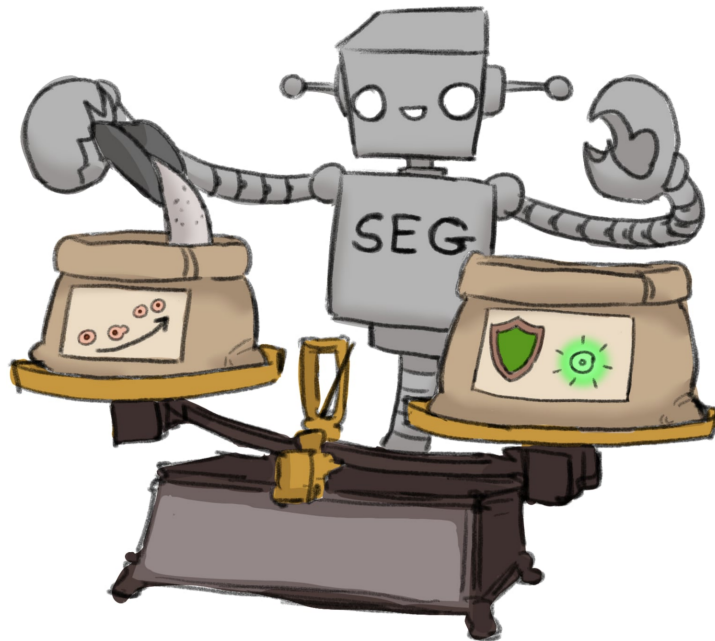


Dynamic control of the trade-off between growth and stress resistance in continuous microbial cultivations



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**DYNAMIC CONTROL OF THE TRADE-OFF
BETWEEN GROWTH AND STRESS RESISTANCE
IN CONTINUOUS MICROBIAL CULTIVATIONS**

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Abstract

Over billions of years of evolution, microbes have colonized nearly every corner of the biosphere. While long-term evolutionary adaptation explains their global success, thriving within a given niche often requires coping with short-term fluctuations. To optimize their fitness under such conditions, isogenic populations rely on phenotype adaptation and diversification. Although beneficial from an ecological perspective, the resulting phenotypic heterogeneity is generally undesirable in applications such as bioproduction, where it makes the process unpredictable and is presumed to contribute to instability and lower yields. Nowadays, microbial cell factories are increasingly employed for the sustainable production of target molecules. It is therefore crucial to better understand diversification dynamics and to develop strains, process designs, and control strategies that optimize microbial production.

Expanding on previous work from our team, we provide a deeper understanding of phenotypic diversification in continuous culture by addressing both its functionality and underlying mechanisms. Notably, we identified and described a fitness-entropy compensation (FEC) mechanism that offsets the activation of gene circuits that compromise survival. We also investigated and successfully applied the Segregostat, a real-time, flow-cytometry-based closed-loop control system, to regulate cell populations in a scale-down (SD) bioreactor mimicking the heterogeneous environmental conditions typical of industrial scale.

Throughout this thesis, we focused on the general stress response (GSR) to study and control phenotypic diversification. It constitutes a particularly relevant framework, as its activation entails trade-offs with growth due to resource redistribution, which influences diversification dynamics. In addition, the GSR represents an important target for bioprocess optimization. Using complementary single-cell approaches, i.e., flow cytometry (FC) and time-lapse microscopy in a microfluidic single-cell cultivation (MSCC) device, we investigated GSR dynamics and control under nutrient limitation in two of the most widely used model organisms, *Escherichia coli* and *Saccharomyces cerevisiae*.

Overall, our findings highlight that microbial populations, although expected to be homogeneous under chemostat conditions, are in fact shaped by continuous feedback between the cells and their environment, which influences their stability and phenotypic diversification. They also emphasize that aligning process conditions with the intrinsic dynamics of regulatory networks (for instance, through the use of the Segregostat) is key to maintaining cellular responsiveness and achieving homogeneous populations. These insights both advocate and pave the way for the development of more cell-centric strategies in bioprocess design and control.

Keywords: stress response, phenotypic heterogeneity, fitness, burden, bet hedging, population control, *Escherichia coli*, *Saccharomyces cerevisiae*, Segregostat, single-cell, automated flow cytometry, microfluidics, microscopy, fluorescent reporter, scale-down, large-scale, bioprocess.

Résumé

Au cours de milliards d'années d'évolution, les microorganismes ont colonisé presque tous les recoins de la biosphère. Si l'adaptation évolutive à long terme explique leur succès global, survivre et prospérer dans une niche donnée requiert souvent de faire face à des fluctuations environnementales à court terme. Pour optimiser leur fitness dans de telles conditions, les populations isogéniques reposent sur l'adaptation et la diversification phénotypique. Bien que bénéfique d'un point de vue écologique, l'hétérogénéité phénotypique qui en résulte est généralement indésirable dans les applications de bioproduction, car elle rend les procédés imprévisibles et est susceptible d'engendrer de l'instabilité ainsi qu'une baisse des rendements. Aujourd'hui, les "usines cellulaires" microbiennes sont de plus en plus utilisées pour la production durable de molécules d'intérêt. Il est donc essentiel de mieux comprendre les dynamiques de diversification et de développer des souches, des procédés et des stratégies de contrôle capables d'optimiser la production microbienne.

Dans la continuité des travaux précédents de notre équipe, nous proposons une analyse approfondie de la diversification phénotypique en culture continue, en abordant à la fois sa fonctionnalité et ses mécanismes. Nous avons notamment identifié et décrit un mécanisme de compensation fitness-entropie (FEC), qui contrebalance l'activation de circuits géniques compromettant la survie cellulaire. Nous avons également étudié et appliqué avec succès le Segregostat, un système de contrôle en boucle fermée basé sur la cytométrie en flux, afin de réguler des populations cellulaires dans un bioréacteur scale-down (SD) reproduisant les conditions hétérogènes typiques des procédés industriels.

Tout au long de cette thèse, afin d'étudier et de contrôler la diversification phénotypique, nous nous sommes focalisés sur la réponse générale au stress (GSR). Ce système est particulièrement pertinent, car son activation met en jeu une balance physiologique entre la croissance et la résistance au stress, liée à la redistribution des ressources, influençant ainsi la dynamique de diversification. Par ailleurs, la GSR représente une cible importante pour l'optimisation des bioprocédés. En combinant des approches monocellulaires complémentaires (à savoir, de la cytométrie en flux (FC) et de la microscopie en temps réel dans un dispositif microfluidique de culture monocellulaire (MSCC)), nous avons étudié la dynamique et le contrôle de la GSR en condition de limitation nutritive chez deux organismes modèles parmi les plus utilisés, *Escherichia coli* et *Saccharomyces cerevisiae*.

Dans l'ensemble, nos résultats mettent en évidence que les populations microbiennes, bien qu'attendues homogènes dans des conditions de chémostat, sont en réalité façonnées par des rétroactions continues entre les cellules et leur environnement, ce qui influence leur stabilité et leur diversification phénotypique. Ils soulignent également que l'adaptation des conditions de culture aux dynamiques intrinsèques des réseaux de régulation et de l'expression des gènes (par exemple, via l'utilisation

du Segregostat) est essentielle pour maintenir la réponse cellulaire et obtenir des populations homogènes. Ces travaux soulignent la nécessité d'adopter une approche davantage centrée sur la cellule dans la conception et le contrôle des bioprocédés.

Mots-clés : réponse au stress, hétérogénéité phénotypique, fitness, fardeau, pari diversificationnel, contrôle de population, *Escherichia coli*, *Saccharomyces cerevisiae*, Segregostat, analyses monocellulaire, cytométrie en flux automatisée, microfluidique, microscopie, rapporteur fluorescent, scale-down, procédés à grande échelle, bioprocédés.

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The idea of pursuing a PhD had actually started to grow during one of his third-year undergraduate courses, which I attended together with Lucas, a friend who was just as enthusiastic about the topic as I was. At the time, we jokingly told ourselves: “*One day, we’ll do a PhD in Prof. Delvigne’s team and have a coffee machine between our desks!*” By the end of my master’s degree, I was preparing to leave academia, but Lucas, who had already started his PhD, together with Frank, convinced me to join them. I am deeply grateful for that, because it allowed me to rediscover my enthusiasm for experimental work and to experience the unique scientific and human adventure that a PhD represents. In the end, we did share an office (and our faithful “writing assistant”, the coffee machine) for several years, and I am certain that our friendship will continue far beyond the PhD.

Although a PhD is ultimately an individual achievement, research and laboratory life are deeply collective endeavors. For this reason, I would like to thank all the former and current members of Frank’s team whom I had the pleasure of working and interacting with : Alizée, Andrew, Constance, Dingrong, Fabian the first, Fabian the second, Fabian the third, Fanny, Georges, The Ghazal, Hannah, Huan, Juan Andrés, “La Puissance”, Laurie, Lucas, Max, Mélanie, Mika, Nathan, Romain, Sam, Satoshi, Thai, Tiphaine, Vincent, and obviously Frank.

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List of acronyms

Note: For consistency across organisms, gene names are written in *italics* and protein names in roman type with an initial capital letter.

σ^{24}	RpoE, sigma factor controlling extracytoplasmic stress response.
σ^{32}	RpoH, sigma factor for the heat shock response.
σ^{54}	RpoN, sigma factor controlling the expression of nitrogen-related genes.
σ^{70}	RpoD, vegetative sigma factor.
σ^S	RpoS, sigma factor for the general stress response (also termed σ^{38}).
A.U.	arbitrary units (also, a.u.).
<i>ada</i>	gene coding for the “adaptive response to alkylation damage” DNA-binding transcriptional dual regulator and DNA repair protein.
AFC	automated flow cytometry.
<i>appC</i>	gene coding for the subunit I of cytochrome <i>bd</i> -II.
<i>araB</i>	gene coding for the ribulokinase, that catalyzes the second step in the arabinose degradation pathway.
<i>araBADefGH</i>	operon arabinose.
AraC	DNA-binding transcriptional dual regulator of the arabinose operon.
aTc	anhydrotétracycline.
Bcy1	“bypass of cyclic AMP requirement” protein, regulatory subunit of PKA.
<i>bolA</i>	gene coding for a DNA-binding transcriptional dual regulator involved in the GSR.

bp	base pair.
cAMP	cyclic adenosine monophosphate.
cas9	CRISPR associated protein 9.
<i>cbpA</i>	gene coding for the “curved DNA-binding protein”.
CcaS/R	light-controlled two-component system formed by the sensor histidine kinase (CcaS) together with its cognate response regulator (CcaR).
CER	core environmental response.
CFD	computational fluid dynamics.
CHO	chinese hamster ovary.
<i>cln2</i>	gene coding for the “cyclin 2” protein.
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats.
CRP	catabolic response protein.
<i>csiE</i>	gene coding for a stationary phase-inducible protein whose physiological role is not clearly defined.
CSR	core stress response.
<i>cyaA</i>	gene coding for the adenylate cyclase enzyme, which catalyzes cAMP synthesis.
CyOFP	cyan-excitable orange fluorescent protein.
Cyr1	“cyclic AMP requirement” protein, adenylate cyclase.
<i>cyr1</i>	gene coding for the adenylate cyclase Cyr1.
d2EYFP	destabilized variant of the enhanced yellow fluorescent protein.
dMSCC	dynamic microfluidic single-cell cultivation.
DNA	deoxyribonucleic acid.
$E\sigma^S$	RpoS-containing RNAP holoenzyme.
EDTA	ethylenediaminetetraacetic acid.
eGFP	“enhanced green fluorescent protein”, variant of GFP used with <i>S. cerevisiae</i> in this work.

<i>elaB</i>	gene coding for a tail-anchored inner membrane protein, implicated in stress-related translation modulation.
ESR	environmental stress response.
<i>evgA</i>	gene coding for a DNA-binding transcriptional activator involved in multi-drug resistance.
FACS	fluorescence-activated cell sorting.
FC	flow cytometry.
FEC	fitness-entropy compensation.
FL1-A	flow cytometry fluorescence channel 1 (area).
FL1-H	flow cytometry fluorescence channel 1 (height).
FLx	flow cytometry fluorescence channel x.
freq _{env}	frequency of environmental fluctuation.
freq _{switch}	frequency of phenotypic switching.
FRET	fluorescence resonance energy transfer.
FSC	forward scatter (in flow cytometry: FSC-A, area; FSC-H, height).
Fw	forward primer.
GadE	DNA-binding transcriptional activator involved in pH homeostasis and in the activation of the glutamate-dependent acid resistance system (GAD).
GadX	DNA-binding transcriptional dual regulator involved in the regulation of the glutamate-dependent acid resistance system (GAD).
<i>gadX</i>	gene coding for GadX.
<i>galI</i>	gene coding for the “galactose metabolism 1” galactokinase.
GFP	green fluorescent protein.
GFPmut2	variant of GFP used with <i>E. coli</i> in this work.
<i>glc3</i>	gene coding for the “glycogen 3” branching enzyme of glycogen.
<i>glgS</i>	synonym of <i>ScoR</i> , gene coding for the “surface composition regulator” protein that negatively regulates production of biofilm polysaccharides and flagellar motility (The name <i>glgS</i> reflects its initial presumed role in the regulation of glycogen synthesis).

glu	glucose.
GRN	gene regulatory networks.
GSR	general stress response.
H	Shannon entropy.
<i>hdeD</i>	gene coding for the “ <i>hns</i> -dependent expression” protein, an acid-resistance membrane protein.
HOG	high osmolarity glycerol pathway.
<i>hsp12</i>	gene coding for the heat shock protein 12.
Hxt	hexose transporter.
i/o	input/output.
IoU	intersection over union.
IPTG	isopropyl β -d-1-thiogalactopyranoside.
K_{switch}	rate of phenotypic switching.
<i>lacAYZ</i>	operon lactose.
LacI	The “lactose inhibitor”, DNA-binding transcriptional repressor.
<i>lacI</i>	gene coding for LacI.
LB	lysogeny broth or Luria-Bertani medium.
MDK	minimum duration required for killing.
<i>met3</i>	gene coding for the “methionine requiring 3” protein.
MI	mutual information.
MIC	minimal inhibitory concentration.
Mig1	“multicopy inhibitor of GAL gene expression” protein, transcription factor involved in glucose repression.
MONKS	Monod-type culture kinetic simulation.
MOPS	3-(N-morpholino)propanesulfonic acid.
mRNA	messenger RNA.

MSCC	microfluidic single-cell cultivation.
Msn2/4	paralogous “multicopy suppressors of Snf1 mutation” proteins, main stress-responsive transcriptional activators.
<i>nlpD</i>	gene coding for the “new lipoprotein D”.
OD ₆₀₀	optical density at 600 nm.
<i>osmC</i>	gene coding for an osmotically inducible peroxiredoxin, implied in oxidative stress resistance.
<i>otsB</i>	gene coding for the “osmoregulatory trehalose synthesis B” trehalose-6-phosphate phosphatase.
<i>oxyR</i>	gene coding for the “oxidative stress regulator” DNA-binding transcriptional dual regulator.
P _{<i>cmv-tet</i>}	minimal <i>cmv</i> (cytomegalovirus) promoter with seven tetracycline-responsive operator motifs (<i>cmv-tet</i>).
<i>pata</i>	gene coding for a putrescine aminotransferase.
PBS	phosphate-buffered saline.
PCR	polymerase chain reaction.
PDMS	polydimethylsiloxane.
PFR	plug flow (bio)reactor.
PKA	protein kinase A.
PLtetO-1	promoter that can be repressed by the protein TetR.
<i>poxB</i>	gene coding for a pyruvate oxidase.
(p)ppGpp	guanosine pentaphosphate and tetraphosphate.
PTS ^{Glu}	phosphoenolpyruvate–glucose phosphotransferase system.
<i>puuA</i>	gene coding for a protein of the “putrescine utilization pathway”, the glutamate-putrescine ligase.
<i>puuD</i>	gene coding for a protein of the “putrescine utilization pathway”, the γ -glutamyl- γ -aminobutyrate hydrolase.
PWM	pulse width modulation.

RBS	ribosomal binding site.
RelA	“relaxed phenotype A” protein, (p)ppGpp synthetase I.
<i>relA</i>	gene coding for RelA.
Rgt2	“regulator of glucose transport 2” protein, glucose sensor regulating expression of glucose transporters (paralog of Snf3).
RNA	ribonucleic acid.
RNAP	RNA polymerase.
<i>rpoH</i>	gene coding for σ^{32} (RpoH).
RpoS	sigma factor for the general stress response (also termed σ^S or σ^{38}).
<i>rpoS</i>	gene coding for RpoS.
rpoSp1	major promoter of <i>rpoS</i> .
<i>rssB</i>	gene coding for an adapter protein that regulates RpoS availability; it prevents the formation of the $E\sigma^S$ holoenzyme by sequestering RpoS and targeting it for proteolytic degradation..
Rv	reverse primer.
SD	scale-down.
SDR	scale-down (bio)reactor.
SerA	phosphoglycerate dehydrogenase that catalyzes the first step in the biosynthesis of L-serine.
<i>serA</i>	gene coding for SerA.
Snf1	“sucrose non fermenting 1” kinase, central regulator of carbon source utilization and stress response.
Snf3	“sucrose non fermenting 3” protein, glucose sensor regulating expression of glucose transporters (paralog of Rgt2).
<i>sodC</i>	gene coding for a superoxide dismutase (Cu-Zn).
SpoT	“spotless phenotype” protein, bifunctional (p)ppGpp synthase/hydrolase.
SSC	side scatter (in flow cytometry: SSC-A, area; SSC-H, height).
STR	stirred-tank (bio)reactor.
STRE	stress-responsive element.

T_{env}	period of environmental fluctuation.
T_{switch}	period of phenotypic switching.
TetR	tetracycline repressor.
TORC1	target of rapamycin complex 1 signaling pathway, nutrient-sensing cascade controlling growth and metabolism.
Tpk	“Takashi’s protein kinase”, catalytic subunits of PKA.
Tr_{Loop}	residence time in the loop.
<i>treF</i>	gene coding for the cytoplasmic trehalase.
tRNA	transfert RNA.
<i>tslI</i>	gene coding for the “trehalose synthase long chain” protein.
<i>uspB</i>	gene coding for the “universal stress protein B”, involved in DNA repair and ethanol tolerance.
<i>wrbA</i>	gene coding for the “tryptophan (W) repressor-binding protein”, whose physiological role remains unclear.
WT	wild type.
<i>yciG</i>	gene coding for a protein involved in motility and biofilm formation.
<i>ycdS</i>	gene coding for a putative transport protein associated with natural and chemical transformations and carbon flux distribution.
<i>yhfG</i>	synonym of <i>ficA</i> , gene coding for a “FIC domain antitoxin”, whose physiological role remains unclear.
YPD	yeast extract peptone dextrose medium.

1

Chapter 1: Introduction

1. Impact of phenotypic diversification dynamics on cell population growth and robustness

The earliest forms of life were microbial and emerged on Earth more than three billion years ago [1, 2]. They played a crucial role in shaping the biosphere and creating the conditions that allowed life as we know it. Since then, they have colonized nearly every corner of the biosphere, from the depths of the oceans to permafrost, and have evolved to thrive even in the most extreme and hostile environments [3]. While long-term evolutionary adaptation explains their global success, survival within a given niche often requires coping with short-term fluctuations. In this context, phenotypic plasticity, the ability of a single genotype to express alternative phenotypes across environments, provides a crucial advantage by enabling cells to withstand and exploit dynamic environments.

The conventional perspective on microbial adaptation holds that cells sense environmental cues and adjust their physiology accordingly. For instance, when preferred carbon sources become scarce, microbes can activate alternative metabolic pathways or shift into more protective states [4, 5]. Yet, this sensing-and-responding strategy is not universally effective: it becomes limiting when the number of possible environmental conditions is too large to be tracked by specific regulatory circuits, or when environmental changes occur more rapidly than cells can reprogram gene expression and establish the appropriate phenotype [6]. These limitations are further compounded by the investment of cellular resources required for the sensing and signaling machinery needed to activate the appropriate response [7]. In such scenarios, an alternative and complementary strategy is to rely on phenotypic diversification, whereby cells stochastically switch between phenotypes even in the absence of external cues [6] (Figure 1.1). This process enables an evolutionary strategy, termed bet-hedging, that relies on phenotypic diversity to spread the risk of maladaptation and increase the likelihood that some individuals will be fit for future environmental conditions [6, 8]. In a heterogeneous yet genetically identical population, a phenotype that is maladaptive under one condition may be preadapted to another and become advantageous when the environment changes (Figure 1.1). This diversification also confers resilience to sudden and severe environmental shifts [6, 8]. Moreover, phenotypic heterogeneity not only enables bet-hedging but also supports additional functionalities, such as division of labor between subpopulations of different phenotypes, both of which enhance the collective fitness of the population [6].

