triggered once dissolved oxygen rose, signalling the transition from exponential to stationary phase.

Bioreactor cultivations were monitored with a custom-made sampling device (the Segregostat) that automatically draws a sample from the reactor and dilutes it with PBS before flow cytometry (FC) analysis (BD Accuri, C6 for the parameter determination and C6+ for the continuous cultures). A total of 40,000 cells was analysed in each sample where the FL1-A channel is used to visualise the GFP content.

2.5.5. Statistical Analysis

The switching and growth parameters were determined in triplicate cultivations. The cultivations to test the impact of dilution rates were performed in duplicates and the dilution rate leading to oscillations was confirmed one last time. During all cultivations, samples were automatically taken every 15 minutes and 40,000 cells were analysed.

5

**The frontier for bioprocesses:
Exploring co-culture design
Via synthetic niches**

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In previous chapters, the focus was on measuring, analysing and predicting biological system, with mainly qualitative analyses. As mentioned in CHAPTER 2, however, the ideal goal would not only be to understand cultures, but also to design them. Therefore, this chapter explores what could be possible by designing a co-culture from the ground up and attempting to predict its behaviour quantitatively.

Besides the co-culture itself, the computational tool allowing for the analysis and prediction of the co-culture is highlighted: a modular modelling framework enabled detailed analysis of distinct parts of the metabolism despite their interactions with each other, paving the way for more detailed analysis and prediction of bioprocesses.

1. Synthetic niches enable co-culture bioprocessing but are prone to mutational escape

This section is adapted from the following publication:

[257] Vincent Vandenbroucke, Juan Andrés Martínez, Lucas Henrion, Andrew Zicler, Samuel Telek, Laurie Josselin, and Frank Delvigne. “Synthetic Niches Enable Coculture Bioprocessing but Are Prone To Mutational Escape”. In: *ACS Synthetic Biology* 15.5 (May 15, 2026). DOI: 10.1021/acssynbio.5c00940

The following resources are associated to this publication:

- doi.org/10.5281/zenodo.17663997
- gitlab.uliege.be/mipi/published-software/2025-syntheticniches

1.1. Abstract

Stabilising microbial co-cultures is a central challenge for bioproduction. While division of labour between strains can enhance efficiency, it often results in population instability over time. Classical strategies, including cross-feeding, quorum sensing, and toxin-antitoxin modules (see GLOSSARY), often rely on complex ecological interactions that are difficult to predict or maintain under bioprocess conditions. We report the first implementation, to our knowledge, of a synthetic-niche-based co-culture operated in a continuous bioreactor, using genetic toggle switches that couple growth to defined phenotypic states. We engineered two auxotrophic strains, TOGGLE_{green} and TOGGLE_{yellow}, in which growth is linked to either GFP- or YFP-expressing states and assessed their behaviour under continuous bioreactor conditions using automated and reactive flow cytometry. Unexpectedly, the introduction of auxotrophic pressure reshaped circuit function, i.e., rather than maintaining bistability as typically reported in batch or microfluidic systems, toggle strains behaved as unidirectional inducible systems that reverted upon inducer withdrawal. This emergent behaviour enabled simplified single-input control at the bioreactor scale, but also revealed a critical limitation for long-term operation, namely rapid mutational escape of the growth-impaired strain occurring within fewer than 10 generations in co-culture, a markedly shorter timescale than the ~50 generations typically reported in the literature. A simple repression-based ODE model recapitulated the reversible dynamics, capturing the effective inducible behaviour observed under continuous cultivation, whereas deviations under prolonged operation highlighted the rapid evolutionary erosion of synthetic control. Our findings demonstrate both the potential and the limitations of synthetic niches as a scalable co-culture control strategy.

1.2. Introduction

Microbial cultures provide a versatile platform for biotechnology, supporting the production of fuels, chemicals, and therapeutics. However, monocultures are often limited by metabolic burden, competing intracellular reactions, and the complexity of balancing multiple enzymatic steps within a single host [72, 73, 78, 141, 258, 259]. To address these constraints, microbial co-cultures have emerged as a promising alternative. By dividing labour between strains, co-cultures reduce metabolic burden, expand the accessible metabolic space, and allow for modular pathway design [146, 260, 261]. Yet, they also introduce new challenges: co-cultures are inherently unstable, as strain ratios drift over time, frequently leading to the dominance of one of the strain and the loss of production [72, 258, 259]. Concepts from microbial ecology have also been applied to co-culture control, including metabolic niche theory and competitive exclusion [262–264]. These approaches are effective only when temporal dynamics are satisfied [146, 265], as niche expansion or contraction often compromises long-term stability [264, 266].

When natural niches and interactions are not possible, synthetic biology has been used to create them. These synthetic interactions include cross-feeding, where at least one strain is fed and therefore limited by another [204, 265, 267], quorum sensing, which has been repurposed to let strains know of their neighbours' cell densities [268], and toxin-antitoxin systems and regulated killing modules which are used to modulate cell concentrations [60, 269, 270]. While effective in certain contexts, these approaches often require tailoring interactions to specific pathways or strains, reducing modularity and making generalisable design difficult [25, 267–269, 271]. Alternatively, synthetic niches create metabolic spaces where microbes do not compete, usually via separation of carbon sources, and often require extensive genetic engineering that may adversely affect production pathways. As a result, focus has turned to creating synthetic niches that interfere minimally with normal cell metabolism and potential production pathways [60]. The aim of these niches is to maintain native cellular function under optimal co-culture conditions, introducing transient metabolic and production changes only when correction of a suboptimal strain ratio is required. A previously reported strategy relies on the transient suppression of antibiotic resistance to momentarily disadvantage the dominant strain and promote the expansion of a secondary population [60]. In this context, toggle switch circuits are particularly attractive: by coupling strain fitness to a bistable regulatory state, they enable orthogonal control of strain ratios while leaving metabolic pathways largely unaffected. Unlike cross-feeding or quorum sensing, this strategy minimises energetic demand by relying on non-metabolisable inducers rather than costly metabolic exchanges. In this way, synthetic niches provide modularity and selectivity without constraining the underlying production pathway [60, 141, 271, 272].

Despite their promise, most studies of synthetic consortia have been conducted under simplified cultivation conditions, such as batch or serial transfer cultures lasting fewer

than 20 generations [73, 78, 258, 259, 271]. Even continuous cultures often remain below 40 generations, and typically in small-volume or microfluidic devices [25, 60, 273]. For instance, Sommer and colleagues observed mutations after ~40 generations in 200 mL batch cultures [29], while Khammash and colleagues reported genetic erosion after ~80 generations in very small-volume consortia [60]. Only a few studies extend beyond 100 generations e.g., an auxotrophic cross-feeding in *E. coli* [267] or a phenol degradation consortium [274], and they are largely ecological rather than production-focused. By contrast, industrial bioprocesses routinely impose more than 60 generations under high-density, large-volume conditions, where the probability of mutational escape scales with total cell divisions [29].

A further limitation lies in methods of observation. Most studies rely on manual sampling and coarse readouts such as CFU counts, DNA quantification, or bulk fluorescence [271]. A few notable exceptions, such as fluorescence-based ratio tracking in microreactors [60, 272], optogenetic cybergnetic control, where cell states are controlled with light [60, 275], or microchemostat platforms, chemostats in microfluidics [273, 276], achieved higher resolution but, to our knowledge, remained restricted to small cell populations or short durations. Understanding the long-term behaviour of synthetic niches under industrially relevant conditions at single-cell resolution remains an open challenge. Overcoming this limitation requires continuous, single cell monitoring tools compatible with bioreactor operation. The Segregostat platform, a cell-machine interface relying on reactive flow cytometry for real-time population monitoring and control, addresses this need by providing real-time, high-resolution feedback on strain ratios during extended cultivation [4, 265].

In this work, we implement synthetic niches based on toggle switch circuits under intensified bioreactor conditions. Using auxotrophic strains and reactive flow cytometry (Segregostat) [248], we explore how auxotrophic pressure reshapes toggle dynamics over prolonged cultivation (> 80 generations). We show that while synthetic niches can achieve selective and simplified control of co-culture composition, they are also prone to mutational escape, highlighting evolutionary robustness as a key requirement for next-generation co-culture design.

1.3. Results

1.3.1. Synthetic niches enable the selective control of the growth rate of microbial species involved in co-cultures

The synthetic niches approach was implemented by combining genetic toggle switches and an auxotrophy to serine via the *serA* gene, which can be deleted from the genome and used for plasmid maintenance [174]. The switches direct *serA* production for the cells and provide a growing, *serA* producing, and a non-growing, *serA* starved phenotypes. In the TOGGLE_green strain, the growth-associated phenotype was linked to the GFP-expressing (Green fluorescent protein) state, repressed by IPTG and inducible by tetracycline (Tc). Conversely, in the TOGGLE_yellow strain, the growth-associated phenotype was coupled to the YFP-expressing (Yellow fluorescent protein) state, which was inducible by IPTG and repressed by Tc (FIGURE 5.1A). Using these genetic constructs, the relative abundance of each strain in the bioreactor could be independently controlled through the periodic addition of IPTG or Tc (FIGURE 5.1B). The timing of inducer addition was guided by a previously developed cell-machine interface, the Segregostat, which relies on reactive flow cytometry for real-time population monitoring and control (FIGURE 5.1C) [248, 265].

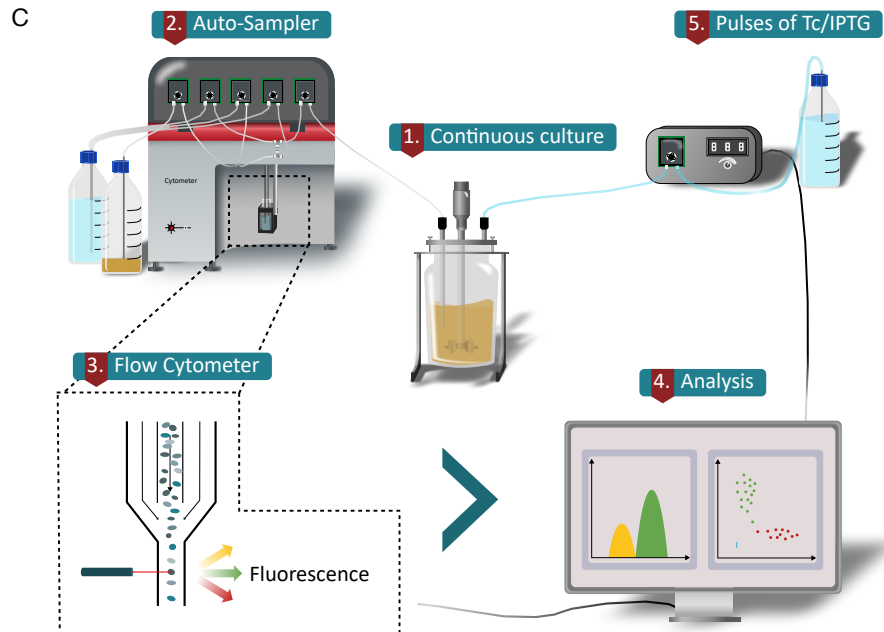
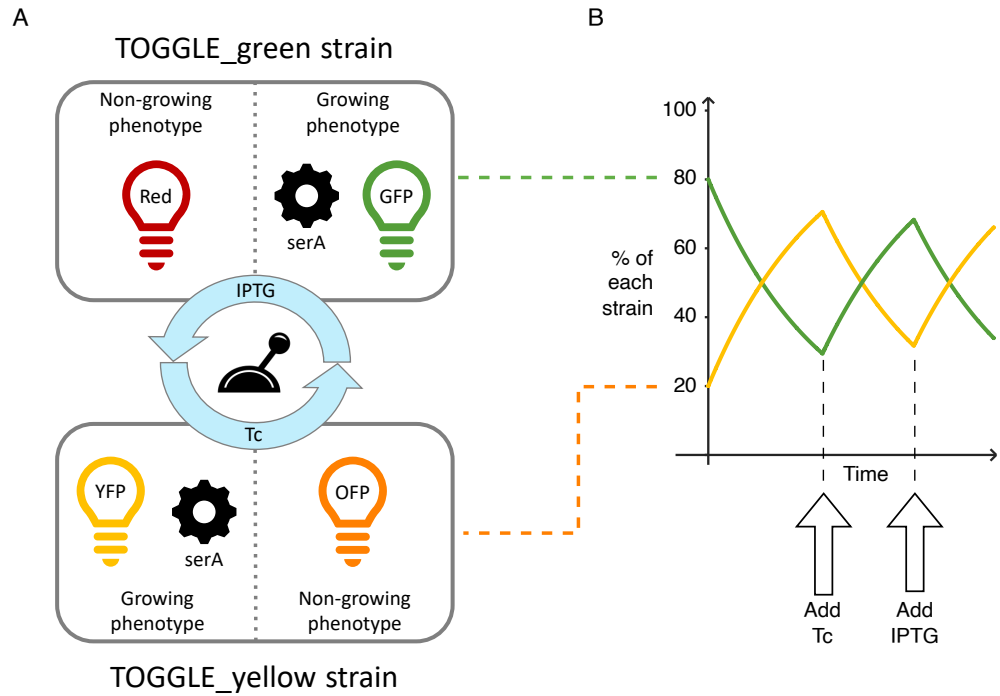


Figure 5.1: Conceptual design for implementing synthetic niches to control co-culture composition. **A** Two strains engineered with growth-dependent genetic toggle switches, where one state of the switch specifically regulates serine biosynthesis in serine auxotrophs. **B** Periodic addition of inducers enables differential control of strain growth, allowing real-time manipulation of co-culture composition. **C** A cell-machine interface (Segregostat) provides feedback-driven inducer addition for optimal co-culture regulation.

The TOGGLE_green and TOGGLE_yellow strains were constructed based on the pECJ3 plasmid, which encodes a toggle switch (FIGURE 5.2A) [173]. In the TOGGLE_green strain, the pECJ3 backbone was retained, and the GFP state was complemented with the *serA* gene, enabling serine biosynthesis upon induction (FIGURE 5.2B). Following sequencing-based validation of the modified plasmid, microplate cultivation revealed a marked growth difference between the GFP and mCherry toggle states, corresponding to a 44% decrease in growth rate (FIGURE 5.2C). Effective switching between red and green fluorescent phenotypes was further confirmed by flow cytometry analysis (SECTION 1.5, ANNEX D).

For the TOGGLE_yellow strain, mCherry and GFP were replaced by sYFP2 (YFP) and CyOFP1 (CyOFP), respectively, with the YFP state coupled to *serA* expression and growth (FIGURE 5.2D). The initial construct, however, showed no significant growth difference between the two states, likely due to leakiness of the P_{trc} promoter driving YFP and *serA*. Replacing this promoter with the tighter $P_{LlacOsys}$ promoter restored toggle functionality, yielding a comparable 42% growth difference between the two states (FIGURE 5.2E). $P_{LlacOsys}$ was derived from the P_{LlacO} promoter, which is tighter than P_{trc} , and the lacOsys binding site, which enhances LacI binding (see SECTION 1.5) [277–279]. Each TOGGLE system was engineered with two distinct fluorescent proteins to mark the alternative states. For subsequent analyses, we focused on the *serA*-associated GFP in TOGGLE_green and YFP in TOGGLE_yellow, as these provide a direct proxy for the growth status of individual cells. In contrast, CyOFP and mCherry served primarily as confirmation that the two switch states were mutually exclusive but offered little additional information.

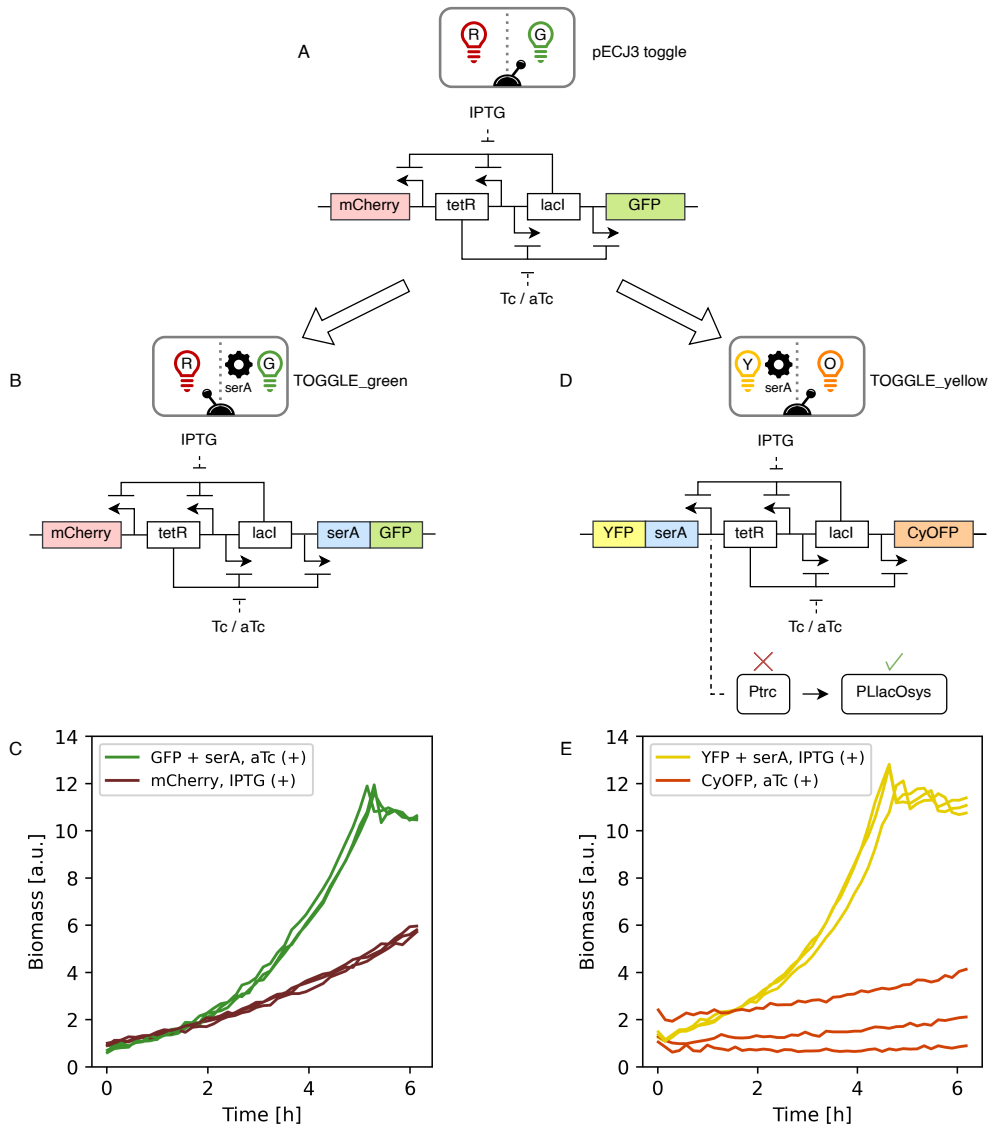


Figure 5.2: Toggle switch design for implementing synthetic niches. **A** Genetic toggle switch architecture carried on the pECJ3 backbone plasmid, used in both strains. The mCherry side of the bistable circuit can be induced with IPTG, the GFP side with tetracycline (Tc) or anhydrotetracycline (aTc). **B** Schematic representation of the TOGGLE_green construct showing the main regulatory elements. **C** Biomass dynamics of TOGGLE_green flipped to either the GFP ($n = 3$ (technical replicates); 0.53 h^{-1} ; standard deviation (SD) = 0.015 h^{-1}) or mCherry state ($n = 3$ (technical replicates); 0.29 h^{-1} ; SD = 0.010 h^{-1} ; one-tailed t-test with the GFP state, assuming unequal population variances: $p = 3 \cdot 10^{-5}$). **D** Schematic representation of the TOGGLE_yellow construct showing the main regulatory elements. **E** Biomass dynamics of TOGGLE_yellow flipped to either the YFP ($n = 3$ (biological replicates); 0.44 h^{-1} ; SD = 0.021 h^{-1}) or CyOFP state ($n = 3$ (biological replicates); 0.26 h^{-1} ; SD = 0.054 h^{-1} ; one-tailed t-test with the YFP state, assuming unequal population variances: $p = 8 \cdot 10^{-3}$).

1.3.2. Auxotrophic pressure induces cell escape from the toggle switch

To validate the predictive capacity of the pECJ3 toggle, the system was first evaluated in continuous culture with flow cytometry monitoring. Subsequently, the derived TOGGLE construct, built from pECJ3, was tested under similar conditions to assess whether it retained similar dynamics. However, unexpected population dynamics emerged upon introducing auxotrophic pressure, i.e., generating serine auxotrophs and coupling *serA* expression to one side of the toggle. This effect is illustrated for TOGGLE_green, with similar behaviour obtained for TOGGLE_yellow (FIGURE 5.3). In the absence of auxotrophic pressure, the strains engineered with the original toggle switch (pECJ3) behaved as expected: IPTG addition triggered a complete switch to the mCherry state, which was stably maintained even after inducer withdrawal (FIGURE 5.3A). Only a small fraction of cells (~1.5%) escaped and reverted to the GFP state, and it started escaping only after 15h without IPTG induction. By contrast, when *serA* dependence was introduced, the escape rate increased dramatically, with more than 90% of cells switching back to the GFP by the time we would expect the escape to start, based on the pECJ3 toggle behaviour (FIGURE 5.3B). Under these conditions, the toggle behaved as a unidirectional inducible system that naturally reverts once the inducer is withdrawn. The difference in timing indicates that it is not only a population-level behaviour: TOGGLE_green escapes faster and is therefore less stable than the original toggle switch. This escape effect simplifies control by enabling unidirectional regulation with a single inducer.

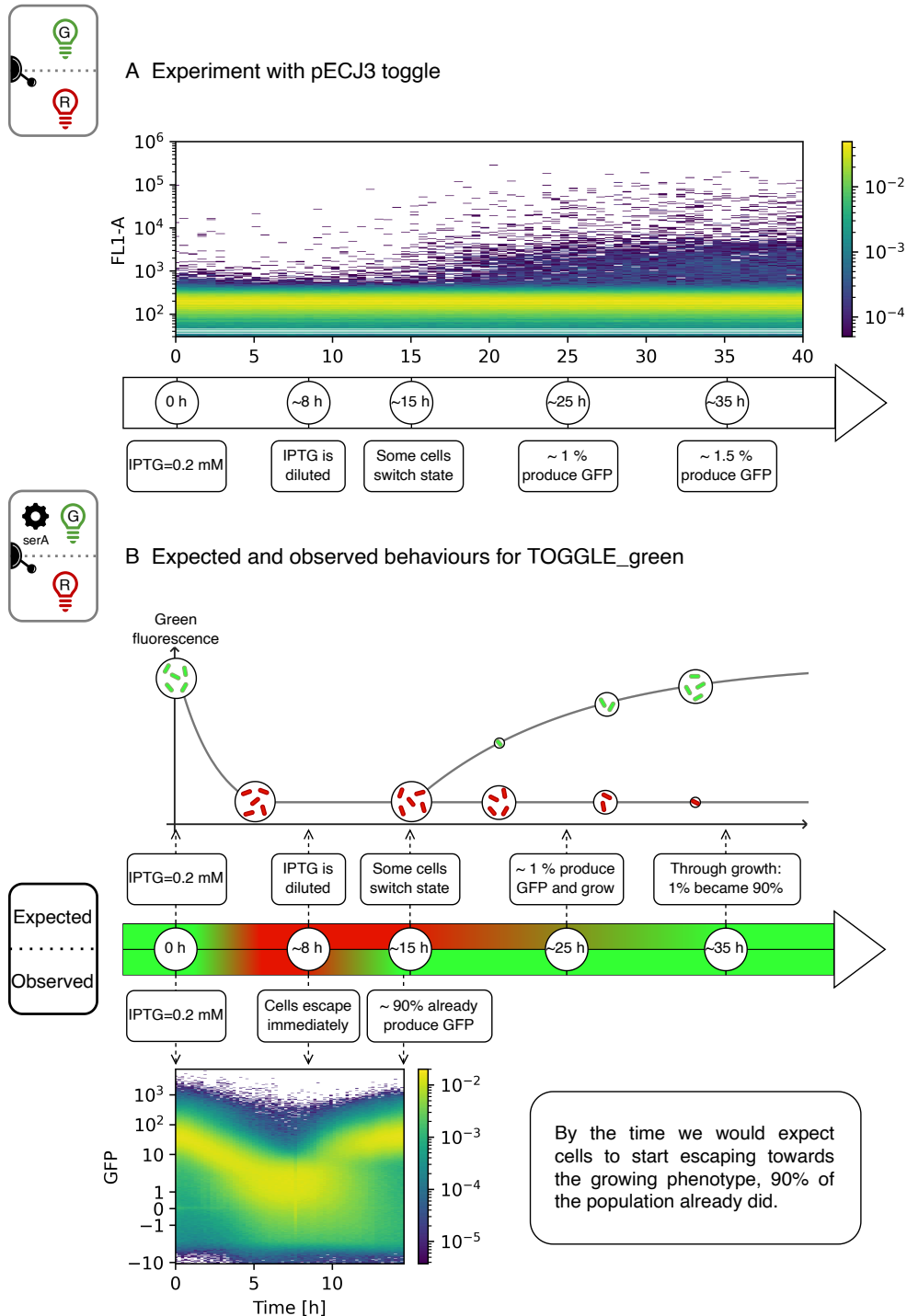


Figure 5.3: Auxotrophic pressure drastically alters population dynamics of the TOGGLE_green strain. **A** Automated flow cytometry (FC) profiling of the plasmid pECJ3 toggle after release from induction with 0.2 mM IPTG (FL1-A = GFP channel). Natural escape from the mCherry-producing phenotype occurs very slowly and stabilises with ~1.5% of the population producing GFP, while the remaining cells continue producing mCherry. **B** Based on the induction dynamics of pECJ3, when IPTG is used to induce the TOGGLE_green strain and inhibit SerA and GFP production, we would expect cells to remain trapped in the non-growing state for a long time. Instead, the SerA and GFP producing state reappears nearly immediately after IPTG is diluted.

1.3.3. Implementation of synthetic niches facilitate parameter inference for simulating mono-culture dynamics

As described above, introducing auxotrophic pressure caused the TOGGLE_green system to behave like a simple inducible gene circuit. To test this hypothesis, we cultivated the strain in a chemostat and applied IPTG pulses (FIGURE 5.4A). As expected, GFP levels decreased following IPTG addition and was accompanied by a drop in biomass. However, once the inducer was diluted, the system naturally reverted to the opposite toggle state. This reversion was also reflected in biomass dynamics, with a natural increase in biomass after IPTG removal (FIGURE 5.4B).

A simple ODE model incorporating only a repression rule (see SECTION 1.5.7) successfully reproduced these dynamics, suggesting that unidirectional control, i.e., modulation with only one inducer, could be sufficient to regulate co-culture composition (FIGURE 5.4A – C) However, our repression-based ODE model captures only short-term dynamics but can show divergence under prolonged cultivation due to phenomena like mutational escape. This point will be explored in the next section.

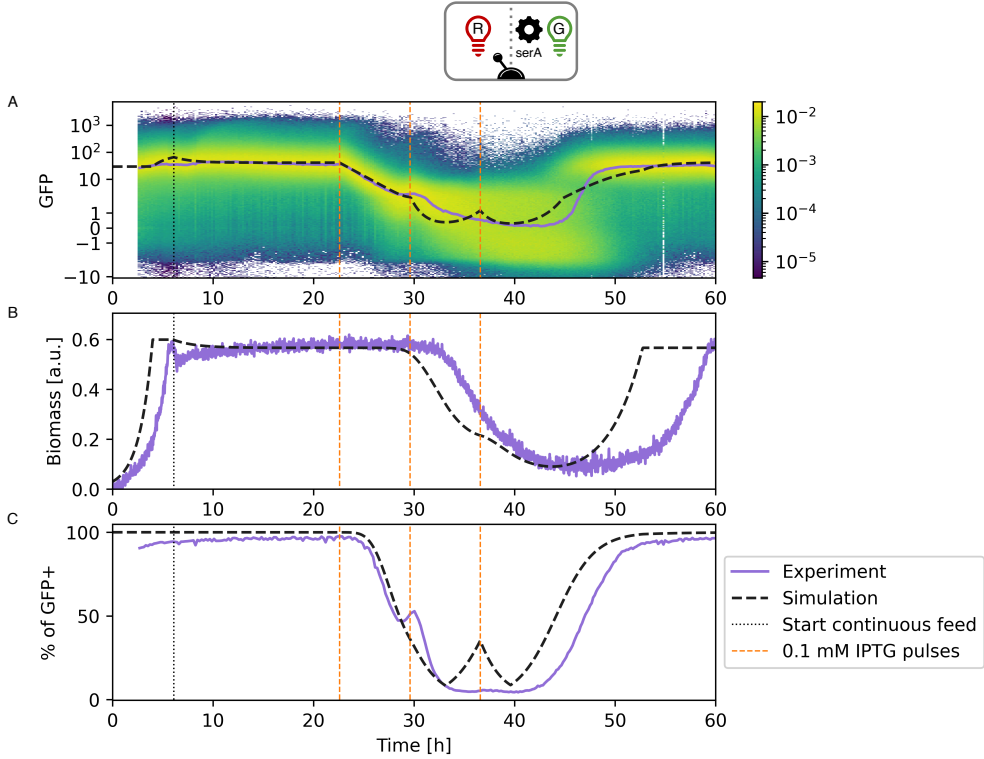


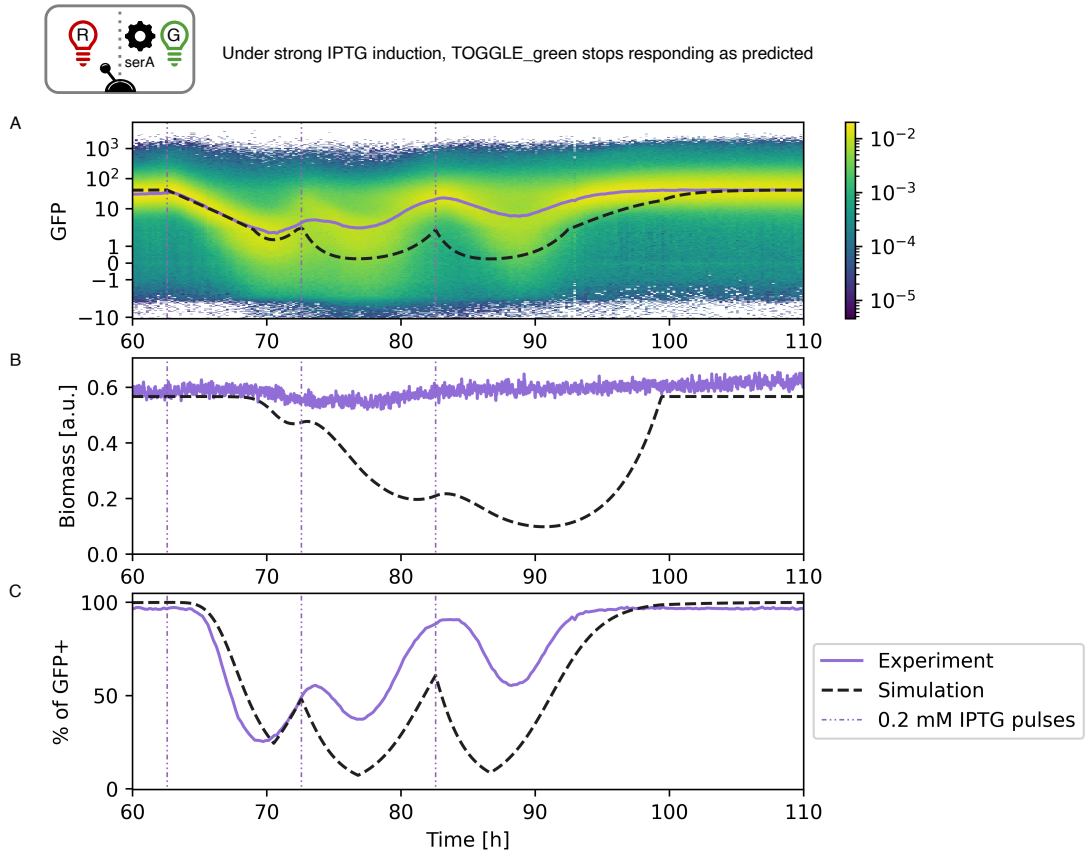
Figure 5.4: Auxotrophic pressure converts the TOGGLE_green circuit into a unidirectional inducible system. **A** Chemostat cultivation of TOGGLE_green with IPTG pulses showing GFP repression and natural reversion upon inducer washout. The experiment and simulation line represent the median fluorescence. **B** Biomass dynamics confirming growth recovery following IPTG removal. **C** Evolution of the percentage of GFP positive cells for the TOGGLE_green strain.