The main driver of such stochastic heterogeneity is the noisiness of molecular processes. Gene expression involves the stochastic binding and interaction of molecules often present in very low copy numbers, making transcription and translation inherently probabilistic [9, 10]. This generates intrinsic noise. At the same time, extrinsic factors contribute to variability: the unequal partitioning of

cellular components during division, differences in the physiological state of cells, or variations in gene copy number (e.g., plasmids) [11, 12]. Moreover, regulators themselves can modulate noise levels; weak binding affinities or particular DNA operator structures can lead to “leaky” expression, as illustrated in the case of LacI in the lac operon [13]. Finally, because genes are embedded in complex regulatory networks, fluctuations in the expression of one gene can propagate through cascades of interactions, amplifying variability across the genome [11]. Together, these mechanisms create a layered architecture of noise that pervades all levels of gene expression. Interestingly, Silander et al. [14] shown that gene expression noise correlates with gene functionality. Essential housekeeping genes, such as those involved in DNA structure or biosynthesis, are expressed relatively homogeneously across the population, whereas genes related to alternative carbon source utilization or adaptation to stress display higher variability, highlighting that gene expression noise is subject to evolutionary selection [6, 14]. Although the mechanisms giving rise to gene expression noise may differ in eukaryotes [14], certain stress-related genes are also heterogeneously expressed even under non-stress conditions [15].

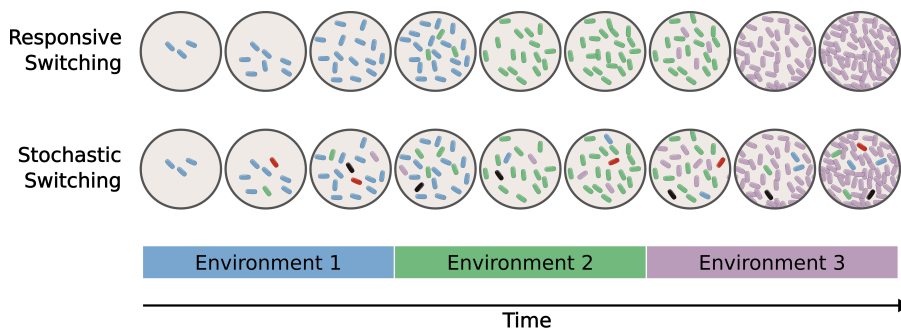


Figure 1.1: Illustration of responsive and stochastic phenotypic switching in isogenic populations exposed to changing environments. The responsive mode reflects phenotype adjustments triggered by environmental cues, whereas stochastic switching generates phenotypic diversity independently of environmental changes. (Adapted from Kussell and Leibler [7])

While phenotypic heterogeneity provides ecological advantages to microbial populations, this feature is generally undesirable in applications such as medicine and bioproduction. In the former, cellular populations within tumors or sites of microbial infection can display substantial variation in growth rates and in sensitivity to drugs or stressors, complicating treatment outcomes [16]. In the latter, heterogeneity tends to make processes unpredictable and is presumed to contribute to instability and reduced production yields [17, 18]. It is therefore crucial to better understand diversification dynamics and to develop strains, process designs, and control strategies that optimize microbial production [17, 19–21].

Our recent contribution has shown that the degree of diversification observed in microbial populations cultivated in continuous bioreactors is mainly dictated by the fitness cost, which is the reduction in growth rate associated with the activation of a specific gene circuit [22]. We found that low fitness costs lead to relatively homogeneous diversification profiles, whereas high fitness costs result in markedly heterogeneous dynamics. These results indicate that, based on fitness cost, we can predict the level of heterogeneity exhibited by the population and its potential controllability [22]. Together, these findings laid the groundwork for the investigations developed throughout this thesis.

A particularly relevant group of genes for studying phenotypic diversification and its control, both from an ecological perspective and for bioprocess applications, are those involved in stress responses. Their expression typically entails a high fitness cost due to resource redistribution, they are often characterized by elevated noise levels, and they belong to complex gene regulatory networks (GRN). For these reasons, we focused on the general stress response (GSR) in two model organisms, *Escherichia coli* and *Saccharomyces cerevisiae*.

2. The general stress response (GSR) : balance between growth and protection

Cell stress commonly refers to environmental conditions that compromise survival or impair optimal cellular function [5]. It can result from sudden changes or prolonged alterations and may stem from either a single specific modification or a combination of multiple factors. Besides, when changes in growth conditions are moderate, the distinction between a stress response and an adaptation to new growth conditions is not sharply defined and likely represents a gradual continuum in practice [23]. Upon exposure to stress, cells first experience immediate consequences, such as disruption or damage, depending on the stress type and intensity. They subsequently activate defense mechanisms by sensing and signaling the perturbation, leading to adjustments in gene expression, metabolism, and other physiological features [5]. The expression of such stress-related genes is governed by a fundamental trade-off: cells can either allocate resources to stress protection or channel them toward rapid growth. Investment in one strategy inevitably comes at the expense of the other, as protective mechanisms are intrinsically incompatible with high metabolic activity [23, 24].

Distinct stressors (e.g., osmotic, pH, temperature) activate specific regulatory circuits, yet most also converge on the GSR [4, 25]. In *S. cerevisiae*, this response is more commonly referred to as environmental stress response (ESR), core stress response (CSR) or core environmental response (CER). The GSR is an emergency reaction functioning as a rapid, broad-spectrum defense program that provides cross-protection against multiple stress conditions, including those not yet encountered,

thereby reducing the risk of potential further damage [4, 25]. This phenomenon of cross-protection implies shared cellular responses to different stress conditions, including adjustment of energy metabolism and the synthesis of protective proteins (e.g., heat shock proteins) or protective compounds (e.g., glycerol or trehalose) [5]. In the context of this thesis, the focus is on the occurrence of the GSR following nutritional stress, specifically depletion of the primary carbon source (glucose), a condition that arises both in natural ecosystems and in industrial bioprocesses [23, 26].

The GSR is an important topic in both fundamental and applied research. From an ecological perspective, it represents a key evolutionary strategy that enables microbes to thrive in fluctuating environments [23, 25, 27], and it plays a role in clinically relevant processes such as virulence and antibiotic persistence [4, 28, 29]. In biotechnology and industrial production, it is of particular importance for bioprocess design and optimization [26, 29, 30]. Moreover, controlling stress-response activity has the potential to mitigate growth arrest and alleviate the metabolic burden associated with its activation [31, 32].

We decided to work with the procaryote model, *E. coli*, and the eukaryote model, *S. cerevisiae*. Owing to their simple growth requirements, rapid doubling times, their early and extensive genetic characterization, and the broad set of molecular tools developed over decades, they have become two of the most widely used model organisms in molecular biology, genetics, and biotechnology [33–44]. They are valued as experimental systems because many of their molecular processes and biological functions are conserved across the tree of life [37, 42]. In addition to the well-known traditional fermentations performed by *S. cerevisiae*, both species are also widely exploited as cell factories to produce pharmaceuticals, biofuels, enzymes, and fine chemicals [2, 35, 37–41, 45], often with higher yields and greater cost-effectiveness than traditional extraction or chemical synthesis methods [2, 39, 41].

Both *E. coli* and *S. cerevisiae* encompass a wide range of strains that thrive in diverse environments and display highly variable properties, from pathogenic to domesticated forms used in laboratory and industrial settings [4, 16, 46, 47]. For this thesis, we selected strains that are widely used in both academic and industrial research, whose popularity stems from their genetic stability (e.g., a haploid yeast unable to mate when cultivated alone, and *E. coli* lacking the F factor, thereby preventing conjugation-mediated gene transfer), their extensive genetic and physiological characterization (including metabolic and regulatory networks) and the wealth of genetic tools available for their manipulation [35, 42, 44, 48–51].

Both species exhibit a GSR, yet they differ in the structure of their regulatory networks and in the physiological consequences of stress induction, making them complementary models for this work. Given the complexity of the underlying molecular networks, only an overview is provided here below, together with references to comprehensive, high-quality reviews.

2.1. Regulation of GSR under nutrient stress

2.1.1. RpoS, the master regulator of the GSR of *E. coli*

When facing depletion of glucose, its favorite carbon source, *E. coli* exhibit two successive and antagonistic mechanisms. First, it invests in nutrient scavenging and motility, trying to find alternative carbon sources, then as soon as nutrient become too scarce, it switch to a lifestyle focussed on resistance and maintenance [4].

The first state is considered more “offensive” and results from the release of catabolic repression exerted by glucose. When decrease of glucose is sensed by the phosphoenolpyruvate–glucose phosphotransferase system (PTS^{Glu}), one of the main glucose transporters, it leads to the accumulation of the nucleotide second messenger cyclic adenosine monophosphate (cAMP) [4, 52, 53]. The cAMP then binds to the catabolic response protein (CRP) to form the cAMP-CRP transcriptional regulator complex that activates or enhances the transcription of genes involved in alternative metabolic pathways and motility [4, 52].

The expression of the second “defensive” state is the most interesting in the context of this thesis since it involves the alternative sigma factor RpoS (σ^S). This subunit of RNA polymerase (RNAP) is the master regulator of the GSR in *E. coli*. It confers promoter specificity to the RpoS-containing RNAP holoenzyme ($E\sigma^S$) and regulates, directly or indirectly, about 10 % of *E. coli*’s genome (i.e., about 500 genes) [4]. The regulation of RpoS activity is impressively complex and occurs at transcriptional, translational and proteolytic levels, as well as at the level of the competition between sigma factors for the RNAP core enzyme. Only some of the key elements of the complex regulation and main functions of RpoS will be presented here bellow (Figure 1.2). For more details, the reader is referred to the related reviews [4, 54–57]. Among these, the review of Hengge [4] and that of Battesti, Majdalani, and Gottesman [54] are particularly comprehensive.

The first layer of this “defensive” program is the stringent response, mediated by rising levels of guanosine pentaphosphate and tetraphosphate ((p)ppGpp). The intracellular concentration of this alarmone is regulated by the RelA/SpoT homolog enzymes, which balance its synthesis and degradation in response to nutrient availability and occurrence of various stresses [4, 58, 59]. The stringent response triggers growth reduction, or even growth arrest, in part by inhibiting ribosome and tRNA synthesis, and by regulating enzymes involved in cell cycle progression [54, 58, 59]. In addition, rising (p)ppGpp levels promote *rpoS* transcription and translation, stabilize RpoS, and enhance its activity, thereby redirecting cellular resources toward stress adaptation through the GSR [4, 54, 58]. Considering the cAMP-CRP complex mentioned before, it plays a dual role at the level of the transcription of *rpoS* which depends on the actual conditions. During exponential and early post-exponential phases of growth, it tends to be an indirect inhibitor, allowing to first try the “offensive” strategy, then it positively regulates the transcription of *rpoS* [4]. The

regulation of transcription, competition for the RNAP and degradation of RpoS are also supported by specific molecules [4].

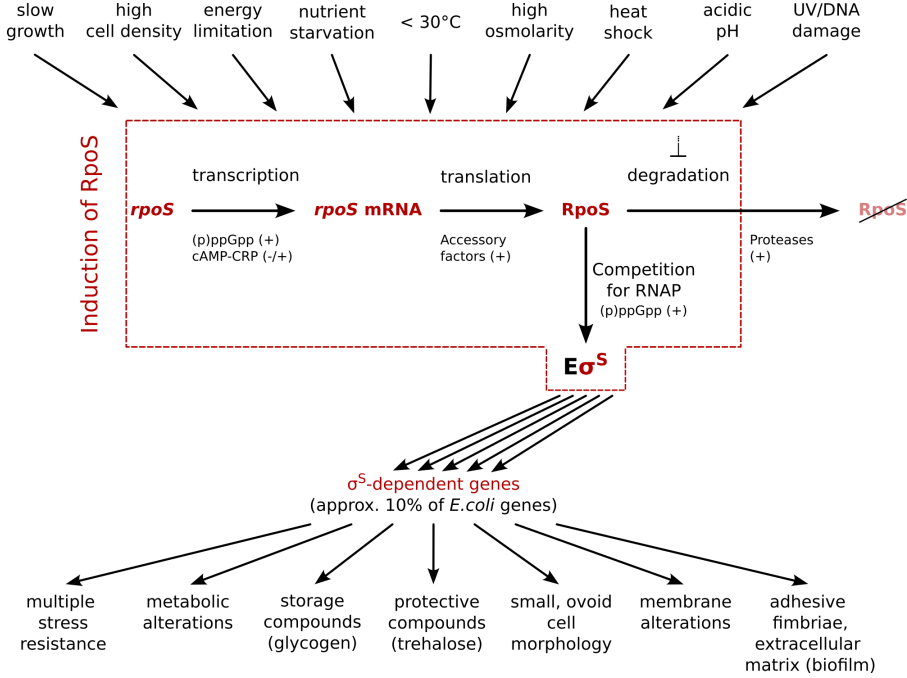


Figure 1.2: Simplified overview of the GSR, showing signal inputs (top), regulation of RpoS (middle), and phenotypic outputs (bottom). Molecules involved at different levels of regulation (i.e., transcription, translation, proteolysis, and association with the RNAP core enzyme) are indicated as non-exhaustive examples. (Adapted from Hengge [4])

Crucially, RpoS-regulated genes differ in their sensitivity to RpoS, resulting in a graded and selective activation of target promoters [60]. This differential responsiveness underlies a coordinated and temporally ordered gene expression program, ensuring that the various cellular functions are activated in a timely manner and that stress adaptation is properly aligned with the growth phase and physiological demands (Figure 1.2) [60]. RpoS levels have been shown to be tightly linked to growth rate, increasing under slow-growth or nutrient-limited growth conditions [4, 54, 61, 62]. While RpoS activation represses functions associated with rapid proliferation, it does not fully inhibit growth. Rather, it enables the expression of stress-adaptive genes that support survival and limited metabolic activity. In certain contexts, RpoS is even required for maintaining slow growth by sustaining expression of central metabolism genes [4, 63]. Taken together, *E. coli* exhibits mutual regulation between growth rate and stress response, allowing it to dynamically adjust its physiology (Figure 1.3) [4, 54, 61, 64, 65].

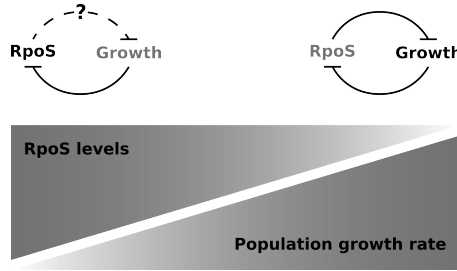


Figure 1.3: Schematic illustrating the anticorrelated relationship between growth rate and RpoS levels. RpoS levels increase as population growth rate decreases, in part due to reduced dilution by cell division. However, at very low growth rates, the ability of RpoS to further inhibit growth appears to diminish. This allows *E. coli* to maintain a slow-growth state supported by a limited metabolic activity, despite high RpoS accumulation. (Adapted from Patange et al. [64])

2.1.2. Msn2, the main activator of the GSR of *S. cerevisiae*

Yeast's GSR program involves a bit less than 15 % of all its genes [5, 23, 66, 67]. Repressed genes are mainly related to protein synthesis and growth, whereas induced genes span a broad range of processes that safeguard cellular homeostasis and promote stress resilience [5, 23, 68]. Examples of cellular processes induced in GSR and stress protective mechanisms are comprehensively described in Hohmann and Mager [5] and Tanghe, Prior, and Thevelein [23].

The main transcription factors involved in the activation of yeast's GSR-upregulated genes are the partially functionally redundant Msn2/4 proteins (Figure 1.4) [5, 23, 25]. Under basal conditions, Msn2 and Msn4 are retained in the cytoplasm. Upon stress, they translocate to the nucleus, bind to stress-responsive elements (STREs) in gene promoters, and activate transcription [69, 70]. Their activity is regulated at multiple levels, including expression, nuclear import and export, as well as protein stability [71]. Notably, Msn2 exhibits stress-dependent nuclear pulses, whose frequency and amplitude determine which target genes are activated [72]. This illustrates how stress responses are tightly integrated with upstream signaling pathways, which differ depending on the environmental condition [71].

Among these pathways, nutrient signaling constitutes a highly intricate and interconnected network. Extensive kinase cross-talk, protein-protein interactions, and layers of feedback and feedforward regulation ensure that gene expression is precisely coordinated with environmental cues [68]. Considering this complexity, only the main components of the signaling pathways contributing to the GSR are highlighted below. More detailed descriptions can be found in dedicated reviews [68, 73, 74].

Yeast has two sensors of external glucose concentrations, Rgt2 for high and Snf3 for low levels of glucose [75, 76]. Depending on the external concentration sensed, different hexose transporters (Hxts) are synthesised according to their affinity for

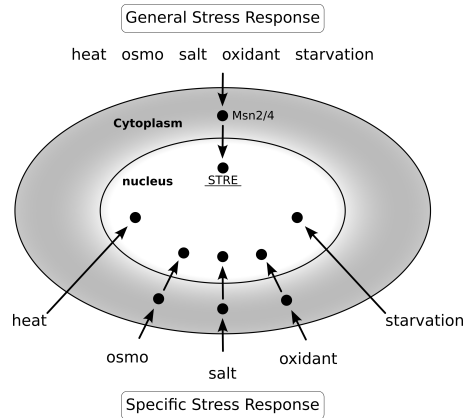


Figure 1.4: Simplified overview of the GSR of yeast, mediated by Msn2/4 transcription factor. (Adapted from Tanghe, Prior, and Thevelein [23])

glucose [75–77]. Besides, some glucose transporters are not directly activated by the glucose concentration, but more by stress in general (e.g., *Hxt5* promotor contains STREs) [78].

In addition to regulating glucose uptake, these sensing systems also initiate catabolite repression when glucose is abundant [68, 73, 79, 80]. When glucose is low, the Snf1 kinase is activated and enables the expression of genes required for the metabolism of alternative substrates, such as ethanol produced during fermentation, and supports the transition to respiration [79, 80]. Beyond this canonical role in carbon metabolism, Snf1 also contributes to cellular stress adaptation and survival since under prolonged glucose limitation it activate Msn2 [79, 80] (Figure 1.5 A).

Among nutrient signaling pathways, protein kinase A (PKA) is one of the most important regulators. In fact, it represents the principal pathway activated by glucose, accounting for nearly 90 % of glucose-induced transcriptional changes [80, 81]. The second messenger cAMP, synthesized in response to both extracellular glucose signals and intracellular glycolytic activity, plays an important role by activating PKA through binding (Figure 1.5 B) [74]. Of note, our model strain carries a point mutation in *cyr1* [44], which does not significantly affect cAMP levels compared to a strain without this mutation [82]. Activated PKA promotes multiple processes required for proliferation while inhibiting stress-associated programs. When glucose availability decreases, cAMP levels fall and PKA-mediated inhibition of the stress response is relieved. The PKA pathway plays a pivotal role in controlling the balance between growth and quiescence in response to the quality and abundance of carbon sources [68].

Although it is not directly regulated by glucose, the TORC1 pathway is worth mentioning because it functionally converges with PKA and acts as a central

coordinator of cellular physiological responses to changes in nitrogen source and availability [68]. Furthermore, it is also inhibited by various stresses, such as oxidative and osmotic ones [83]. Active, it promotes growth and represses stress genes. It has cross-talk with both Snf1 and PKA pathways and indirectly inhibits Msn2/4 [68, 80].

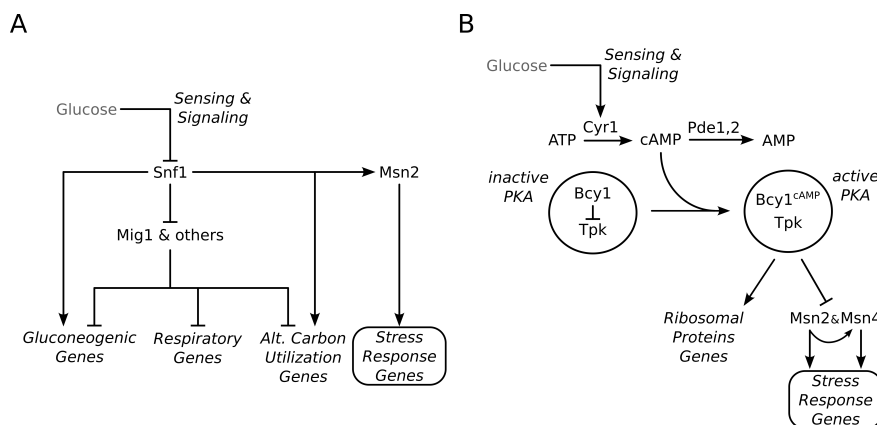


Figure 1.5: Schematic representation of the two main pathways involved in the yeast GSR controlled by glucose concentration. **A** The Snf1 protein kinase is central player in glucose repression pathway. At high glucose concentrations, Snf1 remains inactive, leaving the transcription factor Mig1 unphosphorylated and localized in the nucleus, where it represses genes required for the utilization of alternative carbon sources. When glucose availability decreases, Snf1 becomes active, phosphorylates Mig1, thereby lifting glucose repression, and activate Msn2 [68, 79, 80]. (Adapted from Kayikci and Nielsen [79] and Coccetti, Nicastro, and Tripodi [80]) **B** Repression of the GSR by glucose through the PKA signaling pathway. High glucose availability activates Cyr1, leading to increased intracellular cAMP levels. Binding of cAMP to the regulatory subunit of PKA (Bcy1) releases the active catalytic subunits (Tpk1 and Tpk2). Activated PKA phosphorylates Msn2/4, thereby preventing their nuclear entry and repressing the GSR. (Adapted from Hohmann and Mager [5], Broach [68] and Li, Giardina, and Siegal [16])

While high availability of glucose is advantageous, too high concentrations can be harmful to yeast by imposing osmotic stress, for instance at around 1 mol/L [84]. Under such conditions, the high osmolarity glycerol (HOG) pathway becomes activated [5, 84, 85]. Similar to nutrient stress, this pathway also triggers the GSR via Msn2/4 [5, 85].

Activation of the GSR through Msn2/4 has profound implications for cellular physiology, particularly on growth. Upon stress induction resources are redirected from biosynthetic activities toward protective functions, resulting in a marked reduction in growth rate [23]. Growth-related genes are not directly repressed by Msn2/4, but their expression is indirectly downregulated through the inhibition of

PKA and TORC1 by environmental stresses, most notably nutritional stress [5, 68, 73], but also osmotic, oxidative, and thermal stress [83, 86]. Yeast cells tightly regulate their growth in accordance with nutrient availability by altering cell cycle duration [68]. Under starvation, *S. cerevisiae* cells enter a quiescent state by arresting growth during the less sensitive phase of the cell cycle, resuming proliferation only once nutrients become sufficiently available again. [5, 68, 73]. Compared to *E. coli*, yeast's stress response appears to impose a more pronounced physiological cost, reflecting a deeper metabolic reprogramming and a stronger commitment to protective gene expression at the expense of growth.

3. Impact of environmental fluctuations on GSR and cell population dynamics

The influence of fluctuating environments on phenotypic switching strategies has already been introduced in section 1. Here, we consider this aspect with a perspective focused on bioproduction.

In industrial-scale bioreactors, environmental fluctuations primarily arise from imperfect mixing. As the reactor volume increases, mixing efficiency decreases, and spatial gradients appear [87–90]. Notably, substrate gradients can reach considerable magnitudes, and can expose cells to nutrient excess in some zones and starvation in others, thereby stressing them [26, 32, 89, 91–93]. The time spent in these zones can approach the timescale of gene expression processes such as transcription and translation, making it possible for cells to mount responses to these fluctuations [89, 92].

From the perspective of individual cells, these dynamics are captured by the notion of a “lifeline”, i.e., the sequence of environments experienced by a cell as it travels through the reactor (Figure 1.6) [90, 92]. Because lifelines differ from one cell to another, not all cells experience the same fluctuations at the same time, nor do they necessarily remain long enough in a given zone to respond. When fluctuations are regular and predictable, responsive switching strategies are generally favored, resulting in more homogeneous populations. By contrast, irregular and high-frequency fluctuations, potentially observed in large-scale environments, make responsive adaptation less efficient and can instead promote stochastic switching between phenotypes [7, 94–98]. This combination of heterogeneous exposure and intrinsic cellular noise ultimately shapes the overall phenotypic distribution of the population in industrial bioprocesses.

A potential way to mitigate environmental heterogeneities is to operate continuous bioreactors which, being smaller in size, allow improved mixing. Nevertheless, despite their theoretical advantages [99], several challenges remain before their routine implementation can be considered, including issues of phenotypic and genetic

stability [99–101].

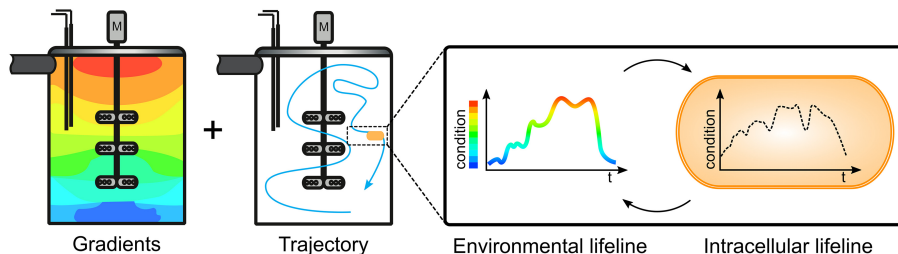


Figure 1.6: Schematic representation of a cell lifeline in large-scale bioreactor. In large-scale bioprocesses, non-ideal mixing generates spatial gradients that individual cells experience as temporal fluctuations along their “lifeline”. The extracellular lifeline represents the environmental input, while the intracellular lifeline reflects the resulting cellular responses over time. (From Blöbaum, Haringa, and Grünberger [90])

These considerations are very important for the design of control strategies. However, most control technologies remain largely untested under the highly dynamic and often harsh conditions encountered at industrial scale [102]. This highlights the importance of scale-down approaches, which mimic large-scale heterogeneities through interconnected compartments (e.g., stirred tank with tubular loop) while allowing systematic investigation of their impact on cells [103, 104]. Furthermore, integrating real-time single-cell monitoring into scale-down studies can help anticipate and manage cellular responses, thereby improving scalability and robustness in industrial fermentation processes [17, 89, 105, 106].

4. Single cell analysis and population dynamics

Single-cell measurements are essential to reveal phenotypic heterogeneity in microbial populations, as bulk measurements (e.g., total fluorescence of the culture broth) provide an average value that masks the underlying phenotypic distributions (Figure 1.7) [105].

Nowadays, a wide range of single-cell analysis tools are available, ranging from microscopy, the oldest and simplest approach, to cutting-edge technologies such as single-cell sequencing [18, 107]. In this work, we focused on two complementary approaches: time-lapse microscopy in microfluidic devices and on-line flow cytometry (FC) [108]. Both rely on fluorescent reporter strains as the fundamental readout, which are therefore presented first, followed by the short descriptions of the two measurement technologies.

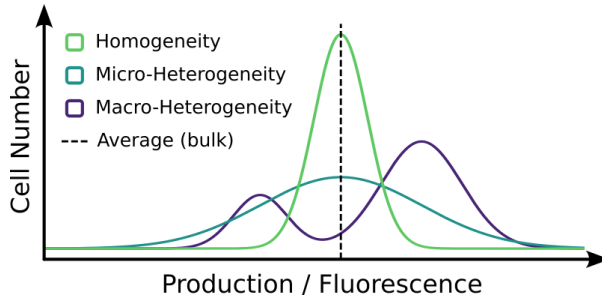


Figure 1.7: Schematic representation of the difference between bulk and single-cell measurements. Single-cell measurements are required to uncover phenotypic heterogeneity within microbial populations, which remains hidden when relying solely on bulk averages. For instance, populations with drastically different distributions can nevertheless share the same average value. (Adapted from Binder et al. [17])

4.1. Fluorescent reporters to monitor stress

Different technologies based on fluorescence enable reporting of phenotypic traits (e.g., dyes, fluorescence resonance energy transfer (FRET), riboswitches). Among them, promoter-based biosensors are the prevalent ones for studying phenotypic heterogeneity in bioprocesses [18, 89, 109]. Such transcriptional reporters are built by insertion of the sequence coding for a fluorescent protein downstream of a selected promoter (either in the chromosome or on a plasmid). The produced fluorescence therefore reflects the activity of the promoter in response to a cellular event of interest (activation of the GSR in this case). Unlike chemical dyes, they do not require sample pretreatment, which makes them particularly well suited for automated single-cell measurements. Even though they usually provide a fast readout, it is important to keep in mind the limitations related to the maturation time of selected fluorescent protein [18, 89, 109].

For *S. cerevisiae*, several GSR reporters have been described, including constructs based on the promoters of *tsl1*, involved in trehalose synthesis, and *hsp12*, encoding a heat-shock protein [71, 110]. In this work, we used a previously established $P_{glc3}::GFP$ transcriptional reporter [111]. The *glc3* gene encodes a glycogen branching enzyme [112] and, like most genes related to trehalose and glycogen metabolism, it is activated under nutrient stress and as part of the GSR [5, 110, 113–115]. In addition, it has been reported to play a role in bet-hedging strategies [110].

For *E. coli*, various GSR reporters have also been employed [64, 65, 116], including constructs that directly monitor *rpoS* transcription [31, 117]. To identify the reporter best suited to our objectives, we carried out a comparative analysis of different designs, the details of which are described in chapter 2.

4.2. Time-lapse microscopy in microfluidics

Microfluidic single-cell cultivation (MSCC) systems are miniaturized culture devices, typically fabricated from a polydimethylsiloxane (PDMS) chip bonded to a glass coverslip [118–120]. They consist of arrays of nanoliter-scale chambers continuously perfused with fresh medium through adjacent supply channels (Figure 1.8). This provides a precisely defined and tightly controlled environment thanks to laminar flow and diffusion-based exchange of nutrients and metabolic products [108, 121]. Furthermore, the high surface-to-volume ratio enable an efficient heat transfer [121].

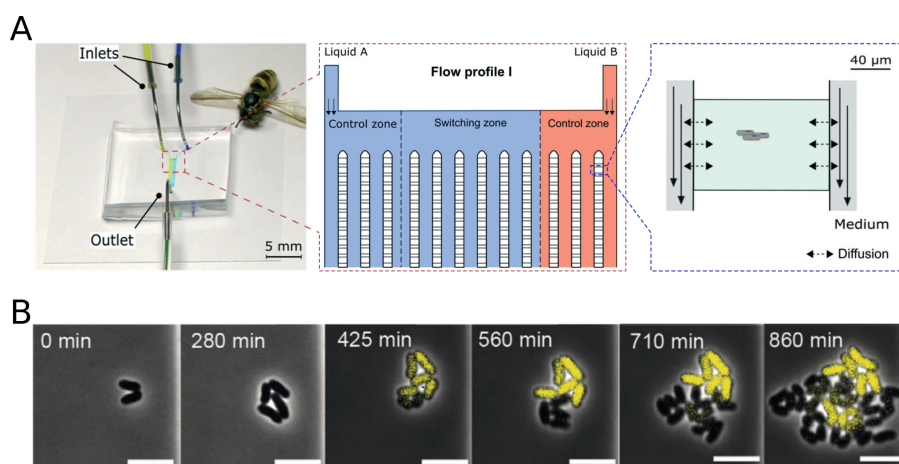


Figure 1.8: MSCC device combined with time-lapse microscopy. **A** dMSCC device showing (left) a photograph of the chip, (middle) a schematic of the design with inlets, supply channels, and cultivation chambers, and (right) a magnified view of a single cultivation chamber. (Adapted from Täuber et al. [122]) **B** Example of time-lapse microscopy of an isogenic *Corynebacterium glutamicum* colony, illustrating cell-to-cell heterogeneity in production (yellow cells) and growth. (Adapted from Ladner et al. [108] and Mustafi et al. [123])

Different chamber geometries have been developed to constrain cellular movement to varying degrees, each with specific advantages and drawbacks (Figure 1.9) [108, 121]. The choice of design depends on the application; for instance, mother-machine systems (0D or 1D) maintain aging cells, which may or may not be desirable. The arrangement of the supply channels is also important: with chips equipped with multiple inlets, it is possible to test several conditions simultaneously. Some chip designs further allow rapid switching between conditions, enabling dynamic microfluidic single-cell cultivation (dMSCC) under fluctuating nutrient regimes (Figure 1.8) [122]. Such platforms make it possible to mimic complex oscillations found in natural habitats [124], as well as to reproduce lifelines that reflect the

heterogeneous conditions of large-scale bioreactors [90]. These features make microfluidics a particularly powerful tool for investigating phenotypic heterogeneity under dynamic environments.

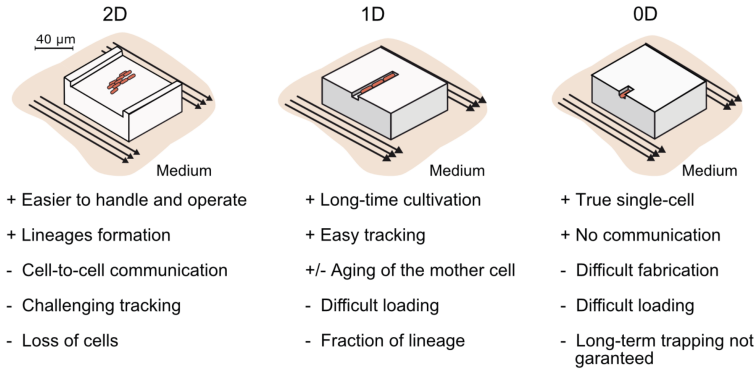


Figure 1.9: Illustration of various microfluidic cultivation chamber geometries and a comparison of their respective advantages and drawbacks. (Adapted from Täuber, Von Lieres, and Grünberger [121])

Cell loading is generally performed by flushing the device with a cell suspension, which results in random seeding of cells into the chambers [119]. Once captured, cells proliferate until the chambers are densely colonized, sometimes containing hundreds of individuals. Throughout the experiment, growth and gene expression (indicated by fluorescent reporters) are monitored via automated time-lapse microscopy [17, 108, 124, 125]. In contrast to FC, microfluidic platforms enable continuous single-cell monitoring with high spatial and temporal resolution, thereby capturing dynamic cellular behaviors (Figure 1.10) [17, 108].

These experiments generate vast amounts of images that are impossible to analyze manually [126]. Indeed, extracting valuable quantitative single-cell data requires several steps after image acquisition, including curation, preprocessing, cell segmentation, tracking, and lineage reconstruction [124]. Recent advances in image analysis tools, particularly those based on artificial intelligence, have substantially reduced this bottleneck [124, 127–130]. Nonetheless, robust and widely available image-analysis pipelines remain scarce [119, 126], and most microfluidic time-lapse images datasets still require labor-intensive manual correction to ensure high-quality results. Ongoing work is steadily improving the efficiency and usability of these tools, ultimately accelerating single-cell data acquisition from MSCC [120, 131–133]. These advances are increasingly moving toward real-time image processing, enabling the implementation of control strategies during ongoing experiments [133–135].

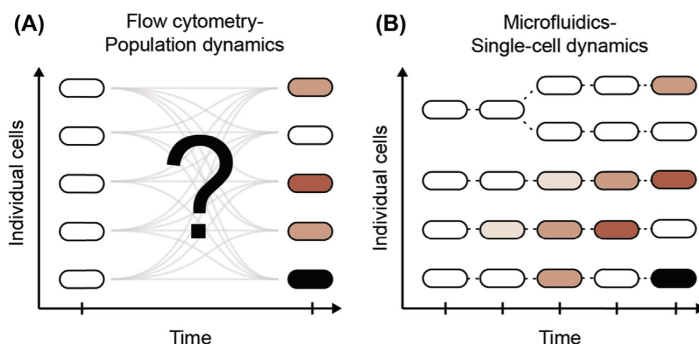


Figure 1.10: Comparison of population versus single-cell resolved analyses. **A** FC enables high-throughput characterization of population dynamics but does not capture the trajectories of individual cells. **B** Time-lapse microscopy, especially when performed in microfluidics, allows tracking of single-cell dynamics over time. (From Ladner et al. [108])

4.3. Automated flow-cytometry

FC is a technique for measuring individual cell optical properties while they are carried in a fluid stream. Within a laminar flow, hydrodynamic focusing aligns the cells in single file so that each passes sequentially through one or more laser beams. As they pass, physical properties such as size and granularity are detected, while fluorescent signals from fluorescent reporters (either produced by the cell or resulting from a labelling such as dyes or antibodies) provide quantitative information on defined cellular components or gene expression (Figure 1.11) [136, 137].

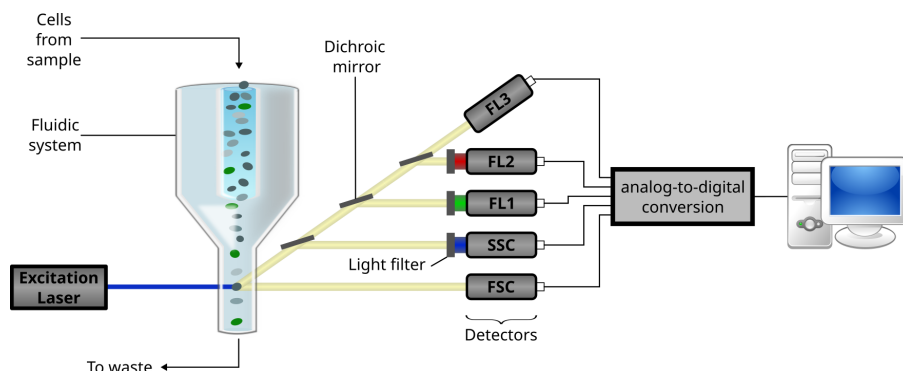


Figure 1.11: Schematic representation of a standard flow cytometer, showing the fluidic, optical, and electronic components. (Adapted from an original figure designed by Kierano [138], with parts from Maximilian Sehrt and Vincent Vandenbroucke)

Initially developed for particle characterization, the technology was later adopted by medical researchers and biologists [136], and has since found various applications,

including the monitoring of drinking water [105]. FC is certainly the most widely used method for single-cell analysis under process conditions [18, 89]. One of its main strengths lies in its high throughput: thousands of individual cells can be analyzed within a single sample. Furthermore coupled with cell sorting (e.g., fluorescence-activated cell sorting (FACS)), it enables the isolation of specific subpopulations for further analyses such as sequencing or microscopy [18, 105], or for continued cultivation [111, 139].

Despite these advantages, FC also has some limitations. Notably, as cells are analyzed as independent events, the method does not provide continuous single-cell dynamics investigation (Figure 1.10) [17, 108]. For this purpose, time-lapse microscopy in microfluidics is a complementary tool [17, 108]. In addition, FC measurements are expressed in relative, instrument-dependent units, which complicates comparisons across devices and requires appropriate calibration [136].

On-line FC systems capable of sampling, diluting, and analyzing cultures in an automated manner have been developed over the years [105, 140]. They allow near real-time monitoring of microbial populations under process conditions [89] and, when coupled with scale-down reactor, make it possible to investigate environments closer to those encountered at industrial scale [17, 106]. Moreover, such automated systems, with their relatively high temporal resolution, provide the foundation for implementing population-level control strategies [140].

4.4. Shannon entropy as a proxy of phenotypic heterogeneity

Once phenotypic heterogeneity can be captured, it becomes essential to quantify it in order to compare population distributions across different conditions [140, 141].

For this purpose, Shannon entropy, a quantifier from information theory, was employed as a metric to describe cell population dispersion in the activation of a given gene circuit [22, 140]. Although its application to microbial populations is relatively recent, entropy has been validated in related biological contexts as an estimator of signal fidelity in gene expression [142]. It can be interpreted as a measure of uncertainty about the population's response (output) given an environmental stimulation (input) [22]. A higher entropy value therefore corresponds to a more heterogeneous population. Shannon entropy (H) is calculated according to the Equation 1.1, where m is the number of bins and p_i is the probability of a data point falling into bin i . A schematic representation and a simplified example of computation are shown in Figure 1.12.

$$H = - \sum_{i=1}^m p_i \cdot \log_2(p_i) \quad (\text{Eq. 1.1})$$

In practice, entropy is computed from fluorescence distributions obtained by FC or microscopy, provided that bin number and boundaries are carefully defined [22,

143]. Key advantages of this approach is that it is independent of the mean expression level and particularly suited to multimodal distributions, outperforming conventional metrics such as the Fano factor or coefficient of variation in capturing complex heterogeneity patterns [14, 144].

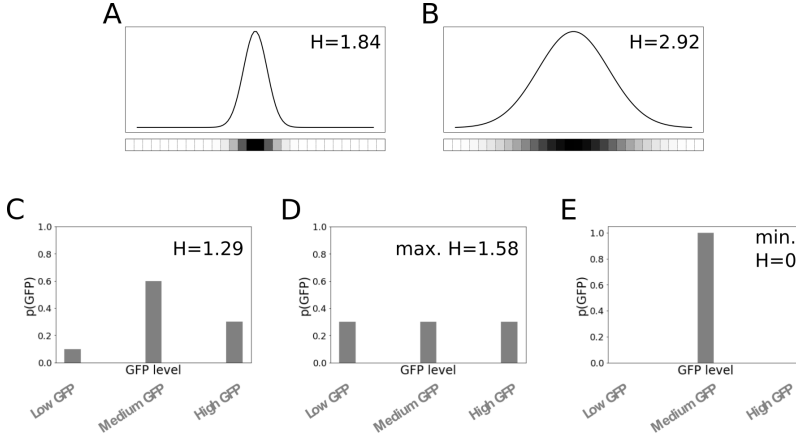


Figure 1.12: Schematic illustration of Shannon entropy. **A-B** Examples of population distributions with lower (A) or higher (B) entropy values. The bins used to compute the entropy are shown below the distributions. **C-E** Simplified example of entropy computation for a population clustered into three fluorescence bins (low, medium, high). These panels are adapted from Henrion et al. [22], where (C) corresponds to an intermediate entropy level, (D) to the maximum entropy attainable for a three-state system, and (E) to the minimum entropy.

5. Strategies to control diversification in cell populations

As already mentioned in the previous section, automated measurements with high (spatio-)temporal resolution, approaching real-time, set the stage for the development of advanced control strategies.

In our laboratory, we have developed a FC-based cell-machine interface enabling feed-back control loop. For several aspects, such as modularity, duration of analysis sequences, and ease of use, this platform can even outperform some commercially available devices. This dedicated setup, termed the Segregostat, is presented below.

The second part of this section provides an overview of control strategies, highlighting how the timing and intensity of applied perturbations (e.g., pulse frequency and amplitude) influence population-level regulation. Concepts from information theory are also introduced as a means to identify optimal stimulation regimes for controlling gene expression.

5.1. The Segregostat

The Segregostat (Figure 1.13) is our in-house, open-access, cell-machine interface [145, 146]. It connects a computer, a flow cytometer (in our case, an Accuri C6 or an Accuri C6+), a bioreactor, and, when required, an actuator for feedback control. The system can operate in two modes. In open-loop mode, it enables automated on-line FC, thereby allowing continuous monitoring of the culture. In closed-loop mode, the acquired data are additionally used to trigger control actions.