1.3.4. Increasing selective/auxotrophic pressure leads to mutational escape

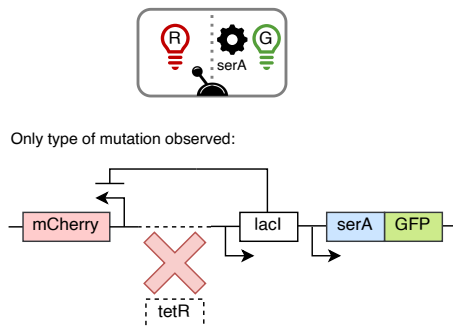
After characterising the short-term dynamics of the toggle switches (FIGURE 5.4), we examined the long-term robustness of the synthetic niche strategy. To this end, we performed continuous culture experiments at a dilution rate of 0.45 h^{-1} , testing each TOGGLE strain independently (FIGURE 5.5). Control was challenged by applying larger IPTG pulses (0.2 mM), triggered based on the fluorescence signal. In the TOGGLE_green strain, control efficiency deteriorated rapidly: after the second IPTG pulse, the system stopped responding as expected (FIGURE 5.5). This loss of responsiveness was evident both at the fluorescence level (FIGURE 5.5A) and in growth dynamics (FIGURE 5.5B). Subsequent IPTG pulses elicited progressively weaker *serA*–GFP responses, while the mCherry signal remained consistent with periodic induction. These experimental results strongly diverged from the simulations, highlighting a breakdown in the designed control logic. A similar decline in control efficiency was observed for the TOGGLE_yellow strain (ANNEX D).

To investigate the genetic basis of this failure, we isolated 5 colonies and performed plasmid extraction and sequencing. In the TOGGLE_green strain, no sample from the experiment described here was available for sequencing. However, sequencing of a subsequent experiment performed under strong IPTG induction revealed a deletion of the *tetR* gene (see FIGURE 5.5D, and FIGURE D.5 in ANNEX D). Although this observation does not directly concern the experiment presented in this study, it indicates that genetic rearrangements can occur under similar induction conditions, and it is consistent with the observed phenotype: loss of GFP response combined with IPTG-induced mCherry expression. Without *tetR*, the *serA*–GFP module is no longer repressed, effectively uncoupling regulatory control and allowing unchecked growth of the strain. The cells are repeatedly subjected to IPTG before the loss of control, which causes a sustained serine starvation. Therefore, before any mutations appear, it is possible that the overall production of TetR produced eventually decreases due to the lack of amino acids. This would increase the leakiness of the repressed promoters and decrease the effect of the inducer.

The TOGGLE_yellow strain exhibited a similar loss of control, but showed a markedly greater diversity of mutations, along with comparable diversity in fluorescence behaviours once these mutations had appeared. (FIGURE 5.5E). Because of the results for the TOGGLE_green strain, we sampled all TOGGLE_yellow experiments for sequencing when a loss of control appeared.



D TOGGLE_green mutation under strong IPTG induction in ulterior experiments



E TOGGLE_yellow also presents mutations under strong tetracycline induction, but with more varied outcomes

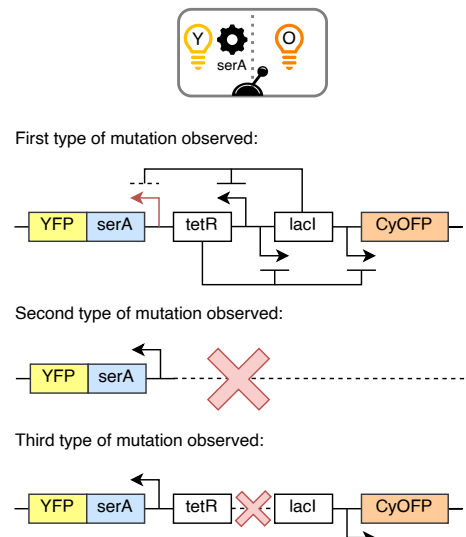


Figure 5.5: Mutational escape in TOGGLE strains under continuous culture. **A–C** Breakdown of control efficiency in the TOGGLE_green strain after IPTG pulsing (0.2 mM, indicated by violet dashed lines). **A** Fluorescence dynamics determined based on automated FC showing rapid loss of GFP response while IPTG still induces mCherry (FIGURE D.3). The experiment and simulation lines represent the median of the fluorescence. **B** Corresponding growth curves highlight the decoupling of induction and growth. **C** Comparison of experimental fluorescence signals with model simulations, showing strong divergence after repeated pulses. **D** Sequencing of isolated colonies from TOGGLE_green during subsequent experiments with IPTG induction revealed deletion of the *tetR* gene, consistent with the loss of GFP regulation and sustained mCherry expression. **E** The TOGGLE_yellow strain exhibited a similar decline in control efficiency but accumulated a broader spectrum of mutations, reflecting higher mutational diversity in this background when auxotrophic pressure was added.

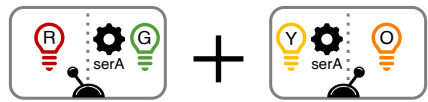
1.3.5. Mutational escape impairs control of co-cultures.

We next co-cultivated the TOGGLE_green and TOGGLE_yellow strains in a continuous bioreactor using the reactive flow cytometry protocol (Segregostat – FIGURE 5.6). Since the strains are both based on the same backbone, there should be no unexpected interaction (see ANNEX D for the absence of cross-feeding between phenotypes), and based on their growth rates, TOGGLE_green naturally dominates. Therefore, IPTG is required to decrease the growth rate of TOGGLE_green and let TOGGLE_yellow temporarily take over.

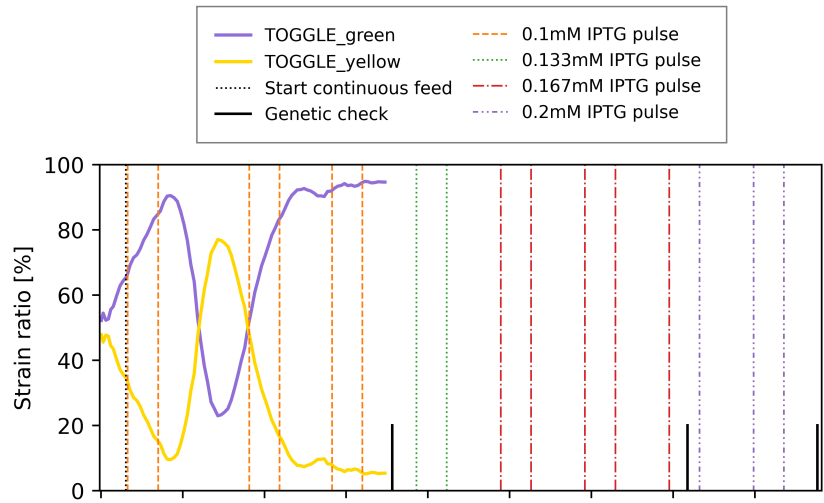
As in monocultures, despite limiting the IPTG concentration, control efficiency deteriorated rapidly, i.e., after the first control cycle, the abundance of the TOGGLE_yellow strain dropped sharply. To test whether stronger induction could restore balance, we progressively increased IPTG pulse amplitudes, but the TOGGLE_yellow strain ratio continued to decline.

To uncover the genetic basis of this instability, we collected samples under low selection pressure at the start of cultivation and under high selection pressure at the end. Sequencing of several colonies in high-pressure samples revealed an approximately twofold reduction in read depth across the *tetR* region in all three reactors, suggesting partial loss of *tetR* sequences within single cells. PCR analyses confirmed this dynamic: under low selection pressure, both wild-type TOGGLE_green cells and mixed populations carrying mutated ($\Delta tetR$) and non-mutated alleles were present. Under sustained high selection pressure, and after extended cultivation that removed most original TOGGLE_green cells, the population consisted mainly of cells harbouring both wild-type and mutant sequences, along with a fraction of fully $\Delta tetR$ mutants.

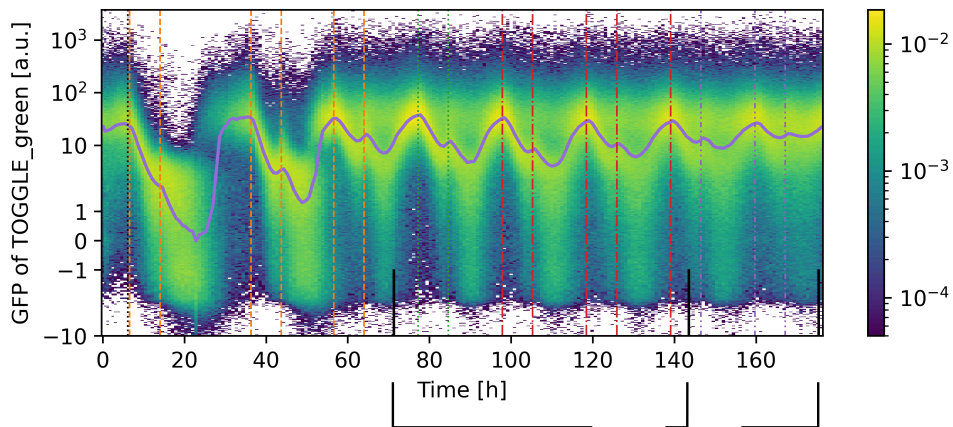
Fitness cost drives complex but predictable cell population dynamics



A TOGGLE_yellow is out-competed in co-culture despite increasing IPTG addition



B The response to IPTG of the TOGGLE_green strain decreases



C

Plasmid sequences found at different time points	Only original	✓	✗	✗
	Co-existing original and Δ tetR	✓	✓	✓
	Only Δ tetR	✗	✓	✓

Figure 5.6: Mutational dynamics of TOGGLE strains in co-culture under selection pressure.

A–B Co-cultivation of TOGGLE_green and TOGGLE_yellow strains was performed in continuous bioreactors with Segregostat control. **A** Proportion of TOGGLE_yellow strain for as long as it can be meaningfully detected. After ~70 h, the proportion of TOGGLE_yellow falls consistently below 5% and are more likely falsely identified TOGGLE_green (see SECTION 1.5).

Control is lost despite the low induction pressure, and the IPTG pulse concentration was increased to try and recover it. **B** GFP fluorescence of the TOGGLE_green population. GFP still responds to IPTG, but with a lower amplitude. **C** Sequencing and PCR analyses were carried out at different levels of selection pressure. Under low selection pressure, single-colony sequencing revealed a mixture of original TOGGLE_green cells, as well as cells carrying both wild-type and $\Delta tetR$ alleles. At high selection pressure and after extended cultivation, the population shifted toward coexistence of mixed alleles and complete $\Delta tetR$ mutants, with original non-mutated colonies largely eliminated. The schematic summarizes the distribution of colony types identified at each stage.

1.4. Discussion

This study establishes synthetic niches as a minimal and modular framework for regulating microbial co-cultures. By coupling cell growth to defined phenotypic states through toggle switch circuits, we effectively transformed metabolic compatibility into a controllable regulatory feature. Under moderate selective pressure, the system provided predictable and reversible control of strain abundance using a single inducer. The introduction of serine auxotrophy converted the bistable toggle into a unidirectional repression logic, simplifying the design while maintaining effective growth control. This architecture behaves functionally as a repressible rather than a bistable system, which may in fact facilitate deployment in industrial settings where simplicity and reliability are preferred [126, 280]. While the exact cause of this phenomenon is not identified, the TOGGLE_green strain induced with IPTG is starving in amino acids specifically, which causes the decreased growth rate, while all other factors are identical between the two strains. This starvation could produce an increase in the relative amount of LacI proteins in the mCherry, non-growing state for several reasons i.e., either a general stress that modifies the metabolism of the cell, a decrease in overall toggle switch protein production due to the lack of resources, which would decrease the $P_{\text{LtetO-1}}$ promoter repression, and an increased LacI concentration as the decreased growth-coupled protein dilution amplifies any leakiness of the promoter. This increased relative concentration could make the mCherry state for TOGGLE_green unstable. Even a small instability would be sufficient to revert the whole population to the growing GFP state in a short time due to the asymmetric growth rates of the two states.

These findings also suggest a design path in which the control circuit could be implemented in only one strain, the faster-growing or genetically tractable partner, while the other strain remains unmodified. Such asymmetric architectures would increase portability and reduce engineering effort, especially in cases where the slower

or more specialised partner is difficult to transform. A notable example is yeast-bacteria co-cultures, where yeast is typically outcompeted by bacteria but has already been extensively engineered for bioproduction [265].

Compared with other approaches, such as cross-feeding, quorum-sensing, or toxin-antitoxin systems, synthetic niches impose minimal energetic burden and preserve metabolic independence of the production pathway, supporting the notion that population control can be achieved without extensive metabolic rewiring.

An additional strength of the synthetic niche framework lies in its quantitative transparency. Because the control dynamics can be described by a single repression rule, key parameters, such as induction thresholds, growth penalties, and recovery kinetics, can be measured on individual strains and then generalised to the co-culture context. This facilitates accurate model-based prediction of system behaviour, in contrast with the empirical tuning required for ecological interactions or cross-feeding systems [264, 266]. However, this predictability depends critically on the genetic integrity of the regulatory circuit: once mutations occur, parameter values lose meaning and the model rapidly diverges from experimental observations.

The principal limitation uncovered by this study is the evolutionary fragility of the control circuit. During extended continuous cultivation, both toggle strains accumulated disabling mutations, most frequently deletions in the *tetR* region, leading to a complete loss of regulation. These mutations decoupled gene expression from growth, causing the escape of unregulated cells and collapse of co-culture control. Similar mutation-driven breakdowns have been reported in other burdensome expression systems [29, 281], in which even minor growth disadvantages accelerate the fixation of escape variants. Our results show that this process can unfold within only a few control cycles under industrially relevant cell densities. This short timescale (ANNEX D, FIGURE D.5) between the interval from the first induction loss to competitive advantage is markedly shorter than the 50 generations typically reported in the literature [29, 282].

The probability of such genetic erosion scales with three parameters: the strength of selective pressure and circuit burden, which determine both the growth penalty and stress-induced mutation rate [283–288]; the population size, which sets the mutational target space [289, 290]; and the number of generations, which defines total replication events [29, 72]. Many published studies of synthetic consortia remain below these thresholds, operating with small populations or limited timescales that may mask instability. The continuous, high-density regime used here therefore provides a stringent benchmark for evaluating co-culture control methods and highlights the necessity of assessing genetic robustness under process-like conditions.

Several avenues could mitigate the instability observed here. Lowering the circuit burden, through tighter promoters, improved repression dynamics, or decoupling growth control from essential metabolic pathways, should reduce selection for escape. Alternative architectures could also be explored, including growth control via conditional

toxin expression [60, 268, 270], optogenetic feedback [60], or hijacking of endogenous cell-cycle arrest modules [281]. Another promising approach would be to impose regulation only on the dominant strain, thereby relaxing selection across the population. Dynamic rejuvenation cycles or periodic strain replacement, already employed in long-term production systems [29, 131], could further delay fixation of mutants. More broadly, integrating circuit design with evolutionary modelling and monitoring should allow the development of “evolutionarily informed” synthetic niches that sustain functionality over industrially relevant timescales. Indeed, our model only integrates repression dynamics from the circuits, but some research shows that some mutation are predictable, even in wild-type strains [291–294]. For example, a resistance to one antibiotic and a sensitivity to another, are correlated with each other even in wild-type strains. It could be possible to add evolution in the model and change the control method based on the phenotype of the mutants.

Taken altogether, the results reported in this work point out that the concept of synthetic niches should be viewed primarily as a short- to medium-term population control strategy and a conceptual design framework, rather than as a solution for fully autonomous long-term stability. Their main practical utility lies in enabling precise, reversible, and quantifiable control of strain ratios over defined operational windows, which are highly relevant in bioprocess engineering involving batch and fed-batch culture. Beyond direct control, synthetic niches provide a minimal and modular test-bed to dissect how growth-coupled regulation shapes community dynamics under defined selective pressures. This simplicity makes them particularly valuable for benchmarking evolutionary robustness, as failures can be unambiguously attributed to genetic erosion rather than emergent ecological complexity. In this sense, synthetic niches function as a stress-test platform for evaluating control architectures under industrially relevant population sizes and generation numbers [295]. While long-term autonomous stability remains challenging [29, 292], the framework is well suited for applications requiring temporal regulation, process initialisation, or controlled production phases.

Overall, our findings emphasise that evolutionary robustness must become a central design criterion in synthetic co-culture engineering. Synthetic niches offer a powerful and generalisable means to modulate strain ratios with minimal metabolic interference, yet their long-term success hinges on maintaining genetic stability. We advocate that validation of co-culture strategies should include extended continuous cultivations where both population size and generational span approach those of real bioprocesses. Only through such intensified and longitudinal testing can we reveal the true durability of control mechanisms. Combining these long-term assays with automated single-cell monitoring platforms such as the Segregostat [4, 248] and real-time adaptive control [60] may ultimately yield microbial communities whose composition can be both programmed and evolutionarily sustained, enabling the transition from proof-of-concept consortia to industrially reliable bioprocesses.

$\text{MnSO}_4 \cdot \text{H}_2\text{O}$: 0.1; $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$: 0.1; $\text{CoSO}_4 \cdot 7 \text{H}_2\text{O}$: 0.21. In bioreactors, the medium was supplemented with TEGO® Antifoam KS 911 ($0.1 \text{ mL} \cdot \text{L}^{-1}$). For plasmid maintenance, kanamycin sulphate (Gibco™, $\geq 95\%$ purity) was added to the medium at a final concentration of $50 \text{ mg} \cdot \text{L}^{-1}$. The trace element solution, thiamine, and kanamycin stock were filter-sterilised ($0.22 \mu\text{m}$) and added aseptically after autoclaving. All other medium components were heat-sterilised at 121°C prior to supplementation.

1.5.3. Preculture

Pre-cultures for both BioLector II and bioreactor experiments were prepared using the same procedure. Single colonies were inoculated into mineral medium containing $10 \text{ g} \cdot \text{L}^{-1}$ glucose and cultured until $\text{OD} > 5$, after which glycerol stocks were prepared by mixing the culture 1:1 with sterile 50% glycerol. These glycerol stocks were then used to inoculate fresh mineral medium in baffled shake flasks, filled to 10% of their nominal volume, and incubated overnight at 37°C at 150 rpm.

1.5.4. BioLector experiments

The BioLector II (m2p-labs, Baesweiler, Germany) is a high-throughput cultivation platform enabling parallel cultivation of microbial strains, with online monitoring of growth and gene expression under well-aerated conditions that mimic small-scale stirred-tank bioreactors [298]. To characterise the growth of each phenotype independently, strains were first cultured under standard conditions for at least 9 h and then pre-induced overnight to force commitment to a single phenotype. Phenotype uniformity was verified before and after induction using an Attune NxT Acoustic Focusing Flow Cytometer (Thermo Fisher Scientific, Waltham, MA, USA, blue and yellow lasers, 530/30 or 510/10 filters were used to detect the growing TOGGLE_green, and 530/30 or 540/20 were used to detect the growing TOGGLE_yellow). Except for the experiment in FIGURE 5.2, BioLector experiments were made as technical replicates, with one pre-culture being used to inoculate three culture wells, with independently prepared inducer solutions. Growth rates used for the model were NOT measured from the BioLector, but from continuous bioreactor data, and confirm the findings from the BioLector. For TOGGLE_yellow, due to the first attempt showing no differences in growth rate, biological triplicates were used.

For TOGGLE_green, induction was performed with $150 \text{ ng} \cdot \text{mL}^{-1}$ anhydrotetracycline (aTc, $\geq 98.0\%$ purity, Thermo Fisher Scientific, Waltham, MA, USA), used when induction time had not yet been optimised, or with 1 mM IPTG (IPTG dioxane-free, $\geq 99.0\%$ purity, Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA). For TOGGLE_yellow, induction was achieved using $25 \text{ ng} \cdot \text{mL}^{-1}$ tetracycline hydrochloride (Tc, $\geq 95\%$ purity, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) or 1 mM IPTG. After induction, cultures were washed twice (centrifugation and resuspension) with glucose-free mineral medium to remove residual inducers, then inoculated into 48-well FlowerPlates (1 mL per well) (Beckman Coulter, Brea, CA, USA) to an initial OD600

of approximately 0.5 and a glucose concentration of 5.0 g·L⁻¹ (OD600 measured with a spectrophotometer Genesys 40, Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA). For each strain, the plate layout included: 3 wells with medium only (baseline and negative control); 3 wells without inducer; 3 wells with inducer (to verify the absence of phenotypic escape). Because no difference in growth or fluorescence was observed between wells with and without inducer (Tc for the GFP- and YFP-expressing states, IPTG for the others), and flow cytometry confirmed the absence of phenotypic escape, only the non-induced conditions are presented. Biomass (scattered light) and fluorescence (520/25 filter, detecting both GFP and YFP signals) were recorded in a BioLector operated at 37 °C, 85% humidity, and 1200 rpm. Additional validation experiments are provided in ANNEX D.

1.5.5. Continuous bioreactor experiments

A detailed list of all continuous bioreactor experiments, including raw data and results, is provided in ANNEX D. The annex also include additional experiments performed with the TOGGLE_{yellow} strain and complementary experiments confirming the basic behaviour of the strains and co-cultures without induction. All bioreactor experiments were carried out at 37 °C and pH 7. Each experiment started with a batch phase until complete glucose depletion, followed by a continuous phase operated at a dilution rate of approximately 0.45 h⁻¹. This dilution rate exceeds the growth rate of non-growing phenotypes but remains below that of actively growing phenotypes. Consequently, chemostat operation at 0.45 h⁻¹ sustains growing populations while washing out non-growing ones. All bioreactors and automated flow cytometry experiments were conducted using a setup adapted from the system described by Sassi et al. [4].

The experiment shown in FIGURE 5.3A was performed in a DASBOX system (original DASGIP system from the DASGIP group, equivalent to the current DASbox system from Eppendorf Hamburg, Germany), equipped with an OXY-4 mini system and OXYPro® oxygen probes (PreSens, Regensburg, Germany) and EasyFerm Plus PHI K8 pH probes from Hamilton (Bonaduz, Switzerland). For the experiments, three vessels were run in parallel (working volume 160 mL). After an initial batch phase using 20 mL of preculture and no IPTG, the culture was shifted to a continuous phase for 17.25 h (>10 generations) with medium containing 0.2 mM IPTG, followed by a continuous phase of more than 75 h without IPTG. Forty hours of this IPTG-free phase are shown in FIGURE 5.3A for one representative replicate, as no further change occurred afterwards. Online monitoring was performed via a custom cell-machine interface coupled to a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (GFP filter 510/15) [7, 265]. Because the Accuri C6 is less sensitive than the Accuri C6+ and cannot accurately detect mCherry, the absolute GFP calibration used for other bioreactors could not be applied here, and raw FL1-A signals are reported.

All other monoculture experiments were performed in a 1 L Bionet F1 bioreactor (Bionet, Murcia, Spain), equipped with EasyFerm Bio PHI Arc pH sensors and VisiFerm

RS485-ECS optical oxygen sensors (Hamilton, Bonaduz, Switzerland). After an initial batch phase using 50 mL of pre-culture and no inducer, an overnight continuous phase without inducer was maintained to reach steady state. Pre-programmed inducer pulses were then applied (0.1 or 0.2 mM IPTG, 15 or 30 ng·mL⁻¹Tc·HCl). Biomass was monitored in situ using a Dencytee Arc sensor (RS485, Hamilton, Bonaduz, Switzerland). Glucose concentrations were determined by sampling, centrifugation, freezing of the supernatant, and analysis using the Megazyme D-Glucose HK assay kit (Megazyme, Bray, Ireland). Flow cytometry monitoring was performed with a BD Accuri C6+ cytometer (4-blue laser configuration; filters 510/15, 540/20, 610/20, 565/20), except for the experiment shown in FIGURE 5.3B, which used filters 510/15, 540/20, 670LP. This alternative configuration accurately measured GFP and mCherry but could not discriminate mCherry from CyOFP1. All monoculture experiments in the Bionet system were performed in duplicate, except the experiment in FIGURE 5.3B, where pulse intervals were sufficiently long to avoid overlap, allowing three consecutive control sequences on the same culture, all yielding similar results. The conclusions from this experiment are consistent with those from other monoculture experiments but are particularly apparent here.

Co-culture experiments were carried out in a DASGIP system (Eppendorf, Hamburg, Germany) using three vessels run in parallel (working volume 400 mL, all vessels were equipped with EasyFerm Plus PHI K8 pH probes (Hamilton, Bonaduz, Switzerland) hence the co-culture experiments were run in triplicate. Flow cytometry measurements were collected using a BD Accuri C6+ cytometer (4-blue laser configuration; filters 510/15, 540/20, 610/20, 565/20). After inoculation with 20 mL of pre-culture, the batch phase was immediately followed by a continuous phase with strain-ratio control through IPTG pulsing (controls without induction are shown in ANNEX D). A pulse size of 0.1 mM IPTG was selected to minimise mutation. Pulses were triggered whenever the TOGGLE_yellow fraction dropped below 50%, with constraints of at most one pulse every 7 h and at most two pulses within 20 h. These constraints were chosen to allow sufficient dilution of IPTG to limit mutational escape and were validated through modelling.

1.5.6. Bioreactor data treatment

The code used for data processing is available at <https://gitlab.uliege.be/mipi/published-software/2025-syntheticniches> and uses Snakemake [299]. Flow cytometry data were analysed using Accuri C6+ reference measurements for each individual fluorescent phenotype and for a non-fluorescent *E. coli* MG1655 strain. Based on these reference signals, each event was decomposed into autofluorescence, GFP, mCherry, YFP, and CyOFP contributions. Fluorescence values were then scaled so that 95% of the events from the non-fluorescent MG1655 reference strain fell below a value of 2 for each fluorophore channel. The value of 2 was therefore used as the detection threshold relative to the wild type autofluorescence background. Note that the expression of one fluorophore slightly increases the baseline signal detected in other

channels (FIGURE 5.8). Negative values may appear after autofluorescence subtraction.

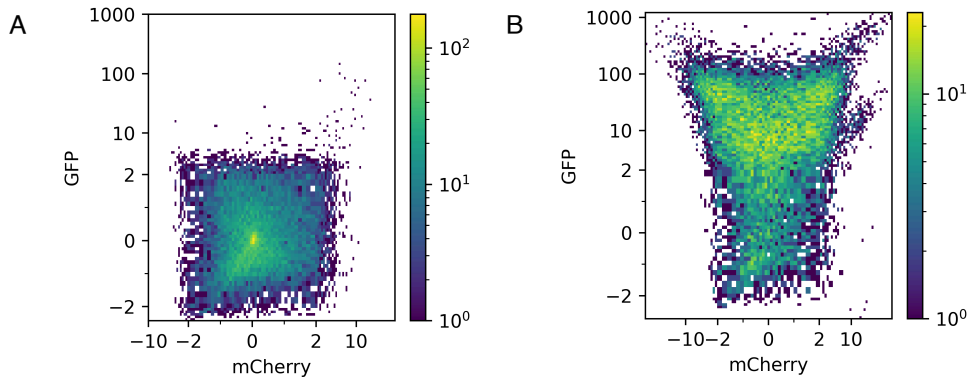


Figure 5.8: Transformation of a flow cytometry sample of (A) MG1655 (wild type) and (B) TOGGLE_green expressing GFP. Light yellow areas indicate a high number of cells, while dark blue areas indicate low cell density. The $[-2, 2]$ interval is where 95% of the cells are present; 2 was an arbitrarily defined threshold. For the few percent of cells with low GFP fluorescence for TOGGLE_green, the mCherry fluorescence is also within $[-2, 2]$. However, for high GFP values, noise produces cells located in a fluorescence range between 2 and -2 in the mCherry channel.

Therefore, a diagonal line is used to separate phenotypes instead of a threshold.

Distinguishing phenotypes in the TOGGLE_green strain was straightforward. Upon IPTG induction, GFP decreases first, followed by an increase in mCherry. A TOGGLE_green cell was therefore classified as GFP-positive (and SerA-positive) when $\text{GFP} > 2$ and $\text{GFP} > \text{mCherry}$. For the TOGGLE_yellow strain, CyOFP increases before YFP reaches its basal level, presumably because CyOFP is detected more easily than mCherry with a 488 nm excitation. Since only low SerA expression is required for growth (consistent with the need for a very tight promoter), a threshold was defined to isolate a pure CyOFP phenotype; any cell above that threshold was considered growth competent. Practically, a TOGGLE_yellow cell was defined as YFP-positive when $\text{YFP} > 0.38 \times \text{CyOFP}$ and $\text{YFP} > 1$.

In co-cultures, strains were distinguished based on the fluorophore vector space: cells closer to the autofluorescence–GFP–mCherry plane were assigned to TOGGLE_green, and cells closer to the autofluorescence–YFP–CyOFP plane to TOGGLE_yellow. Because CyOFP and mCherry emit weakly under 488 nm excitation, monoculture controls confirmed partial overlap between CyOFP and mCherry signals. This overlap was expected, as these fluorophores correspond to phenotypes that should not coexist during co-culture: under IPTG, mCherry (induced), GFP (repressed but growing), and YFP (induced and growing) are present; under Tc, CyOFP (induced), YFP (repressed but growing), and GFP (induced and growing) are present. Both inducers are never applied simultaneously, as this would activate both sides of the toggle switch. Consequently,

in IPTG-controlled co-cultures, all cells that could correspond to either CyOFP or mCherry were classified as mCherry. Verification using monocultures confirmed that this classification identified more than 90% of events correctly in all cases, and more than 95% except under prolonged or strong induction.

All monoculture and co-culture experiments were performed on flow cytometers of the same model. A calibration was therefore applied to convert co-culture measurements into their equivalent monoculture measurement space (correlation of the shape $y = ax^b + c$ between the intensity of identical samples passed in both cytometers, for all used channels, with the various fluorescent proteins, individual correlation points were found by comparing identical measurement percentiles between machines).

1.5.7. Model parameter estimation

A complete description of the parameter inference procedure is provided in SECTION 2. Briefly, growth of the productive (growing) phenotype was described using a classical Monod equation:

$$\mu = \mu_{\max,i} \frac{S}{K_{S,i} + S} \quad (5.1)$$

where μ is the instantaneous growth rate a strain, $\mu_{\max,i}$ is its maximum growth rate, S is the glucose concentration, and $K_{S,i}$ is the glucose concentration at half-maximum growth.

For the non-growing phenotype, we considered:

$$\mu = \min \left(\mu_{\max,i} \frac{S}{K_{S,i} + S}, \mu_{\text{limited},i} \right) \quad (5.2)$$

where $\mu_{\text{limited},i}$ represents the maximum growth rate of the non-growing phenotype of strain i .

Maximum growth rates were not identical in BioLector and bioreactor batch. Therefore, we deduced the growth rates ($\mu_{\max,i}$, $\mu_{\text{limited},i}$) of individual from the biomass evolution during a series of 0.1 mM pulses for TOGGLE_green and during a series of 15 ng/mL Tc pulses for TOGGLE_yellow, while taking into account the measured phenotype ratio over time. $K_{S,i}$ was determined using the residual glucose concentrations at steady-state. The biomass yield was determined based on the biomass obtained in batch and the provided glucose concentration. Besides the maximum growth rates, growth parameters were considered equal between phenotypes and different between strains.

The induction parameters were determined by analysing the fluorescence and phenotype ratio behaviour based on the inducer concentration, and assuming the previously determined growth parameters are correct. For TOGGLE_green, the GFP

median of the population could be reproduced by an IPTG-repressible production equation:

$$\frac{dGFP}{dt} = k_{GFP} \frac{K_{IPTG}}{IPTG + K_{IPTG}} (GFP_0 - GFP) - \mu GFP \quad (5.3)$$

where GFP is the GFP concentration, K_{IPTG} is the repression constant, IPTG is the IPTG concentration in the reactor, k is the production constant, GFP_0 is the GFP level at which production stops, μ is the growth rate.

1.5.8. Detection of mutational escape

To detect and isolate mutations, culture samples were withdrawn from the bioreactor and immediately mixed 1:1 with sterile 50% glycerol before storage at -80°C . Frozen samples were subsequently streaked on LB agar, and single colonies were picked for liquid culture. Plasmid DNA was extracted using the corresponding commercial miniprep kit (NucleoSpin Plasmid EasyPure by Macherey-Nagel, Düren, Germany) and sequenced the whole plasmid (Eurofins Genomics, Luxembourg, Luxembourg).

To determine the presence or absence of the *tetR* gene, PCR was performed on colonies obtained from the LB plates (six colonies per time point and per biological replicate). One PCR used a primer located inside *tetR*; no amplification indicated loss of the gene (primers: GGCGAGTTTACGGGTTGTTA and GGCATACTCTGCGACATCGT). A second PCR amplified the genomic region encompassing *tetR*, yielding a long amplicon when *tetR* was present and a shorter fragment when *tetR* was absent (primers: GGCATACTCTGCGACATCGT and TTGGTCACCTTCAGCTTGG). Colonies containing both the wild-type and the deleted allele produced two amplicons.

2. A modular modelling framework paves the way for better population dynamics predictions

In the previous section, a modular modelling approach was used to determine individual growth parameters and predict mono- and co-culture behaviours. This unusual approach, enabling an easy transfer of results from characterisation of mono-cultures to prediction of co-cultures by separating the different components of the models, is detailed in the following section.

This section is expanded from the supplementary information for the following publication:

[257] Vincent Vandenbroucke, Juan Andrés Martínez, Lucas Henrion, Andrew Zicler, Samuel Telek, Laurie Josselin, and Frank Delvigne. “Synthetic Niches Enable Coculture Bioprocessing but Are Prone To Mutational Escape”. In: *ACS Synthetic Biology* 15.5 (May 15, 2026). DOI: 10.1021/acssynbio.5c00940

The following resources are associated to this publication:

- doi.org/10.5281/zenodo.17663997
- gitlab.uliege.be/mipi/published-software/2025-syntheticniches

2.1. Rationale for modular modelling

When modelling *in silico* parameters, one common hurdle is the determination of multiple unrelated parameters with different units and scales based on diverse sources of information. To infer these parameters, it is possible to define a single model of the system, define a loss function, which summarises the various predictions into a single error number, and minimise that loss function to find the best possible parameters for the system.

To create such a loss function, choices are necessarily made regarding the relative importance of these unrelated parameters. For example, for the case of this co-culture, a model would include both fluorescence and biomass data, and a single loss function would have to weigh the fluorescence error against the biomass error. This would lead to ‘optimal parameters’ that depend on how the loss function was chosen.

However, in practice, different measurements are associated to different parts of the process, and to different model parameters. Therefore, another approach is to consider the modelled system as a collection of smaller, independent models, each representing part of the process and accounted for independently from each other. This was the approach chosen here, as depicted in FIGURE 5.9: first, the growth parameters were estimated considering the fluorescence data as ground truth and optimising the model to

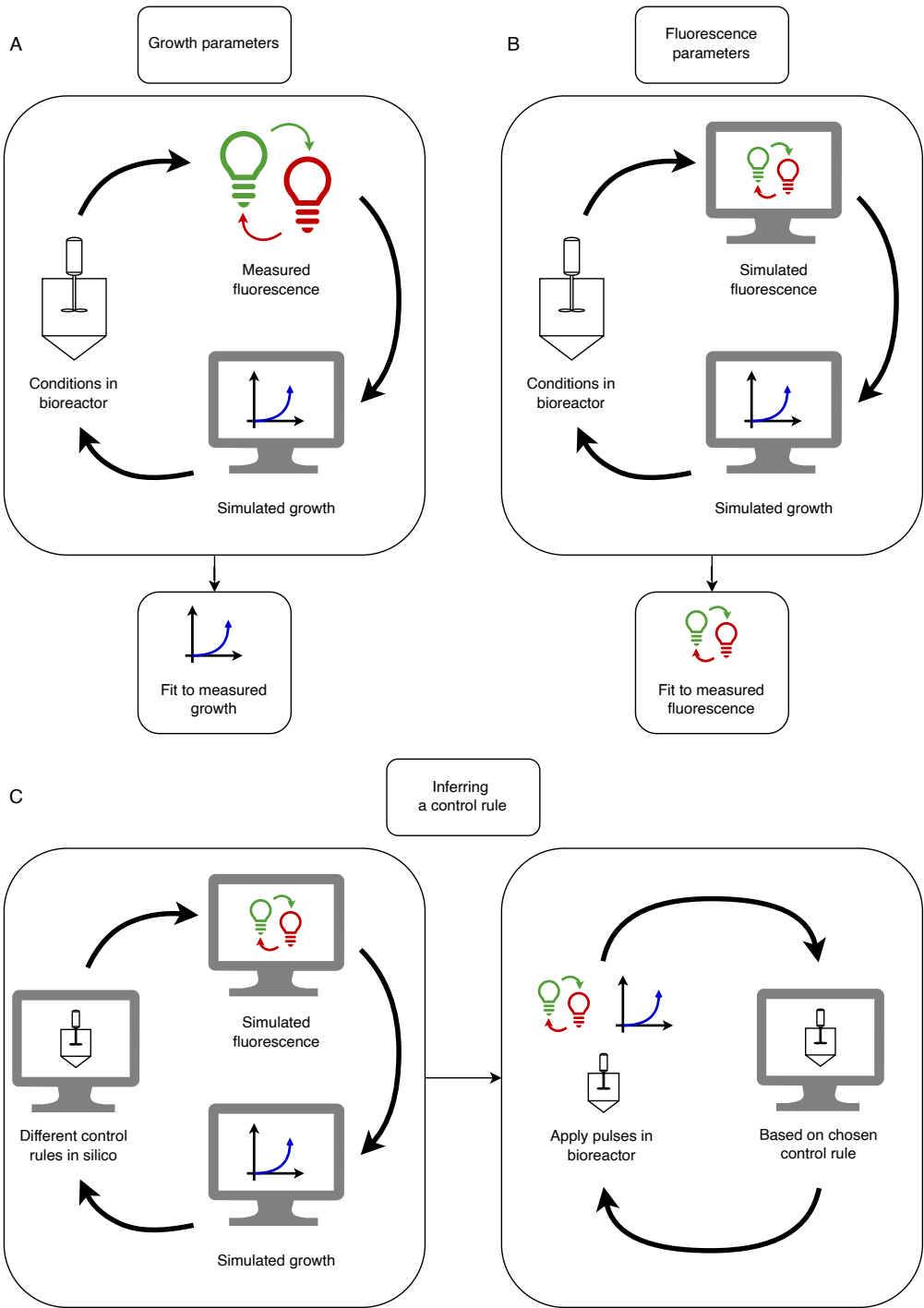


Figure 5.9: Modular models make it possible to represent different parts of the process separately. **A** To estimate growth parameters, the conditions in the bioreactors and the fluorescence, measured or set during the process, were considered as input data. The model represented the growth of the population based on the measured phenotypes and was optimised using the measured biomass. **B** The fluorescence parameters are directly impacted by the concentration of inducer in the bioreactor, but the differences in growth rates between phenotypes also impacts their relative abundance. Based on the previously determined growth model, a fluorescence model was adjusted to represent reality. **C** Once this is done, it is possible to represent any experiment *in silico*, and possibly validate them *in vivo*.

fit biomass observations. Then, the growth model was used to determine a fluorescence model suitably representing fluorescence measurements. Finally, the full model was applied both to confirm it could represent all performed experiments – it was only valid when mutations were not present – and provide an estimation for the behaviour of a co-culture, with different inducer pulse profiles.

To achieve this modularity, the models were implemented using a custom interface (available at <https://gitlab.uliege.be/mipi/published-software/2025-syntheticniches>), allowing to define parts of the model separately and combine them as needed. This lets us easily replace the module containing the fluorescence of an experiment by a simulation of the fluorescence, or a pulse profile from an experiment by a control rule that uses the simulated fluorescence profile to determine when to add inducer. Differential equations were simulated using a Euler step of 0.01 h, which was sufficient to ensure numerical stability.

2.2. Implementation of modular modelling

In practice, a model consists of different computing modules, each with their own time and time steps. For example, a simplified continuous bioreactor model could have an input and a content modules – more modules can be added if required. The input could be a constant value, or change arbitrarily. The content could be a differential equation, that depends on the input module. The modules have different time scales, and therefore need to be able to change independently from each other.

When running a simulation for the model, all modules start at the initial time. They each have a time interval, for which their values are considered valid, and all modules that depend on each other need to advance in time in parallel with each other (FIGURE 5.10). In our example, the input would have a long validity time, and could not advance in time because it would outpace the content. Therefore, the content simulation would be ran until it reaches the end of the validity interval of the input, at which point the latter can be stepped forward.

This strategy can be extended to as many compute modules as required and does not presuppose anything about the modules themselves besides the basic law of causality,

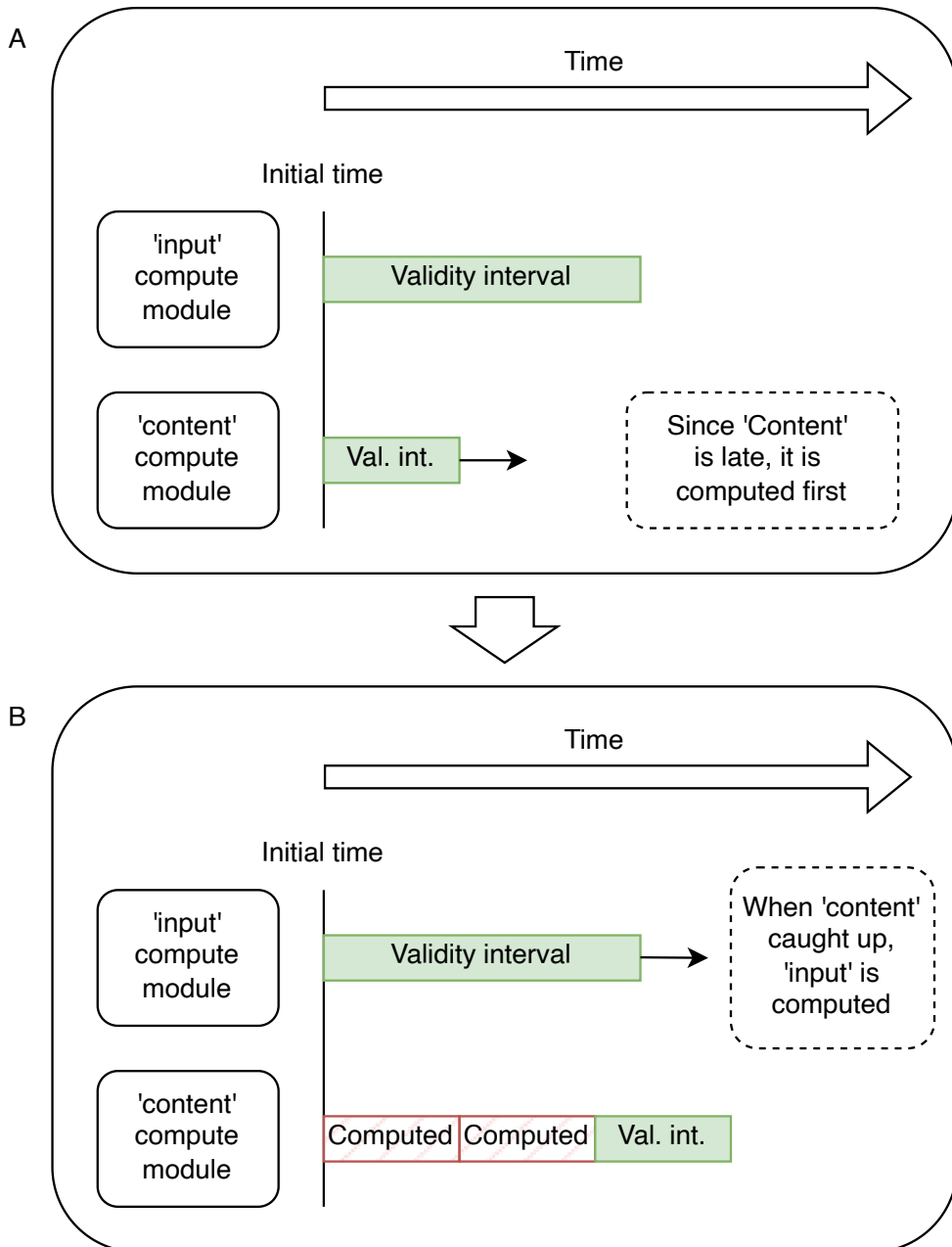


Figure 5.10: Example of modular modelling with two compute modules. **A** The input module starts out valid for longer than the content module. Therefore, the content module needs to be updated. **B** Once the content module catches up to the input module, the latter can be updated until it overtakes the content module again. This process is repeated until the end of the simulation time is reached.

modules can only know what happened in the past and not the future.

2.3. Determination of growth parameters

For both TOGGLE_green and TOGGLE_yellow, the growth model considered was a classical Monod equation for the growing phenotype:

$$\mu_i = \frac{\mu_{\max,i} S}{K_{S,i} + S}, \quad (5.4)$$

where μ_i is the normal growth rate for the strain i , $\mu_{\max,i}$ is the corresponding maximum growth rate, S is the substrate (glucose) concentration in the bioreactor, and $K_{S,i}$ is the substrate concentration at half-maximum growth.

For the non-growing phenotype, a maximum limit was applied to the growth rate with a simple threshold, considering that the limit in growth rate due to SerA auxotrophy should be independent from the glucose metabolism:

$$\mu_{i,l} = \min(\mu_i, \mu_{i,\text{limited}}), \quad (5.5)$$

where $\mu_{i,l}$ is the growth rate of the SerA-limited phenotype, μ_i is the growth rate of the growing phenotype (EQUATION 5.4), and $\mu_{i,\text{limited}}$ is the maximum growth rate of the limited phenotype.

In addition to these equations, to represent growth, the biomass yield $Y_{\frac{X}{S},i}$ (in $\text{TCD}_{\text{biomass}}/\text{g}_{\text{substrate}}$, to correspond to the measured biomass data which the probe provides in $\text{TCD}_{\text{biomass}}$) of each strain was considered identical for both phenotypes. This biomass yield was measured by considering the increase in biomass during batches, before the start of the continuous phase, where all $5 \text{ g}\cdot\text{L}^{-1}$ of glucose provided are consumed.

The growth rates of each strain in bioreactor did not exactly match the initial measurements made in microplates. Therefore, they were re-calculated based chemostats with periodic inducer pulses (2024-08-08-TSGPulses1 and 2024-07-11-TSY2Pulses1, see ANNEX D), where, during the first series of pulses, biomass decreases, there is no mutation yet (as there is a biomass response), and the growth rate should be close to its maximum for both phenotypes. By considering the ratio of phenotypes as it is provided by fluorescence and the observed derivative of the biomass, an approximation of the maximum growth rates in the continuous phase could be made (see TABLE 5.1).

The last parameter, $K_{S,i}$, was determined based on glucose measurements during steady-state of single strains before any inducer was added.

Based on the measured parameters, the biomass curves could be reproduced given the fluorescence. This held true even when mutations appeared, as the fluorescence of mutated cells still indicated their ability to grow, except in one case. Towards

Table 5.1: Maximum growth rates of each phenotype, determined based on BioLector and continuous bioreactor data.

Strain	Phenotype	Growth rate in BioLector (in h ⁻¹)	Growth rate in bioreactor (in h ⁻¹)
TOGGLE_green	Growing	0.53	0.75
	Non-growing	0.29	0.23
TOGGLE_yellow	Growing	0.44	0.66
	Non-growing	0.26	0.32

the end of that experiment (2024-10-02-TSY2Pulses2, see ANNEX D), a mutated, growing population exhibiting no fluorescence appeared, which would be counted as non-growing for the model. The predicted overall biomass stayed stable since the non-growing population was small enough to be compensated by an increased growth of the growing phenotype. Notably, for TOGGLE_yellow, while we could reproduce the observed overall decrease in biomass, the experiments reached a stable (and not oscillating) biomass during the series of 15 ng·mL⁻¹Tc pulses. This means that the assumption made to find the maximum growth rate (the growth rate of each phenotype is close to its maximum at this point) is not entirely valid. When running co-culture model, we adjusted the growth rate of TOGGLE_yellow to match the observed relative strain ratio evolution in co-culture when no inducer was added.

2.4. Determination of induction parameters

Using the previous growth parameters, it is possible to isolate the behaviour of fluorescence in the bioreactor. For the TOGGLE_yellow strain, when tetracycline is added, a portion of the population switches to the non-growing phenotype. While we did make a model for this behaviour (see code at <https://gitlab.uliege.be/mipi/published-software/2025-syntheticniches>), it is never useful for the co-culture as TOGGLE_yellow grows more slowly than TOGGLE_green and is never induced in co-cultures. This model breaks similarly to TOGGLE_green when mutations appear.

For the TOGGLE_green strain, the behaviour of the median fluorescence can be reproduced using an inhibition model based on IPTG concentration:

$$\frac{dGFP}{dt} = k_{GFP} \frac{K_{IPTG}}{IPTG + K_{IPTG}} (GFP_0 - GFP) - \mu GFP, \quad (5.6)$$

where GFP is the fluorescence of the growing phenotype, associated to growth, k_{GFP} is the maximum production rate of GFP, K_{IPTG} is the IPTG concentration at half-maximum production rate, GFP_0 is the maximum fluorescence theoretically possible, and μ is the growth rate of the growing phenotype.

These parameters could be determined based on the fluorescence data during one

experiment (2023-10-26-TSGPulseTest, see ANNEX D) in order to reproduce the fluorescence behaviour for two others (2024-08-08-TSGPulses1 and 2024-09-05-TSGPulses2). Since the first consists of pulses where the fluorescence has time to decrease and recover to base levels, it provides both the loss and recovery dynamics of GFP. In particular, the parameters could be determined as follows:

- If $\text{GFP}_{\max}$ is the maximum measured GFP median, then the recovery part of the fluorescence, where IPTG is absent and cells produce GFP, can be expressed as:

$$\text{GFP}_{\max} - \text{GFP}(t) = c \exp(-(k_{\text{GFP}} + \mu)t) \quad (5.7)$$

And the parameter k can be inferred by fitting an exponential to the data.

- Then, based on the fluorescence at steady-state, the parameter GFP_0 can be determined as:

$$\text{GFP}_0 = \text{GFP}_{\max} \frac{k_{\text{GFP}} + \mu}{k_{\text{GFP}}} \quad (5.8)$$

- Finally, K_{IPTG} can be determined by considering the fluorescence at the minimum median fluorescence of each pulse, where $d\text{GFP}/dt = 0$:

$$0 = k_{\text{GFP}} \frac{K_{\text{IPTG}}}{\text{IPTG}_{\min} + K_{\text{IPTG}}} (\text{GFP}_0 - \text{GFP}_{\min}) - \mu \text{GFP}_{\min}, \quad (5.9)$$

leading to:

$$k_{\text{apparent}} = \mu \frac{\text{GFP}_{\min}}{\text{GFP}_0 - \text{GFP}_{\min}} \quad (5.10)$$

$$= k_{\text{GFP}} \frac{K_{\text{IPTG}}}{\text{IPTG}_{\min} + K_{\text{IPTG}}} \quad (5.11)$$

$$K_{\text{IPTG}} = \frac{k_{\text{apparent}}}{k_{\text{GFP}} - k_{\text{apparent}}} \text{IPTG}_{\min} \quad (5.12)$$

This provides the required data to reproduce the fluorescence median, but in order to use that fluorescence and to determine the growth rate, there needs to be a relationship between the median and the percentage of growing cells. As an approximation, we decided to consider the increase in non-growing cells as a direct function of the median, such that if X_1 and X_2 are the growing and non-growing biomasses respectively, then:

$$\frac{dX_1}{dt} = (\mu_{\text{TSG}} - D)X_1 - X_1 \exp\left(-\frac{\text{GFP}}{\tau}\right) \quad (5.13)$$

$$\frac{dX_2}{dt} = (\mu_{\text{TSG},l} - D)X_2 + X_1 \exp\left(-\frac{\text{GFP}}{\tau}\right) \quad (5.14)$$

when the median is decreasing. It can be determined from a linear regression of experimental data during the decrease in GFP as everything in EQUATION 5.14 can be extracted from biomass and fluorescence data except for τ . The resulting parameters work surprisingly well to represent other experiments despite the simplicity of the model, as long as there is no mutation.

2.5. *Co-culture model*

With both models established, it is possible to combine them to represent a co-culture of TOGGLE_green and TOGGLE_yellow. A co-culture without any inducer pulses closely reproduces the observed behaviour, provided we adjust the growth rate of TOGGLE_yellow, as mentioned in SECTION 2.3. Furthermore, by applying pulses *in silico*, the model indicates that the TOGGLE_yellow strain can be maintained in the medium, provided there is no mutation in the culture (see FIGURE 5.11 for the control rule that was applied in practice). As experiments showed, mutations make this model invalid very quickly, despite applying at most 2 pulses in 7 h and 3 pulses in 20 h.

2.6. *Perspectives for modular models*

This case study demonstrates the utility of a fully modular modelling framework: by separating different parts of the model, it becomes possible to investigate individual parts of the process without interference from the others. Different parts of the process involving different types of models, such as stochastic simulations and ordinary differential equations, and even experimental data, could be directly inserted in the same framework.

This flexibility opens up the field of possibilities, as was demonstrated here: parameters were determined independently from each other and the resulting bacterial models were directly inserted in a prospective digital twin of a bioprocess, complete with an external control rule of the system.

At this point, the modularity of the system is implemented on the computation side of the model: once a model part is designed, it needs to manually be integrated with the others in order to be used. This can be limiting: for now, it would be complicated to easily manage multiple internal cell processes at once. The integration of these processes with each other would need to be done manually, and replacing a part with a different model could lead to a full re-design of the system.

There could be, however, a second part to this modelling framework: the description side. Biological systems can be described with the Synthetic Biology Markup Language [300], an "extensible format for the exchange and reuse of biological models". The language is modular and designed for biochemical reaction networks; it could be extended to include the implementation details of the model as well. From there, the structure of the model itself could be modified easily, and automatically translated to a working implementation, making modular modelling convenient and useful.

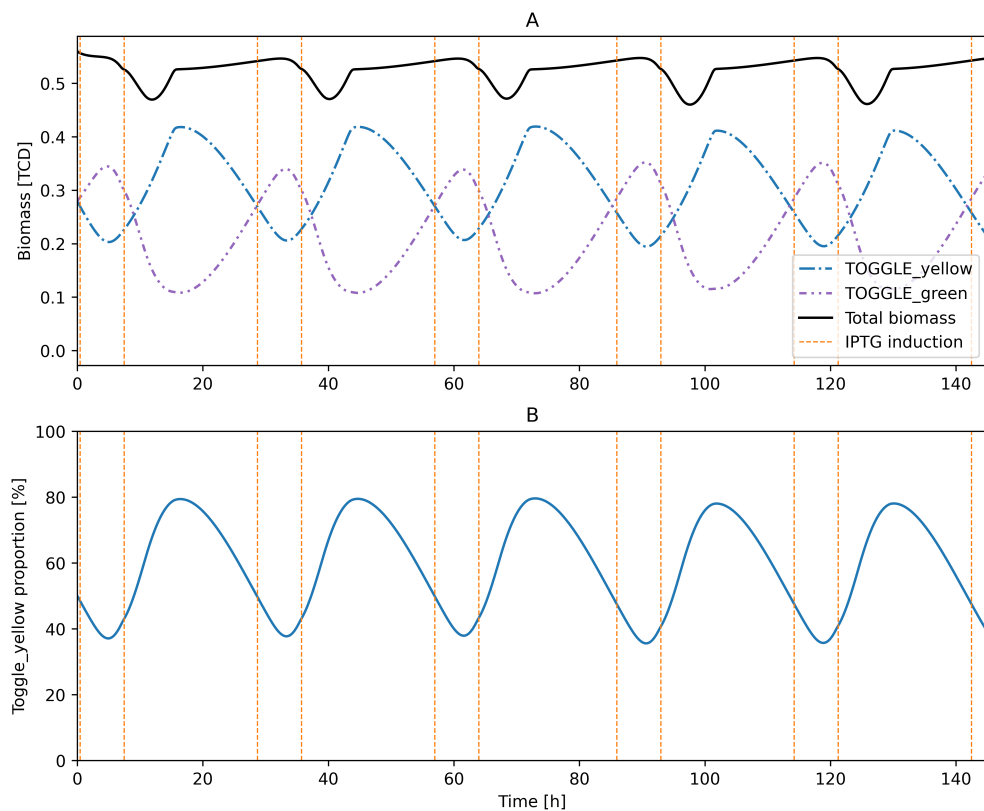


Figure 5.11: **A** Predicted biomass behaviour in continuous culture when the control rule is applied. **B** Corresponding percentage of TOGGLE_yellow in the co-culture.

6

Conclusion and perspectives

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1. Fitness as a systemic approach integrating both biology and process engineering

Throughout CHAPTER 4, two cases of unexpected population-level behaviours were investigated, and both were shown to arise from the interactions between the biological systems and their culture environment.

In the case of the Fitness-Entropy compensation effect (SECTION 1), the population structure was shown to be directly dependent on the selection of fitter phenotypes in the culture. When a gene circuit causes a fitness cost, the selection of the fittest cells naturally leads to an increase in population heterogeneity, if the cells can escape their unfit phenotype. The dynamics and amplitude of this selection process directly depend on the culture device and conditions, and observations from different culture systems would need to be adapted to be comparable.