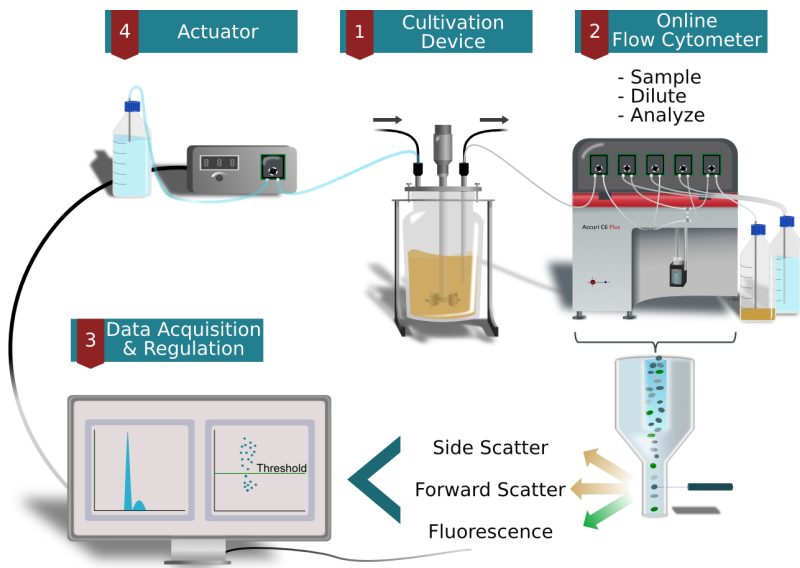


Figure 1.13: Schematic representation of the Segregostat setup. The device connecting the different elements of the system is represented as the box with peristaltic pumps above the flow-cytometer. Original figure designed by Maximilian Sehart.

A typical automated flow cytometry (AFC) measurement takes approximately ten minutes and proceeds as follows. Sampling is performed through a capillary tube connected to a peristaltic pump, which transfers culture from the bioreactor to a dilution and analysis chamber. To avoid bias from residual cells, each run begins with a flushing step, followed by cleaning of the chamber with filtered phosphate-buffered saline (PBS). A fresh sample is then introduced and diluted using a sequence of PBS additions and partial withdrawals, with the protocol adjusted according to the cell concentration measured in the previous analysis. Once the dilution is done, FC analysis is performed. The resulting data are then cleaned of artefacts and basic statistics are computed using a custom Octave script. Both raw and processed data are stored. When operating in closed-loop mode, the data are compared against a predefined set point (e.g., the distribution of events across a fluorescence threshold)

and, if necessary, the actuator is triggered (e.g., peristaltic pump for glucose pulsing).

The Segregostat is continuously improved by our skilled technicians. It is modular, and the control conditions are determined by custom scripts, enabling the implementation of alternative or more complex control strategies than those exemplified here.

To efficiently process the large amount of single-cell data generated by on-line FC, our colleagues have developed a dedicated toolbox to standardize, accelerate, and simplify the data analysis workflow [147, 148].

All these tools have been of paramount importance for data acquisition and analysis throughout this thesis.

5.2. Exploiting Information and Control Theory for Directing Gene Expression in Cell Populations

This subsection is an adapted version of the article of the same title.

Henrion, L., Delvenne, M., Bajoul Kakahi, F., Moreno-Avitia, F., and Delvigne, F. “Exploiting Information and Control Theory for Directing Gene Expression in Cell Populations”. In: *Frontiers in Microbiology* 13 (2022). DOI: 10.3389/fmicb.2022.869509

The parallel advances made at the level of cell culturing procedures (i.e., microfluidics [150] and cell machine interfaces [151]), as well as the manipulation of gene circuits [152–154], have paved the way for the design of efficient cell population control procedures. It is now possible to act either on cell population [145, 155, 156] or on individual cells within population [157, 158] for directing gene expression and cellular functions. However, these control strategies have been applied mostly to synthetic gene circuits, limiting their applicability to natural circuits. In this review, we argue that it is possible to expand these control strategies, and we focus more precisely on a generic approach that could be used to control gene expression in individual cells within populations.

A critical aspect that must be taken into account before being able to manipulate gene expression in cell population is related to the inherent noise of cellular systems [159]. This noise induces cell-to-cell variability in gene expression, and a potential control procedure must be designed by taking into account the inherent functionality exhibited by noise on the cellular system [6, 153]. Indeed, it is known that biological noise is a mechanism exploited by cell population in order to increase its fitness in front of fluctuating environmental conditions [7, 94]. As an example, in natural ecosystems, microbial populations are often exposed to unpredictable environmental changes such as nutrient starvation, exposure to antibiotics, temperature variations, and many other

sources of stress [6] that can fluctuate periodically or randomly. Cellular systems have then evolved accordingly by adapting different cellular components in order to accommodate such fluctuations involving different timescales [27]. If environmental conditions change slowly and regularly, a responsive switching strategy leads to increased fitness for the cell population [7]. On the other hand, if environmental conditions are fast and erratic, a random switching mechanism, leading to pre-adapted cells, is more suited for optimizing population fitness. The study of phenotypic diversification mechanisms involved in antibiotic persistence in bacteria has pointed out that cellular systems can take benefit from both stochastic and responsive switching [97]. It is clear that, for designing an efficient population control procedure, stochastic switching must be minimized and responsive switching must be favoured. Such responsive mechanisms typically involve gene circuits, able to record environmental changes and to respond accordingly. A spectacular realization of the inference of periodic environmental changes by gene circuits is the implementation of circadian (oscillation with a period of 24 h [160]) or ultradian (oscillation with a period < 24 h [161]) rhythms by cellular systems. Many other gene circuit architectures or motifs are known to be able to infer extracellular signals and trigger appropriate biological responses [162, 163]. Even if we have now access to a classification of the motifs and their possible dynamics [164], it is still a challenge to infer the dynamics when several motifs are combined to each other or when the response interferes with many other cellular components. Indeed, in some case, gene circuit architectures can involve overlaps between different stress response pathways, allowing cells to anticipate environmental changes [165, 166]. This anticipatory switching arises in ecosystems where different environmental changes exhibit a strongly correlated time profile. As an example, *Escherichia coli* has evolved in order to be able to grow inside and outside a host (i.e., a mammals) [167]. When invading the host, *E. coli* is exposed to heat shock where temperature increases from 20 to 37°C. This heat shock is then followed by oxygen limitation as bacteria are reaching the gastrointestinal tract. The gene circuits involved in heat shock response and oxygen limitation have been found to share common inputs and outputs in *E. coli*, elevation of heat leading to the adaptation to oxygen limitation in order to anticipate correlated environmental changes.

Given all these elements, it is then difficult to infer the mode of switching, i.e., stochastic, responsive, or anticipatory (or a combination of them) based on the gene circuit architecture. Accordingly, we propose in this work, a generalizable approach aiming at stimulating the responsive component of switching for directing gene expression in cell population. Such approach could be made possible through the use of a universal metrics aiming at quantifying the information transfer efficiency in cells and leading to the design of robust cell machine interfaces.

5.2.1. Using information theory for determination the optimal stimulation frequency leading to coordinated gene expression in cell population

Cells are intrinsically programmed in order to react to external stimuli and to adapt appropriately by switching to different phenotypic states [96, 168]. It is then unrealistic to try to keep these cells into a specific phenotypic state, even if this would be a nice outcome for several applications, such as the optimization of cell factories for bioprocessing [17]. Indeed, these phenotypic states are linked to specific environmental states through selection pressure and the resulting fitness advantage, environmental condition being under constant evolution [94]. A more realistic alternative is to control cell switching itself, which is now technically feasible through the use of cell machine interfaces [141, 145, 156]. In order to make this control strategy successful, two specific aspects must be taken into account, i.e., the efficiency in information transmission through the targeted gene circuits and the timing at which cells commit to phenotypic switching. These two aspects will be illustrated through a case study recently addressed, i.e., the synchronization for the activation of the gene circuit responsible for the induction of the arabinose operon in *E. coli* [156]. The relevance of this case study is also justified by the fact that the arabinose operon has been long used as a biological case study for the characterization of the functionality of biological noise in cell population [169] and also by the fact that the genes belonging to the arabinose operon are widely used for synthetic biology applications and notably for the synchronization of cell response [170, 171]. Finally, the arabinose operon is known to exhibit strong cell-to-cell variability both in the timing for activation [169, 172] and also the level of expression of the corresponding genes [173], suggesting that the underlying cell switching mechanisms involves a mix of responsive and stochastic components. This make this system very interesting to be considered for possible coordination at the population level.

The first step in the cell-to-cell coordination for the activation of the arabinose operon is to know the possible effector for the underlying gene circuit. The activation of the arabinose operon is under the control of a feedforward loop (Figure 1.14 A) combining the glucose depletion signal (through the accumulation of cAMP inside cells) and the presence of arabinose (through the activation of the transcription factor AraC) [174]. Under glucose-limiting conditions, it is then possible to activate or deactivate this gene circuit based on arabinose pulsing (Figure 1.14 A) [156]. At this stage, the first drawback exhibited by biological noise can be observed. Indeed, upon arabinose pulsing, cells will commit to the activation of the feedforward circuit leading to the synthesis of the different proteins involved in arabinose assimilation. However, due to biological noise, timing in commitment will exhibit cell-to-cell heterogeneity [175, 176]. Timing in cellular commitment to alternative phenotypes depends on the accumulation of regulatory proteins at the single cell level. Transcription and translation processes in individual cells are prone to biological noise [177, 178]. These processes can be simulated based on the resolution of the chemical master equation

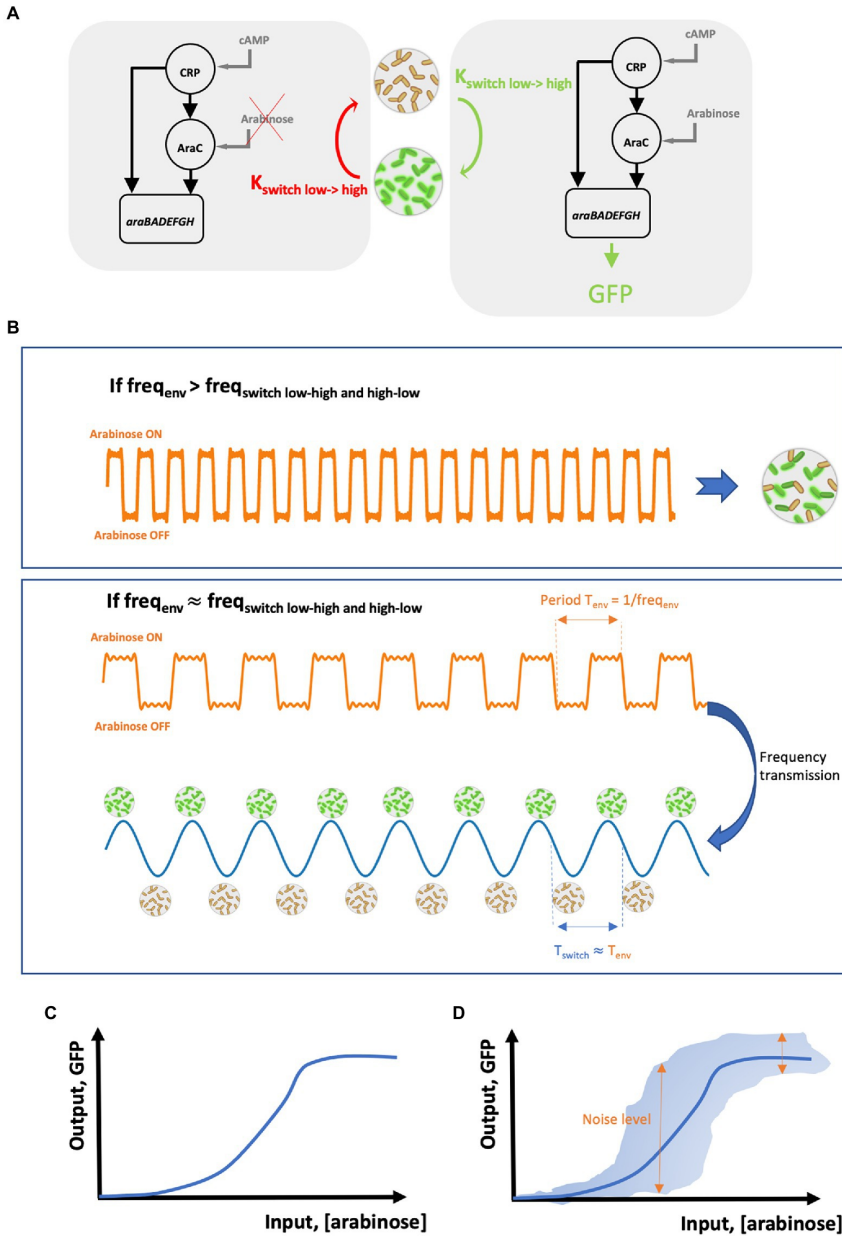


Figure 1.14: A Scheme of the feedforward loop motif involved in the regulation of the arabinose operon. On the left, arabinose is not available and the AraC branch cannot be induced. Accordingly, cell switching does not take place and, eventually, previously induced cells are relaxed back to the uninduced (low) state at a rate $K_{\text{switch low} \rightarrow \text{high}}$. On the right, arabinose is available and the AraC branch, together with the cAMP-CRP branch, is activated leading to the induction of the genes *araBADEFGH* involved in arabinose metabolism. Under these conditions, cells from the low-state switch actively to the high state at a rate $K_{\text{switch high} \rightarrow \text{low}}$.

Figure 1.14: B Proper coordination/synchronization of gene expression can be achieved based on periodic stimulations (or environmental fluctuations) made at a specific frequency freq_{env} . If freq_{env} is too high by comparison with the frequency for cell switching freq_{switch} , then cells are not coordinated and exhibit strong variability in gene expression. However, when freq_{env} is set close to freq_{switch} , coordination in gene expression is possible leading to synchronized gene expression. **C** Typical shape of a Hill relationship between an input (here the concentration of arabinose in the medium) and its resulting output (here, detected based on the synthesis of GFP based on a $P_{araB}::\text{GFP}$ transcriptional reporter). **D** Impact of biological noise (represented by double arrows) on the probability for delivering an output based on a given input.

or, more practically, based on the Gillespie algorithm [177]. These simulations have been shown to lead to very realistic pictures for mRNA and protein synthesis in individual cells [163] and pointed out that these processes follow Poisson statistics. Accordingly, the transition of cells between two adjacent phenotypic states (for example, the two states, GFP negative and GFP positive, drawn in Figure 1.14 A) can also be represented by a Poisson process. One key property of the Poisson process is that the timing between two consecutive events (e.g., the time between the synthesis of two mRNAs from the same DNA sequence in a single cell) follows an exponential distribution. Based on this statement, the residence time distribution of cells in a given phenotypic state can be represented by an exponential distribution [179]. It is thus very critical to take into account this residence time distribution for coordinating cell switching at the population level. One way to overcome this use is to rely on the use of a cell machine interface allowing the on-line monitoring of the switching process at the level of individual cells in the population and to react accordingly. This principle has been notably adopted for developing the segregostat [145, 156]. This system is based on the use of on-line FC for recording the cell switching rate and to trigger environmental switching accordingly. The fact that the frequency of environmental perturbation must be set based on the phenotypic switching frequency has been previously deduced from numerical simulation [94]. In a similar way, another study has pointed out that the control of gene circuits is dramatically reduced above a critical stimulation frequency [95]. Under these conditions, the frequency of the extracellular signal is effectively transmitted, leading to a cell population with synchronized gene expression (Figure 1.14 B). Effective entrainment of cell population can be assessed based on the oscillatory gene expression profile exhibiting a frequency close to the one of the input stimulations. Such oscillations were experimentally observed during segregostat experiments carried out for controlling the activation of the arabinose operon [156]. It is also important to point out that in this case, square waves are used as stimulatory input. This strategy is also called pulse width modulation (PWM) [98, 180]. We will see in the next section that this strategy has been used several times for controlling different cellular systems (listed in Table 1.1). In the present case,

population oscillates according to a frequency corresponding to the one of the input square waves. This is represented in Figure 1.14 B based on the period T_{env} ($T_{env} = 1/\text{frequency}$) of the input square wave stimulation, which is transmitted to the population and lead to oscillation in gene expression with the same period $T_{switch} = T_{env}$.

All these observations point out that cells are able to deduce changes in their surroundings based on diverse sensory mechanisms. We do not want here to discuss about the biological diversity of these mechanisms, but rather to quantify the efficiency at which a cell is able to infer extracellular perturbation. A universal way to quantify information transmission through biochemical network can be derived from Shannon theory or information theory [181]. In order to be able to understand the importance of information theory in biochemical signal processing, it is important to introduce the concept of input output (i/o) or dose response relationship. For many gene circuits, this i/o relationship can be represented by a sigmoidal curve (Figure 1.14 C), also called Hill equation [182]. For example, Hill equation can be used to infer the response of the feedforward loop involved in the regulation of the arabinose operon [183]. This i/o correlation tells us what will be the output of the gene circuit according to a given input. However, we have seen that cell switching mechanism involves a random component in addition to the responsive one. This random component can be represented by the error bars on the i/o correlation (Figure 1.14 D). Accordingly, one input can drive different output trajectories, leading to cell-to-cell heterogeneity. It can be seen that some input leads to a very heterogeneous response, making cell unable to properly infer the state of the environment. This is exactly where information theory can be useful, i.e., by providing a metric for quantifying the amount of information transmitted by the gene network for some specific input environmental conditions. This metric, mutual information (MI), corresponds to the logarithm of the number of distinct, input-dependent, states that can be reached by cells [182] and is quantified in bits. For example, a gene network exhibiting a MI of one bit means that only two physiologically distinct states can be resolved by cells based on the input conditions. Generally speaking, most of the gene circuits are corrupted by noise and can carry only a limited amount of information, and most of the studies carried out so far in this area have pointed out that MI equals to only 1-2 bits for different gene networks and model organisms [72, 162, 181, 184, 185]. This leads to the conclusion that only these states have to be targeted when designing a cell population control strategy.

The next section will be dedicated to the description of some realization in the field of cell population control (also termed cybergenetics), pointing out that the above-mentioned methodology could help at this level by providing a general framework aiming at developing further cell population control procedures.

5.2.2. The contribution of control theory and the nascent field of cybergenetics

The fact that cell population can be controlled based on pulsatile inputs has been reported a long time ago. Indeed, long before the advent of single-cell technologies, Goodwin [186] observed that it was possible to synchronize cell cycle in *E. coli* cells by periodically pulsing phosphate in a phosphate-limited chemostat. This pioneering work has led to the establishment of a robust modeling framework for the understanding of the impact of external conditions on the synchronization of cell cycle for many types of organisms [187–189]. However, these studies have been carried out based on an open-loop control approach and the application of regular pulses with varying frequencies and amplitudes. More recently, the application of control theory to the manipulation of cellular systems, i.e., cybergenetics, has set the ground for a more rational design of cell population control procedures [155, 190]. Cybergenetics is an entirely new and exciting field of research at the interface between control engineering and synthetic biology, and emerged with the recent advances made in genetic engineering combined with the works initially derived from cybernetics [191]. A distinction can be made between “internal cybergenetics” (also called in vivo and involving genetic controllers directly embedded in cells) and “external cybergenetics” (also called in silico controllers) [134, 151, 157, 192]. In the context of this review, we will be focused more on the latter technology, since it involves cell machine interface and pulsatile inputs used as actuators.

Remarkably, although different systems have been used (e.g., various model organisms, type of gene circuits to be controlled, and single cell techniques), all the data accumulated point out that it is possible to effectively control gene circuits at the level of individual cells by applying external periodic signals (Table 1.1). Evidences have been provided suggesting that pulses of inducers tend to decrease noise in biochemical network, leading to synchronized gene expression [193, 194]. This effect can be explained based on the dose response relationship (Figure 1.14 D) where input concentrations at the extremities of the dynamic range lead to a homogenous response at the population level. In contrast, input concentrations at the center of the dynamic range produce a heterogeneous population. This strategy, known as PWM, seems to be generalizable for the effective control of diverse gene circuits in diverse cellular systems. Most of the experiments involving the control of gene expression in cellular systems have been performed in microfluidic devices (Table 1.1). This type of cultivation device allows the acquisition of single cell data with a high spatio-temporal resolution, but with a low experimental throughput due to the time and computational power required for image analysis [195] and with possible technical biases by comparison with conventional cultivation devices [196, 197]. Nonetheless, there is a growing interest in using standard cultivation devices (e.g., flasks, bioreactors, etc.) for studying and controlling cell populations [198]. In this case, single-cell analyses can be performed based on AFC, leading to the rapid accumulation of data at the

population level. In this context, the use of cell machine interface relying on FC can lead to the automated and systematic determination of the optimal stimulation frequency for the effective synchronization of gene expression at the population level [156].

Table 1.1: Range of environmental stimuli used for controlling gene expression in cell population and range of periodic signal associated with these environmental perturbations.