Genetic oscillations without oscillator, presented in SECTION 2, were shown to be an emergent property of the interactions between the biological system and its continuous culture. A previously unknown feedback loop between population growth, nutrient availability and dilution in the chemostat was uncovered. This feedback loop is a system-level oscillating circuit, similar to a repressilator system but requiring feedback with the environment instead of between genes. The chemostat is the simplest continuous culture device, and the case study of interest had a single actuator. It is unknown what kind of system-level dynamics could arise from the interactions of more complex genetic and culture systems.

These two cases show that considering the interactions between biological systems and their culture environment is necessary to understand population-level behaviours, as soon as the phenotypes of interest impact fitness. Unfortunately, this covers a large part of biological applications, and more complex phenomena, such as biofilm formation or bacterial persistence when producing toxic compounds, would add a layer of spatial or temporal complexity to the system. Fortunately, if we understand these phenomena, they could also be an opportunity to enable new bioprocesses or treatments.

Fitness itself, in the cases studied here, was easily defined, as, in a continuous culture, faster growing cell sub-populations are the ones taking over. If the experimental setup were to change, for example to a mother-machine, a microfluidics device where single cells can be studied as daughter cells are pushed out of the observation area [301], the fitter subpopulations would become the slowest growing ones and the entire population structure would change. In this sense, phenotype fitness acts like a filter between what the population is doing and what is observed. In a lot of cases, however, fitness is not so simply defined. If there was a biofilm in a liquid culture, for example, even with a single species, there might be specialist cells that produce extracellular matrix, cells that detach from the biofilm to seed new biofilms, or cells that join existing biofilm layers. In this relatively simple case, the fitness of each subpopulation would depend on the fitness of the others, and of how much biofilm is already present. This is not an entirely new idea [302–305], but, to my knowledge, it is mostly applied at the scale of genotypes, species and microbial communities. I do believe that bringing the insights from ecology regarding species interaction and fitness (e.g., [12, 306]) to the way single species and phenotypic shifts could make it possible to predict the behaviours of these complex populations beyond liquid cultures.

2. Modelling cell population measurements

Throughout the manuscript, various analysis tools were applied in innovative ways to understand the observed phenomena.

In CHAPTER 3, section 3, a method to infer fitness cost from automated flow cytometry data was presented. It uses the natural dilution of fluorescent proteins in cells to infer their growth rate. Currently, there are precious few tools to infer such dynamics parameters for individual phenotypes. Most of them rely on microfluidic devices, which while powerful, have time-consuming data to analyse, do not fully represent bioreactor conditions, and do not reproduce the phenotype selection process, which could complicate result transfers to other scales when coupled with the low number of cells available. Since we cannot directly follow individual cell lines in a bioreactor (barring new scientific developments [307, 308]), in an ideal world, we would be able to infer these kinetic parameters for subpopulations from multiple sources of indirect measurements. The method presented in CHAPTER 3, section 3 is a step in that direction.

In CHAPTER 3, section 4, the Shannon entropy was mechanistically linked to the population structure in a microbial culture via the Fitness-Entropy compensation mechanism. This reasoning does not rely on biological changes, but rather on the selection process occurring in the culture: when the culture system does not favour the dominant phenotype in the culture, the latter's proportion decreases compared to any other phenotype and population heterogeneity increases. This let us identify one source of phenotypic heterogeneity, which we observed in chemostats in CHAPTER 4, section 1 and which would need to be accounted for when trying to determine the biological

sources of heterogeneity

In CHAPTER 4, section 2, a minimal mathematical model was developed to understand the emergence of oscillations in gene expression without genetic oscillator or external forcing. The model re-uses standard chemostat equations, and adds a single term describing an inducible switch between non-stressed and stressed phenotypes. It is, overall, a small modification to the standard chemostat model.

These three applications of analysis tools demonstrate that as automated flow cytometry provides a large amount of data, it is possible to extract meaningful insights from it, and it represents a viable approach to study biological processes, not only to control them as described by Sassi et al. [4]. These tools represent a small step towards a better understanding of biological processes, and I suspect varied alternatives will emerge to refine these methods and develop new ones.

One important point required to refine any analysis method, however, is their comparison with alternative measurement techniques. Because biological data can come from many different sources in different culture devices, and because the type of culture directly impacts the population structure, it is crucial to take it into account when comparing results. That was one motivation for developing the modular modelling framework of CHAPTER 5, section 2: extracting biological parameters, then simulating cultures in different process conditions makes it possible to properly apply an induction curve to both a continuous culture in the lab and a fed-batch at scale. While the Fitness-Entropy compensation mechanism and the mathematical model for oscillations are conceptual simplifications and limited in how much quantitative insight they can provide, modelling has the potential to address that shortcoming and quantify how much entropy comes from which mechanism for noise, or how long the oscillations in the bioreactor should last based on current knowledge.

This assumes that there is enough knowledge available to model the strains, which is not always the case. This was illustrated in CHAPTER 5, section 1 where a growth-limiting toggle switch caused the appearance of mutations within a few generations of restrained growth. That section underlies the importance of making experimental conditions as close to the system they represent as possible. In this case, mutations were the problems, and they are rare events. To account for them when designing strains, there needs to be experiments containing enough biomass and generations to observe them. Besides mutations, there could be other factors affecting bioprocesses at scale or other co-cultures, and these factors are often difficult to account for in any single experiment. In addition to models enabling comparison of biological data, a good strain characterisation requires a diverse set of experiments going from microfluidics to scale-down in order to have a chance of catching all potential scaling issues.

3. Which models and measurements are required for quantitative predictions?

More specifically, based on the findings of this thesis, one possible avenue to reach better models can be suggested. Biological models are often either ODE approximations or stochastic simulations. The latter is often unworkable when the number of cells becomes too high, such as in bioreactors. The former enables qualitative insights, quantitative thresholds (such as with *Bacillus subtilis* in CHAPTER 4, section 2) and predictions in simple cases (as was used for co-cultures in CHAPTER 5, section 2). However, they can break down when it comes to representing more complex population structures. For *B. subtilis*, the cells were considered to be either 'sporulating' or not, but in practice, the switching decision is also dependent on competence, which represses sporulation, and the entire cell population may not sporulate with the same kinetic constant [8, 170, 309]. For the co-culture, the model could predict the toggle-switch before mutations appeared because the population mostly did not split into multiple sub-populations with different phenotypes, and the GFP level in the cells could be represented with a single median value. If the model had needed to represent an arbitrary number of sub-populations, each at a different stage of the transition between phenotypes, that is, if we had used much lower inducer concentrations or more frequent induction, ODEs would probably have been insufficient. In these cases, once the general insights into the dynamics of the process are understood and the key parameters of the process are known, it would be useful to come back to hybrid *in silico* models, such as the one from Henrion et al. [7], which sparked CHAPTER 4.

These hybrid models use ODEs to represent the external environment of the cells, where concentrations are high enough that changes in concentrations are deterministic and continuous. By contrast, in cells, where concentrations of some proteins, DNA, and other molecules can be extremely low, the system behaves stochastically, as rare events such as binding a DNA polymerase can decide the fate of the cells. To represent them, the population can be split into a very high number sub-populations, each behaving stochastically. This approach let us reproduce the Fitness-Entropy compensation mechanism and the emergent oscillations observed in CHAPTER 4 – although it did not tell us what caused these oscillations – and would be perfectly suited to solve the problems mentioned in the previous paragraph. In addition, unlike purely stochastic simulations, this kind of model is computationally tractable, as the size of the population simulated can be limited compared to the number of actual cells in a culture.

Within each simulated cells, the model can start off very simple, and be refined through experiments, although full cell models are probably out of reach for now. If the model starts with a single fluorescent reporter for a single gene circuit, as in this work, the logical next step would be to represent the parts of the metabolism directly impacting the genes of interest. That would often include the carbon metabolism, the general energy balance, or the stress response, which all impact the cell's metabolism widely. Studying

these sections of the metabolism could be done first through double or triple fluorescent reporter systems, as well as more detailed analysis of the population bulk consumption or production of key metabolites, using gas analysis or Raman spectroscopy, for example. Ideally, these measurements could be taken both in microfluidics and in liquid cultures, to ensure their validity.

There are many other potential avenues to improve the models, and how easy, effective and relevant they are will likely be context and case-dependent. Omics techniques, sometimes applied to single cells, would provide large amounts of data, and could also help coming at the problem from the other direction. While the approach I propose builds up knowledge, one key, well-known system at a time, statistical techniques make it possible to find patterns in large amounts of data, sometimes where no pattern was expected, whittling down the amount of unknowns rather than building up the amount of 'knowns', in a sense; both approaches are complementary.

4. Studying mutational dynamics with different biological systems

In CHAPTER 5, section 1, mutations thwarted attempts at co-culture control within a few generations. They occurred because of a very high selection pressure, which was the basis of the control mechanism for the co-culture. However, a single genetic design was tested, a mid- to high-copy plasmid with a toggle-switch controlling SerA auxotrophy. Now that these rapid mutations are known, potential mitigation strategies could be applied, for this system or for genetic constructs which cause very high fitness cost in general.

The first element that can be modified is the integration strategy for the genetic construct: a mid-copy plasmid is convenient to insert in cells, but high expression of the control system may be unneeded, and, in this case, even undesirable as leakiness of the promoter driving SerA caused the non-growing phenotype to be slow-growing instead. A low copy plasmid or even a genomic integration could, on the one hand, help decrease spurious expression, improve the granularity of the control, and maybe even cause potential future mutating cells to grow so slowly that they would be washed out before they can mutate. On the other hand, genomic integration or the choice of origin of replication produce different mutation profiles [310], and it is entirely possible that some of these profiles decrease the loss of function.

The construct itself may also be optimised to avoid mutations. With our toggle switch system, the primary kind of mutations observed was the recombination of identical promoters in the plasmid. It would be possible to remove such redundancy by having all proteins on a single promoter, or by using bidirectional promoters¹, which can be used

¹Thanks to the member of the jury who suggested bidirectional promoters, I would not have thought of them.

to enhance genetic stability [311]. The design could also be simplified by removing the part of the switch that is unused in each strain, since the construct behaves as a push-button instead of a switch regardless. Or the genomic *serA* promoter itself may be modified with a LacO operator, instead of working with a $\Delta serA$ strain, which would help ensure that no excess serine is produced compared to the cell's original mechanism.

Finally, the stress applied to the cell can help cause mutations, and different kinds of stresses, different amino acid starvations, oxygen limitation, and more, cause different mutation profiles [310, 312]. Therefore, changing the essential gene used for growth control, or even producing a toxin could help improve the stability of the system. We had initially settled on auxotrophy as it ensured that the plasmid was conserved, that no species was eliminated by the inducers, and that the cells would not become resistant to the produced molecule, but it is possible that a toxin causing slow growth or partial population death would lengthen control in the culture overall, if it reduces mutation rates in the cell.

5. Integrating mutational dynamics with population control?

As mentioned previously, in CHAPTER 5, section 1, the key issue towards co-culture control was the occurrence of mutations. For one of the strains, these mutations were surprisingly reproducible, both in terms of which mutation appeared and in terms of timing – duplicate experiments showed very similar phenotypes over time. This was also observed in *Escherichia coli* BL21 with a lactose-inducible T7 production system [180, 313]. More generally, in long term cultures for burdensome gene circuits, the timing of function loss appears generally stable as well [29], although it is usually longer than the <10 generation observed here.

This hints at mutational escape being more predictable than individual random events causing population shifts, in some cases at least. The BL21 case is particularly striking as the same modifications of three nucleotides in two locations on the lac promoters are always observed and were already present in the literature for the T7 system [314].

Stress-induced mutations are indeed a known phenomenon: microbes can decrease DNA-replication fidelity and increase their mutation rate when stressed [285, 288, 315–319]. In addition, recent findings indicate that the phenotypic traits of bacteria mutating under selection pressure are reproducible, even for traits that are not selected for [292, 293, 320–322]. In other words, when directed evolution focuses on one trait in a microorganism, unrelated traits reliably evolve in parallel. Taken together, these two observations show a future where it might be possible to predict, prevent, and maybe use mutations to steer populations towards desired outcomes. When combatting bacterial resistance/persistence, rotating antibiotics in the right order could dramatically increase effectiveness. For co-culture control, cell-machine interfaces measuring how much the

culture adapted to one type of control could switch control strategy and prolong the culture. As an example, there are some antibiotic resistances that are anticorrelated with each other [293]. By having a slow microorganism insensitive to two antibiotics, and a fast microorganism sensitive to both, antibiotics could be used to directly affect the strain ratio, and alternating antibiotics would prevent adaptation.

Annex A

**Supplementary table: literature pertaining
to synthetic or systems biology**

This section is a table of the literature reviewed for the mini-review presented in CHAPTER 2, adapted from:

[70] Vincent Vandenbroucke, Delphine Soyeurt, Christian Dusny, Frank Delvigne, and Juan Andres Martinez. “Enabling Continuous Co-Culture Bioprocesses (C3Bio): A Systems and Synthetic Biology Approach.” Unpublished manuscript. Nov. 28, 2022

Table A.1: Comprehensive table of works pertaining to systems or synthetic biology. A number of reviews are mentioned instead of exact papers, as the point is the approach taken towards the problem and not the exact examples. Here, a work tackling “stability” is considered to tackle population stability, phenotypic heterogeneity and noise in gene expression. A work tackling “production” is defined as related to the optimisation and management of pathway expression. The classification of some works in one or another category may be arbitrary in edge cases, and the categorisation is then only indicative.

Reference	What	Systems biology			Synthetic biology			Stability	Production
		Analyze	Design	Control	Analyze	Design	Control		
1986, Davison and Stephanopoulos [36]	Oscillating pH can stabilise an unstable co-culture with competing microorganisms.			x				x	
1987, Stephens and Lyberatos [37]	In silico determination of when a cycling environment could stabilise a co-culture even though one organism grows faster than the other in all conditions (because it might be slower to adapt).			x				x	
1993, Dikshitulu et al. [115]	Determination of the oscillating conditions in which two species can coexist in a fed-batch reactor.			x				x	
1995, Lenas and Pavlou [120]	In silico determination of whether a co-culture of three organisms can be maintained stable through periodic oscillations.			x				x	
1998, Yokoi et al. [101]	Enhance the growth of an obligate anaerobe with a facultative anaerobe to protect from oxygen.	x	x					x	

Year	Authors	Model Description	Model Type	Simulation	Experiment	Analysis	Design	Control	Optimization
2000	Elowitz and Leibler [47]	Genetic oscillator based on three genes repressing each other.							
2000	Gardner, Cantor, and Collins [48]	Two genes repressing each other constitute the toggle-switch, a memory circuit.							
2001	Namjoshi and Ramkrishna [323]	Method to obtain cybernetic variables for phenotypes at steady-state in system models.	x						
2001	Sohn and Kuriyama [112]	Periodic and constant environmental nutrient conditions modify natural metabolic oscillations.	x						
2002	Hasty et al. [324]	In silico coupling of oscillating cell metabolism and synthetic genetic oscillator to improve oscillations. (m model)				x			x
2004	Garcia-Ojalvo, Elowitz, and Strogatz [325]	Simulation of an oscillator synchronized through quorum sensing to study how coupling works.	x						
2004	Kobayashi et al. [125]	Use of sensors, a toggle-switch, and quorum sensing, to program cell behaviours in specific conditions. The system is designed to be controlled.				x	x		x
2004	Thattai and van Oudenaarden [152]	Simulation of the possible advantage of phenotypic heterogeneity in unstable environments.	x						x
2004	You et al. [270]	Regulate population density through quorum sensing and induced cell lysis.		x			x		x
2005	Collins, Arnold, and Leadbetter [326]	Use directed evolution to produce new quorum sensing systems.		x					x
2005	Fung et al. [327]	Metabolic oscillator analog to genetic oscillators.					x		x
2005	Hooshangi, Thiberge, and Weiss [328]	Artificial transcription cascade and noise analysis. Synthetic biology can be used to reproduce and study natural circuits.				x			x

2005, Kussell [20]	Simulation of the possible advantage of phenotypic heterogeneity in unstable environments.	x						x		Fitness cost drives complex but predictable cell population dynamics
2005, Lipan and Wong [329]	Study in silico the effect of different types of oscillating signals on genetic circuits.					x			x	
2005, Pedraza and van Oudenaarden [330]	In silico study of how noise propagates in gene networks.	x							x	
2007, Brenner et al. [331]	Use of logic gates and quorum sensing in a co-culture to start production only when each strain has grown enough in the culture device.						x		x	
2007, Tan, Reza, and You [332]	Study in silico the existence of a limiting frequency when genes receive periodic inputs.	x							x	
2007, Ullner et al. [333]	Study in silico how a repressilator can be repressed by quorum sensing.					x			x	
2008, Balagaddé et al. [27]	A synthetic predator-prey ecosystem where dynamics in the system are analysed based on culture conditions.	x	x			x	x		x	
2008, Brauer et al. [334]	Study of the coordination between growth, metabolism, and different kinds of stresses.	x								
2008, Brenner, You, and Arnold [335]	Review of cell interaction engineering and division of labour for co-cultures.						x		x	
2008, Cai, Dalal, and Elowitz [336]	Study of cell behaviour in silico and in vivo by modifying the cells.					x				
2008, Kim et al. [38]	Study whether cell interaction engineering through spatial segregation makes it possible to get a stable co-culture in microfluidics in vivo and in silico.	x	x						x	
2008, Stricker et al. [241]	An alternative genetic oscillator to the repressilator.						x		x	
2008, Veening, Smits, and Kuipers [8]	Review of examples of phenotypic diversity and their purpose.	x							x	

2008, Zhou et al. [337]	Study synchronisation of various oscillators in silico, including with quorum sensing.	x					x	
2009, Eiteman et al. [136]	Production of organic compounds through division of labour by making substrate-selective strains.					x	x	x
2009, Hsiao et al. [338]	Improve production by choosing complementary strains in terms of carbon source (division of labour).		x				x	x
2009, Siegal-Gaskins, Ash, and Crosson [339]	Mathematical analysis of signals to obtain single-cell-like data.	x						
2009, Wagemakers et al. [340]	In silico synchronisation of a hypothetical oscillator using external inputs.					x	x	
2009, Zeidan and Van Niel [341]	Improve production by choosing complementary strains in terms of carbon source (division of labour).		x				x	x
2010, Bader et al. [78]	Case studies of industrial co-cultures and their advantages.	x	x				x	x
2010, Chuang, Rivoire, and Leibler [342]	Investigation of behaviours of artificial cheater populations in a culture. Synthetic biology is used to investigate system biology.	x			x		x	
2010, Danino et al. [343]	Synchronized genetic oscillator using quorum sensing.					x	x	
2010, Massie et al. [10]	Investigation of cell cycle synchronisation in plankton thanks to nutrient based communication.	x						
2010, Tsao et al. [344]	Exploit existing quorum sensing to trigger production		x			x		x
2011, Ferrell, Tsai, and Yang [345]	Review of mathematical models to reproduce oscillations of the cell cycle.	x						
2011, Gupta et al. [346]	Study of the phenotypic equilibrium in cancer cells.	x						
2011, Mondragón-Palomino et al. [144]	Entrain a genetic oscillator.					x	x	x

2011, Strelkova and Barahona [347]	Study generalised in silico model of the repressilator.					x				x
2012, Chakraborty, Wu, and Hazen [348]	How to elucidate cell interactions (review).	x								x
2012, Dai et al. [107]	Method to measure the tipping points of disturbances in systems, beyond which they may not recover stability.	x								x
2012, Geng et al. [103]	Predicting the improved performance of a co-culture compared to single cultures based on a cybernetic model.	x	x							x
2012, Kerner et al. [25]	Induced nutrient based communication to couple growth of two different strains, the strain ratio can be tuned based on inducer addition. The system is designed to be controlled.						x	x		x
2012, Mee and Wang [148]	Review of synthetic ecology designs.	x	x			x	x			x
2012, O'Brien, Van Itallie, and Bennett [124]	Review of synthetic gene circuit models for design.						x			x
2013, Bechtel and Abrahamsen [349]	Case-studies of models explaining oscillating cell behaviours.	x								
2013, Minty et al. [87]	Systems and synthetic biology are used to design and optimize a co-culture system for production, an approach which could benefit C3BIO if it is combined with the need for long-term stability, which can be addressed through external control and specific design not only for production, but also for stability.			x	x		x	x		x
2013, Shong and Collins [350]	Tune sensitivity and response time of quorum sensing system.						x			x

Fitness cost drives complex but predictable cell population dynamics

2013, Zuroff, Xiques, and Curtis [98]	Production of organic compounds through division of labour and cell interaction engineering through toxin-based communication by choosing the right strains.		x						x	x
2014, Goers, Freemont, and Polizzi [102]	Review of techniques to design and control a stable co-culture, from natural interactions to external environment.	x	x	x	x	x			x	
2014, Hung et al. [351]	Modulating in silico and in vivo a genetic switch to control cell phenotype.							x		x
2014, Jagmann and Philipp [92]	Review regarding cell interaction engineering, including the use of biofilms for spatial segregation.		x			x			x	
2014, Jaruszewicz, Kimmel, and Lipniacki [108]	In silico model of influence of growth rate on toggle-switch noise.	x			x				x	
2014, Prindle et al. [352]	Post-translational coupling of oscillators using degradation tags, in silico and in vivo.					x			x	
2014, Shong and Collins [353]	Apply a logic gate to induce production only when an inducer is added and quorum sensing is activated.					x	x			x
2015, Carvalho-Netto et al. [354]	Study of the transcriptional profile of a contaminated culture.	x								
2015, Chen et al. [355]	Quorum sensing is used to create oscillating population dynamics thanks to a system model.					x			x	
2015, Gebreselassie and Antoniewicz [104]	Use C-13 markings to elucidate metabolic fluxes in a co-culture.	x								
2015, Kellogg and Tay [356]	Entrainment of mammalian cells using periodic induction with transcription factors.	x							x	
2015, Wang, Barahona, and Buck [357]	Regulate sensor sensitivity through tuning of intracellular receptor concentrations					x				x

2015, Weiße et al. [153]	Modeling of the trade-off between gene expression and growth, as they consume the same resources.	x			x				x
2015, Zargar et al. [97]	Controller cells manage quorum sensing intensity to modify phenotype.	x			x	x			x
2015, Zhang et al. [358]	Production of organic compounds through division of labour by splitting the production pathway.					x			x
2015, Zhang et al. [137]	Production of organic compounds through division of labour by strain modifications considering both a split pathway and substrate-selective strains.					x		x	x
2015, Zhou et al. [359]	Production of organic compounds through division of labour by splitting the production pathway.					x			x
2016, Briand et al. [360]	Self-inducible delayed gene induction.					x			x
2016, Camacho-Zaragoza et al. [361]	Production of organic compounds through division of labour by splitting the production pathway.					x			x
2016, Cuba Samaniego et al. [362]	Study oscillations of a molecular titration system in silico.					x			x
2016, Dolinšek, Goldschmidt, and Johnson [28]	Review of synthetic microbial ecology and how to define and exploit microbial interactions.	x	x	x				x	x
2016, Ghosh, Chowdhury, and Bhattacharya [26]	The authors prone a systems biology approach to identify cell interactions then manage them.	x	x					x	
2016, Jones et al. [363]	Production of organic compounds through division of labour by splitting the production pathway and optimisation through variation of the induction time.					x	x		x
2016, Potvin-Trottier et al. [243]	Rational improvements on top of the repressilator. The model is used to confirm design viability.					x		x	

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2016, Servinsky et al. [364]	Use quorum sensing to create bimodality, in order to study the fitness advantage of bimodal populations. Cells can be designed to study systems.	x		x	x		x	
2016, Zargar, Quan, and Bentley [365]	Couple quorum sensing and product sensing to improve production and reduce heterogeneity.				x		x	
2016, Zhang and Stephanopoulos [366]	Production of organic compounds through division of labour by splitting the production pathway.				x			x
2017, Baumgart, Mather, and Hasty [367]	Combine quorum sensing and a genetic oscillator to get synchronized oscillations in microfluidics and in silico.	x			x		x	
2017, Binder et al. [15]	Review on how to manage phenotypic heterogeneity in large scale bioreactors.	x	x		x			x
2017, Ganesan et al. [368]	Production of organic compounds through division of labour by splitting the production pathway and optimisation through variation of the induction time.				x	x		x
2017, Gupta et al. [135]	Redirect metabolism thanks to quorum sensing to improve production.				x			x
2017, Gutiérrez et al. [95]	Agent-based model to simulate and validate synthetic consortium design.		x		x		x	
2017, Haringa et al. [34]	CFD simulation of a bioreactor. Understand the system the cells are subjected to.	x					x	
2017, Jones et al. [369]	Production of organic compounds through division of labour by splitting the production pathway.				x			x
2017, Lemoine et al. [370]	Summary of measurement tools that can be used to assess the effect of the oscillating environment at large scales and the phenotypes.	x					x	
2017, Liu et al. [138]	Production of organic compounds through division of labour by making substrate-selective strains.				x		x	x

Fitness cost drives complex but predictable cell population dynamics

2018, Heins and Weuster-Botz [190]	Review of the impacts of scale on phenotypic heterogeneity.	x				x
2018, Kelliher et al. [377]	Study of cell cycle in yeasts.	x				
2018, Kylilis et al. [378]	Study of cell interactions, in particular cross-talk between implanted quorum sensing systems, and associated software.	x		x		x
2018, Li et al. [379]	Promoter selection for the best induction profile, based on transcriptomics in the system of interest. Design of the system based on measured cell behaviour.	x	x			x
2018, Li, Giardina, and Siegal [380]	Study of phenotypic heterogeneity in yeasts by acting on inducers, either by adding them or through genetic modifications.	x		x		x
2018, Liu et al. [49]	Review of how synthetic biology can be used to redirect carbon fluxes.				x	x
Liu et al. [381]	Production of organic compounds through division of labour by splitting the production pathway.				x	x
2018, Merritt and Kuehn [382]	Impact of environment dynamics on phenotypes and heterogeneity.	x				x
2018, Niu et al. [383]	Production of organic compounds through division of labour by strain modification, with directed evolution and error-prone PCR to improve production in the system and resistance of the strain to the product.		x		x	x
2018, Özkaya et al. [12]	Systems and synthetic biology are used to create cheater populations and investigate their population stability. Cells can be designed to study systems.	x		x	x	x
2018, Perez-Carrasco et al. [384]	In silico combined memory circuit and oscillator and how to control the switch between the two regimes.					x

2018, Rugbjerg et al. [29]	Study the interaction between the system, the metabolic load, and the appearance of cheaters, and use them to design more stable systems.	x	x	x	x	x	
2018, Rugbjerg et al. [52]	Self-selecting monoculture by coupling production and essential cell processes.				x	x	x
2018, Sasaki et al. [140]	Production of organic compounds through division of labour by making substrate-selective strains.				x	x	x
2018, Thuan et al. [385]	Production of organic compounds through division of labour by splitting the production pathway.				x		x
2019, Akdemir et al. [386]	Production of organic compounds through division of labour by strain modifications and optimisation through variation of the induction time.				x	x	x
2019, Chen et al. [387]	Development of a sensor to a new molecule.				x		x
2019, Gao et al. [388]	Protease-based toggle-switch to apply dynamic metabolic control.				x	x	x
2019, Guarino et al. [389]	In-silico feedback control of a toggle-switch.					x	x
2019, Jawed, Yazdani, and Koffas [73]	Review on how to design co-culture mainly for an efficient production, with notes on how to keep them stable.		x		x		x
2019, Lawson et al. [145]	Method to design the system based on rational knowledge. Interactions are studied and adjusted based on desired properties.	x	x	x	x		x
2019, Li, Wang, and Zhang [390]	Production of organic compounds through division of labour by splitting the production pathway.				x		x
2019, Liao et al. [131]	Produced toxins are used to regulate population dynamics in a rock-paper-scissor dynamic.				x		x

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2020, Canon et al. [394]	Positive interactions in lactic acid bacteria co-cultures.	x					x	
2020, Fontanarro et al. [129]	Computer-aided logic gate design to control cells in dynamic conditions.					x	x	x
2020, Johnston et al. [39]	Design of a system of spatial segregation using hydrogels to manage a co-culture, and providing a way to easily control of the strain ratio.		x	x				x
2020, Li et al. [110]	Creations of a mutualistic co-culture using existing strains by leveraging natural metabolic pathways.		x					x
2020, Miano, Liao, and Hasty [395]	Inducible quorum sensing enables periodic cell lysis. The system is designed to be controlled.					x	x	x
2020, Naseri and Koffas [396]	Review of combinatorial optimisation methods to produce optimal synthetic gene circuits. Computer-aided design.					x		x
2020, Perrino et al. [122]	On-line measurement and analysis of a microfluidics chip for feedback control based on a cell model, allowing to manage cell cycle.			x				
2020, Rugbjerg and Olsson [130]	Review of self-selecting cultures.					x		x
2020, Shannon et al. [397]	In vivo and in silico feedback control of molecular titration system to control gene expression.					x	x	x
2020, Wang et al. [398]	Production of organic compounds through division of labour by splitting the production pathway.					x		x
2020, Wang et al. [56]	System design principles for integration of measurement and actuation in bioreactors.			x				x
2020, Yuan et al. [399]	Production of organic compounds through division of labour by splitting the production pathway.					x		x

Fitness cost drives complex but predictable cell population dynamics

2020, Zhai, Palazoglu, and Sun [400]	Mathematical tools to analyse internal and external oscillations for cells in a bioreactor for optimizing production.			x				x
2020, Zhang et al. [401]	Measurements of effect of growth on memory of synthetic genetic circuits.	x			x		x	
2021, Chun et al. [402]	Artificial redox-based cell-cell communication, creating a type of artificial quorum sensing.					x		x
2021, Costello and Badran [403]	Review of ways to create/test orthogonality between synthetic gene circuits and normal cell function.		x			x		x
2021, Das et al. [93]	Review on microalgae co-cultures, mention of cell choice for de-toxification, cell interaction engineering, toxin and nutrient based communication, and quorum sensing in general, as well as the impact of those interactions on production.		x				x	x
2021, English, Gayet, and Collins [127]	Review on synthetic biology tools to design gene circuits.					x		x
2021, Gargatte et al. [134]	Combination of a self-selecting culture, division of labour, and cell interaction engineering (through export of pathway intermediates) to improve production.					x		x
2021, González et al. [94]	Review of white rot fungi co-cultures, study of cell interactions, cell interaction engineering, and division of labour are key to a good process; they note that competition often boosts production in those systems. Study then design of the system based on what is known.	x	x					x

2021, Hartline et al. [50]	Review of dynamic metabolic control, including a section on sensors and ways to apply that control, to redirect carbon fluxes or tackle phenotypic heterogeneity.			x	x	x	x	Fitness cost drives complex but predictable cell population dynamics
2021, Heltberg et al. [404]	Mathematical analysis of clocks and noise in biology.	x				x		
2021, Kusuda, Shimizu, and Toya [272]	On-line measurement used for feedback control of the strain ratio in a co-culture.			x	x	x		
2021, Liao, Niu, and Liu [405]	Review of productions of organic compounds through division of labour.			x		x	x	
2021, Liu, Caruso, and Zhang [406]	Production of organic compounds through division of labour by splitting the production pathway, and some process conditions adjustments.	x		x			x	
2021, Machado et al. [149]	Identification of competing or cooperative communities and environmental factors related to both, thanks to 'omics tools.	x				x		
2021, Mancuso et al. [106]	Impact of environmental fluctuations on community diversity.	x				x		
2021, Nguyen et al. [116]	Dynamic metabolic control based on on-line measurements and analysis.		x			x		
2021, Perrino et al. [100]	Review of control strategies for gene expression by design (synthetic biology in single or multiple strains) or external control.		x		x	x	x	
2021, Ruolo et al. [99]	Review of control strategies for gene expression by design (synthetic biology in single or multiple strains) or external control.			x	x	x	x	
2021, Winkle et al. [90]	Agent-based model to simulate regulation of strain ratio via cell length regulation.	x					x	

2021, Xu and Yu [85]	Review on how to design stable microbial co-cultures, using systems and synthetic biology, without external control.	x			x		x	x
2021, Zhang et al. [407]	Production of organic compounds through division of labour by modifying one strain and using the wild-type capabilities of the other.	x			x			x
2021, Zorzan et al. [408]	Review of in vivo and in silico tools for tuning gene circuits. The system can be designed for control.				x	x		x
2022, Bloxham, Lee, and Gore [119]	Determine how a cycling environment stabilises a co-culture even though one organism grows faster than the other in all conditions, because it is slower to adapt.	x	x				x	
2022, Finger et al. [105]	Study of the behaviour of a co-culture and tuning of cell proportions through abiotic factors.	x	x				x	x
2022, Henrion et al. [143]	Review on how to use to analyse and control gene expression via control of the extracellular environment.	x	x	x		x	x	
2022, Hoffman, Tang, and Avalos [128]	Review on how to control genes with optogenetics.					x	x	
2022, Jaramillo-Madrid, Lacchini, and Goossens [409]	Review of organelle engineering for terpene production.					x		x
2022, Jiang et al. [132]	Cell interaction engineering, using quorum sensing to impact cell survival, and manage population proportion of 2 strains in co-culture.					x	x	
2022, Kapoore et al. [79]	Review of bioreactor designs for co-cultures.		x				x	x
2022, Martinez et al. [147]	Design of co-culture control scheme based on mono-culture data.	x	x				x	

2022, Qiao et al. [410]	Production of organic compounds through division of labour by strain modifications.	x				x
2022, Ray, Nayak, and Ghosh [411]	Review of co-cultures with microalgae, mention cell interaction engineering and the control of culture conditions to accommodate all members of the system.	x			x	
2022, Ronda and Wang [146]	Synthetic biology methods to engineer spatio-temporal dynamics in communities.		x	x	x	
2022, VanArsdale et al. [412]	Electricity sensor for cells.		x			x
2022, Zhang et al. [413]	Division of labour to degrade pollutants, with investigation of how changing inducer intensity, and inoculation time interval and ratios impact the system. In this case, the system is designed then controlled, not designed to be controlled.		x	x		x
2022, Zhu, Ai, and Jia [142]	Production of organic compounds through division of labour, through natural complementary pathways and alteration of the carbon fluxes to create substrate-selective strains.	x		x	x	x

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Annex B

**Supplementary data: a Fitness-Entropy
compensation effect is set by the trade-off
between growth and gene expression in cell
populations**

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This Appendix is part of the supplementary materials for CHAPTER 4, section 1, related to Fitness-Entropy compensation, adapted from:

[179] Mathéo Delvenne, Vincent Vandenbroucke, Lucas Henrion, Maximilian Sehr, Juan A. Martínez, Alizée Sloodts, Samuel Telek, Andrew Zicler, and Frank Delvigne. *A Fitness–Entropy Compensation Effect Set the Trade-off between Growth and Gene Expression in Cell Populations*. July 9, 2025. DOI: 10.1101/2025.07.05.663304. Pre-published

The following resources are associated to this publication:

- doi.org/10.5281/zenodo.14273182
- gitlab.uliege.be/mipi/published-software/2024-fitnessentropycompensation

1. Calibration of the automated flow cytometry

Three instruments were used for the automated flow cytometry measurements, two Accuri C6+ and one Accuri C6. For the T7 system in *E. coli* and the *glc3* system in *S. cerevisiae*, all three machines were used for different experiments, while for all other

systems, only one machine per system was used. Since measurements are not identical between machines, we performed a calibration between them and transformed all the measurements of any machines into what one of our C6+ cytometers, which we are using as reference, would have measured.

To do so, identical samples of both yeast and bacteria were passed through either both C6+ machines, or our reference C6+ cytometer and the C6. For *S. cerevisiae*, the calibrations were made using precultures of our GFP-producing strain with different levels of stress, due to starvation (cells in exponential growth or in stationary phase) or heat shock (culture in stationary phase let 30min, 1h and 1h20 at 37°C). For the *E. coli* T7 system, the calibrations were made using cultures on defined medium with glucose, with or without induction of the T7 system with IPTG.

The measurements were split in percentiles and a correlation was determined between the percentiles of the reference and the other cytometer, for both forward scatter (FSC-A) and fluorescence (FL1-A), which are the measurements we use for our analyses. The codes are available in the files `ConversionFromC6.py` and `ConversionFromC6plus_Left.py`.

For the Accuri C6 to C6+ transformation, the correlation that best fitted all data was a power law of the form $y = ax^b$, where y is the measurement of the reference cytometer, x is the measurement of the other cytometer, and a and b are parameters fitted to the data. A linear fit would have sufficed for the FSC-A measurements and for FL1-A for *S. cerevisiae* (FIGURE B.1A), but for the T7 system the relationship was not linear at all (FIGURE B.1B). We chose our fit so that high fluorescence values were correct, while low values in the C6, gave an over-estimated fluorescence after transformation. The high values were prioritised because they are the ones used to determine the growth rate in segregostat. Nevertheless, the exponent in this power law means that the determination of growth rate for the T7 system needs to be adjusted to account for the non-linearity of the correlation.

For the transformation between the two C6+ machines, we used a linear correlation for all measurements, although the correlation for the T7 system fluorescence was not as good as others (FIGURE B.2). This is likely because, for this system, the cells are very close to the detection limit of the cytometer, and different amounts of noise are measured by the two machines. This doesn't seem to affect the entropy significantly, however, since the entropy for each replicate of the Xylose/lactose and Glucose/lactose experiments were very similar despite coming from different machines (FIGURE B.3).

2. A look at the numbers for the different test cases

We've seen in CHAPTER 3, section 4 that, in order to fully prove the Fitness-Entropy compensation effect, we need to show that the entropy of the selected phenotype is larger than both the entropies of the individual phenotypes. The entropies of the T7,

Appendix B. Supplementary data: a Fitness-Entropy compensation effect is set by the trade-off between growth and gene expression in cell populations

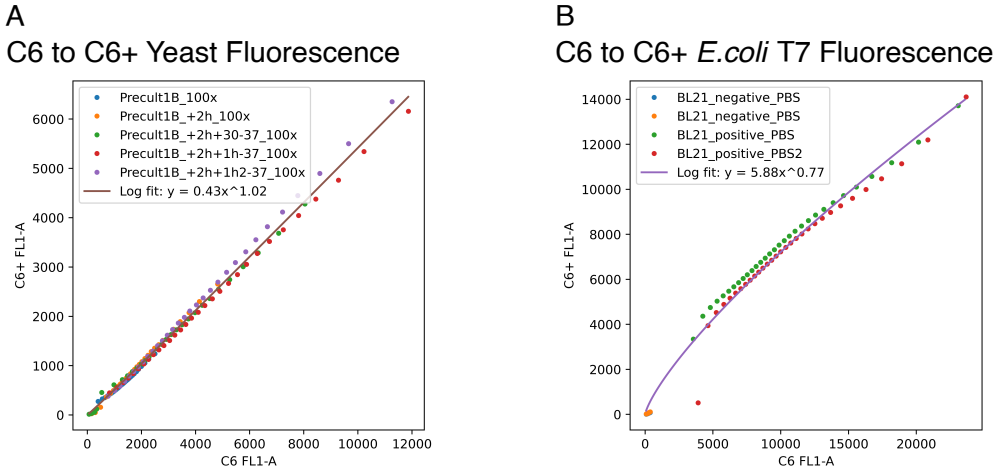


Figure B.1: Calibration of the Accuri C6 cytometer with the Accuri C6+ cytometer. The data points are the percentiles of the measurements, and the curves are the fitted correlations.

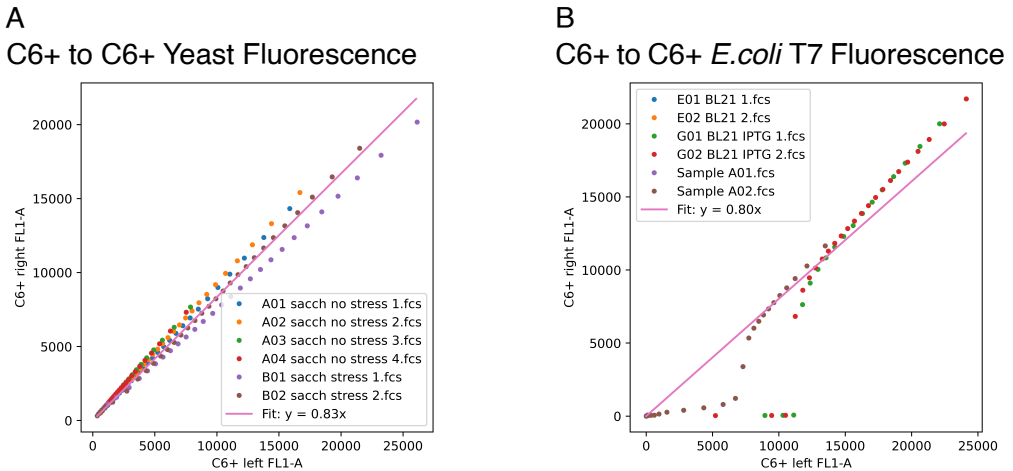


Figure B.2: Calibration of the Accuri C6+ cytometer with another Accuri C6+ cytometer. The data points are the percentiles of the measurements, and the curves are the fitted correlations.

ydcS, and glc3 systems were measured in different conditions to assess the validity of the effect more quantitatively. The results are presented in FIGURE B.3.

The entropy of the pure phenotypes for the T7 system was measured as T7 without inducer on one side, and as T7 with IPTG (in flask) or with xylose+lactose feed (from our bioreactors) on the other side. By comparison, the entropy of the chemostat on glucose+lactose, where we claim Fitness-Entropy compensation, is 25 to 50% higher

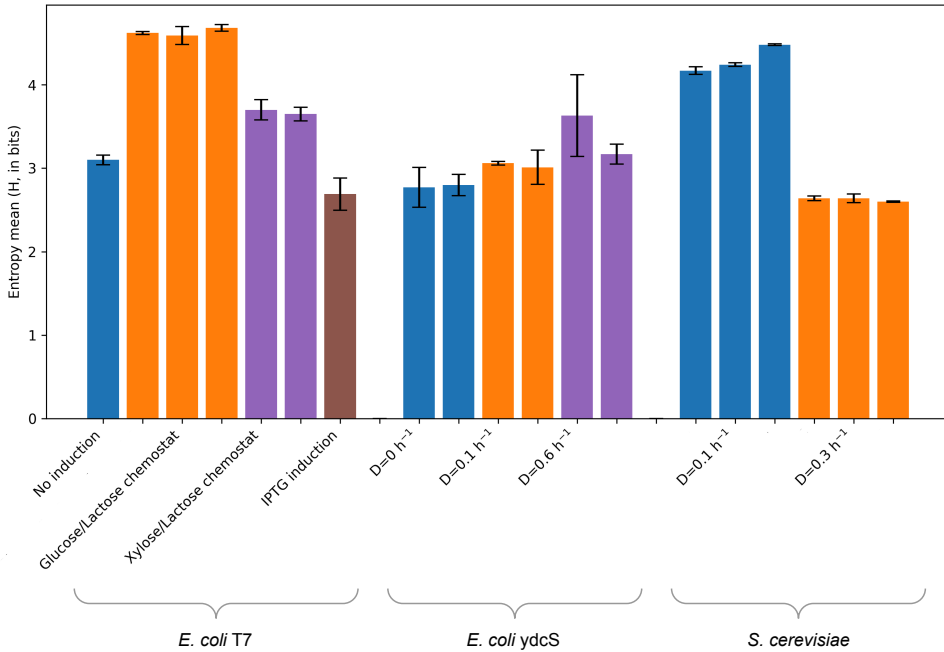


Figure B.3: Entropy of the different systems measured in different conditions. Error bars are three standard deviations on 6 samples (technical replicates), except for the Uninduced and IPTG induced samples for the T7 system, which only have 2 samples each and come from flasks instead of bioreactors. Biological replicates are juxtaposed and in the same colour. The entropies of the conditions where we would expect Fitness-Entropy are shown in the middle for each system. For the T7 system, the xylose/lactose and IPTG conditions are both purely induced phenotypes but have different entropies, primarily because the noise (measured as uninduced) in the xylose/lactose condition becomes more prevalent over time as cells are washed out of the bioreactor. For the ydcS system, $D=0 \text{ h}^{-1}$ indicates the stationary phase in bioreactors without any feeding. There is no Fitness-Entropy compensation for that system, and the entropy at 0.1 h^{-1} is in between the other conditions. For the yeast, only one of the unstressed reference point is available (0.3 h^{-1}), but it is much lower than the entropy observed in chemostat at a lower dilution rate (0.1 h^{-1}).

than the entropy of the pure phenotypes¹, showing that we are indeed measuring the Fitness-Entropy compensation effect.

For the ydcS system, there should be no noticeable Fitness-Entropy compensation effect, as the stressed phenotypes have a higher growth rate than the dilution rate and no significant trade-off was observed. As expected, the entropy of the low dilution

¹Note that the difference with the xylose is only so low because the background noise of the cytometer (up to 50 events/ μL) is larger in that case (only 300 events/ μL because of the washout of the population) than in other bioreactors (about 1000 events/ μL). This increases entropy as the noise does not overlap with the measurements of GFP.

rate ($D=0.1 \text{ h}^{-1}$) is in between and of a similar magnitude to the entropies of the pure phenotypes ($D=0 \text{ h}^{-1}$, a stationary phase with no feeding causing starvation stress, and $D=0.6 \text{ h}^{-1}$, a chemostat with a high dilution rate eliminating stress).

Finally, for the yeast, we were unfortunately not able to measure a purely induced phenotype in bioreactor. Batch cultivation with prolonged stationary phase to induce stress expression caused a bimodal distribution unlike what we observe in microfluidics. In flask, cells experienced successively conditions of the Crabtree effect (ethanol production), respiro-fermentation conditions (respiration of glucose and re-consumption of ethanol) and finally conditions with very low concentration of glucose. We are therefore not confident that this distribution was a reliable indicator of the population as it would happen if the *glc3* system was induced by a uniform glucose starvation. The microfluidics measurements in stressed state are more representative and do indicate a well-defined phenotype, but we currently have no way to compare the entropy measurements from flow cytometry with microfluidics. As a result, we cannot conclude with full certainty that the Fitness-Entropy compensation effect is present in the *glc3* system, but we believe that the increase in entropy between the high and low dilution rates (about 60%), the entropy at $D=0.1$ being higher than any entropy of a pure phenotype we measured, the uniform induction of the system in microfluidics, and the growth trade-off measured, are strong indicators in favour of the effect being present.

3. Characterisation of the genetic toggle switches with and without trade-off

Individual phenotypes of the toggle switch with influence on growth (i.e., with auxotrophy to its plasmid) were characterised before bioreactor experiments. The cultures were performed in a BioLector (M2PLabs, Germany), a microfermentation system that enables online monitoring of biomass and fluorescence in microtiter plates, as described below in Extended materials and methods. The measurements showed that the growth rate of the growing phenotype was $0.531 \pm 0.014 \text{ (std) h}^{-1}$, while the non-growing phenotype had a growth rate of $0.295 \pm 0.010 \text{ (std) h}^{-1}$ (data available in the supplementary dataset and code available in the `ToggleSwitchBiolectorAnalysis.py` file). The latter measurement is much smaller than the dilution rate used in bioreactor, and therefore using IPTG should wash the culture out.

As described in CHAPTER 4, section 1, continuous cultures of both toggle switch strains were carried out in lab-scale bioreactors. While the cultivation of the strain with growth control is shown in FIGURE 4.4, that of the negative control strain, which has no effect on growth, is presented below (FIGURE B.4).

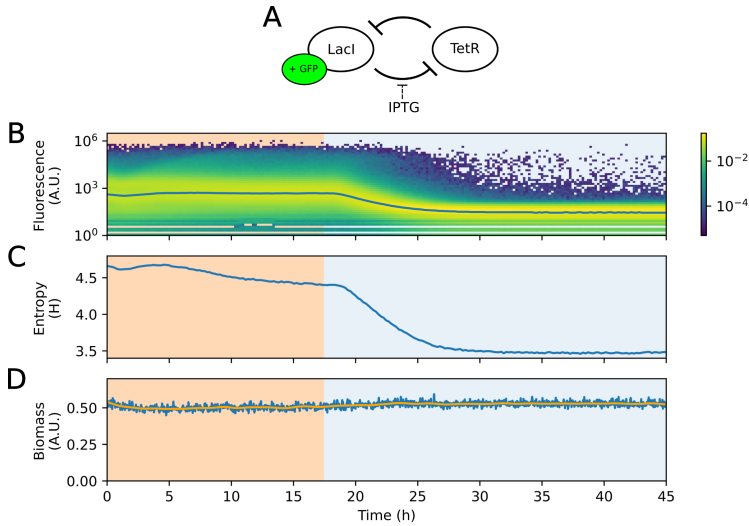


Figure B.4: Continuous cultivation of the negative control toggle switch (without serine auxotrophy), upon addition of IPTG in the feed (blue background), the production of GFP was inhibited. **A** Schematic representation of the toggle switch. **B** Single-cell fluorescence distributions over time, the blue line indicates the median fluorescence. **C** Shannon entropy computed from the fluorescence distribution over time. **D** Biomass over time, the blue line indicates raw data and the orange line indicates smoothed data.

3.1. Extended materials and methods

3.1.1. Characterisation in BioLector platform

Precultures in defined medium from glycerol stocks were first made without inducer. These precultures were then used to inoculate flasks with either 150 ng·mL⁻¹ anhydrotetracycline (less toxic than tetracycline) or 1 mM IPTG to produce pure cultures of each phenotype. These cultures were incubated overnight and washed twice in defined medium without sugar to remove the inducer. The OD was then adjusted to inoculate a BioLector plate at an OD of 0.5. In the BioLector, each pure phenotype was grown in triplicate with and without inducer. For the growing phenotype, tetracycline was used instead of anhydrotetracycline in the BioLector at a concentration of 25 ng·mL⁻¹, as it had been found enough to induce the phenotype but below the MIC. The non-growing phenotype was induced with 1 mM IPTG.

Growth curves for each well were produced by removing the baseline from the measurements then fitting an exponential to the exponential phase of growth by linear regression (see supplementary code).

4. Selection of the general stress reporter for *E. coli*

We conducted a comprehensive search for suitable transcriptional reporters to detect the activation of the general stress response. Given that RpoS is the master regulator (transcription factor) of this response, we initially selected 26 genes from its regulon. These genes were chosen to represent the approximately 500 genes in the RpoS regulon based on the following criteria: (i) noise level [194], (ii) regulatory complexity (including σ S exclusivity, co-regulation by other transcription factors, position in the regulation network (regulator/regulated), (p)ppGpp dependency, etc.), (iii) sensitivity to RpoS concentrations [414], (iv) functions in response to various stress (pH, osmotic, etc.), (v) anti-correlation with growth, and (vi) correlation with antibiotic persistence.

The initial screening was performed using the BioLector platform to evaluate the basal expression levels, dynamic expression changes during the transition to stationary phase (i.e., entry into stressful conditions), and the dynamic range of expression (difference between basal and maximum expression levels) (FIGURE B.5). Samples were taken during the exponential growth phase and late stationary phase for cytometry analysis and Shannon entropy computation (FIGURE B.6). Based on these results, we shortlisted candidates using the following selection criteria: (i) expression levels (to be detectable by our flow cytometer), (ii) dynamic range (to clearly differentiate stress stages), and (iii) dynamic expression patterns during the transition to stationary phase. The selected reporters were further tested in a continuous cultivation device across various dilution rates, which correspond to different RpoS (i.e., stress) levels [211] (FIGURE B.7). From these experiments, we ultimately identified YdcS as the optimal reporter. YdcS is a putative transport protein associated with natural and chemical transformations [166] and carbon flux distribution [167]. Its transcription is specifically initiated by σ S-containing RNA polymerase ($E\sigma$ S) [166]. Follow-up analysis using Microfluidic Single-Cell Cultivation (MSCC) confirmed that YdcS is a reliable reporter of the general stress response (SECTION 5).

One may ask why we did not work with RpoS transcriptional reporter directly. There are three main reasons. First, the RpoS transcriptional reporter of the Zaslaver's collection seems deficient. Probably because the major *rpoS* promoter (*rpoSp1*) that is located within the *nlpD* gene, over 500 nucleotides upstream the *rpoS* gene, is missing in the plasmid [159, 164, 189]. Secondly, since few molecules of RpoS are produced per cell, detecting its transcriptional reporter with our cytometry setup would be challenging. Finally, RpoS transcriptional reporter may be a bit tricky since RpoS can accumulate without proper assembly into the active RNAP holoenzyme ($E\sigma$ S) which directly regulates stress-related gene expression [164]. These challenges necessitated the use of an alternative approach. An ideal reporter gene would be exclusively regulated by $E\sigma$ S and exhibit a simple regulatory system—criteria met by *ydcS*.

To verify that *ydcS* is a good indirect reporter of $E\sigma$ S activity, we used a dual-reporter strain (*E. coli* MG1655 $P_{rpoS}::\text{CyOFPI } P_{ydcS}::\text{GFPmut2}$). This strain combines an RpoS

transcriptional reporter integrated into the chromosome (capturing the major promoter) with a plasmid-based *ydcS* transcriptional reporter from the Zaslaver collection (see CHAPTER 4, section 1.10.2). Using microfluidics, we demonstrated that *ydcS* (GFP) expression increases under stressful conditions and correlates well with *rpoS* (CyOFP) levels (FIGURE B.8). This finding confirms that *ydcS* is a reliable and convenient reporter for general stress response activation.

Appendix B. Supplementary data: a Fitness-Entropy compensation effect is set by the trade-off between growth and gene expression in cell populations

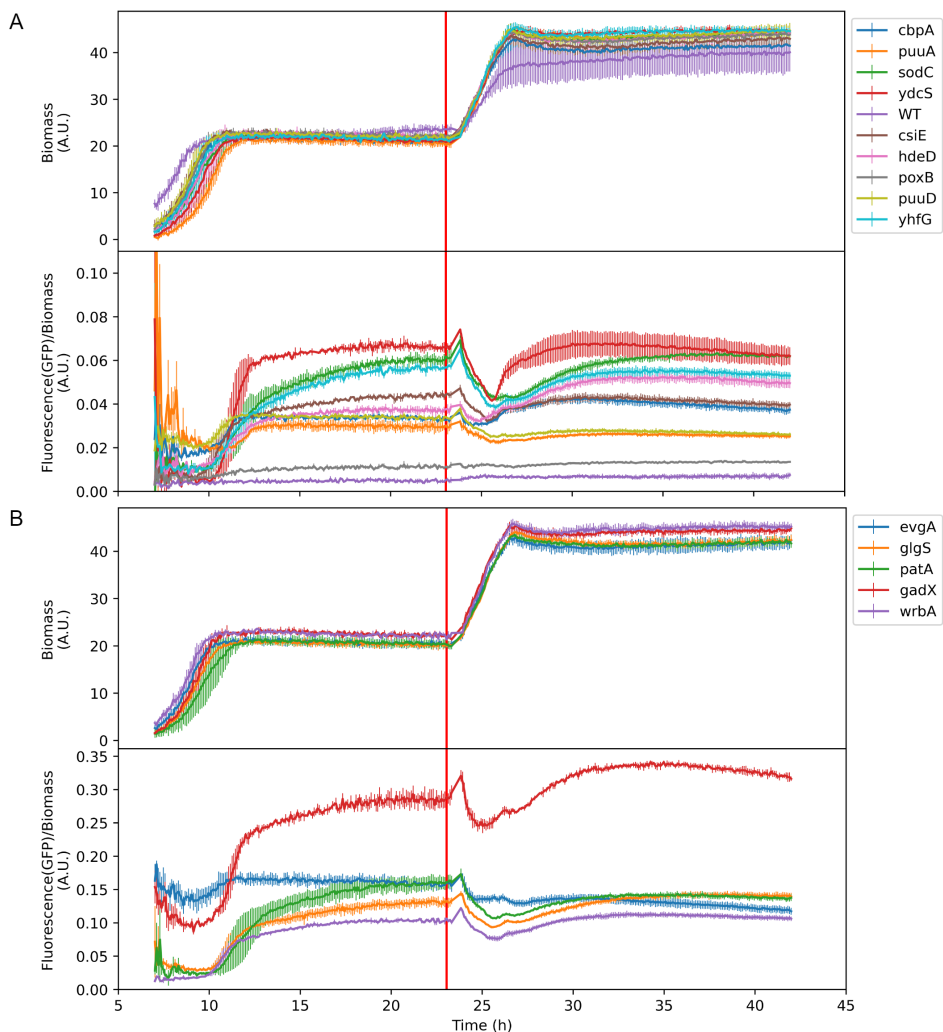


Figure B.5: Example of visualization of data from the BioLector screening for reporters with low to intermediate expression levels (A) and reporters with higher expression levels (B). The top panel represents the biomass over time, while the second panel shows the relative fluorescence (GFP signal normalized by biomass) over time. Due to the low initial biomass, measurements are noisy at the beginning of the culture. Therefore, data collected before most wells display monotonal exponential growth have been excluded. Vertical lines represent the standard deviation ($n = 3$). The red vertical line at approximately 23 hours marks the time of sampling for cytometry analysis and the addition of glucose to the micro-wells.

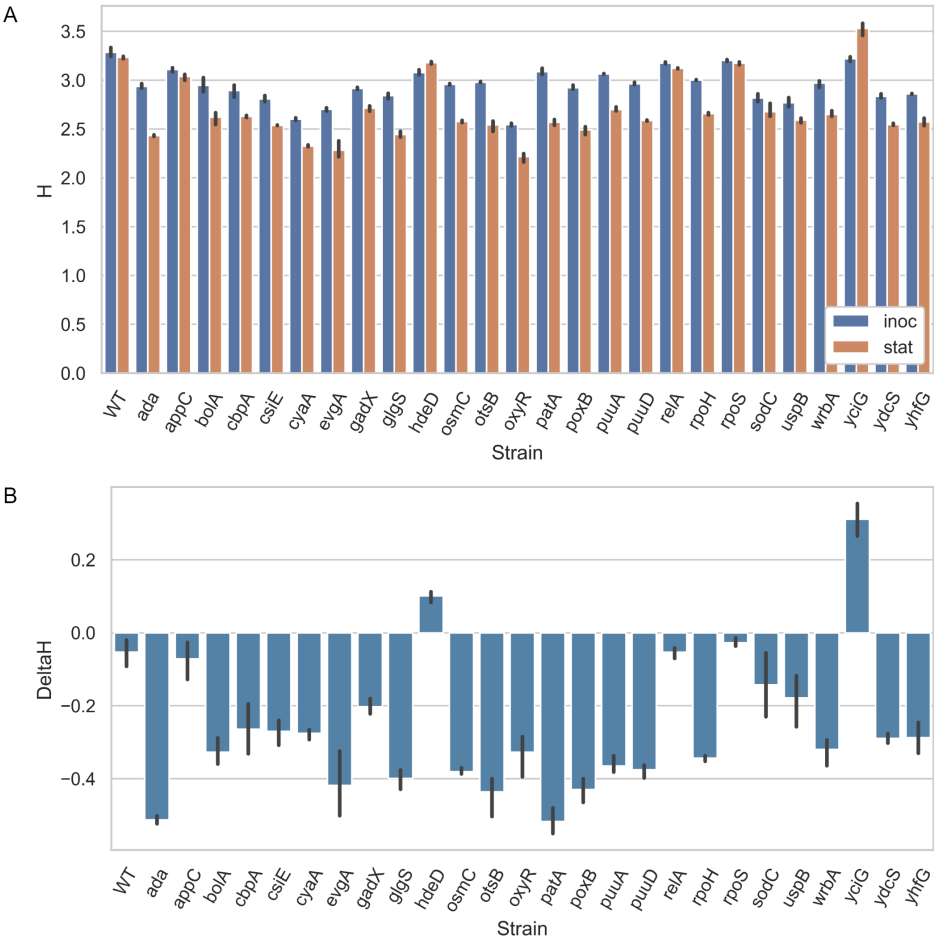


Figure B.6: Shannon entropies of different transcriptional reporters sampled in the BioLector platform during exponential growth and stationary phase (A), and the differences of entropies between these phases (B).