Approximated period T for the input stimulation ^A	Type of perturbation ^B	Controlled trait	Organism	Culture system	Single-cell analysis tool	References
83 min*	Pulse of phosphate in phosphate-poor medium	Cell cycle	<i>E. coli</i>	Bioreactor 240 ml	None	[186]
98 min*	Pulses of methionine to induce ($P_{met3-cln2}$)	Cell cycle	<i>S. cerevisiae</i>	Microfluidics	Microscopy	[199]
110 min* 150 min*	Nutrient availability, i.e., poor-rich	Cell cycle	<i>S. cerevisiae</i>	Microfluidics	Microscopy	[200]
16 min 45 min	Red-far red	<i>gal1</i> -responsive genes through optogenetic	<i>S. cerevisiae</i>	Microplates with 96 wells	FC	[201]
28 min 88 min	Pulses of sorbitol-enriched medium in normal medium	Osmostress (1,500 a.u.)	<i>S. cerevisiae</i>	Microfluidics	Microscopy	[193]
142 min	Glucose-galactose	$P_{gal1}::GFP$	<i>S. cerevisiae</i>	Microfluidics	Microscopy	[202]
990 min	Tetracycline	Tetracycline-inducible system $PP_{cmv-tet}::d2EYFP$	Mammalian (CHO)	Microfluidics	Microscopy	[203]
120 min	Green light intensity (in relation with red light intensity)	CcaS/R gene expression system at a varying level of expression	<i>E. coli</i>	Bioreactor 20 ml	AFC	[155]

Table 1.1 – *Continued from previous page*

Approximated period T for the input stimulation ^A	Type of perturbation ^B	Controlled trait	Organism	Culture system	Single-cell analysis tool	References
6 min	Computed open-loop green-red light	CcaS/R-based system	<i>E. coli</i>	Microfluidics	Microscopy	[204]
150-210 min (depending on amplitude/concentration)	IPTG—aTc	Maintain a toggle switch at an unstable intermediary level	<i>E. coli</i>	Microfluidics	Microscopy	[157]
10 min	High/low red intensity, blue light	Transcription	<i>S. cerevisiae</i>	Microfluidics	Microscopy	[158]
Cycles OFF/ON 5/80 s 8/80 s 11/80 s	Blue light	GFP expression	<i>S. cerevisiae</i>	Microplates 24 wells	None	[205]
Cycles OFF/ON 65/15 s	Blue light	Production of isobutanol and 2-methyl-1-butanol	<i>S. cerevisiae</i>	For isobutanol /butanol: Bioreactor 0.5 L	None	[205]
5-60 min	Pulses of galactose to stabilize synuclein concentration at different values	a-synuclein formation	<i>S. cerevisiae</i>	Microfluidics	Microscopy	[206]

Table 1.1 – Continued from previous page

Approximated period T for the input stimulation ^A	Type of perturbation ^B	Controlled trait	Organism	Culture system	Single-cell analysis tool	References
90/105/180 min	Methionine concentration in cultivation medium	Yeast cell cycle coordination	<i>S. cerevisiae</i>	Microfluidics	Microscopy	[207]
600 min	Arabinose pulses	Arabinose operon induction	<i>E. coli</i>	Bioreactor 1 L (continuous mode)	On-line FC	[156]

^A This column indicates the range of periodic signal associated with these environmental perturbations. In most of the case, the input signal can be approximated by a square wave of period T and frequency 1/T (see Figure 1.14 B for more details).

^B This column indicates the range of environmental stimuli used for controlling gene expression.

* These stimulation periods have been determined without monitoring and feedback control (open-loop control).

5.2.3. Perspective: Exploiting intrinsic frequency of gene circuits

Taken altogether, the elements assembled in the previous sections point out that a lot of different gene circuits architectures can exhibit periodic behavior (and not only the motifs reported to behave as natural oscillators, such as the repressilator [208] or the oscillator motif [170, 171]) if stimulated at the appropriate frequencies [95]. Development made in information theory and in cybergenetics provides the computational framework and the experimental tools in order to generalize this concept to many biological systems. Impressive achievements can be expected from these field of research such as the control of complex cell regulatory program (e.g., control of cell cycle program [207]) and the control of microbial communities composition [209, 210], with applications in various field from bioproduction [198, 211] to biomedicine [98, 154].

6. Thesis objectives

Previous work in our group has shown that microbial populations cultivated in continuous, theoretically homogeneous bioreactors undergo diversification processes that strongly depend on the associated fitness costs. We have also demonstrated that the Segregostat control strategy can be successfully implemented at the laboratory scale in various systems.

Building on these findings, this thesis pursues two main objectives: one of a more fundamental nature, and the other oriented toward potential applications.

1. To gain a deeper understanding of phenotypic diversification in continuous culture, by investigating both its functionality and underlying mechanisms. In particular, we test the hypothesis that the increase in entropy observed in systems with high fitness costs correlates with the overall fitness of the population.
2. To assess whether the Segregostat can be applied to control cell populations under conditions mimicking large-scale bioreactors, through experiments performed in a dedicated scale-down system.

To address these objectives, a reliable reporter of the general stress response in *E. coli* was first established through a multi-scale screening approach, complementing the one already available for *S. cerevisiae* and providing a robust foundation for subsequent analyses. We then addressed the first research aim by applying a novel on-line FC-based framework to quantify the trade-off between growth and gene expression, thereby deepening our understanding of phenotypic diversification in continuous culture. Finally, we explored the use of the Segregostat for population-level control under scale-down bioreactor conditions, revealing how the frequency and amplitude of environmental perturbations shape cellular responses. Altogether, this work offer an integrative view of stress-response dynamics across scales and organisms, and open new perspectives while suggesting new approaches for future bioprocess design, single-cell monitoring, and population control.

2

Chapter 2: Selection of a suitable reporter for the GSR in *E. coli*

This chapter is an extended version of Supp. Notes A.5 & A.6 from the Supplementary Material of the preprint referenced below.

Delvenne, M., Vandenbroucke, V., Henrion, L., Sehrt, M., Martinez, J. A., Sloodts, A., Telek, S., Zicler, A., and Delvigne, F. *A Fitness Entropy Compensation Effect Is Set by the Trade-off between Growth and Gene Expression in Cell Populations*. 2025. DOI: 10.1101/2025.07.05.663304. URL: <https://www.biorxiv.org/content/10.1101/2025.07.05.663304>. Pre-published

1. Introduction

For *Escherichia coli*, various reporters of the general stress response (GSR) have been employed [64, 65, 116], including constructs directly monitoring *rpoS* transcription [31, 117].

One may wonder why we did not use an *rpoS* transcriptional reporter directly. There are three main reasons. First, as only a few RpoS molecules are produced per cell [213], detecting its transcriptional activity with our cytometry setup would be challenging. Second, caution is required when using *rpoS* transcriptional reporters from available plasmid collections, as not all constructs necessarily include the full set of *rpoS* promoters. In many cases, the major promoter (*rpoSp1*), located within the *nlpD* gene more than 500 nucleotides upstream of *rpoS*, is absent [4, 57, 214]. These two points together may explain why, according to preliminary results and subsequent screenings, the fluorescence signal from our *rpoS* reporter was indistinguishable from the autofluorescence of the wild type (WT) strain (Figure 2.3 A). Finally, using an *rpoS* transcriptional reporter is inherently tricky, since RpoS activity is subject to multi-level regulation [4, 54, 215]. For example, in exponentially growing cells RpoS is expressed at a basal level but rapidly degraded. Under other conditions, it may accumulate without necessarily forming the active RpoS-containing RNAP holoenzyme ($E\sigma^S$). Consequently, a transcriptional reporter of *rpoS* provides a potentially biased representation of the actual GSR activation state.

For these reasons, it is more reliable to monitor the expression of a gene regulated by $E\sigma^S$ as a proxy for the activation of the GSR. To identify the reporter best suited to our objectives, we implemented a multi-stage screening process (summarized in Figure 2.1). This approach progressively narrowed down candidates from the broad RpoS regulon to a single validated reporter through the following four steps (described in more detail in the following sections):

1. Identification of the candidates: Out of the ~ 500 genes in the RpoS regulon, a representative subset was first identified based on specific criteria leveraging literature-based data.
2. First screening: This initial pool was evaluated in the Biolector, a microfermentation system enabling online monitoring of biomass and fluorescence in microtiter plate, to assess basal expression levels, induction kinetics during the transition to stationary phase, and the dynamic range of expression.
3. Second screening: Shortlisted candidates were further tested under chemostats at various dilution rates. This step ensured that the reporter remains a reliable proxy for $E\sigma^S$ activity across a gradient of stress levels. Additionally, it confirmed that the signal is clearly detectable by automated flow cytometry (AFC) under our standard laboratory cultivation conditions.
4. Verification of the selected reporter: The final selected reporter was validated by monitoring single-cell dynamics in a microfluidic single-cell cultivation (MSCC) device.

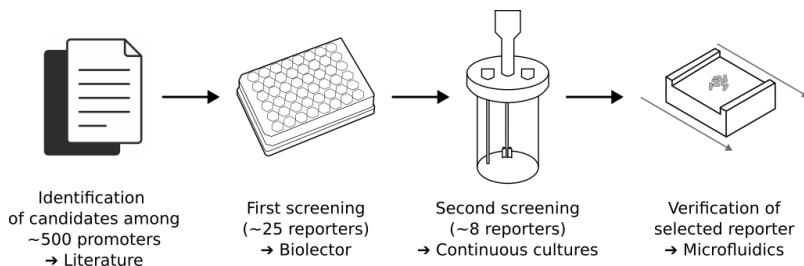


Figure 2.1: Workflow for the selection of a reliable GSR transcriptional reporter in *E. coli*

2. Identification of the candidates

The regulon controlled by the master regulator of the GSR, the transcription factor RpoS, encompasses approximately 500 genes. Given this large number of potential candidates, we selected a representative subset of genes according to the following criteria:

1. Expression noise [14]: Prioritizing genes with high noise levels, as stochasticity in gene expression is a primary driver of phenotypic diversification.
2. Regulatory complexity and topology: Selecting genes with diverse regulatory architectures, including (i) RpoS (σ^S) dependency and co-regulation by other transcription factors, (ii) position within the regulatory network (regulators vs. downstream targets), and (iii) integration of global signals such as cAMP or (p)ppGpp (based on the EcoCyc database [48]).
3. Response dynamics [60]: Including genes that span a wide range of activation thresholds and sensitivity profiles to varying RpoS concentrations.
4. Functional diversity: Covering different functions involved in response to various stresses (pH, osmotic, etc.) (based on the EcoCyc database [48]).

Because the expression of many *rpoS*-regulated genes is anticorrelated with growth rate, this property also guided our selection. Such anticorrelation has been demonstrated in microfluidics for *bolA*, *evgA*, *gadX*, *oxyR*, and *rpoH* [64, 65]. The complete set of candidates selected for the first step of the screening is listed in Table 2.1. In addition to these RpoS-regulated genes, we simultaneously analyzed *cyaA* and *relA* reporters. These genes encode enzymes involved in the synthesis of cyclic adenosine monophosphate (cAMP) and guanosine pentaphosphate and tetraphosphate ((p)ppGpp), respectively, two secondary messengers that play key roles in the regulation of *rpoS*.

Initially, the candidate genes were selected to capture diverse and representative features within the RpoS regulon. This strategy was motivated by the idea of exploring potential relationships between the expression dynamics of different genes (see Discussion), as well as enabling further investigations under various stress conditions beyond nutrient limitation. As a consequence, some of the selection criteria (such as those favoring complex regulatory architectures) were not fully aligned with the primary goal of identifying a reliable reporter of $E\sigma^S$ activity. Therefore, these criteria were progressively refined throughout the successive screening steps to better match this objective.

Table 2.1: Summary of potential reporters for the GSR in *E. coli*

Promotor	Function	σ factors	ppGpp	cAMP -CRP	Target genes	RpoS sensitivity	Noise
<i>ada</i>	adaptive response against DNA alkylating agents	σ^S, σ^{70}	ind.	ind.	4	linear +	0,24
<i>appC</i>	subunit of cytochrome bd-II, role in respiration	σ^S, σ^{70}	ind.	ind.			0,40
<i>bolA</i>	GSR	σ^S, σ^{70}	+	ind.	11	sensitive +	0,17
<i>cbpA</i>	curved DNA-binding protein	σ^S, σ^{70}	+	ind.		linear +	0,28
<i>csiE</i>	unclear physiological role	σ^S, σ^{70}	ind.	+		linear +	0,11
<i>cyaA</i>	catalyze synthesis of cAMP	σ^{70}	ind.	-			0,02
<i>evgA</i>	multi-drug resistance	σ^S, σ^{70}	ind.	ind.	19		0,25
<i>gadX</i>	regulation of acid resistance system	σ^S, σ^{70}	+	-	34	linear +	0,28
<i>glgS</i>	motility and biofilm formation	σ^S	+	+			0,34
<i>hdeD</i>	inner membrane protein related to acid response	σ^S, σ^{70}	+	ind.		insensitive +	0,91
<i>osmC</i>	osmotically inducible peroxiredoxin, implied in oxidative stress resistance	σ^S, σ^{70}	+	ind.		linear +	0,10
<i>otsB</i>	trehalose metabolism	σ^S	+	ind.		linear +	0,39
<i>oxyR</i>	oxidative stress regulator	σ^S, σ^{70}	ind.	+	45		-0,14
<i>patA</i>	putrescine pathway 1	σ^S, σ^{54}	+	ind.		linear +	0,62
<i>poxB</i>	pyruvate oxidase	σ^S, σ^{70}	+	ind.		linear +	0,29
<i>puuA</i>	putrescine pathway 2	σ^S	ind.	ind.		linear -	0,49
<i>puuD</i>	putrescine pathway 2	σ^S	ind.	-		linear -	0,49

<i>relA</i>	stringent response: activate synthesis of (p)ppGpp	σ^{70}, σ^{54}	ind.	+			
<i>rpoH</i>	σ^{32} , transcription factor of heat shock response	$\sigma^S, \sigma^{70},$ σ^{54}, σ^{24}	ind.	+ or -	174		0,09
<i>rpoS</i>	σ^S , transcription factor of general stress response	σ^{70}	+ or -	+ or -	342	linear +	
<i>sodC</i>	convert superoxide radicals to hydrogen peroxide and water	σ^S	+	ind.		linear +	0,32
<i>treF</i>	trehalase	σ^S	+	ind.		linear +	1,00
<i>uspB</i>	DNA repair and ethanol tolerance	σ^S	+	ind.		linear +	0,17
<i>wrbA</i>	unclear physiological role	σ^S	+	ind.		linear +	0,41
<i>yciG</i>	involved in motility	σ^S	ind.	ind.		linear +	0,46
<i>ydcS</i>	DNA transport across the inner membrane during transformation	σ^S	ind.	ind.		sensitive +	0,27
<i>yhfG</i>	toxin-antitoxin unclear physiological role	σ^S				sensitive +	0,41

+ : upregulation, - : downregulation, ind. : indirect regulation.

Values in the 'Target genes' column indicate the total count of regulated downstream targets (also called regulatees) as identified in the EcoCyc database [48].

sensitive : induction at low RpoS level, insensitive : high RpoS levels required to induce the gene, linear : linear correlation between RpoS level and induction strength [60].

The noise values are taken from Silander et al. [14] and normalized to the highest value among the investigated promoters (i.e., *treF*), which itself ranks as the fourth highest among all genes tested. With the exception of *cyaA*, all selected genes fall within the 50 % noisiest genes measured by Silander et al. [14].

3. Screening and selection of the general stress reporter for *E. coli*

3.1. Initial screening of the candidates

The initial screening was performed in batch cultures using the Biolector platform to evaluate (i) basal expression levels, (ii) dynamic expression changes during the transition to stationary phase (i.e., entry into stressful conditions), and (iii) the dynamic range of expression, defined as the difference between basal and maximum expression levels (Figure 2.2).

Samples were taken during the exponential growth phase and late stationary phase for cytometry analysis and Shannon entropy computation (Figure 2.3).

Based on these results, we shortlisted candidates to be tested in the second screening step considering the following criteria:

1. High expression levels: Candidates had to exhibit fluorescence levels sufficiently high to be clearly distinguished from cellular autofluorescence by flow cytometry (FC), while remaining within the detector's range.
2. Observable dynamic range: We sought a noticeable difference between basal expression (exponential growth) and induced expression (stationary phase) to ensure a clear detection of stress response activation.
3. Diversity of kinetic profiles: We intentionally selected candidates exhibiting a variety of expression patterns during the transition to stationary phase (e.g., differences in activation timing and induction trends throughout the stationary phase).

Beyond these general criteria, specific biological and contextual factors also guided our selection. For instance, *hdeD* was chosen to be paired with *gadX* as it is directly regulated by the latter, allowing for a more integrated analysis of this regulatory circuit. Additionally, *bolA* and *yciG* were included to ensure comparability with previous studies conducted by our group.

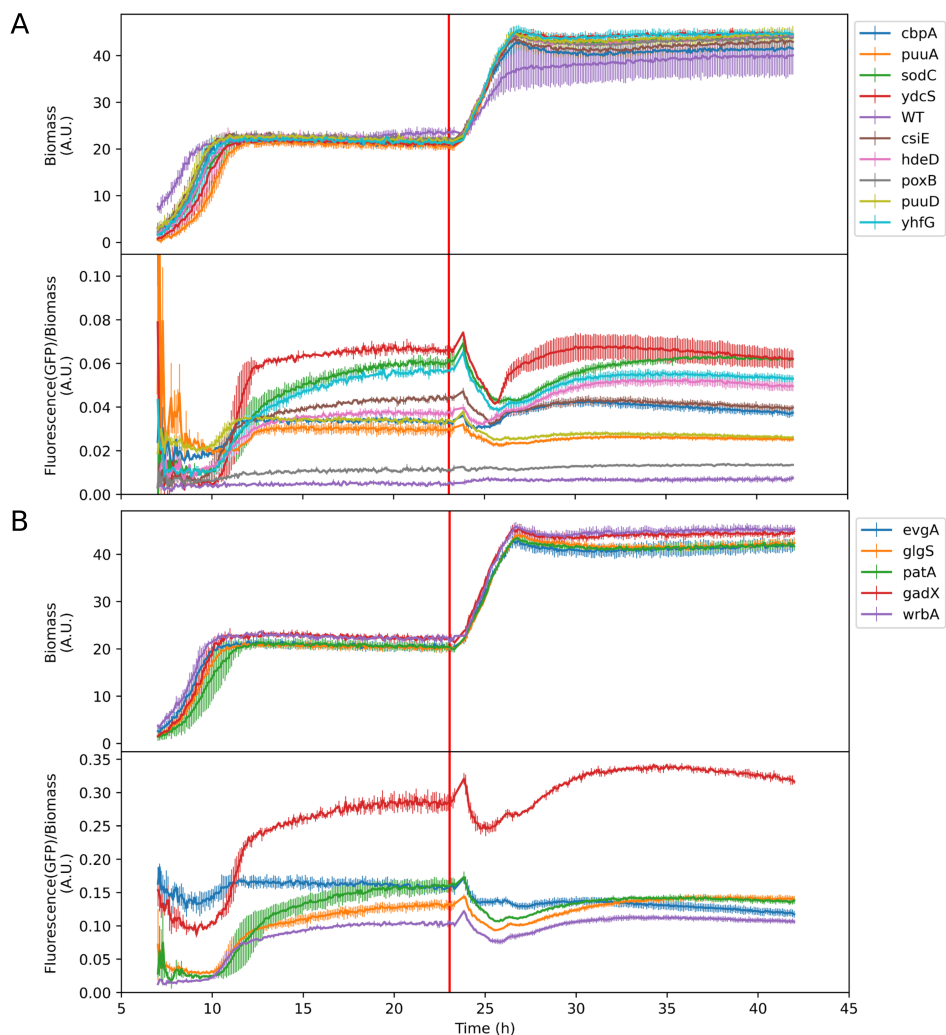


Figure 2.2: Example of visualization of data from the Biolector screening for **A** reporters with low to intermediate expression levels and **B** reporters with higher expression levels. The top panel represents the biomass over time, while the second panel shows the relative fluorescence (GFP signal 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 monotonic 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.