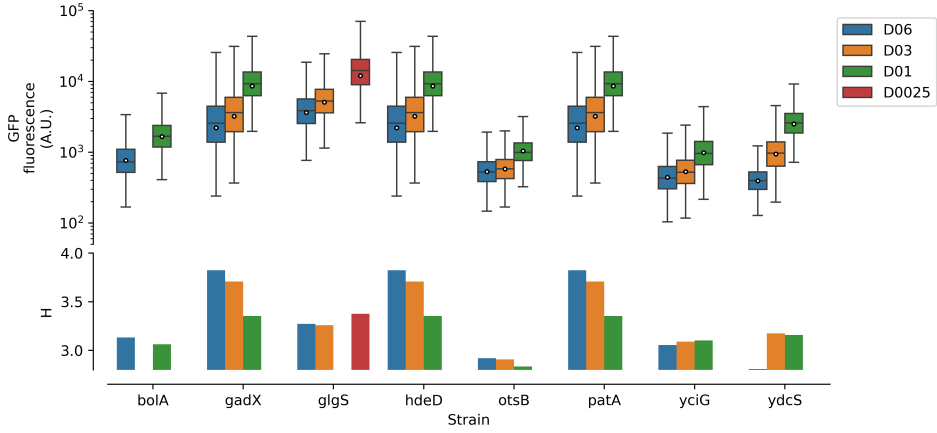


Figure B.7: Summary plot of continuous cultures at different dilution rates performed with a subset of transcriptional reporters. The top panel represent the distribution of fluorescence as boxplots. The mean is indicated as a white dot and whiskers are drawn to the farthest datapoint within 1.5 * Interquartile range from the nearest hinge. The second panel represent the shannon entropies of corresponding samples.

4.1. Extended materials and methods

4.1.1. Strains and plasmids

E. coli MG1655 strains from the Zaslaver collection [159] were used for the reporters screenings. Each strain carries a plasmid containing a transcriptional reporter (production of GFPmut2 controlled by the reported promoter) and confers kanamycin resistance. Each plasmid used was verified by sequencing the promoter region using a primer annealing to the GFP sequence (5'-CAAGAATTGGGACAACTCCAGTG-3'). The promoters analysed during the first screening in the BioLector platform are : P_{ada} , P_{appC} , P_{bolA} , P_{cbpA} , P_{csiE} , P_{cyaA} , P_{evgA} , P_{gadX} , P_{glgS} , P_{hdeD} , P_{osmC} , P_{otsB} , P_{oxyR} , P_{patA} , P_{poxB} , P_{puuA} , P_{puuD} , P_{relA} , P_{rpoH} , P_{rpoS} , P_{sodC} , P_{uspB} , P_{wrbA} , P_{yciG} , P_{ydcS} and P_{yhfG} . In addition, a *E. coli* MG1655 strain without plasmid has been used as control. The strains used for the second step of screening in continuous cultures were the ones with the following promoters: P_{bolA} , P_{gadX} , P_{glgS} , P_{hdeD} , P_{otsB} , P_{patA} , P_{yciG} and P_{ydcS} . The dual reporter strain used in a Microfluidic Single Cell Cultivation device and the related experiments are described in CHAPTER 4, section 1.10.2 and 1.10.6.

4.1.2. Screening in BioLector platform

4.1.2.1 Cultivation in the BioLector platform: The first screening was performed in a BioLector (M2PLabs, Germany) with 48-deepwells microplates allowing measurement of Biomass (filter module 401: Exc. 620/10 nm | scattered light) and GFP (filter module 404: Exc 480/10 nm | Em. 520/25 nm). The mineral medium used was the same as described in CHAPTER 4, section 1.10.3, with the addition of 10 g/L

of MOPS as buffer (pH=7). Medium without kanamycin was used with the strain without plasmid. The strains were precultured in two steps to avoid initial induction of the reporters. The first precultures were inoculated in 50 ml flasks (with 5ml of the corresponding medium) with one single colony from a LB plate (supplemented with 50 mg/L kanamycin, for strains with plasmid). It was then incubated overnight at 37 °C at a shaking speed of 150 rpm. 8 µL of each of the first precultures were added to three different wells (triplicates) of black bottom BioLector FlowerPlates (ref: MTP-48-OFF) containing 800 µL of fresh medium and were cultivated in BioLector (shaking frequency = 1400 rpm, Temperature = 37°C). After 4 hours, 8 µL of each of these second precultures were transferred in 800 µL of fresh medium in corresponding wells of readable BioLector FlowerPlate (ref: MTP-48-B) and incubated in the same conditions. Three wells were used as blank and contained only medium. For each well, biomass and GFP were measured every approximately 5.5 min. The remaining cell suspensions of the second precultures were analysed in flow-cytometry as the sample for exponential growing phase (supposedly with the minimum fluorescence) and their OD₆₀₀ have been measured to approximate the initial OD₆₀₀ of the cultures. After more than 20 hours, when the culture reached stationary phase, 20 µL of each well was sampled for cytometry analysis. This volume was replaced by 20 µL of a concentrated glucose solution (200 g/L) and the plate was further incubated for about 20 hours. The data are available in the supplementary dataset and the code is available in the Supp_Biolector_Process_Data.py file.

4.1.2.2 Off-line cytometry analysis: Flow cytometry analyses were performed using a benchtop flow cytometer (BD Accuri C6) with the medium pre-set flow rate (35 µL/min) and core sizes (16 µm). The primary threshold was set at 20 000 units of FSC-A. The FL1-A channel (exc. 488 nm, em. 533/30 nm) was used for GFP quantification. Flow cytometry data was cleaned before being used by removing zeros and doublets. A second threshold was set at 10 000 units of FSC-A to correct an artefact in the measurements. To compute the entropy, 50 fixed bins in log scale between 0 and 10⁶ a.u. of GFP fluorescence were used. It is important to note that the values of entropies cannot be compared between different cytometers (e.g., BD Accuri C6 and BD Accuri C6+) and different fluidics parameters (i.e., flow-rates, core-size and thresholds). Since the other cultivations and the calibrations were not carried out with the exact same parameters, they cannot be compared to these measurements. The data are available in the supplementary dataset and the code is available in the Supp_Biolector_FCA.py file.

4.1.3. Screening in chemostat experiments at three different dilution rates

4.1.3.1 Cultivation in the DASGIP bioreactors: Precultures were performed in two steps, as described in CHAPTER 4, section 1.10.4. Chemostat experiments were performed in DASGIP bioreactors (Eppendorf, Germany) with 150 mL of working

volume at controlled pH 7, 1000 rpm, and 37°C. Three dilution rates, i.e., 0.6, 0.1, and 0.3 h⁻¹, were sequentially imposed on the system. Details about samplings and timings of experiments can be found in the supplementary document "Supp_EcoliStressReporterSelection/Dasgip/Notes-MetaData.docx".

4.1.3.2 Online cytometry analysis: For automated flow-cytometry analyses of 3 DASGIPs reactors running in parallel, the “multi-stat” system was used. It is an adaptation of our in-house Segregostat [4] connected to a benchtop flow cytometer (BD Accuri C6). Every approximately 15 min, it takes a sample from one of the bioreactor and dilutes it with PBS before analysis. The following measure is performed in the next reactor, meaning that a sample is taken every approximately 45 min in each reactor. The fluidics flow rate was custom (24 µL/min, 8 µm core size) and a threshold was set either to 20 000 units of FSC-A. The FL1-A channel (exc. 488 nm, em. 533/30 nm) was used for GFP quantification. Flow cytometry data was cleaned before being used by removing zeros and doublets. To compute the entropy, 50 fixed bins in log scale between 0 and 10⁶ a.u. of GFP fluorescence were used. The cytometry files (’.fcs’) used to make the FIGURE B.7 correspond to the last measure in the last retention time for each dilution rate. They are listed in the supplementary document "Supp_EcoliStressReporterSelection/Dasgip/Notes-MetaData.docx". The data are available in the supplementary dataset and the code is available in the Supp_Dasgip_FCA.py file.

5. Time-lapse microscopy in a Microfluidic Single Cell Cultivation (MSCC) device

The dual-reporter strain *E. coli* MG1655 P_{rpoS}::CyOFP1 P_{ydcS}::GFPmut2 was characterised using time-lapse microscopy in a Microfluidic Single Cell Cultivation (MSCC) device (FIGURES B.8 to B.10).

As described in CHAPTER 4, the trade-off between growth and gene expression of *Saccharomyces cerevisiae* P_{glc3}::GFP was also monitored in this device (FIGURE B.11).

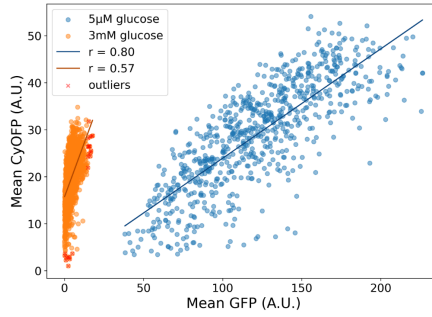


Figure B.8: Correlations between $P_{rpoS}::\text{CyOFP1}$ and $P_{ydcS}::\text{GFPmut2}$ expression in limiting (5 μM glucose, blue) and non-limiting (3 mM glucose, orange) conditions in a MSCC device. Each dot represent a single-cell (from a total of 5 cultivation chambers per glucose concentration condition). Outliers are datapoint with a $|z\text{-score}| > 3$.

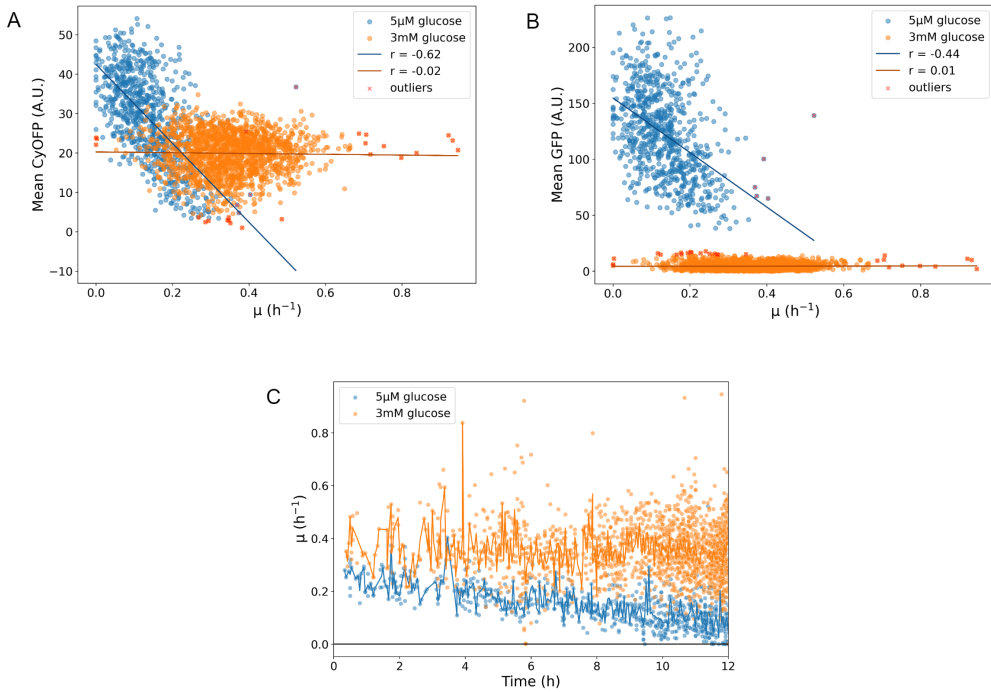


Figure B.9: Single-cell growth in limiting (5 μM glucose, blue) and non-limiting (3 mM glucose, orange) conditions in a MSCC device. Each dot represent a single-cell (from a total of 5 cultivation chambers per glucose concentration condition). Outliers are datapoint with a $|z\text{-score}| > 3$. **A** Correlations between $P_{rpoS}::\text{CyOFP1}$ expression and growth rate, **B** Correlations between $P_{ydcS}::\text{GFPmut2}$ expression and growth rate, **C** Evolution of single-cell growth rate over time.

Appendix B. Supplementary data: a Fitness-Entropy compensation effect is set by the trade-off between growth and gene expression in cell populations

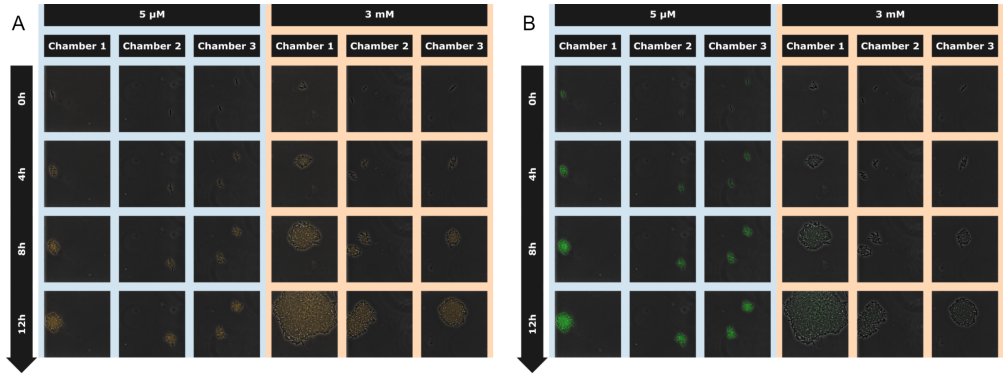


Figure B.10: Time-lapse microscopy pictures of *E. coli* $P_{rpoS}::\text{CyOFP1}$ $P_{ydcS}::\text{GFPmut2}$ cultivated in MSCC, three microfluidic chambers per condition. Stressful condition ($[\text{glu}] = 5 \mu\text{M}$) is indicated with blue background and non-stressful condition ($[\text{glu}] = 3 \text{ mM}$) is indicated with orange background. **A** CyOFP fluorescence. **B** GFP fluorescence.

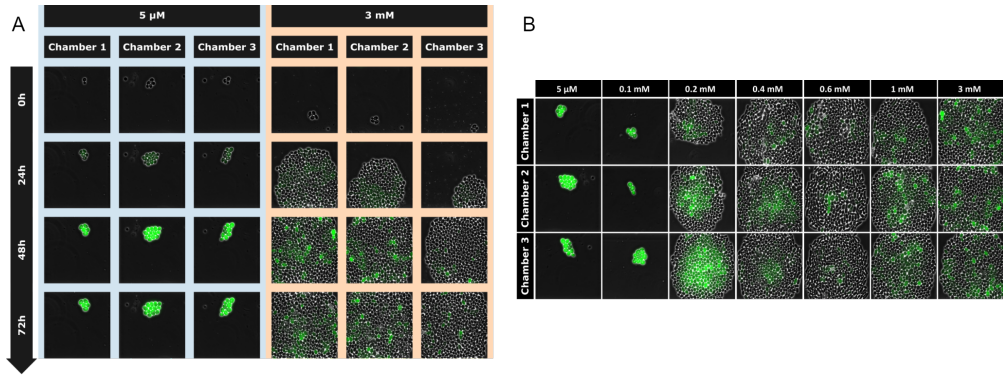


Figure B.11: Microscopy pictures of *S. cerevisiae* $P_{glc3}::\text{GFP}$ cultivated in MSCC, three microfluidic chambers per condition. **A** Time-lapse in stressfull condition ($[\text{glu}] = 5 \mu\text{M}$) indicated with blue background, and in non-stressful condition ($[\text{glu}] = 3 \text{ mM}$), indicated with orange background. **B** Microscopy pictures at different GLU feed concentrations (0.005, 0.1, 0.2, 0.4, 0.6, 1 and 3 mM) after 48 h of cultivation (from Martinez et al. [204]).

6. Cultivations of *S. cerevisiae* $P_{glc3}::\text{GFP}$ at various dilution rates

Residual glucose concentrations at different dilution rates were obtained through changestat (continuous cultures where the dilution rate is increased/decreased over time) experiments [415]. In these continuous cultures, the feed pump speed was either increased (accelerostat, A-stat) or decreased (decelerostat, D-stat) in steps of 1 rpm

every two hours, resulting in a dilution rate change of 0.004 h^{-1} per step. This strategy was chosen to ensure a quasi-steady state at each step.

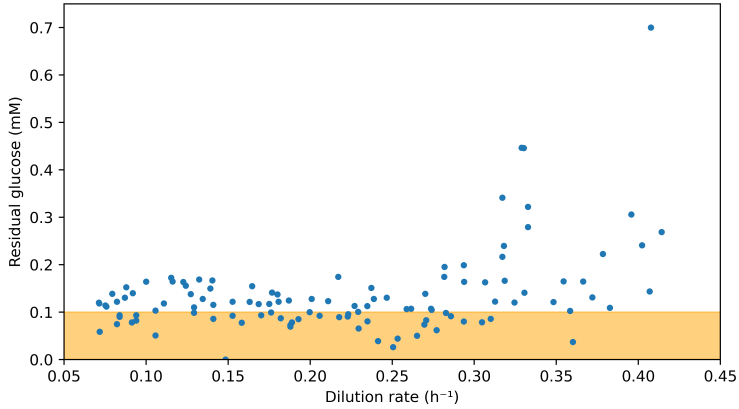


Figure B.12: Residual glucose concentrations at different dilution rates (data from Maximilian Sehart's work [415])

7. Tables

7.1. *Strains and plasmids*

Table B.1: Primers used for the construction of the *E. coli* MG1655 $P_{rpoS}::\text{CyOFP1}$ strain.
Note: The uppercase sequences in the pTarget primers are the selection markers for the CRISPR-cas9 system.

Related Strain	Role	Primer name	Sequence (5'-3')
<i>E. coli</i> MG1655 $P_{rpoS}::\text{CyOFP1}$	Cassette construction	Fw Homo1	cgcagagcaactggataagcc
		Rv Homo1 + Tail-RBS	ctagtatttctcctctttctctagattactcgcggaacagcgc
		Fw CyOFP1 + Tail-RBS	tctagagaaaaggagagaaatactagatgggtgagcaaggggcgagg
		Rv CyOFP1	ttacttgtacagctcgtccatgcc
		Fw Homo2 + tail-CyOFP1	ggacgagctgtacaagtaagtaagcatctgtcagaaagg
		Rv Homo2	agccgcatttattttcactgg
	Construction of pTarget		agctagctcagtcctaggtataataactagt
		Fw pTarget-rpoS	GACAGATGCTTACTTACTCG
			gttttagagctagaaatagcaagttaaaa
		Rv pTarget	actagtattatacctaggactgagctagctg
		Fw verif_pTarget-rpoS	gacagatgcttacttactcg
	Verification /Sequencing	Rv verif_pTarget	ttgccgactaccttggtgat
		Fw verif_prpoS-CyOFP1	tggttactgggtgctggagg
		Rv verif_prpoS-CyOFP1	ccgtactattcgtttgccg

Table B.2: Primers used for the construction of the *E. coli* MG1655 Δ lacAYZ Δ lacI and *E. coli* MG1655 Δ lacAYZ Δ lacI Δ serA strains.

Note: The uppercase sequences in the pTarget primers are the selection markers for the CRISPR-cas9 system. For the *lacI* transformation, selecting a *lacI* pTarget makes the pTarget and the pCas plasmid mutually exclusive. It is possible to select for the modification with the second antibiotic, but pCas is removed from the selected cells. Then, the pTarget can be cured by electroporating the pCas plasmid back.

Related Strain	Role	Primer name	Sequence (5'-3')
<i>E. coli</i> MG1655 Δ lacAYZ Δ lacI	Cassette construction	Fw Homo1_lacAYZI	cgggtattgtgggcgatggt
		Rv Homo1_lacAYZI	gagtcaattcagggtggtgaatattataaaaaatgcctgatacgtgcg
		Fw Homo2_lacAYZI	attcaccaccctgaattgactc
		Rv Homo2_lacAYZI	ggtgatgactatcaactggcacgg
	Construction of pTarget	Fw pTarget-lacI	agctagctcagtcctaggtataatactagt AACGGTCTGATAAGAGACAC gttttagagctagaaatagcaagttaaaa
		Rv pTarget	actagtattatacctaggactgagctagctg
		Fw verif_pTarget-lacI	AACGGTCTGATAAGAGACAC
		Rv verif_pTarget	ttgccgactaccttggtgat
	Verification /Sequencing	Fw verif_lacAYZI	tatggctcgccatcaggatcgg
		Rv verif_lacAYZI	ttatccttcaccgggcaatggt
<i>E. coli</i> MG1655 Δ lacAYZ Δ lacI Δ serA	Cassette construction	Fw Homo1_serA	gtggtgaccgataatggcaactgat
		Rv Homo1_serA	ttaccaatcctgtcttttgaaatgtgtgtgc
		Fw Homo2_serA	gacaggattgggaattccccttctctgaaaaatcaacgggc
		Rv Homo2_serA	ctgcggtggttacaccgtcgaa
	Construction of pTarget	Fw pTarget-serA	agctagctcagtcctaggtataatactagt AGACAAGATTAAGTTTCTGC gttttagagctagaaatagcaagttaaaa
		Rv pTarget	actagtattatacctaggactgagctagctg
		Fw verif_pTarget-serA	agacaagattaagtcttctgc
		Rv verif_pTarget	ttgccgactaccttggtgat
	Verification /Sequencing	Fw verif_serA	gtattgcagacgcttccaagcagg
		Rv verif_serA	cggattagacgtgctgatcacacc

7.2. Lab-scale bioreactors cultivations

8. Supplementary material legends

8.1. Code

Relevant code for data analysis is available at <https://gitlab.uliege.be/mipi/published-software/2024-fitnessentropycompensation>.

The code to determine the growth trade-off based on segregostat measurements is available in the file `Approx_mu_fFLUO_Seg_functions.py` (`decay_percentiles` function), it provides an estimate for the growth rate as a function of fluorescence at the start of the time interval considered for each percentile. It is used in the file `Approx_mu_fFLUO_Seg_fitfct.py` to find a function fit. This code takes into consideration the methods to mitigate the various errors described in CHAPTER 3, section 3.

The adapted procedure for the T7 production system is available in the file `Approx_mu_fFLUO_Seg_Ecoli_T7.py`.

The procedure for finding the trade-off based on chemostat data for *ydcS* is available in the file `Approx_mu_fFLUO_varD_fitfct.py`.

8.2. Data

There are 4 folders attached to this supplementary material:

Microfluidics contains the raw data from the microfluidics experiments.

Reactors contains the raw data from the reactors experiments; the code to analyse this data is available at <https://gitlab.uliege.be/mipi/published-software/2024-fitnessentropycompensation>. Figures and notes describing the experiments are also included.

Supp_ReporterSelection contains the raw data from the reporter selection experiments (supplementary).

Toggle-switch_plasmid_maps contains the plasmid maps of the toggle switch plasmids.

biolector_growth_rate_determination contains the data related to the characterisation of the growth rate of individual phenotypes for the switch with effect on growth. In the BioLector data, the three first rows are our strains of interest, in triplicate, and the final three rows are irrelevant for this work. The first column is a blank of sterile medium. Columns 2 and 3 are the growing phenotype, without and with Tc added, respectively. Columns 4 and 5 are the

Table B.3: Summary of specific operating conditions of the continuous cultures performed in lab-scale bioreactors.

Culture type and strain	Operating conditions	Replicates	Publication
Segregostat <i>E. coli</i> W3110 $P_{\text{araS}}::\text{GFPmut2}$	Bioreactor : Biostat B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) $D = 0.45 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation = 1000 rpm Regulation threshold = 1000 A.F.U. $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$ Regulation pulse = 0.15 g arabinose	2021-09-29_LHE_Seg_Ecoli_Ara 2022-01-05_LHE_Seg_Ecoli_Ara	Henrion et al. (2023)
Segregostat <i>E. coli</i> BL21(DE3) pET28::GFP	Bioreactor : Biostat B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) $D = 0.45 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation = 1000 rpm Regulation threshold = 1000 A.F.U. $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$ Regulation pulse = 0.15 g lactose	2022-09-20_LHE_Seg_Ecoli_BL21 2022-10-11_LHE_Seg_Ecoli_BL21	Henrion et al. (2023)
Chemostat <i>E. coli</i> BL21(DE3) pET28::GFP	Bioreactor : Biostat B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) $D = 0.45 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation = 1000 rpm $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$ $[\text{lactose}]_{\text{feed}} = 1 \text{ g/L}$	2022-08-10_LHE_Chem_Ecoli_BL21	Henrion et al. (2023)
	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) $D = 0.45 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation = 1000 rpm $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$ $[\text{lactose}]_{\text{feed}} = 1 \text{ g/L}$	2023-02-15_LHE_Chem_Ecoli_BL21 2025-03-11_ALS_Chem_Ecoli_BL21 2025-04-01_ALS_Chem_Ecoli_BL21	Henrion et al. (2023) This paper
Chemostat xylose <i>E. coli</i> BL21(DE3) pET28::GFP	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) $D = 0.45 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation = 1000 rpm $[\text{xylose}]_{\text{feed}} = 5 \text{ g/L}$ $[\text{lactose}]_{\text{feed}} = 1 \text{ g/L}$	2025-05-05_ALS_Chem_Ecoli_BL21_xylose 2025-05-28_FDE_Chem_Ecoli_BL21_xylose	This paper
Chemostat <i>E. coli</i> MG1655 $\Delta\text{lacAYZ} \Delta\text{lacI} \text{ pECJ3}$	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) $D = 0.5 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation = 1000 rpm $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$	2024-02-14_VIV_ToggleControl1 2024-05-09_VIV_ToggleControl2	This paper
Chemostat <i>E. coli</i> MG1655 $\Delta\text{lacAYZ} \Delta\text{lacI} \Delta\text{serA} \text{ pECJ3 (PLtetO-1::serA::GFP)}$	Induction : after 12h of continuous culture Inducer : medium with 0.1 mM IPTG	2024-03-13_VIV_ToggleTSG1 2024-03-27_VIV_ToggleTSG2	This paper
Segregostat <i>E. coli</i> MG1655 $P_{\text{ydcS}}::\text{GFPmut2}$	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) $D = 0.3 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation : 1000 & 1200 rpm $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$ Regulation threshold = 1000 & 1150 A.F.U. Regulation pulse = 1 g glucose (5.7 ml)	2024-09-11_FDE_Seg_Ecoli_ydcS 2025-01-29_MaD_Seg_Ecoli_ydcS	This paper
Segregostat <i>S. cerevisiae</i> CEN.PK 113-7D $P_{\text{GLC3}}::\text{eGFP}$	Bioreactor : Biostat B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) $D = 0.1 \text{ h}^{-1}$ $\text{pH} = 5$ $T = 30^\circ\text{C}$ Aeration = 1 L/min Agitation : 1000 rpm $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$ Regulation threshold = 5000 A.F.U. Regulation pulse = 0.4 g glucose	2020-11-09_FDE_Seg_Sacch 2021-04-15_FDE_Seg_Sacch	Henrion et al. (2023)
	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) $D = 0.1 \text{ h}^{-1}$ $\text{pH} = 5$ $T = 30^\circ\text{C}$ Aeration = 1 L/min Agitation : 1000 rpm $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$ Regulation threshold = 3000 A.F.U. Regulation pulse = 0.4 g glucose (5.7 ml)	2025-02-10_MaD_Seg_Sacch	This paper
Chemostat <i>E. coli</i> MG1655 $\text{PydcS}::\text{GFPmut2}$	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) $D = 0.6 \text{ \& } 0.1 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation = 1000 rpm $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$	2024-06-12_MaD-FDE_Chem_Ecoli_ydcS 2024-06-26_MaD_Chem_Ecoli_ydcS	This paper
	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) $D = 0.3 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation : 1000 $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$	2025-01-29_MaD_Chem_Ecoli_ydcS	This paper
Chemostat <i>S. cerevisiae</i> CEN.PK 113-7D $P_{\text{GLC3}}::\text{eGFP}$	Bioreactor : Dasgip (Eppendorf, Germany) Cytometer : BD Accuri C6 (BD Biosciences) $D = 0.6, 0.3 \text{ \& } 0.1 \text{ h}^{-1}$ $\text{pH} = 7$ $T = 37^\circ\text{C}$ Aeration = 1 L/min Agitation : 1000 $[\text{glucose}]_{\text{feed}} = 5 \text{ g/L}$	See the "Supp_EcoliStressReporterSelection" folder in provided data	This paper
	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) $D = 0.3 \text{ \& } 0.1 \text{ h}^{-1}$ $\text{pH} = 5$ $T = 30^\circ\text{C}$ Aeration = 1 L/min Agitation : 1000 rpm $[\text{glucose}]_{\text{feed}} = 7.5 \text{ g/L}$	2024-07-04_MaD_Chem_Sacch 2024-08-19_MaD_Chem_Sacch	This paper

non-growing phenotype, without and with IPTG added, respectively. Similarly, growth rate data related to YFP or CyOFP is irrelevant for CHAPTER 4. The two sets of growth rates are calculated from different measurement amplification intensities on the same experiment.

Annex C

**Mathematical and *In Silico* analysis:
biological oscillations without genetic
oscillator or external forcing**

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This Appendix is the supplementary material for CHAPTER 4, section 2, related to spontaneous oscillations in gene expression, adapted from:

[416] Vincent Vandenbroucke, Lucas Henrion, and Frank Delvigne. *Biological Oscillations without Genetic Oscillator or External Forcing*. Sept. 23, 2024. DOI: 10.1101/2024.09.20.614027. Pre-published

The following resources are associated to this publication:

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1. Stress model in continuous culture

This section describes the reasoning behind and the analytical solution for the model described in CHAPTER 4, section 2, where *Bacillus subtilis* is shown to have oscillations based on dilution rate in a chemostat. Instead of being limited to sporulation, the model is generalised for a stress response. Additional notes on the model are provided in SECTION 2, and a generalisation to a fed-batch culture (where a steady-state is possible during exponential feeding) is available in SECTION 3.

A chemostat is usually modelled with the following equations:

$$\frac{dS}{dt} = (S_{in} - S)D - \frac{\mu X}{Y} \quad (C.1)$$

$$\frac{dX}{dt} = (\mu - D)X \quad (C.2)$$

However, often, cells have a second state that can be induced when they are stressed (in the case described in CHAPTER 4, section 2, the switching of *B. subtilis* to spore

formation), in which case their growth rate decreases. We can represent those two states in a chemostat with the following equations, if we consider that the stressed state is irreversible and cannot go back to non-stressed. As the time required to dilute the stress proteins is quite long, and made longer by the lower growth rate caused by stress, switching to a stress state is much faster and this is a reasonable assumption for a simplified approach. That is without considering the fact that, in a chemostat with non-stressed cells, the stressed-cells would be overtaken slowly and, if not renewed, are likely to simply be washed out before they can go back to their non-stressed state. This general stress model can be reduced further in the case of *B. subtilis*, as spores do not grow at all and the growth rate of the stressed phenotype is zero ($k = 0$ below), but the general case is solved here.

$$\frac{dS}{dt} = (S_{in} - S)D - \frac{\mu X_1}{Y_{X1}} - \frac{k\mu X_2}{Y_{X2}} \quad (C.3)$$

$$\frac{dX_1}{dt} = (\mu - D)X_1 - H_1(S)X_1 \quad (C.4)$$

$$\frac{dX_2}{dt} = (k\mu - D)X_2 + H_1(S)X_1 \quad (C.5)$$

$$\mu = \mu_{max} \frac{S}{K_S + S} \quad (C.6)$$

$$\mu_{max,1} = \mu_{max} \quad (C.7)$$

$$\mu_{max,2} = k\mu_{max} \quad (C.8)$$

$$H_1(S) = \begin{cases} H & \text{if } S \leq K_I \\ 0 & \text{if } S > K_I \end{cases} \quad (C.9)$$

Where the variables are defined as:

X_1	the concentration of the fast growing phenotype in the reactor. (in the case of <i>B. subtilis</i> , vegetative, non-stressed cells)
X_2	the concentration of the slow growing phenotype in the reactor. (in the case of <i>B. subtilis</i> , dormant spores)
Y_{X1}	the yield of the fast growing phenotype.
Y_{X2}	the yield of the slow growing phenotype.
D	the dilution rate.
k	the ratio of the growth rate of the slow growing phenotype to the growth rate of the fast growing phenotype.
K_I	the threshold substrate concentration for the switch.
K_S	the half-saturation constant of the Monod equation.
H	the switching rate of the fast growing to the slow growing phenotype.
$H_1(S)$	the switch function from the fast growing to the slow growing phenotype.
μ	the instantaneous growth rate of the fast growing phenotype.
μ_{max}	the maximum growth rate of the fast growing phenotype.
S	the substrate (usually glucose) concentration in the reactor.
S_{in}	the substrate concentration in the feed.
t	the time.

We can note the following conditions on the parameters based on the parameters:

- All variables are positive due to the constraints of reality and considering growing phenotypes.
- $D, Y_{Xi}, \mu_{max}, K_S, K_I$ and H are strictly positive.

Considering those equations for a chemostat, the question that now occurs is: What happens at steady state?

Due to the switch to a stress conditions, we have two kinds of steady-state to consider: ones where the substrate concentration is above the threshold K_I , and ones where the substrate concentration is below the threshold K_I . Separating those cases let us replace $H_1(S)$ by H or 0 in the equations, greatly simplifying the system. Note that by definition, the steady-state requires the residual substrate concentration S to be constant, therefore all the possible steady-state can be found by splitting the equations this way.

To jump to the solution of the steady-state, go to SECTION 1.3.

1.1. When $S > K_I$

In this case, at steady-state (derivatives are null), there is no switching and we can replace EQUATIONS C.3 to C.5 by the following:

$$0 = (S_{in} - S)D - \frac{\mu X_1}{Y_{X1}} - \frac{k\mu X_2}{Y_{X2}} \quad (C.10)$$

$$0 = (\mu - D)X_1 \quad (C.11)$$

$$0 = (k\mu - D)X_2 \quad (C.12)$$

According to the latter 2 equations, there are 4 possible cases:

1. $X_1 = 0$ and $X_2 = 0$.
2. $X_1 = 0$ and $D = k\mu$.
3. $X_2 = 0$ and $D = \mu$.
4. If $k = 1$, $D = \mu$

These are the expected steady-states and show already known results: in the absence of switching, at steady-state, either the two populations are washed out or not initially present (case 1); and there is a marginally stable case where if both phenotypes have exactly equal growth rates, they can both survive, in any proportion as they behave as a single population and this whole model becomes irrelevant (case 4).

For the sake of this argument, we can consider $k \neq 1$ as that is a trivial and uninteresting case.

Both phenotypes cannot coexist in this case if $k \neq 1$, as one existing, in the absence of switching, imposes a specific dilution rate that implies, at steady-state, the other no being there.

The last case, a wash-out of both/either phenotype, will always happen for a population if $(\mu - D) = 0$ is impossible. To show when it is forced, finding out when it is possible is the easiest way.

Then, re-writing equations EQUATIONS C.10 to C.12 for a steady-state with a single phenotype:

$$(S_{in} - S)D = \frac{X_i}{Y_{Xi}} \mu_{max,i} \frac{S}{K_S + S} \quad (C.13)$$

$$\mu_{max,i} \frac{S}{K_S + S} = D \quad (C.14)$$

where i indicates either phenotype, whichever is present since we established that in this case only one can be present.

Since Y_{Xi} , D and K_S are strictly positive, we can deduce:

$$\mu_{max,i} \frac{S}{K_S + S} = D \quad \Rightarrow \quad \frac{D}{\mu_{max,i}} = \frac{S}{K_S + S} \quad (C.15)$$

$$S_{in} - S = \frac{X_i}{Y_{Xi}} \quad \Rightarrow \quad S = S_{in} - \frac{X_i}{Y_{Xi}} \quad (C.16)$$

Therefore:

$$\begin{aligned} \frac{D}{\mu_{max,i}} &= \frac{S}{K_S + S} \\ &= 1 - \frac{K_S}{K_S + S} \\ \Leftrightarrow \frac{K_S}{K_S + S} &= 1 - \frac{D}{\mu_{max,i}} \\ &= \frac{\mu_{max,i} - D}{\mu_{max,i}} \\ \Leftrightarrow K_S + S &= \frac{\mu_{max,i} K_S}{\mu_{max,i} - D} \\ \Leftrightarrow S &= \frac{\mu_{max,i} K_S}{\mu_{max,i} - D} - K_S \\ &= \frac{DK_S}{\mu_{max,i} - D} \end{aligned} \quad (C.17)$$

Plugging EQUATION C.17 into EQUATION C.16 and solving for X_i gives:

$$\begin{aligned} S_{in} - \frac{DK_S}{\mu_{max,i} - D} &= \frac{X_i}{Y_{Xi}} \\ X_i &= Y_{Xi} \left(S_{in} - \frac{DK_S}{\mu_{max,i} - D} \right) \end{aligned} \quad (C.18)$$

Thus, eventually, from EQUATIONS C.15 to C.18 we get:

$$\begin{cases} D &= \mu_{max,i} \frac{S}{K_S + S} \\ S &= \frac{DK_S}{\mu_{max,i} - D} = S_{in} - \frac{X_i}{Y_{Xi}} \\ X_i &= Y_{Xi} \left(S_{in} - \frac{DK_S}{\mu_{max,i} - D} \right) = Y_{Xi}(S_{in} - S) \end{cases} \quad (C.19)$$

at steady states, for cases 2 and 3. And since $S \geq 0$ and $X_i \geq 0$, for this to be possible, we need:

- $\mu_{max,i} > D$ for $S \geq 0$. When a given phenotype is initially present, a wash-out and no biomass is leftover at steady-state (it becomes case 1) if the maximum growth rate of the phenotype is lower than the dilution rate (as expected).
- $S_{in} > DK_S/(\mu_{max,i} - D)$ for $X_i > 0$. This can be rearranged (as per standard chemostat equations) as $D < \mu_{max,i} S_{in}/(K_S + S_{in}) < \mu_{max,i}$, dilution rate above which there is no glucose consumption at equilibrium as there is not enough substrate to sustain the population.

Those equations and deductions are only valid when $S > K_I$, or:

$$\frac{DK_S}{\mu_{max,i} - D} > K_I \quad (\text{C.20})$$

Firstly, we established that, when there is no wash-out, and there is biomass at steady-state, $\mu_{max,i} > D$ is required. Therefore, the left hand-side of the inequality is always positive.

We can examine when transform the inequality to examine when it is possible or:

$$\begin{aligned} & \frac{DK_S}{\mu_{max,i} - D} > K_I \\ \Leftrightarrow & DK_S > K_I(\mu_{max,i} - D) && \text{since: } \mu_{max,i} > D \\ \Leftrightarrow & DK_S + DK_I > K_I\mu_{max,i} \\ \Leftrightarrow & D(K_S + K_I) > K_I\mu_{max,i} \\ \Leftrightarrow & D > \frac{K_I\mu_{max,i}}{K_S + K_I} && \text{since: } K_S, K_I > 0 \end{aligned} \quad (\text{C.21})$$

Therefore, steady-state without switching can be reached when $\mu_{max,i} S_{in}/(S_{in} + K_S) > D > \mu_{max,i} K_I/(K_S + K_I)$ for each phenotype if the three parameters allow (although, for the stressed phenotype, we are assuming that there is no switch back to the first one, which is probably wrong if waiting long enough in this high substrate concentration case).

As a summary of this section, we have for each case described at the start:

1. Wash out of all the cells initially present, when $D > \mu_{max,i} \frac{S_{in}}{(S_{in} + K_S)}$ for all i phenotypes non-zero in the initial conditions.
2. a steady state with only the second phenotype present, which could happen if all cells switched to the second phenotype, and never switched back, maybe in the

case of differentiation:

$$\begin{cases} X_1 = 0 \\ D = k\mu_{max} S / (K_S + S) \\ S = DK_S / (k\mu_{max} - D) = S_{in} - X_2 / Y_{X2} \\ X_2 = Y_{X2}(S_{in} - S) \\ 0 \leq S \leq S_{in} \\ k\mu_{max} S_{in} / (S_{in} + K_S) > D > k\mu_{max} K_I / (K_S + K_I) \end{cases} \quad (C.22)$$

3. A steady state with only the first phenotype present:

$$\begin{cases} X_2 = 0 \\ D = \mu_{max} S / (K_S + S) \\ S = DK_S / (\mu_{max} - D) = S_{in} - X_1 / Y_{X1} \\ X_1 = Y_{X1}(S_{in} - S) \\ 0 \leq S \leq S_{in} \\ \mu_{max} S_{in} / (S_{in} + K_S) > D > \mu_{max} K_I / (K_S + K_I) \end{cases} \quad (C.23)$$

1.2. When $S \leq K_I$

A similar reasoning can be applied to the alternative case, where the switching always occurs.

At steady-state, we get:

$$0 = (S_{in} - S)D - \frac{\mu X_1}{Y_{X1}} - \frac{k\mu X_2}{Y_{X2}} \quad (C.24)$$

$$0 = (\mu - D - H)X_1 \quad (C.25)$$

$$0 = (k\mu - D)X_2 + HX_1 \quad (C.26)$$

The equations can be solved easily when $X_1 = 0$ and $X_2 = 0$, as this means there is no biomass. Notably, this case is incompatible with the assumption that there is switching regardless as $S_{in} = S$ in that case, and in a bioreactor $S_{in} > K_I$, so we come back to the previous section.

In case $X_1 = 0$, the equations become identical to the previous section. The only difference is the condition of existence derived at the end, which becomes $D < \mu_{max,i} K_I / (K_S + K_I)$ instead. This completes all cases where only the second phenotype is present with a region where it is actually likely that all cells stay in the second phenotype, as the substrate concentration would be too low to permit switching back to the first phenotype.

Thus the only case of interest to discuss here is when both X_1 and X_2 are non-zero. Re-arranging EQUATIONS C.24 to C.26 with those assumptions:

$$(S_{in} - S)D = \frac{\mu X_1}{Y_{X1}} - \frac{k\mu X_2}{Y_{X2}} \quad (C.27)$$

$$\mu = \mu_{max} \frac{S}{K_S + S} = D + H \quad (C.28)$$

$$(D - k\mu)X_2 = HX_1 \quad (C.29)$$

Interestingly, EQUATION C.28 implies that the first phenotype, through D , H , μ_{max} and K_S , fully determines the substrate concentration, with only H differing from a normal chemostat and no direct influence of the phenotype ratio in the culture.

Based on EQUATION C.28, we can deduce:

$$\begin{aligned} S &= \frac{(K_S + S)(D + H)}{\mu_{max}} \\ \Leftrightarrow S \left(1 - \frac{D + H}{\mu_{max}}\right) &= K_S \frac{D + H}{\mu_{max}} \\ S \frac{\mu_{max} - D - H}{\mu_{max}} &= \\ \Leftrightarrow S &= \frac{K_S(D + H)}{\mu_{max} - D - H} \end{aligned} \quad (C.30)$$

EQUATION C.29 can easily be re-arranged into:

$$X_1 = \frac{D - k\mu}{H} X_2 \Leftrightarrow X_2 = \frac{H}{D - k\mu} X_1 \quad (C.31)$$

From there, we can plug into the remaining EQUATION C.27:

$$\begin{aligned} (S_{in} - S)D &= \frac{\mu X_1}{Y_{X1}} + \frac{k\mu}{Y_{X2}} \frac{H}{D - k\mu} X_2 \\ \Leftrightarrow X_1 &= \frac{(S_{in} - S)D}{\frac{\mu}{Y_{X1}} + \frac{k\mu H}{Y_{X2}(D - k\mu)}} \end{aligned} \quad (C.32)$$

$$\begin{aligned} \text{and} \quad (S_{in} - S)D &= \frac{(D - k\mu)\mu}{HY_{X1}} X_2 + \frac{k\mu X_2}{Y_{X2}} \\ \Leftrightarrow X_2 &= \frac{(S_{in} - S)D}{\mu \left(\frac{(D - k\mu)}{HY_{X1}} + \frac{k}{Y_{X2}} \right)} \end{aligned} \quad (C.33)$$

We can thus write the solution for this case of the steady-state as:

$$\begin{cases} S &= \frac{K_S(D+H)}{\mu_{max}-D-H} \\ \mu &= \mu_{max} \frac{S}{K_S+S} = D + H \\ X_1 &= \frac{(S_{in}-S) D}{\mu/Y_{X1} + k\mu H/Y_{X2} (D - \mu k)} \\ X_2 &= \frac{(S_{in}-S) D}{\mu ((D - k\mu)/(HY_{X1}) + k/Y_{X2})} \\ \frac{X_1}{X_2} &= \frac{D - k\mu}{H} \end{cases} \quad (C.34)$$

For this solution to be possible, we need:

- $S > 0$
- $X_1 > 0$
- $X_2 > 0$

Since $X_1 = X_2^{(D - k\mu)/H}$, we need for both to be positive:

$$D > k\mu \quad (C.35)$$

Since $D + H = \mu$, we actually need:

$$\frac{D}{D + H} > k \quad (C.36)$$

Note that this condition can only be true if $k < 1$, as the left hand side is always smaller than 1. Thus, if the induced phenotype were to grow faster than the initial one, it would be impossible for the two phenotypes to coexist in the reactor. In this case, we are considering a stress phenotype, so $k < 1$ is a reasonable assumption.

In other musings, this condition means at its origin that the dilution rate will be faster than the instantaneous growth rate of the second phenotype.

The condition can be easily re-arranged in terms of D as well:

$$\frac{D}{D + H} > k \quad \Leftrightarrow \quad D > \frac{kH}{1 - k} \quad (C.37)$$

This ensures that both X_1 and X_2 have the same sign. To ensure they are positive, we need:

$$S_{in} > S \quad (C.38)$$

which is always true if cells grow.

Finally, we need for $S \geq 0$:

$$D < \mu_{max} - H \quad (\text{C.39})$$

The latter also implies that, for both phenotypes to coexist,

$$\mu_{max} > H \quad (\text{C.40})$$

is required.

Those conditions, combined with the one required by this section, $S \leq K_I$, are the only conditions required for the existence of a steady-state with both phenotypes present.

The latter condition can be developed:

$$\begin{aligned} & \frac{K_S(D+H)}{\mu_{max} - (D+H)} < K_I \\ \Leftrightarrow & K_S(D+H) < K_I (\mu_{max} - (D+H)) \quad \text{"<" sign unchanged since (C.39)} \\ \Leftrightarrow & DK_S + HK_S < \mu_{max} K_I - DK_I - HK_I \\ \Leftrightarrow & D(K_S + K_I) < \mu_{max} K_I - H(K_S + K_I) \\ \Leftrightarrow & D < \frac{\mu_{max} K_I}{K_S + K_I} - H \end{aligned} \quad (\text{C.41})$$

Similarly to condition C.40, we can deduce that

$$\mu_{max} \frac{K_I}{K_S + K_I} > H \quad (\text{C.42})$$

from the previous condition.

Note that condition C.41 is stronger than condition C.39, as:

$$\begin{aligned} & \mu_{max} - H > \frac{\mu_{max} K_I}{K_S + K_I} - H \\ \Leftrightarrow & \mu_{max} > \mu_{max} \frac{K_I}{K_S + K_I} \end{aligned} \quad (\text{C.43})$$

Inequation C.43 is always true, as $\frac{K_I}{(K_S + K_I)}$ is smaller than one, so condition C.39 is irrelevant. The deduced condition on the relationship is similarly stronger in (C.42) than in (C.40).

In summary, the steady-state with both phenotypes present is possible with the non-trivial conditions:

- $(\mu_{max} K_I) / (K_S + K_I) - H > D > kH / (1 - k)$
- $\mu_{max} K_I / (K_S + K_I) > H$

1.3. Solution of the steady-state

Overall, the steady-state of the cells can be described as a function of an increasing dilution rate:

$$\begin{aligned}
 X_2 \text{ only} : & \begin{cases} D < k\mu_{max}^{K_I/(K_S + K_I)} & \text{Only } X_2 \text{ is present,} \\ & \text{no induction} \\ k\mu_{max}^{K_I/(K_S + K_I)} < D < k\mu_{max}^{S_{in}/(K_S + S_{in})} & \text{Only } X_2 \text{ is present} \\ & \text{but } S > K_I \\ k\mu_{max}^{S_{in}/(K_S + S_{in})} < D & \text{Wash-out of } X_2 \text{ only} \end{cases} \\
 X_1 \text{ and } X_2 : & \begin{cases} kH/(1-k) < D < \mu_{max}^{K_I/(K_S + K_I)} - H & X_1/X_2 = (D - k\mu)/H \end{cases} \\
 X_1 \text{ only} : & \begin{cases} \mu_{max}^{K_I/(K_S + K_I)} < D < \mu_{max}^{S_{in}/(K_S + S_{in})} & \text{Only } X_1, \text{ no induction} \\ \mu_{max}^{S_{in}/(K_S + S_{in})} < D & \text{Wash-out} \end{cases}
 \end{aligned}$$

For X_2 only, while there are multiple cases, when those cases are overlapping with another region, then that other region is most likely to be reached instead: other regions include X_1 , and while our assumptions include that there is no switch from X_2 to X_1 , that assumption would likely prove wrong in the long term. In particular, the region where X_2 is the only phenotype present and $S > K_I$ (inhibiting the switch from X_2 to X_1) is probably not stable and thus we will not consider it further.

Interestingly, since no assumption can be made on the relative values of the different parameters, the different regions do not always define the entire range of dilution rates.

Between the regions where only X_2 and both phenotypes are present, there may be a region without steady-state if $kH/(1-k) > k\mu_{max}^{K_I/(K_S + K_I)}$. Between those two thresholds of dilution rates, the substrate concentration would be too high to keep inducing the second phenotype.

Between the regions where only X_1 and both phenotypes are present, there may be a region without steady-state if $\mu_{max}^{K_I/(K_S + K_I)} > \max\{\mu_{max}^{K_I/(K_S + K_I)} - H, k \cdot (\mu_{max}^{K_I/(K_S + K_I)})\}$, where the second term of the maximum is in case the X_2 only region reaches beyond the cohabitation region. Whichever is the maximum, this inequality is always true (because of the $-H$ or because $k < 1$). Therefore, there is always a region without steady-state when decreasing the dilution rate and the stressed phenotype starts to be induced. This interval is the one discussed in CHAPTER 4, section 2.

In those regions without steady-state, any attempt to reach steady-state when $S > K_I$ causes the biomass to increase and S to decrease. When $S < K_I$, the cells start switching and substrate consumption decreases, causing S to increase above K_I again. Since cells do not respond instantaneously to change, and in this region where S is close

to K_I small changes in concentration can cause large changes in responses, the cells may oscillate between the two states.

2. Limitations of the model and additional notes

There are a few additional things that can be added on this model, in no particular order:

- While it was derived for a chemostat, it can be applied to an exponential fed-batch reactor as well (see SECTION 3). For a fed-batch with less than exponential feeding, the effective growth rate decreases over time. In other words, it would not be a question of whether the region without equilibrium is reached, but when. Then the second question is whether that will cause oscillations or not, which will depend on the exact biological system, and how long the cells stay in the problematic region.
- This model predicts that there is a region where there is no steady-state when there are sharp transitions in cell metabolism, such as with stress induction, or induction of burdensome production with lactose. Oscillations may appear thanks to the delay in response time of the cells, and very small changes in substrate concentration causing large changes in cell response. However, the switch is not a perfect step function, and the delay will be shorter or longer depending on the cells. Further, a constant switching speed may not be a good approximation, at least when close to the switching threshold. All in all, oscillations may appear, but they may be dampened, or the system may stabilise quickly in an intermediate state that this model is incapable of representing.
- In the demonstration, we considered what happens when $K_I < S$ and $K_I > S$. When $K_I = S$, the details of what happen in the model depend on how $H_1(S)$ was defined (here, $H_1(K_I) = H$, therefore the $K_I = S$ would be the limit of the $K_I < S$ case). In practice, however, reality is not completely discontinuous, and this is the edge of an undefined region regardless, so the mathematics of this particular fictional point do not really matter, only the limits of the region between cases does.
- We consider here that both phenotypes are growing, no matter how slowly. If the slow growing phenotype were to have a death rate instead ($k < 0$), several things would happen:
 - No region would exist where only the second phenotype can survive, as even a batch culture would eventually have no cells left. This is expressed by condition C.37 being always true.

- Nothing else would change, as in no place in SECTION 1.2 is there any division by k for any condition. We can therefore expect exactly the same dynamics and region of uncertainty as with $k \geq 0$.
- For a case such as the induction of metabolic burden with a second carbon source (such as the induction of BL21 with lactose (co-feeding of glucose and lactose), where oscillations were observed as well [7]), considering co-consumption of carbon sources, the inequations are inverted such that the set of equations C.19 is valid only when $D < \mu_{max}^{K_I/K_S + K_I}$, and the set C.34 is valid only when $\mu_{max} - H > D > \mu_{max}^{K_I/K_S + K_I} - H$ and $D > H^k/(1-k)$. Further, the case of only the second phenotype being present should be stable between $k\mu_{max}^{K_I/(K_S + K_I)}$ and $k\mu_{max}$. Therefore, a steady-state (or more than one) always exists at least as long as the induced phenotype is not washed out, and oscillations may only be an intermediate state that will eventually stabilise. Accounting for those oscillations would require investigation of the exact dynamics of the system of interest, and probably require taking into account carbon catabolite repression (When induced, cells stop growing, this increases both glucose and lactose concentrations. Increasing glucose concentration would inhibit induction by carbon catabolite repression, from which similar dynamics to the stress case would follow).

3. Exponential fed-batch

The traditional equations for a fed-batch reactor are:

$$\frac{d(SV)}{dt} = F(t) S_{in} - \frac{\mu XV}{Y} \quad (C.44)$$

$$\frac{d(XV)}{dt} = \mu XV \quad (C.45)$$

$$\frac{dV}{dt} = F(t) \quad (C.46)$$

Where $F(t)$ is the flow rate added to the reactor, and D is the expected growth rate of the cells. In case of exponential feeding, V and dV/dt are defined as:

$$V(t) = C_0 + C_1 e^{Dt} \quad (C.47)$$

$$\frac{dV}{dt} = C_1 D e^{Dt} \quad (C.48)$$

Where C_0 is the integration constant, so that $V(0) = V_0$ the initial volume, and C_1 modulates the state of the exponential. Traditionally, C_1 is defined as V_0 (see [417]),

which makes $C_0 = 0$ and simplifies the equations significantly. In Öztürk, Çalık, and Özdamar [418], however, the authors report a common value of $C_1 = V_0 X_0 / S_{in} Y$ for *B. subtilis*. We can note that, if the initial glucose concentration is equal to S_{in} , then we can approximate $C_1 \approx V_0$. Regardless of the exactitude of that approximation, for the fed-batch to make sense, we need $V_0 > C_0 > 0$. Therefore, since we are here interested in steady-states only, we can t_0 the original time of the reactor such that $V(t_0) = V_0$, which means that C_0 can arbitrarily set to 0 and the steady-states will be identical.

The equations for the fed-batch can be adapted like for the chemostat:

$$\frac{d(SV)}{dt} = F(t) S_{in} - \frac{\mu X_1 V}{Y_{X1}} - \frac{k\mu X_2 V}{Y_{X2}} \quad (C.49)$$

$$\frac{d(X_1 V)}{dt} = V X_1 \mu - H_1(S) X_1 V \quad (C.50)$$

$$\frac{d(X_2 V)}{dt} = V X_2 k\mu + H_1(S) X_1 V \quad (C.51)$$

$$\frac{dV}{dt} = F(t) = C_1 D e^{Dt} \quad (C.52)$$

$$V(t) = C_1 e^{Dt} \quad (C.53)$$

Those equations can be set back into concentrations by using the chain rule for $A = S, X_1, X_2$ in the following equation:

$$\frac{d(AV)}{dt} = \frac{dA}{dt} V + A \frac{dV}{dt} = V \frac{dA}{dt} + AF(t) \quad (C.54)$$

The equations become:

$$\frac{dS}{dt} = \frac{F(t)}{V} (S_{in} - S) - \frac{\mu X_1}{Y_{X1}} - \frac{k\mu X_2}{Y_{X2}} \quad (C.55)$$

$$\frac{dX_1}{dt} = \left(\mu - \frac{F(t)}{V} - H_1(S) \right) X_1 \quad (C.56)$$

$$\frac{dX_2}{dt} = \left(k\mu - \frac{F(t)}{V} \right) X_2 + H_1(S) X_1 \quad (C.57)$$

Since $F(t)/V = D$, these equation become exactly identical to a chemostat, and the same conclusions can be drawn.

4. Growth and switching parameter determination

A triplicate batch cultivation in the DASbox was performed to estimate μ_{max} and switching (K_I, H) parameters of our strain. To this end, each bioreactor was inoculated

with a OD of roughly 0.08 and samples were collected throughout the batch to assess the OD and the residual glucose concentration. The residual glucose concentration was determined using a D-glucose enzymatic assay kit from Megazyme in 98-well plates with a plate reader. During the batch, FC analysis was performed with our custom platform, the Segregostat, to observe when and at what speed cells were sporulating. The K_S was defined based on the analysis the residual glucose concentration at steady state at a high dilution rates (where cells are not sporulating) during the chemostats with varying dilution rates.

The following parameters were measured or estimated:

- $H = 1.1 \text{ h}^{-1} \rightarrow$ Average from 0.83, 1.2, and 1.3 with $r_2 = 0.98, 0.99$ and 0.99 respectively (computed by linear regression over the time there is switching)
- $K_S = 0.14 \text{ g} \cdot \text{L}^{-1} \rightarrow$ Based on the concentration at $D=0.32 \text{ h}^{-1}$, measured as 0.2761 and $0.2432 \text{ g} \cdot \text{L}^{-1}$. From $D = \mu_{max} S / (K_S + S)$, we get $K_S = \mu_{max} S / D - S$
- $K_I \in [0.01, 0.1] \text{ g} \cdot \text{L}^{-1} \rightarrow$ Explained below
- $\mu_{max} = 0.49 \text{ h}^{-1} \rightarrow$ Average from 0.52, 0.47 and 0.48 with $r^2 > 0.99$ for all three (computed by linear regression over the time of exponential growth, see code)
- $k = 0 \rightarrow$ Sporulating cells are considered non-growing

To determine the start of sporulation, we considered the time at which the measured optical density dropped. That drop likely corresponds to a change in morphology of the cells. For that stretch of time, samples were taken more often than the automated flow cytometry, providing a better time resolution, in addition to having no absolutely no cross-contamination (using the segregostat with our DASGIP in triplicate setup, a few percent leftover from each sample may be measured with the next sample), which is relevant when looking at very low percentages of induction. Based on these OD measurements, we considered the induction threshold was passed during the preceding measured time interval. This corresponds to 0.013 to $0.097 \text{ g} \cdot \text{L}^{-1}$, 0.009 to $0.068 \text{ g} \cdot \text{L}^{-1}$ and 0.010 to $0.282 \text{ g} \cdot \text{L}^{-1}$ for reactors 1, 2 and 3 respectively. Thus we estimated K_I to be in the range of 0.01 to $0.1 \text{ g} \cdot \text{L}^{-1}$, although considering the cells need time to switch, K_I 's order of magnitude is likely the higher end of the range.

Based on these measurements, we could compute the oscillations' range of dilution rates for *B. subtilis*. The lower end of the range is at $D = 0 \text{ h}^{-1}$, as $k = 0$ and $(\mu_{max} K_I) / (K_S + K_I) - H < 0$ ($\mu_{max} < H$). The upper end depends on K_I . Based on the estimated range of K_I , it should be between $(\mu_{max} K_I) / (K_S + K_I) \in [0.03, 0.2] \text{ h}^{-1}$. The first replicate of the experiment showcasing a range of dilution rates showed the start of the oscillations at $D = 0.17 \text{ h}^{-1}$, which would correspond to a K_I of $0.075 \text{ g} \cdot \text{L}^{-1}$.

5. Code and Data Analysis

Three different codes were used for CHAPTER 4, section 2, all are available in the supplementary dataset. First, a short Matlab code was used to simulate the system with a delay. Second, a data analysis Python script was used to estimate the parameters from the experimental data. Third, a small data analysis Python script was used to plot the experimental data. The Python data analysis relies on the previously published toolbox [265] at <https://gitlab.uliege.be/mipi/published-software/mbiomas-core>. All the datasets and related code used in this chapter are available at <https://doi.org/10.5281/zenodo.13803913>.

The exact script used are also available as supplementary material of the original publication [416].