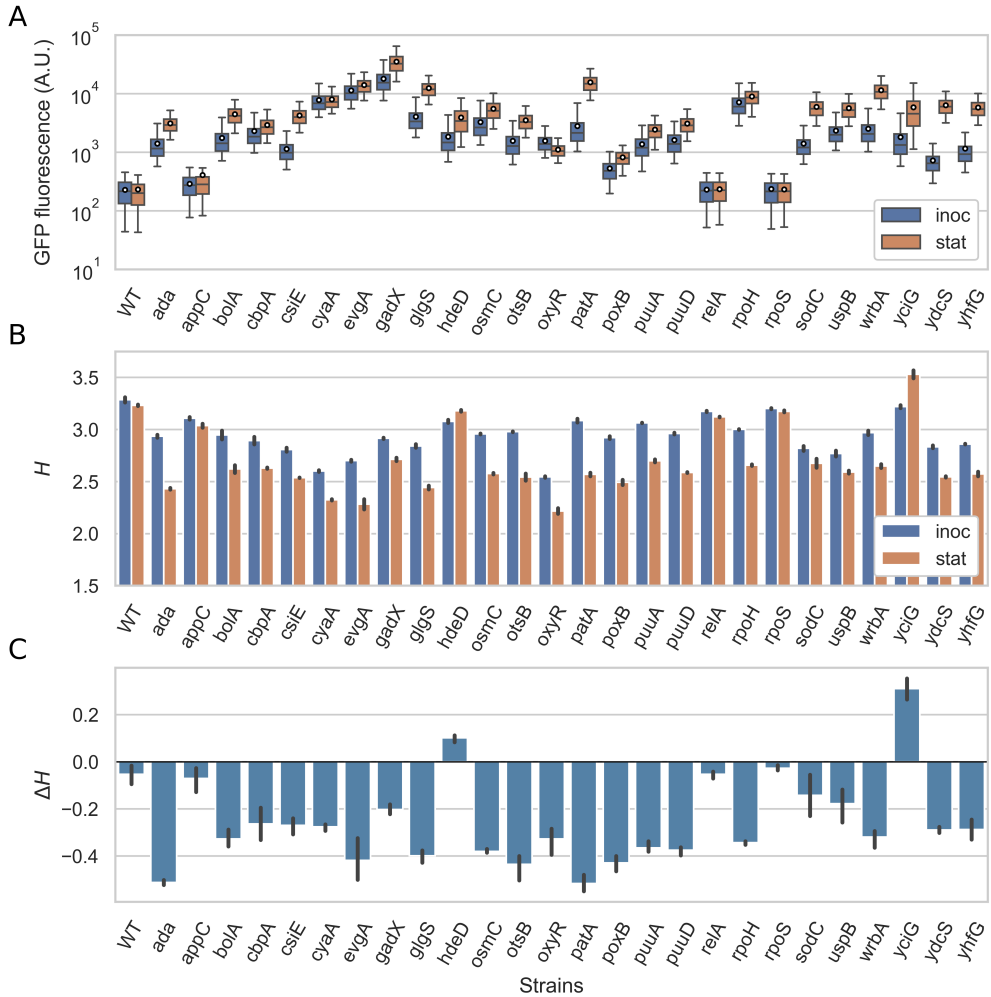


Figure 2.3: Fluorescence distributions and Shannon entropy values of different transcriptional reporters sampled in the Biolector platform from inoculum at exponential growth (inoc, $n = 3$) and at stationary phase (stat, $n = 3$). **A** Distribution of fluorescence represented as boxplots; the mean is indicated as a white dot and whiskers extend to the 5th and 95th percentiles. Boxplots were generated by pooling single-cell measurements from the three biological replicates. **B** Shannon entropy (H) values for each strain under both conditions. **C** Differences in entropy values between both conditions ($\Delta H = H_{\text{stat}} - H_{\text{inoc}}$).

3.2. Comparison of reporters under various continuous culture conditions

To reflect the cultivation conditions typically studied in our laboratory, the selected reporters were further evaluated in a continuous cultivation device across various dilution rates, corresponding to different stress levels (Figure 2.4). In chemostat cultivations, the dilution rate sets the specific growth rate and is directly correlated with residual substrate concentrations, while being inversely correlated with the expression of stress-related genes [62, 216].

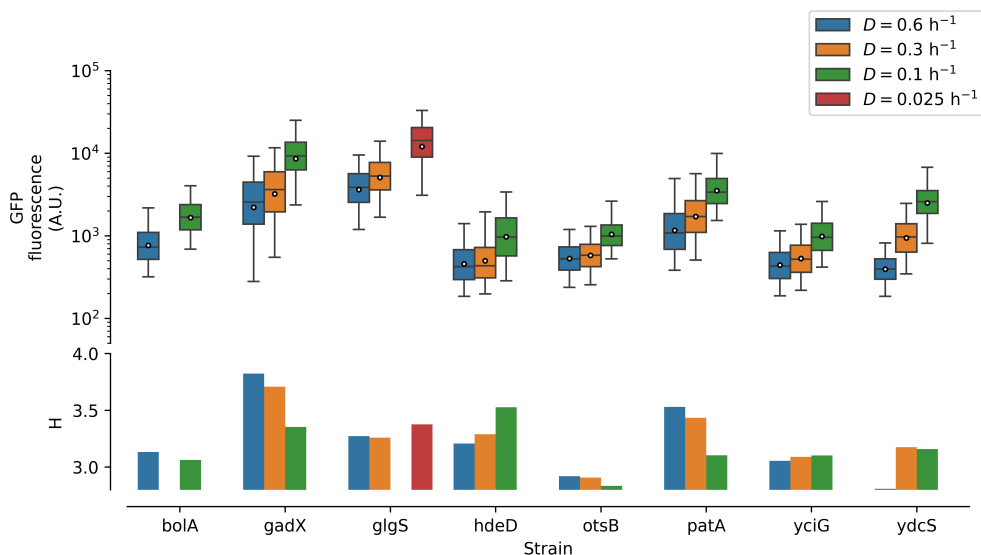


Figure 2.4: Summary plot of continuous cultures at different dilution rates performed with a subset of transcriptional reporters. The top panel represents the distribution of fluorescence as boxplots, where each boxplot corresponds to a single cytometry file from the final measurement of each dilution rate (see Material and methods). The mean is indicated as a white dot and whiskers extend to the 5th and 95th percentiles. The second panel represents the Shannon entropies of corresponding samples. Note that the results presented here are based on single experiments without replicates and should therefore be interpreted with caution. In the case of *ydcS*, subsequent experiments (presented in the next chapters) showed that population heterogeneity increases at higher dilution rates and decreases at lower dilution rates.

From these experiments, we ultimately identified *ydcS* as the optimal reporter. The *ydcS* gene encodes a putative transport protein associated with natural and chemical transformations [217] and carbon flux distribution [218]. It was selected due to its low basal expression, its large dynamic range with a clear distinction between different expression levels across dilution rates (i.e., stress levels), and its strong dependence on $E\sigma^S$ [219, 220]. To the best of our knowledge, it has a relatively simple regulatory

circuit, which facilitates biological interpretation by minimizing potential cross-talk with other regulatory networks or environmental signals. Indeed, RpoS is currently the only transcription factor known to initiate its expression (EcoCyc [48]). However, low levels of *ycdS* transcripts have been detected in $\Delta rpoS$ strains [219, 220], suggesting a minor $E\sigma^S$ -independent expression. Nevertheless, given its high sensitivity to $E\sigma^S$ availability [60] and its minimal background signal in comparison to other potential reporters, *ycdS* serves as a reliable proxy for the physiological activity of RpoS.

The single-cell dynamics of expression of *ycdS* were then verified in a MSCC device.

4. Verification of the correlation between *ycdS* and *rpoS* expression

To validate *ycdS* as a reporter of $E\sigma^S$ activity, we constructed a dual-reporter strain (*E. coli* MG1655 $P_{rpoS}::\text{CyOFP1 } P_{ycdS}::\text{GFPmut2}$) and observed its single-cell dynamics. This strain combines a chromosomally integrated *rpoS* transcriptional reporter, capturing the major promoter absent from the available plasmid, with a plasmid-based *ycdS* reporter from the Zaslaver collection.

Time-lapse microscopy in a MSCC device, under limiting (5 μM) and non-limiting (3 mM) glucose concentrations, revealed that *ycdS* (GFP) expression increases under stressful conditions and strongly correlates with *rpoS* (CyOFP) levels (Figure 2.5). Thanks to its low basal expression, *ycdS* provides a clear resolution between stressed and non-stressed phenotypes. By contrast, the *rpoS* reporter shows a high basal expression, confirming that a direct transcriptional reporter of *rpoS* is not a reliable proxy for the actual activity of the protein.

Interestingly, *ycdS* differed from the stress-related reporters described by Sampaio et al. [65], showing almost no expression under non-limiting conditions, likely due to its stronger dependency on $E\sigma^S$, while its distribution under limiting conditions ($\text{CV} = 0.32$, skew = 0.25) resembled theirs in non-limiting environments (Figure 2.5 C).

Simultaneously, we monitored single-cell growth rates (Figure 2.6). Reporter expression was moderately anticorrelated with growth under glucose-limiting conditions, while, as expected, no anticorrelation was observed under non-limiting conditions.

Together, these findings indicate that *ycdS* is a reliable and practical reporter of the GSR.

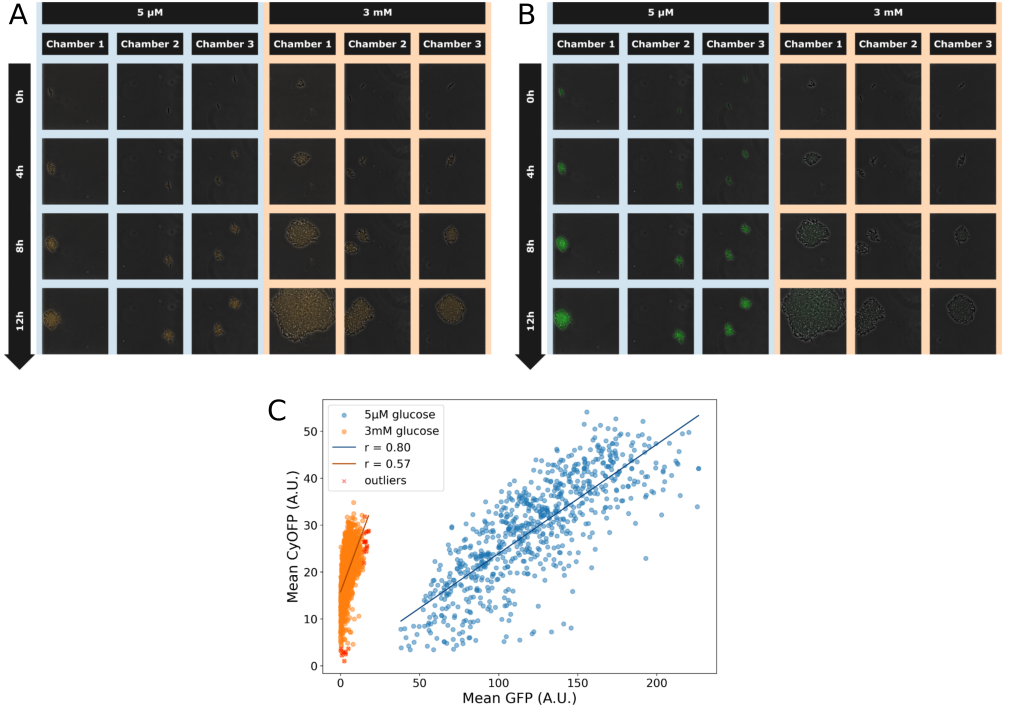


Figure 2.5: Time-lapse microscopy of *E. coli* $P_{rpoS}::\text{CyOFP1}$ $P_{ydcS}::\text{GFPmut2}$ cultivated in a MSCC device under stressful (5 μM glucose, blue) and non-stressful (3 mM glucose, orange) conditions, and correlation between the expression levels of the two transcriptional reporters. **A-B** Time-lapse microscopy of three microfluidic chambers per condition. For clarity, images displaying (A) CyOFP fluorescence and (B) GFP fluorescence are shown separately. **C** Correlations between $P_{rpoS}::\text{CyOFP1}$ and $P_{ydcS}::\text{GFPmut2}$ mean single-cell fluorescence values. Each dot represents a single-cell (pooled from a total of 5 cultivation chambers per glucose concentration condition). Outliers correspond to datapoint with a $|z\text{-score}| > 3$.

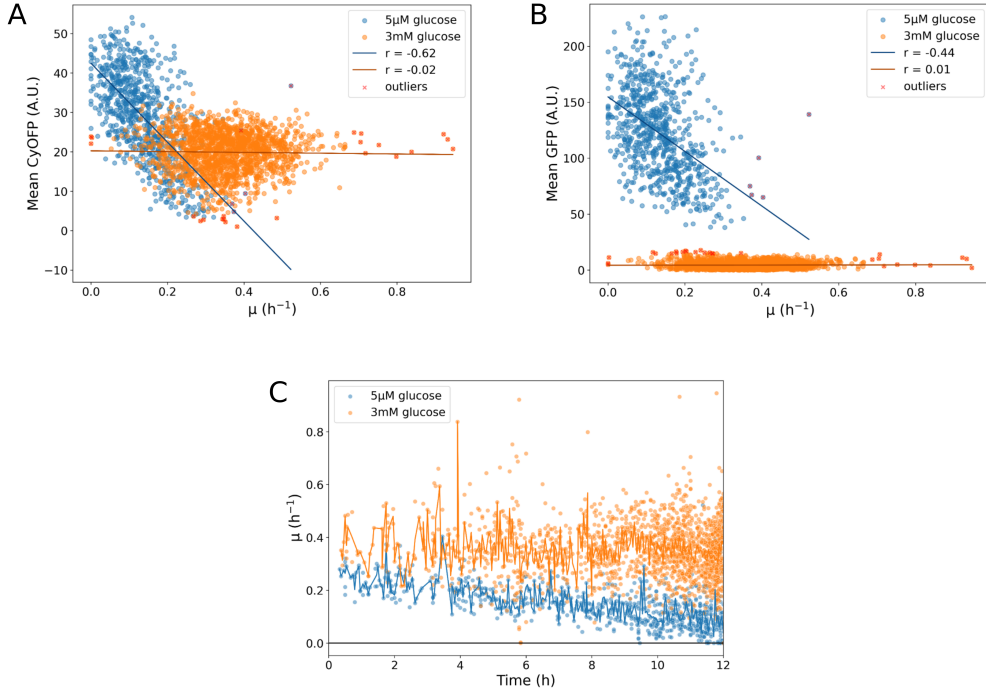


Figure 2.6: Single-cell growth in limiting (5 μM glucose, blue) and non-limiting (3 mM glucose, orange) conditions in a MSCC device. Each dot represents a single-cell (from a total of 5 cultivation chambers per glucose concentration condition). Outliers are datapoint with a $|z\text{-score}| > 3$. **A** 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.

5. Discussion

5.1. Validation of *ydcS* as a robust proxy for *RpoS* activity: limitations and perspectives

Before using our chosen reporter, *ydcS*, as a proxy for the activation of the GSR, it was essential to ensure that it accurately reflects the activity of $E\sigma^S$. While *RpoS* is the primary sigma factor initiating its expression, the detection of trace amounts of *ydcS* transcripts in $\Delta rpoS$ strains [219, 220] suggests a minor level of $E\sigma^S$ -independent transcription. However, the fact that *ydcS* expression levels correlated with stress intensity (demonstrating low basal expression under low-stress conditions) and the strong correlation observed in our dual-reporter strain between *rpoS* and *ydcS* expression, corroborate that *ydcS* induction is a reliable indicator of the master regulator's activity. Nonetheless, as previously noted, a transcriptional reporter of *rpoS* is not a reliable proxy for the actual activity of the protein itself due to complex post-translational regulations. Therefore, further characterization using strains with impaired or enhanced $E\sigma^S$ activity (such as $\Delta rpoS$ or $\Delta rssB$ [4], respectively) could provide an even finer resolution of its induction dynamics and resolve any remaining ambiguity.

Another potential concern in our experimental design is the use of a plasmid-based reporter, which may influence observed population heterogeneity. Fluctuations in plasmid copy number can indeed introduce baseline noise. To quantify this effect, a plasmid-based constitutive reporter could be used as a control. Furthermore, a chromosomal integration of the *ydcS* reporter would ideally eliminate any plasmid-specific artifacts. Sampaio et al. [65] performed these two controls with *gadX* reporters, showing that fluctuations in a constitutive reporter do not reach the level of variation observed for stress-related reporters. They also confirmed that signal dynamics remain comparable between plasmid-based and chromosomally integrated versions, despite lower expression levels in the latter. Although these findings were obtained with a different reporter, they provide a strong methodological precedent that supports the validity of our approach and suggests that the biological signal of the GSR remains the dominant driver of the observed heterogeneity.

5.2. Single-cell dynamics of multiple reporters

Time-lapse microscopy in microfluidic systems offers a powerful framework to study single-cell dynamics. Notably, it enables the direct linking of gene expression and growth rate at the single-cell level [64, 65]. Using multiple reporters, it is also possible to examine the relationship between the expression of different genes, as we did with our dual-reporter strain (*E. coli* MG1655 $P_{rpoS}::\text{CyOFP1}$ $P_{ydcS}::\text{GFPmut2}$), and to study information transmission between genes within the same pathway [143].

The possibility of applying such multi-reporter strategies was one of the reasons

why we initially considered a relatively broad range of reporter candidates instead of restricting the selection to putative RpoS-exclusive genes. By selecting genes occupying different positions within the regulatory network (e.g., regulator versus regulated), we kept the opportunity to investigate the temporal relationships between a regulator and its targets, and to quantify the dynamic influence of RpoS on its regulon. The *gadX* promoter, for instance, appeared to be an attractive candidate because it had already been characterized [65] and plays an important regulatory role in the acid stress response, which would have allowed us to investigate an additional stress condition in greater detail. However, strain construction is time-consuming, and microfluidics data analysis is even more so. We therefore chose to put aside complex regulatory systems and selected *ycdS* as a reliable indirect reporter of RpoS activity.

Indeed, all the opportunities offered by microfluidic systems come with their own challenges. Tracking single-cell lifelines and analyzing them precisely with sufficient replicates is labor-intensive and time-consuming. Moreover, few ready-to-use pipelines exist for microfluidic image processing; we therefore assembled one based on existing tools (see Material and methods). This scarcity likely stems from the fact that several steps are setup-dependent, such as image preprocessing before applying cell-segmentation algorithms. Nevertheless, despite the absence of standardized pipelines, it is important to highlight the considerable efforts made by several research groups to develop efficient microfluidic systems and associated protocols [120], as well as image-analysis tools for cell segmentation[128, 129], tracking, and lineage reconstruction [221, 222]. These significant contributions, shared openly with the scientific community, greatly accelerate data acquisition and research in general.

5.3. Impact of conditions on heterogeneity of stress response

Several studies have investigated the expression of GSR-related genes during exponential growth. For instance, Valencia et al. [28] examined the impact of RpoS levels on antibiotic resistance, tolerance, and persistence. However, gene expression and associated physiological traits clearly depend on growth conditions. Under such conditions, RpoS may remain inactive [4, 54, 215], which justifies the use of indirect reporters of its activity rather than monitoring transcription or protein concentration directly (although concentration is already more informative than transcription, as it integrates post-transcriptional regulation). For example, Patange et al. [64] employed *bolA* as an indirect RpoS reporter. In our case, *ycdS* was selected as a relevant reporter because of its lower basal expression, larger dynamic range, and stronger dependence on $E\sigma^S$. Both *bolA* and *ycdS* therefore serve as reporters of RpoS activity and, more broadly, of the GSR in *E. coli*. Nonetheless, it is worth noting that they are not among the best reporters of metabolic burden in the context of heterologous protein production [223], and should not be used for that purpose.

As expected, our BioLector screenings indicated that most tested reporters displayed

similar dynamics, activating approximately upon entry into stationary phase, with differences in timing (Figure 2.2). They also suggested that GSR-related gene expression tends to be more homogeneous under stressful conditions (stationary phase) than during exponential growth (Figure 2.3). In both cases, population distributions remained unimodal. This is consistent with noise measurements in exponentially growing cells by Silander et al. [14], and supports the idea of a bet-hedging-like strategy. Conceptually, it is coherent that upon transition to stress, cells respond collectively by activating the GSR, while in the absence of stress signals expression is more stochastic. Mechanistically, reduced dilution of sensing and signaling components at slower growth may enhance the responsiveness of regulatory circuits [224].

Nevertheless, this interpretation requires caution. For example, *hdeD* and *yciG* displayed opposite behaviors in both BioLector and continuous cultures at different dilution rates (Figure 2.3 and Figure 2.4). Thus, genes such as *patA* and *hdeD*, identified as noisy in exponential phase [14], could either homogenize or become more heterogeneous under stress. Comparable patterns have been observed in other contexts, including nutrient-limited conditions [225, 226]. Such “decisions” occur mainly at the promoter level, depending on sigma factor competition, as well as additional regulatory inputs [226]. This highlights that, although under the control of the same master regulator RpoS, uniform responses cannot be assumed. Notably, RpoS-regulated genes differ in their sensitivity to RpoS, resulting in a graded and selective activation of target promoters [60]. This differential responsiveness underlies a coordinated and temporally ordered gene expression program, ensuring that the various cellular functions are activated in a timely manner and that stress adaptation is properly aligned with the growth phase and physiological demands [60]. Similar principles likely apply to other regulators.

From a functional standpoint, it is plausible that some genes become noisier under stress when they are not immediately required, but may confer an advantage if additional or stronger stresses occur. Such heterogeneity thus adapts the cross-protection strategy of the GSR to more specific responses. This seems to be the case for *hdeD*, which is not essential for acid resistance but beneficial during prolonged exposure to low pH [227, 228]. Acid stress provides a particularly good illustration of complex regulatory circuits, with several genes exhibiting heterogeneous expression [65, 229, 230]. Notably, GadE and GadX engage in both self- and mutual activation, while GadX also interacts with RpoS as both regulator and target. Such positive feedback loops can enhance heterogeneity in the expression of downstream genes [230]. This phenomenon could be considered a form of “responsive diversification”, because the response to stress increases phenotypic heterogeneity within the population [8, 225, 231]. Whether responsive diversification should be considered a form of bet-hedging depends largely on definitions: in the strict sense, bet-hedging refers to heterogeneity generated prior to stress, whereas responsive diversification arises

directly from stress exposure [8]. In either case, this concept is closely related to the mechanism we term Fitness-Entropy Compensation, which will be introduced in the next chapter.

6. Material and methods

The materials and methods presented here partially overlap with those described in chapter 3 and appendix A, which correspond to the related prepublication. Key methodological details are repeated here to ensure readability and coherence within this chapter.

6.1. 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.

6.2. Strains and plasmids

E. coli MG1655 strains from the Zaslaver collection [214] 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 analyzed 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_{yjiG} , 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_{yjiG} and P_{ydcS} . The dual-reporter strain used in MSCC experiments was constructed by introducing the P_{ydcS} plasmid into the *E. coli* MG1655 $P_{rpoS}::\text{CyOFP1}$ strain. *E. coli* MG1655 $P_{rpoS}::\text{CyOFP1}$ was constructed using the same CRISPR-cas9 enhanced lambda red phage mediated homologous recombination method described by Jiang et al. [232]. The CyOFP1 gene was inserted downstream of the *rpoS* gene with a synthetic ribosomal binding site (RBS) 5'-AAAGAGGAGAAA-3' [208, 233]. 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 400 bp around the site of insertion. Tailed primers have been used to include the synthetic RBS in the cassette. The primers used are described in Supp. Table A.1.

6.3. Growth media

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

E. coli strains have been cultivated in a mineral medium containing (in g/l), K_2HPO_4 : 14.6, $NaH_2PO_4 \cdot 2H_2O$: 3.6, Na_2SO_4 : 2, $(NH_4)_2SO_4$: 2.47, NH_4Cl : 0.5, $(NH_4)_2$ -H-citrate: 1, glucose: 5, thiamine: 0.01. The medium is supplemented with a trace element solution totaling 11 ml/l assembled from the following solutions (in g/l), 3/11 of $FeCl_3 \cdot 6H_2O$: 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 g/L): $CaCl_2 \cdot 2H_2O$: 0.74, $ZnSO_4 \cdot 7H_2O$: 0.18, $MnSO_4 \cdot H_2O$: 0.1, $CuSO_4 \cdot 5H_2O$: 0.1, $CoSO_4 \cdot 7H_2O$: 0.21. For plasmid maintenance, it has been supplemented with kanamycin (50 mg/l). The trace element solution, the thiamine and the antibiotic were filter-sterilized (0.22 μm) before supplementation of the other component that were heat-sterilized (at 120°C).

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

6.4. Screening in Biolector platform

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 the main document, 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 pre-cultivated 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 flower-plates (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 correspondings wells of readable biolector flower-plate (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 analyzed in flow-cytometry as the sample for exponential growing phase (supposedly with the minimum fluorescence) and their OD_{600} have been measured to approximate the initial OD_{600} 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.

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 $\mu\text{L}/\text{min}$) 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 10^6 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.

6.5. Screening in chemostat experiments at three different dilution rates

Cultivation in the Dasgip bioreactors: Chemostat experiments were performed in Dasgip bioreactors (Eppendorf, Germany) with 150 mL of working volume at controlled pH 7, 1000 rpm, and 37°C. Precultures were performed in two steps to avoid the induction of the reporters. Both steps were performed in 500 ml or 1 L flasks (with respectively 50 or 100 ml of the corresponding medium), at 37 °C (*E. coli*) 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 mg/L kanamycin) 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 (~ 3 h). The batch phase was initiated with an $\text{OD}_{600} \approx 0.1$ and lasted until full consumption of the carbon source (marked by an increase in oxygen concentrations). For the chemostat, 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`".

Online cytometry analysis: For AFC analyses of 3 Dasgips reactors running in parallel, the “multi-stat” system was used. It is an adaptation of our in-house Segregostat [145] 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

phosphate-buffered saline (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 $\mu\text{L}/\text{min}$, 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 10^6 a.u. of GFP fluorescence were used. The cytometry files (‘.fcs’) used to make the Figure 2.4 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.

6.6. Microfluidic cultivations and time lapse microscopy

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

The media used is the same as described above, but with modified glucose concentrations (5 μM 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 μm x 90 μm x 700 nm) [120, 122]. The temperature was set at 37°C. The chambers were inoculated with a few cells by flushing the device with a cell suspension ($\text{OD}_{600} \approx 0.1$). Three biological replicates have been performed for each glucose concentration condition. For two of them, one chamber was analyzed while 3 chambers (technical replicate) were analyzed in the last biological replicate (for a total of 5 chambers for each glucose concentration condition).

Microscopy images 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).

Microscopy images were analyzed 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 preprocessed (normalization, stabilization, 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) [128, 129] 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) [221, 222] with the “Label image detector” and the “Overlap tracker” options (precise IoU calculation, min. IoU = 0.1 and scale factor = 1.1). Singles-cells data and lineages were then exported as tables and further analyzed. For instance, different approximations of the growth rates have been performed. The one used in Figure 2.6 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).

3

Chapter 3: *E. coli* and *S. cerevisiae* exhibit distinct GSR-related population dynamics in continuous culture driven by the trade-off between growth and gene expression

This chapter is an adapted version of the preprint article referenced below.

Delvenne, M., Vandenbroucke, V., Henrion, L., Sehr, M., Martinez, J. A., Sloodts, A., Telek, S., Zicler, A., and Delvigne, F. *A Fitness Entropy Compensation Effect Is Set by the Trade-off between Growth and Gene Expression in Cell Populations*. 2025. DOI: 10.1101/2025.07.05.663304. URL: <https://www.biorxiv.org/content/10.1101/2025.07.05.663304>. Pre-published

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 genetic circuits in continuous culture. Our results reveal that it effectively stabilizes cell populations by mitigating the detrimental trade-offs between growth and gene expression. This stabilization 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 stabilization 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.

2. Introduction

Individual cells within a population activate specific gene circuits in response to stress conditions, often at the expense of cellular growth [54, 57, 234]. Similarly, gene circuits employed in bioproduction can compromise cellular growth and both productivity and predictability [18, 235]. 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 [17, 18, 89, 236].

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 [22]. We observed that low fitness costs result in relatively homogeneous cell population diversification profiles (Figure 3.1 A), whereas high fitness costs lead to more heterogeneous diversification dynamics

(Figure 3.1 B) [22].

To quantify the degree of cell population dispersion in the activation of a given gene circuit, we utilized 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 [142, 237] 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 [14, 144].

We hypothesized 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 to various environmental conditions [6, 7, 11, 94, 153]. 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 FEC 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 FEC can also occur in natural gene circuits, focusing on genes involved in the general stress response (GSR) in *Saccharomyces cerevisiae* and *E. coli*, as well as its potential sources.

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 [238, 239], 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 (AFC) [22]. 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 3.1 A), whereas burdensome gene circuits displayed significantly higher entropy (Figure 3.1 B).

Chapter 3. *E. coli* and *S. cerevisiae* exhibit distinct GSR-related population dynamics in continuous culture driven by the trade-off between growth and gene expression

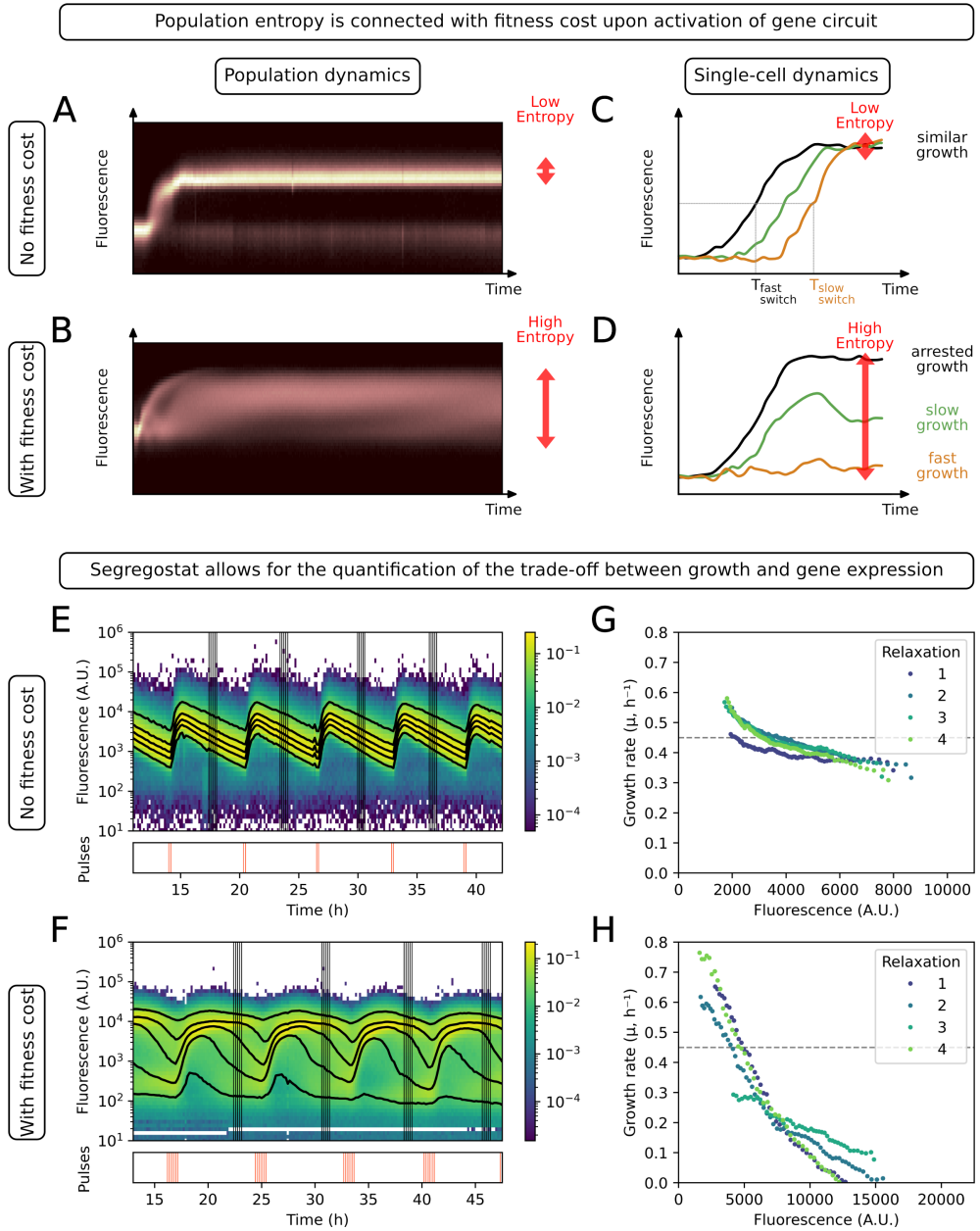


Figure 3.1: Relationship between gene expression, fitness cost, and population entropy. **A-B** Population diversification dynamics obtained based on AFC (single-cell fluorescence distributions over time) of (A) *E. coli* W3110 $P_{araB}::GFP$, without fitness cost, and (B) *S. cerevisiae* $P_{glc3}::GFP$, 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). (Adapted from Henrion et al. [22])

Figure 3.1: **C-D** Schematic representation of single-cell traces for circuits (C) without or (D) with fitness cost. **E-F** Fluorescence distributions over time for (E) *E. coli* W3110 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.

This observation led us to hypothesize 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 3.1 A & C) [169]). However, these differences were quickly leveled out as the system reached steady state (Figure 3.1 C). In contrast, burdensome gene circuits result in growth arrest and/or reduction, further amplifying the differences in fluorescent protein accumulation (Figure 3.1 D). 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 [22, 145]. This system was used to generate successive induction and relaxation phases with *E. coli* cells containing either a non-burdensome arabinose utilization reporter system (strain W3110 P_{araB}::GFP) or a burdensome T7 expression system (strain BL21(DE3) pET28:GFP) (Figure 3.1 E & 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 this latter phase, where fluorescence levels decrease due to protein dilution caused by cell growth and division [240], we investigated the relationship between fluorescence intensity (GFP signal) and growth rate (Figure 3.1 G & H, Supp. Notes A.2 & A.3). In accordance with our previous fitness cost analysis [22], our results pointed out that the arabinose system had no significant impact on growth (Figure 3.1 G), whereas the T7 system displayed a pronounced fitness cost (Figure 3.1 H).

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

The aim of this work 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 organization 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 [241–245]. 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 analyze the potential function of entropy as a population fitness stabilizer, 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, characterized by bursts of diversification, where cells periodically activated the T7 system, incurred a fitness cost, and were progressively washed out (Figure 3.2 A & B). Despite this repeated washout, the cell population exhibited a robust behavior in terms of cell density, with growth sustained by cells displaying lower induction levels (Figure 3.2 C). Using AFC data, we computed the time profiles of entropy (Figure 3.2 D) and population fitness (Figure 3.2 E), the latter being estimated as the mean fitness based on a trade-off curve generated from Segregostat data and fluorescence measurements (Figure 3.1 H, Supp. Note A.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 3.2 C).

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 [246, 247]. 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.

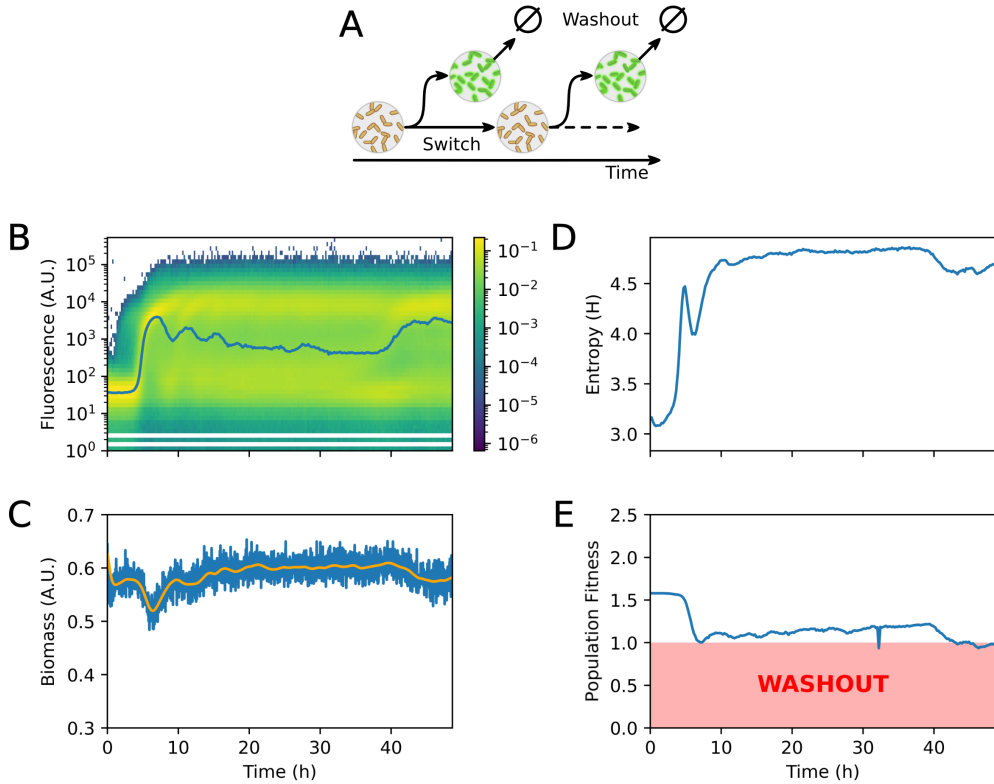


Figure 3.2: FEC for *E. coli* with a T7 expression system in continuous culture (chemostat). **A**

Schematic representation of the phenotypic diversification dynamics with bursts of diversification. **B** Fluorescence distributions over time determined based on AFC (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 AFC.

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 behavior 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 3.3 A). 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 3.3 B). Xylose is not able to exhibit catabolite repression and is co-consumed with lactose [248], explaining the lack of environmental escape in this case. We then compared the measured biomass (Figure 3.3 C) and entropies (Figure 3.3 D) 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 3.2 C-E and Figure 3.3 C & 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 3.3 C & D).

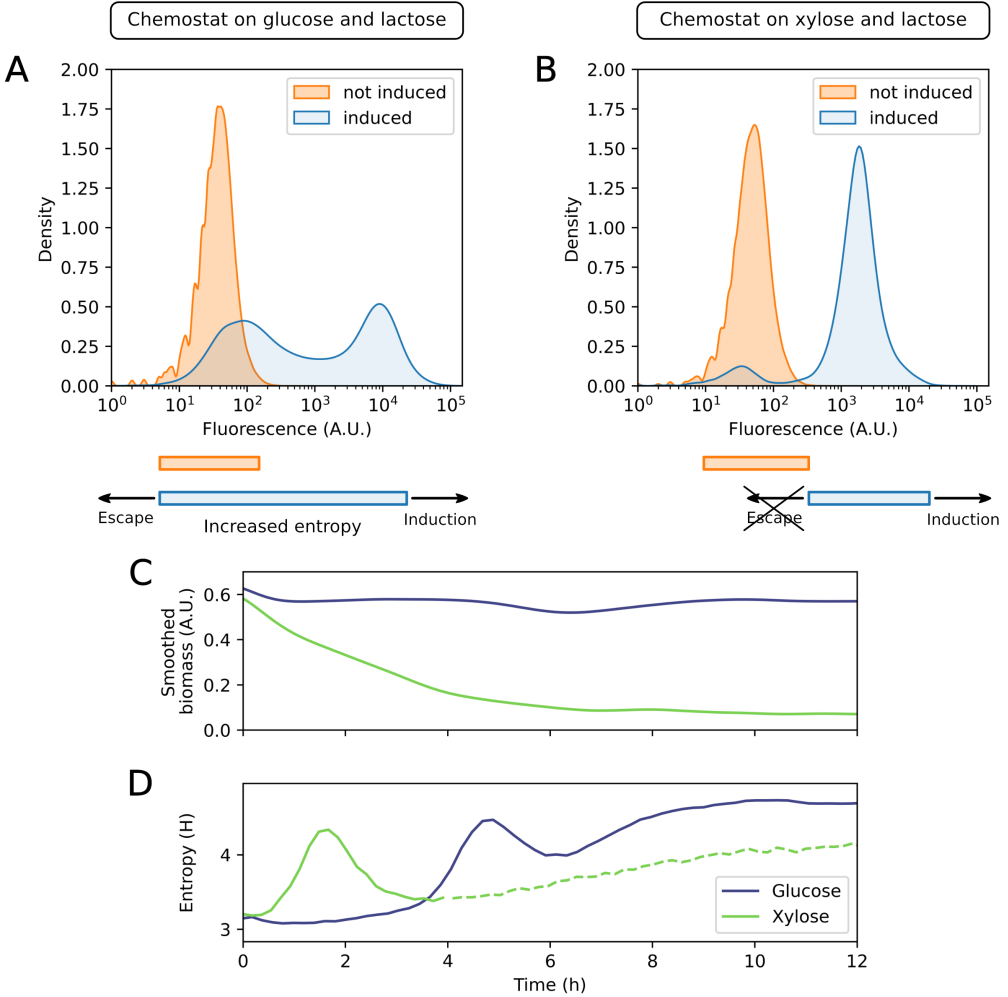


Figure 3.3: **A-B** Fluorescence distributions of *E. coli* cells with a T7 expression system cultivated in chemostat on (A) glucose and lactose, and (B) xylose and lactose. Orange: before induction, blue: after induction (20 h for glucose+lactose, and 2.5 h, i.e., just before the start of washout as measured by event numbers in FC, for xylose+lactose). The schematic below the fluorescence plots illustrates the (A) presence or (B) absence 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.

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 metabolized 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 (Supp. Note A.4). 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, AFC 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 (Figure 3.4 A). 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, Supp. Note A.4), as the cells were unable to synthesize the essential amino acid serine. We subsequently analyzed the population dynamics of this *E. coli* strain using AFC (Figure 3.4) 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.

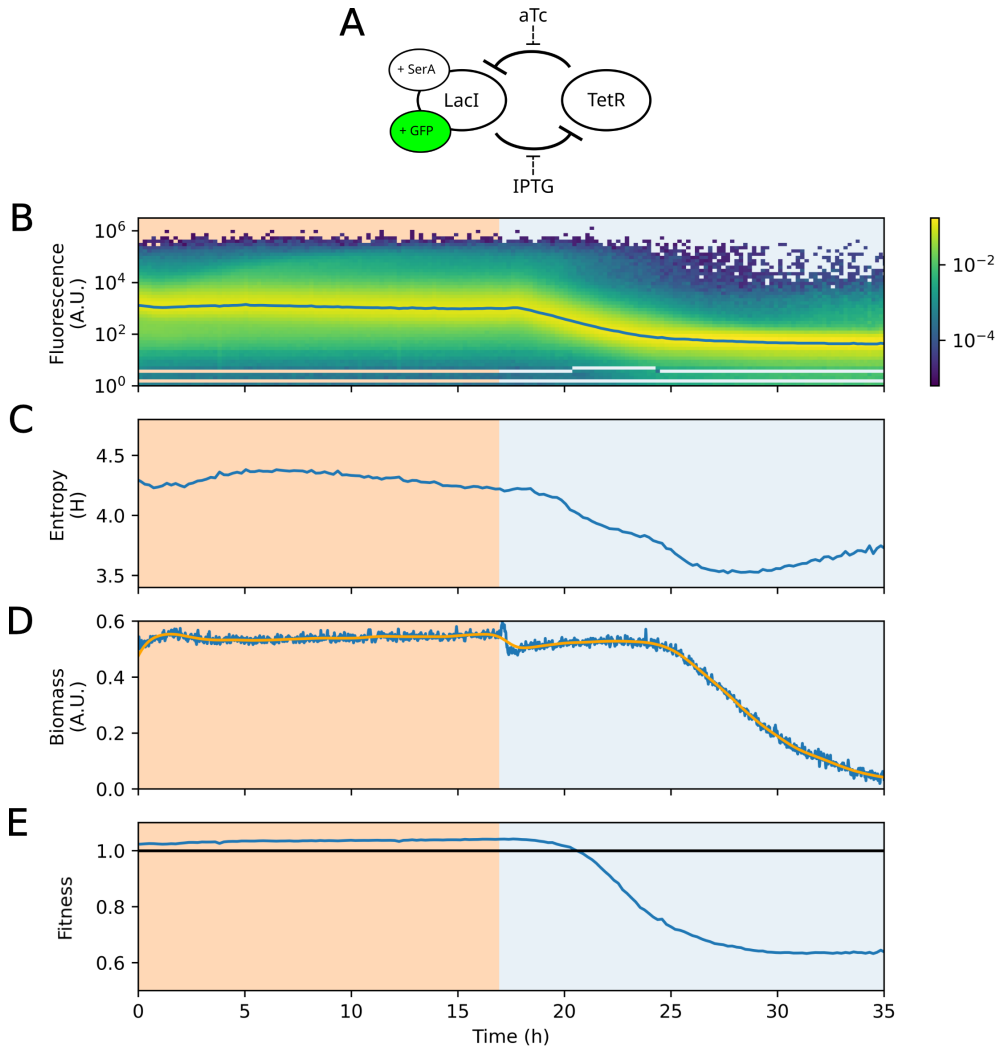


Figure 3.4: Continuous cultivation of a toggle switch controlling serine auxotrophy exhibits no environmental escape and no FEC. Upon addition of IPTG in the feed (lighter, blue background), the production of serine was inhibited. **A** Schematic representation of the toggle switch, **B** Fluorescence distributions over time as determined based on AFC (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 AFC and the independent measurement of the growth rate for each state of the switch.