Annex D

**Additional experiments: synthetic niches
enable co-culture bioprocessing but are
prone to mutational escape**

Contents

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This Appendix is the supplementary material for CHAPTER 5, adapted from:

[257] Vincent Vandenbroucke, Juan Andrés Martínez, Lucas Henrion, Andrew Zicler, Samuel Telek, Laurie Josselin, and Frank Delvigne. “Synthetic Niches Enable Coculture Bioprocessing but Are Prone To Mutational Escape”. In: *ACS Synthetic Biology* 15.5 (May 15, 2026). DOI: 10.1021/acssynbio.5c00940

The following resources are associated to this publication:

- doi.org/10.5281/zenodo.17663997
- gitlab.uliege.be/mipi/published-software/2025-syntheticniches

1. Experiments performed

TABLE D.1 reports the list of the experiments carried out for CHAPTER 5 and their main results. It is followed by additional practical information about some of these experiments where necessary, and results mentioned in the CHAPTER 5 concerning TOGGLE_{yellow}.

Table D.1: List of experiments performed for CHAPTER 5

Experiment	Figure	Strain and Culture device	Induction profile	Main results	Note (next page)	Relevant section in main text	
1 2022-09-26-serineSweep		<i>ΔserA E.coli</i> in Biolector	Culture with varying amounts of serine supplementation.	The final biomass is directly proportional to amount of serine provided.			
2 2023-07-22-inductionSweep		pECJ3 toggle in Biolector	Pre-culture without induction and culture with range of IPTG and Tc concentration.	Measured induction range for IPTG and Tc and verified no growth reduction with Tc at induction concentrations.	Note 1		
3 2023-07-09-TSGCharacterisation	5.2C	TOGGLE_green in Biolector	Induction with one inducer in pre-culture, no inducer in culture.	Measured growth rates of isolated phenotypes.			
4 2024-02-22-TSY2Characterisation	5.2E	TOGGLE_yellow in Biolector				1. Synthetic niches enable the selective control of the growth rate of microbial species involved in co-cultures	
5 2023-07-23-TSGCross-feeding	D.1A	TOGGLE_green in DasBox equivalent (triplicate)	Continuous culture with inducer towards growth. Once at steady-state, the culture was supplemented with <i>ΔserA E.coli</i> and the resulting strain ratio was monitored.	The TOGGLE_green strain did not meaningfully export nutrients for another strain to survive.	Note 2		
6 2024-07-25-TSY2Cross-feeding	D.1B	TOGGLE_yellow in DasBox equivalent (triplicate)		The TOGGLE_yellow strain enabled the growth of ~1-2% of <i>ΔserA E.coli</i> .			
7 2024-10-16-TSBBaselineNoise	5.3A	pECJ3 toggle in DasBox equivalent (triplicate)	Batch without induction, continuous culture overnight with induction 0.1 mM IPTG, then no more induction.	After the release of induction, at most 1.5% of the population changed state through noise.			
8 2023-10-26-TSGPulseTest	5.3B, D.2	TOGGLE_green in Bionet	Series of 3 0.2 mM IPTG pulses, then 3 0.1 mM pulses, spaced wide enough that the culture came back to its steady-state between pulses.	Individual pulses over time had reproducible effects, and there is no need for the second inducer.	Note 3	2. Auxotrophic pressure induces cell escape from the toggle switch	
9 2024-08-08-TSGPulses1		TOGGLE_green in Bionet	Series of 0.1 mM IPTG pulses, then series of 0.2 mM pulses.	Measured induction and growth parameters in continuous culture, and observed reduced effectiveness of biomass control during the second pulse series. For TOGGLE_yellow, mutations were checked and detected at the end of the culture.	Note 4	3. Implementation of synthetic niches facilitate parameter inference for simulating mono-culture dynamics And 4. Increasing selective/auxotrophic pressure leads to mutational escape	
10 2024-09-05-TSGPulses2	5.4, 5.5, D.3						
11 2024-07-11-TSY2Pulses1	D.4						
12 2024-10-02-TSY2Pulses2		TOGGLE_yellow in Bionet	Series of 15 ng·mL ⁻¹ pulses, then series of 30 ng·mL ⁻¹ pulses.		Note 5		
13 2025-01-30-TSGMutation	D.5	TOGGLE_green in Bionet	Continuous feed with i) no IPTG, ii) 0.1 mM IPTG, iii) no IPTG, and iv) 0.1 mM IPTG.	After the first loss and recovery of biomass, the <i>ΔtetR</i> mutation was detected, and the GFP was no longer regulated.	Note 6	4. Increasing selective/auxotrophic pressure leads to mutational escape	
14 2025-03-12-freeCoCulture	D.6	Co-culture in DasGIP (triplicate)	No inducer.	TOGGLE_yellow was, as expected based on growth rates, always the losing strain.	Note 7		
15 2025-05-21-ControlledCoCulture	5.6	Co-culture in DasGIP (triplicate)	Pulses of inducer based on control rule. After TOGGLE_yellow was completely lost with 0.1 mM pulses, pulse concentration was gradually increased.	Despite reasonably low induction concentration, the TOGGLE_green mutated and escaped control.		5. Mutational escape impairs control of co-cultures	

The following section contains additional practical information about the experiments from TABLE D.1 where necessary, and results mentioned in CHAPTER 5 concerning TOGGLE_{yellow}:

Note 1, BioLector experiments: The first BioLector experiments were used to ensure the strains behaved as expected. In particular, the induction range of IPTG and tetracycline (Tc) were determined with the 2023-07-22-inductionSweep experiment.

Note 2, 2023-07-23-TSGCross-feeding, 2024-07-25-TSY2Cross-feeding: One potential concern when there are phenotypes that cannot produce an amino acid, is cross-feeding from producing cells towards non-producing. Serine is the basis of all amino acid metabolism in *Escherichia coli*. Therefore, if the non-producing cells can scavenge serine, other amino acids, or cell debris produced by the growing phenotype in the culture, it could allow the non-growing phenotype to grow as well. To ensure that no cross-feeding effectively happened, growing cultures of TOGGLE_{green} and TOGGLE_{yellow} were supplemented with a $\Delta serA$ *E. coli* without plasmid. If there is any cross-feeding, then the $\Delta serA$ strain should be able to survive in the culture. To get a worst case scenario, the continuous culture was supplemented with 25 ng·mL⁻¹ Tc for TOGGLE_{green} or 0.1 mM IPTG for TOGGLE_{yellow}. Precultures in minimal medium with 1 g·L⁻¹ serine (an excess of serine) were added to reach about 5% of the total events, enough to be measurable and credibly survive for long enough in case of cross-feeding, but not enough to meaningfully alter bioreactor concentrations in serine or other metabolites. For TOGGLE_{green}, no $\Delta serA$ survived, while for TOGGLE_{yellow}, 1 to 2% of $\Delta serA$ were sustained, indicating that, under normal conditions, the non-growing phenotypes should be nearly totally eliminated from the culture (FIGURE D.1).

Note 3, 2023-10-26-TSGPulseTest: For 0.2 mM pulses, there was a small effect on biomass for each pulse and the fluorescence took longer than the biomass to come back to normal. Pulses were spaced 14.5 h apart. For 0.1 mM pulses, a single pulse was not enough to affect biomass. Pulses were spaced 12.5 h apart. A summary of the experiment is presented in FIGURE D.2.

Note 4, 2024-08-08-TSGPulses1, 2024-09-05-TSGPulses2: As mentioned in CHAPTER 5, even when control is lost over GFP production, mCherry still responds to IPTG. This was observed in all experiments with TOGGLE_{green} and a loss of control (FIGURE D.3).

Note 5, 2024-07-11-TSY2Pulses1, 2024-10-02-TSY2Pulses2: A behaviour similar to TOGGLE_{green} was observed when pulsing Tc for TOGGLE_{yellow}. However, the concentrations of Tc employed were lower in the induction curve so that only

a fraction of TOGGLE_yellow switched to the non-growing phenotype. When the induction pressure was high enough for all cells to switch, all cells switched to the non-growing phenotype, quickly switched back to growing, and stopped responding to tetracycline. Towards the end of the culture, one culture exhibited a population with constitutive YFP and CyOFP expression (depicted in FIGURE D.4, while the other exhibited a small non-fluorescent population, which prompted the sequencing and revealed diverse mutations).

Note 6, 2025-01-30-TSGMutation: Based on the results with TOGGLE_yellow, we wanted to check for mutations with TOGGLE_green. An experiment with constant 0.1 mM induction (IPTG in the feed) showed initial wash-out, then recovery of the population (FIGURE D.5). The resulting cell population still responded to IPTG by modulating mCherry production, but not by modulating GFP, just like the reduced sensitivity in the previous repeated pulse experiments with the strain. Sequencing revealed the *tetR* deletion.

Note 7, 2025-03-12-freeCoCulture: A basic co-culture without induction was first performed, to verify that the expected strain was winning. As shown in FIGURE D.6, TOGGLE_yellow is outcompeted in the co-culture, as expected due to their lower growth rate.

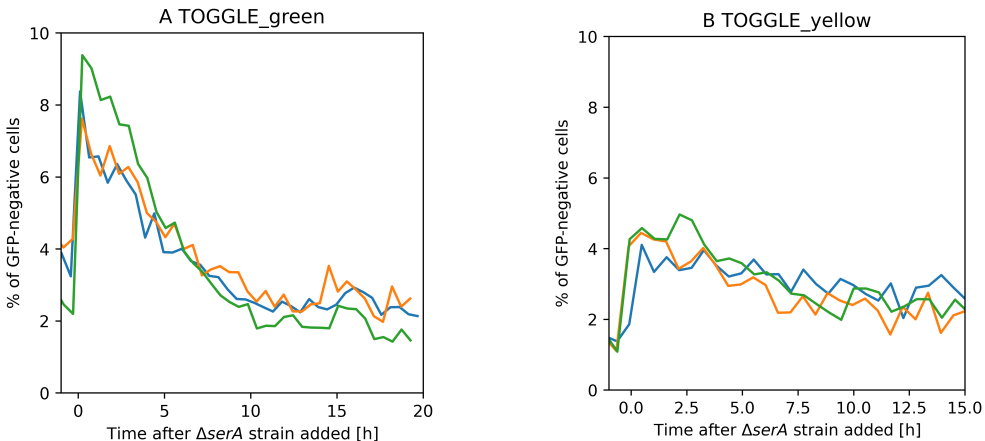


Figure D.1: Evolution of GFP-negative population after injecting $\Delta serA$ *E.coli* in a TOGGLE_green (A) or TOGGLE_yellow (B) culture to test for cross-feeding, in triplicate (experiments 5 and 6 in TABLE D.1). A The measurements are reduced to background noise indicating that $\Delta serA$ *E.coli* totally disappears from the culture. B The measurements get to a level slightly higher than background noise, indicating small amounts of cross-feeding.

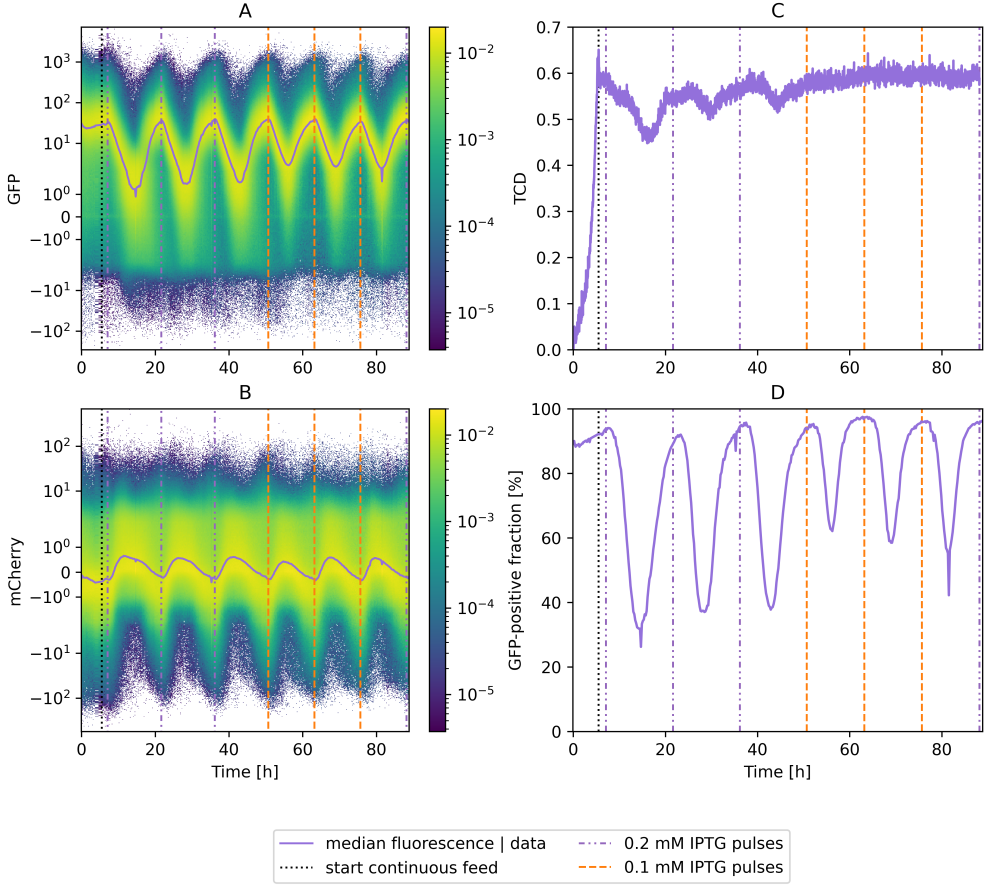


Figure D.2: Summary of the 2023-10-26-TSGPulseTest experiment (row 8 in TABLE D.1), referenced in CHAPTER 5, section 1.3.2, and zoomed in in FIGURE 5.3B. **A** Evolution of GFP over time, the line indicates the median fluorescence. Light yellow indicates a high proportion of cells at a given time and fluorescence, dark blue indicates a low proportion. **B** Similarly, evolution of mCherry over time. **C** Measured cell density over time (from Hamilton Dencyttee probes, $0.5 \text{ TCD} \approx 5 \text{ OD}$). **D** Percentage of GFP-positive (growing) cells over time.

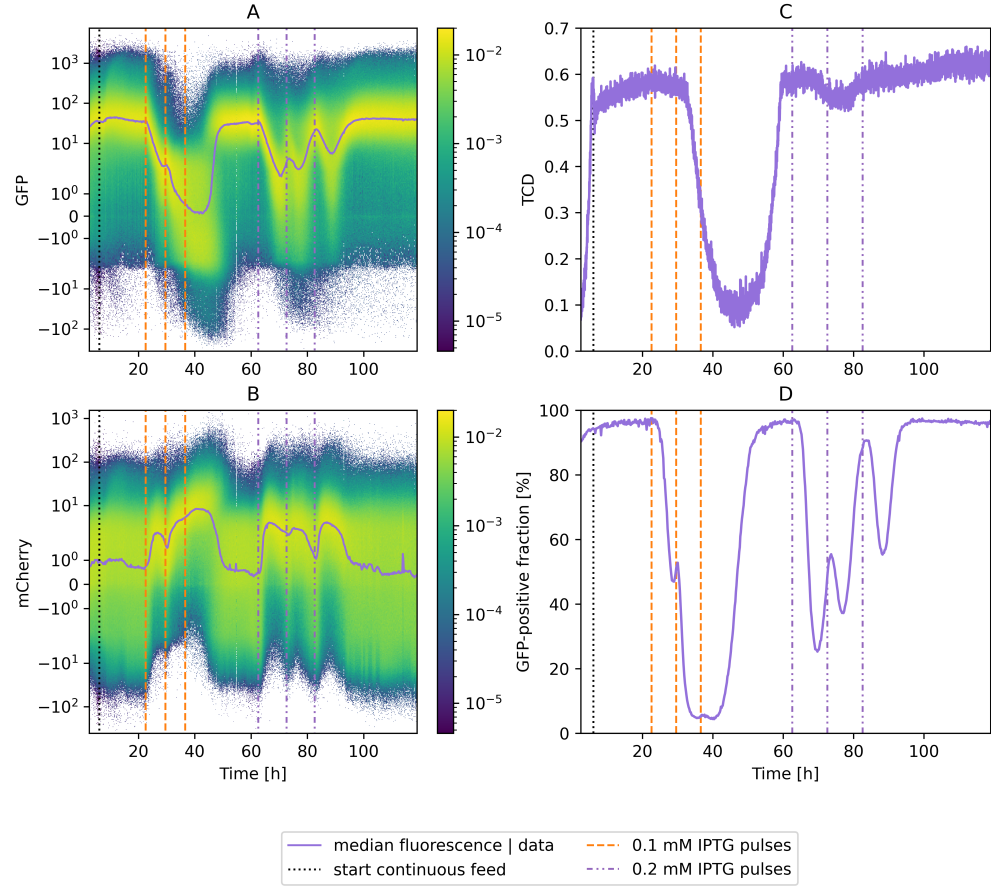


Figure D.3: Summary of the 2024-09-05-TSGPulses2 experiment (row 10 in TABLE D.1). Figure referenced in CHAPTER 5, section 1.3.4. **A** Evolution of GFP over time, the line indicates the median fluorescence. Light yellow indicates a high proportion of cells at a given time and fluorescence, dark blue indicates a low proportion. **B** Similarly, evolution of mCherry over time. **C** Measured cell density over time (from Hamilton Dencytte probes, $0.5 \text{ TCD} \approx 5 \text{ OD}$). **D** Percentage of GFP-positive (growing) cells over time.

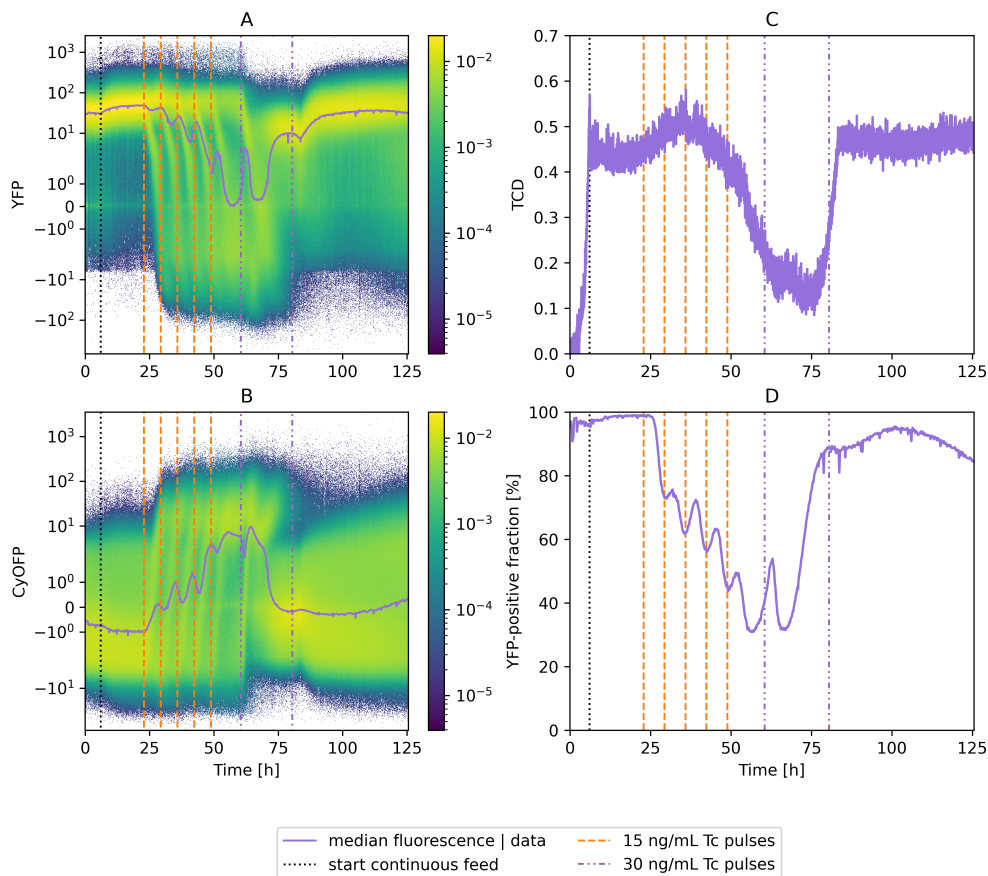


Figure D.4: Summary of the 2024-07-11-TSY2Pulses1 experiment (row 11 in TABLE D.1), mentioned in CHAPTER 5, section 1.3.4. **A** Evolution of YFP over time, the line indicates the median fluorescence. Light yellow indicates a high proportion of cells at a given time and fluorescence, dark blue indicates a low proportion. **B** Similarly, evolution of CyOFP over time. Unlike TOGGLE_green, TOGGLE_yellow stopped responding to high concentrations of inducers, including for CyOFP. After the second pulse of 30 ng·mL⁻¹ Tc, a population exhibiting both and fluorescent proteins appeared in this experiment, causing a gradual increase in the CyOFP values. **C** Measured cell density over time (from Hamilton Dencytte probes, 0.5 TCD≈5 OD). **D** Percentage of YFP-positive (growing) cells over time.

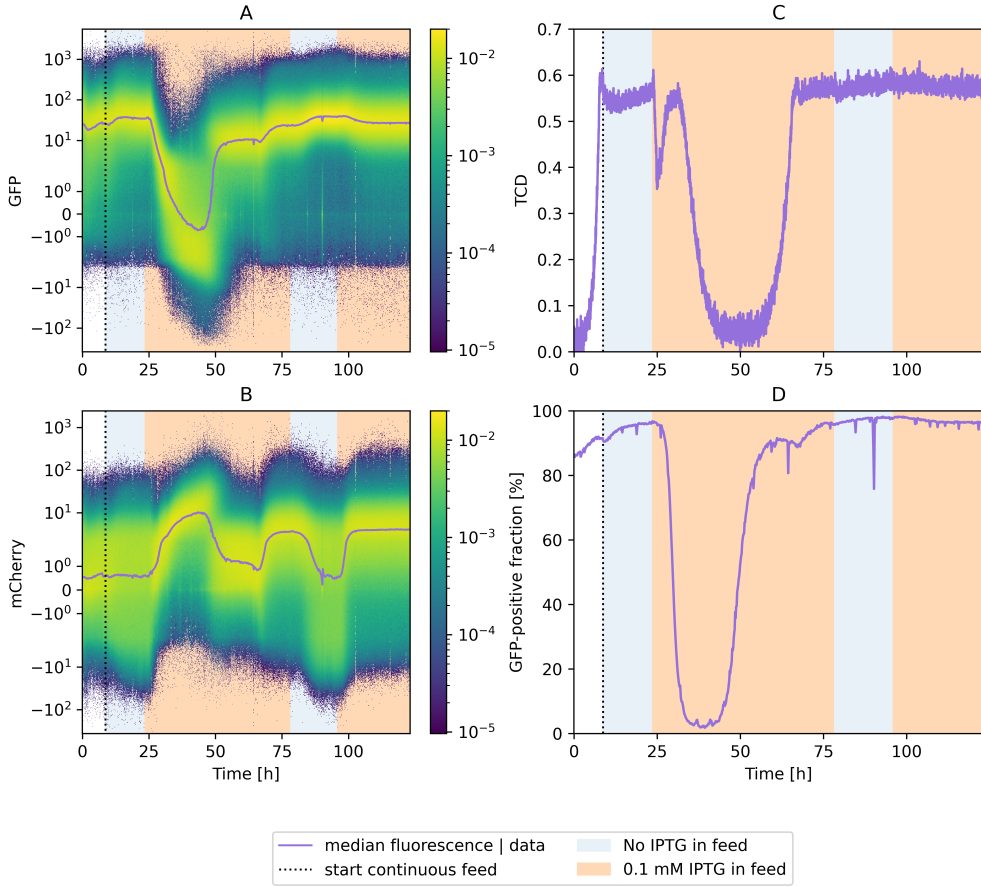


Figure D.5: Summary of the 2025-01-30-TSGMutation experiment (row 13 in TABLE D.1), mentioned in CHAPTER 5, section 1.3.4. After a loss of biomass until 50 h of experiments, cells recover both growth capability and GFP expression, and a second induction period shows no loss of growth capacity. **A** Evolution of GFP over time, the line indicates the median fluorescence. Light yellow indicates a high proportion of cells at a given time and fluorescence, dark blue indicates a low proportion. **B** Similarly, evolution of mCherry over time. **C** Measured cell density over time (from Hamilton Dencytte probes, $0.5 \text{ TCD} \approx 5 \text{ OD}$). Due to a technical error, the first hour with IPTG did not receive any carbon, hence the initial loss of biomass. **D** Percentage of GFP-positive (growing) cells over time.

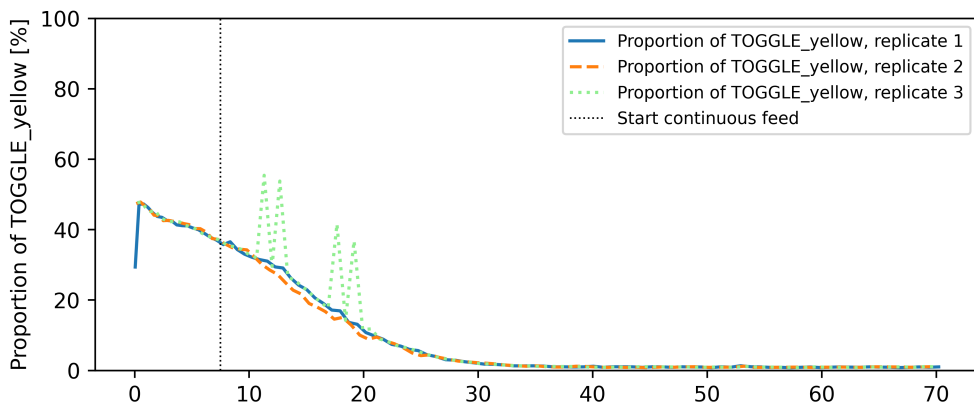


Figure D.6: Proportion of TOGGLE_yellow in three co-cultures without inducer (2025-03-12-freeCoCulture, experiment 14 in TABLE D.1).

2. Supplementary file list

The supplementary files available at doi.org/10.5281/zenodo.17663997 contain the following documents:

- `genetic_constrcuts.7z` contains the sequences obtained for the TOGGLE_green and TOGGLE_yellow plasmids, as well as the sequences for the modified genomic DNA regions $\Delta lacAYZI$ and $\Delta serA$ in *E.coli* MG1655.
- `cytometer_calibration.7z` contains the fcs files used for calibration of fluorophores and differences between cytometers.
- Each `experiment_*.7z` file contains the corresponding experiment data, as well as associated notes and plots relevant to understand it.
- `mut_sequences.7z` contains the sequences obtained when checking for mutations.

All the relevant code for data analysis is available at <https://gitlab.uliege.be/mipi/published-software/2025-syntheticniches>.

Annex E

Overview of publications

- (First author) [257] Vincent Vandenbroucke, Juan Andrés Martínez, Lucas Henrion, Andrew Zicler, Samuel Telek, Laurie Josselin, and Frank Delvigne. “Synthetic Niches Enable Coculture Bioprocessing but Are Prone To Mutational Escape”. In: *ACS Synthetic Biology* 15.5 (May 15, 2026). doi: 10.1021/acssynbio.5c00940
- (Preprint, co-first author) [179] Mathéo Delvenne, Vincent Vandenbroucke, Lucas Henrion, Maximilian Sehr, Juan A. Martínez, Alizée Sloodts, Samuel Telek, Andrew Zicler, and Frank Delvigne. *A Fitness–Entropy Compensation Effect Set the Trade-off between Growth and Gene Expression in Cell Populations*. July 9, 2025. doi: 10.1101/2025.07.05.663304. Pre-published
- [419] Pedro Pessoa, Juan Andres Martinez, Vincent Vandenbroucke, Frank Delvigne, and Steve Pressé. “Simulation-Based Inference Captures Non-Markovian Effects as Exemplified in Protein Production Kinetics through Cell Division”. In: *Proceedings of the National Academy of Sciences* 123.15 (Apr. 14, 2026). doi: 10.1073/pnas.2517309123
- (Preprint) [180] Lucas Henrion, Vincent Vandenbroucke, Juan Andres Martinez, Julian Kopp, Samuel Telek, Andrew Zicler, and Frank Delvigne. *Lowering the Switching Cost Related to the Activation of Burdensome Gene Circuits Promotes Cell Population Homogeneity and Productivity*. Oct. 16, 2024. doi: 10.1101/2024.10.14.618176. Pre-published
- (Co-first author) [232] Vincent Vandenbroucke, Lucas Henrion, and Frank Delvigne. “Biological Oscillations without Intracellular Oscillator or External Forcing”. In: *npj Systems Biology and Applications* (Aug. 5, 2026). doi: 10.1038/s41540-026-00801-4
- [420] Alvaro R. Lara, Flavio Kunert, Vincent Vandenbroucke, Hilal Taymaz-Nikerel, Luz María Martínez, Juan-Carlos Sigala, Frank Delvigne, Guillermo Gosset, and Jochen Büchs. “Transport-controlled Growth Decoupling for Self-induced Protein Expression with a Glycerol-repressible Genetic Circuit”. In: *Biotechnology and Bioengineering* 121.6 (June 2024). doi: 10.1002/bit.28697
- [7] Lucas Henrion, Juan Andres Martinez, Vincent Vandenbroucke, Mathéo Delvenne, Samuel Telek, Andrew Zicler, Alexander Grünberger, and Frank Delvigne. “Fitness Cost Associated with Cell Phenotypic Switching Drives Population Diversification Dynamics and Controllability”. In: *Nature Communications* 14.1 (Oct. 2, 2023). doi: 10.1038/s41467-023-41917-z
- [421] Frank Delvigne, Lucas Henrion, Vincent Vandenbroucke, and Juan Andres Martinez. “Avoiding the All-or-None Response in Gene Expression During E.

Coli Continuous Cultivation Based on the On-Line Monitoring of Cell Phenotypic Switching Dynamics”. In: *Inclusion Bodies*. Ed. by Julian Kopp and Oliver Spadiut. Vol. 2617. New York, NY: Springer US, 2023. ISBN: 978-1-07-162929-1 978-1-07-162930-7. DOI: 10.1007/978-1-0716-2930-7_7

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