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 [4, 54, 64, 65]. 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 (Supp. Note A.5). The strong correlation between *ydcS* and *rpoS* expression confirmed its relevance as a reporter of the GSR (Supp. Figure A.19). For *S. cerevisiae*, we employed a previously developed $P_{glc3}::GFP$ transcriptional reporter [22, 111, 249]. The *glc3* gene is associated with the GSR in yeast and has been reported to be involved in bet-hedging [5, 110, 114].

To visualize 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 [17, 118, 122]. The activation of the *ydcS* gene in *E. coli* system did not induce growth arrest in cells exhibiting high expression levels (Figure 3.5 A & 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 3.5 C & D, Supp. Note A.3). Given the significant impact of the stringent response on growth [58], 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 [4, 54, 61, 62, 64, 65]. 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 [4, 57]. 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 [4, 63].

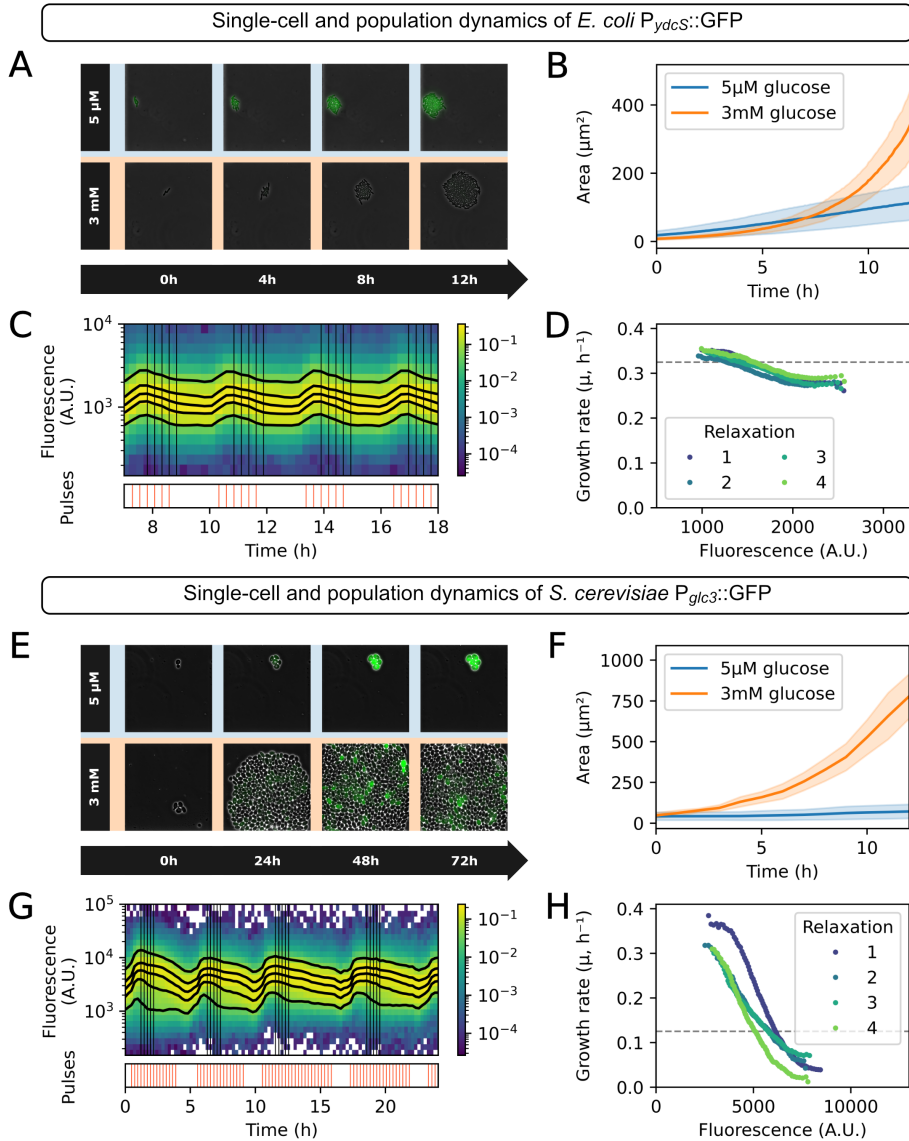


Figure 3.5: Single cell and population dynamics in MSCC and in Segregostat, for *E. coli* MG1655 $P_{ydcS}::GFP$ and *S. cerevisiae* $P_{glc3}::GFP$. **A & E** Time-lapse fluorescence microscopy images of colonies grown in mineral medium with 5 μ M (top, blue) or 3 mM (bottom, orange) glucose concentration. **B & F** Colony growth in microfluidic chambers, quantified as the total cell area over time (for (B) *E. coli*, $n = 3$ for both 5 μ M and 3 mM glucose; for (F) *S. cerevisiae*, $n = 4$ for 5 μ M 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.

We carried out similar analyses on the $P_{glc3}::GFP$ system in yeast at high and low nutrient conditions [22, 249]. 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 3.5 E & F). This drastic growth arrest was confirmed based on Segregostat experiments (Figure 3.5 G), with a trade-off curve pointing out a substantial reduction in growth rate upon full activation of the reporter system (Figure 3.5 H, Supp. Note A.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 [5, 23, 234].

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.

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 AFC. 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 3.6 A & 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 [72]. Furthermore, entropy did not increase upon switching to the stressed state; it may even be slightly reduced (Figure 3.6 B & E). Notably, fitness remained unaffected (Figure 3.6 D). 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.

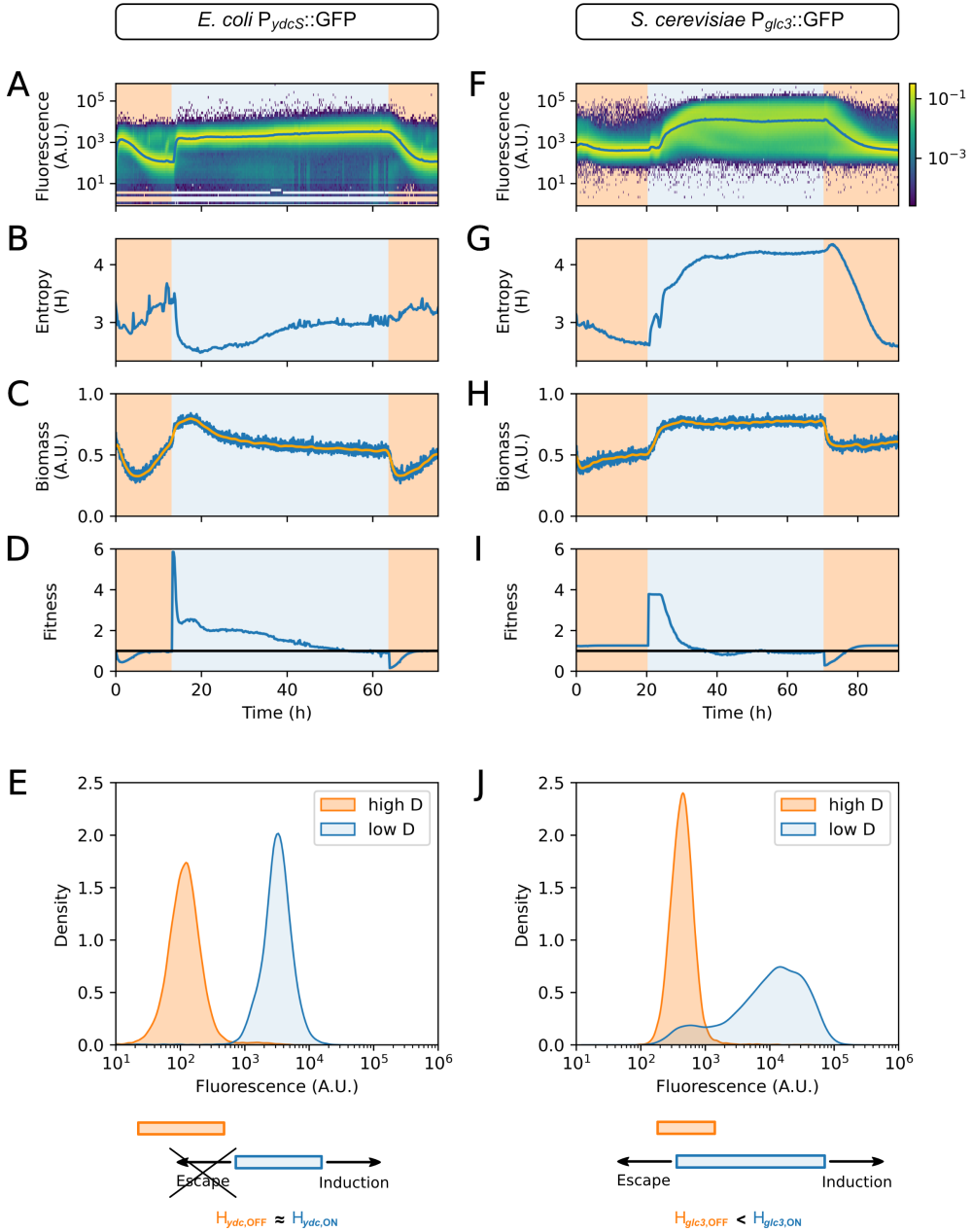


Figure 3.6: Single cell and biomass analysis of **A-E** *E. coli* MG1655 P_{gdcS}::GFP and **F-J** *S. cerevisiae* P_{glc3}::GFP cultivated in continuous cultures carried out at high (darker, orange background, $D = 0.6 \text{ h}^{-1}$ for *E. coli* and $D = 0.3 \text{ h}^{-1}$ for *S. cerevisiae*) and low (lighter, blue background, $D = 0.1 \text{ h}^{-1}$ for both strains) dilution rates. **A & F** Fluorescence distributions over time determined based on AFC (the blue line indicates the median fluorescence). **B & G** Shannon entropy computed from the corresponding fluorescence distribution over time.

Figure 3.6: 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 (E) absence or (J) presence of environmental escape.

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 3.5 E-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”, characterized by overlapping gene expression distributions across varying stress intensities [72](Figure 3.6 F & 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 3.6 G). Notably, population fitness did not decrease (Figure 3.6 I), and biomass concentration remained stable (Figure 3.6 H). 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 metabolic activity and tend to shift toward a more quiescent state, thereby decreasing substrate consumption [5, 23, 234]. 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 3.5 E & F, Supp. Figure A.22). In microfluidics, cell physiology is decoupled from environmental conditions, thereby eliminating environmental feedback. Residual glucose concentrations were measured in

continuous cultures across various dilution rates and compared to those in the MSCC device (Supp. Note A.7). At low dilution rates ($D < 0.3 \text{ h}^{-1}$), residual glucose concentrations remained very low around 0.1 mM, the threshold concentration at which the entire population exhibited growth arrest in the MSCC device (Supp. Figure A.22 B). This supports the hypothesis that transient environmental conditions enable cells to escape the stressed state.

9. Discussion

Gene expression noise and phenotypic switching enables microbial populations to adapt and thrive in dynamic environments [6, 7, 9, 11, 250, 251]. 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 [7, 252, 253], 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 [6, 7, 11, 251], 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 [7]. 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 [254]. 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 3.6). This heterogeneity is quantitatively captured by an increase in population entropy (as shown mathematically in Supp. Note A.1).

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 labor-intensive and does not accurately replicate the growth conditions of our systems of interest. By contrast, our approach using AFC 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 [117, 233] or via omics approaches on subpopulation or even at the single cell level [15].

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 [254, 255]. 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.

10. Material and methods

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.

10.2. Strains and plasmids

A strain of *E. coli* W3110 with a plasmid from the Zaslaver collection ($P_{araB}::GFPmut2$ with kanamycin resistance [214]) was used as an example of arabinose utilisation system with low fitness cost. The data acquired with this strain were already published [22].

The *E. coli* BL21(DE3) strain, one of the most important industrial production backbone, carrying the pET28:GFP plasmid (Addgene #60733)[256] was used to investigate the burdensome T7 expression system. Part of the data acquired with this strain were already published [22].

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)[257]. 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 ribosomal binding site (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. [232]. *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 Supp. Table A.2. 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 (Supp. Note A.4).

For the study of the general stress response of *E. coli* in chemostat, we utilized the *E. coli* MG1655 $P_{ydcS}::GFPmut2$ strain from the Zaslaver collection [214]. 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 CRISPR-cas9 system as above. The CyOFP1 gene was inserted downstream of the *rpoS* gene with a synthetic RBS 5'-AAAGAGGAGAAA-3' [208, 233]. 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 Supp. Table A.1.

For the study of the general stress response of *S. cerevisiae* in chemostat and in microfluidics, we utilized *S. cerevisiae* CEN.PK 113-7D strain with the chromosomal integration of a reporter cassette $P_{glc3}::\text{eGFP}$ [111, 258].

10.3. Growth media

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

E. coli strains have been cultivated in a mineral medium containing (in g/l), 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 is supplemented with a trace element solution totaling 11 ml/l assembled from the following solutions (in g/l), 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 g/L): $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 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 has been supplemented with kanamycin (50 mg/l). The trace element solution, the thiamine and the antibiotic were filter-sterilized (0.22 μm) before supplementation of the other component that were heat-sterilized (at 120°C).

S. cerevisiae has been cultivated in a mineral medium based on the recipe of Verduyn et al. [259]. 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 sterilization (at 120°C) of those components, the filter-sterilized (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.

10.4. Continuous cultivations

Continuous cultivations were performed in either Biostat B-Twin (Sartorius, Göttingen, Allemagne) 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 (FC) 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}::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 summarized in Supp. Table A.3.

E. coli P_{araB}::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 OD₆₀₀ 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 [169].

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 g/L glucose medium and an OD₆₀₀ above 5 (which is the maximal cell density reached with 5 g/L glucose). The pre-cultures (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 pre-cultivated 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 mg/L 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 $OD_{600} \approx 0.5$, and was incubated until it reached enough biomass to inoculate the bioreactor (~ 3 h for *E. coli*, ~ 6 h for *S. cerevisiae*). The batch phase was initiated with an $OD_{600} \approx 0.1$ (or 0.5 for experiments already published in previous paper [22]). 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 g/L.

Dissolved oxygen and pH were monitored in all experiments. Excepted for experiment previously published [22], biomass was monitored with Hamilton Dencytte RS485 probes. All cultures were also monitored at the single-cell level using AFC. The AFC system is the combination of an in-house sampling device (part of the Segregostat [22, 145]) and a benchtop flow cytometer (BD Accuri C6 or BD Accuri C6+). Every ~ 12 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 $\mu\text{L}/\text{min}$, 8 μm 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 μm 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 analyzed 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.

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 (Supp. Note A.2). 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 (Supp. Note A.3). Regarding *E. coli* $P_{ydcS}::GFP$, 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 (Supp. Figure A.13). 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:

$$Population\ Fitness_{Toggle} = \frac{p(GFP^+) \cdot \mu_{GFP^+} + p(GFP^-) \cdot \mu_{GFP^-}}{D} \quad (\text{Eq. 3.1})$$

Where D is the dilution rate and $p(GFP^+)$ and $p(GFP^-)$ represent the proportions of GFP^+ and GFP^- cells in the population. Their respective growth rates $\mu_{GFP^+} = 0.531 \pm 0.014$ (std) h^{-1} and $\mu_{GFP^-} = 0.295 \pm 0.010$ (std) h^{-1} were determined during the toggle-switch phenotypic characterization (Supp. Note A.4).

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 μM 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 $\mu m \times 90 \mu m \times \sim 850$ nm for *S. cerevisiae* and 80 $\mu m \times 90 \mu m \times 700$ nm for *E. coli*) [120, 122, 260]. The experiments on *S. cerevisiae*, were already published [22, 249]. 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 analyzed while 3 chambers (technical replicate) were analyzed 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 [261]. Microscopy images of *E. coli* were analyzed 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 preprocessed (normalization, stabilization, 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) [128, 129] 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) [221, 222] with the "Label image detector" and the "Overlap tracker" options (precise IoU calculation, min. IoU = 0.1 and scale factor = 1.1). Singles-cells data and lineages were then exported as tables and further analyzed. For instance, different approximations of the growth rates have been performed. The one used in Supp. Figure A.20 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).

Chapter 4: Control of stress expression in scale-down continuous cultures using the Segregostat

This chapter is an adapted version of the article referenced below.

Delvenne, M., Martinez, J. A., Haringa, C., Noorman, H., Minden, S., Takors, R., and Delvigne, F. “Overriding Bioprocess Perturbations With a Cell–Machine Interface for Reliable Microbial Stress-Response Control”. In: *Microbial Biotechnology* 19.4 (2026). DOI: 10.1111/1751-7915.70329

1. Abstract

Controlling cell population dynamics and phenotypic diversification is a key objective in systems and synthetic biology, particularly for ensuring uniform responses from engineered gene circuits. While cell-machine interfaces have been employed to modulate host-gene circuit interactions, environmental perturbations typical of industrial bioreactor conditions remain underexplored. In this study, we investigate the impact of such perturbations on the general stress response in *Escherichia coli* and *Saccharomyces cerevisiae*. Using scale-down (SD) bioreactor experiments, we evaluate the performance of the Segregostat, a real-time control system that leverages automated flow cytometry (AFC) to induce dynamic nutrient shifts. The Segregostat achieves robust stress response control, even under severe perturbations such as extended residence times in a two-compartment reactor. We hypothesize that this robustness arises from the system’s ability to amplify host-compatible fluctuations beyond bioreactor-induced perturbations. Our findings highlight the importance of integrating environmental factors into control strategies for reliable gene circuit behavior in industrial bioprocessing environments.

2. Introduction

Isogenic microbial populations are now well known to display phenotypic heterogeneity. It is observed in homogeneous environments as a consequence of stochastic gene expression [9], and can be further shaped by single-cell responses to fluctuating environmental conditions [94, 149]. Such diversification leads to the apparition of functional differences between cells, potentially increasing the fitness of the population [6]. However, in bioproduction contexts, phenotypic heterogeneity is generally undesirable, as it tends to make processes unpredictable and is presumed to contribute to instability and reduced production yields [17, 18]. Developing strains, process designs and strategies to control population heterogeneity and optimize microbial production therefore represent key challenges in biotechnology [17, 19–21].

Systems and synthetic biology have significantly advanced our ability to design and control synthetic or natural gene circuits, yet most work has focused on their interactions with the cellular host [263, 264], addressing challenges such as

metabolic burden [223], switching dynamics [253], and population heterogeneity [22]. Meanwhile, bioprocess engineering has concentrated on optimizing the interplay between cells and their physical environment within bioreactors [87, 92]. On top of these parallel advances, progress in fields such as industrial biotechnology, therapeutic protein production, and biosensing increasingly depends on bridging these two domains to create scalable and controllable bioprocesses [103]. A key step toward this integration is the development of robust frameworks that account for the full spectrum of interactions i.e., between gene circuits, host physiology, and bioreactor conditions. Recent innovations have introduced cell-machine interfaces capable of connecting microbial populations to external computers and controllers, enabling real-time modulation of gene expression [151, 155, 158]. However, these technologies remain largely untested under the dynamic and often harsh conditions relevant to industrial-scale bioprocessing[102].

In this work, we address this gap by evaluating the Segregostat, a real-time control platform for microbial populations [145], under SD bioreactor conditions. This approach enables us to assess the robustness, responsiveness, and scalability of cell-machine interfaces, paving the way for their deployment in industrial biotechnology applications. More specifically, we employed the Segregostat to modulate the general stress response in *S. cerevisiae* and *E. coli*. These regulatory networks, previously shown to generate substantial population heterogeneity [64, 65, 110], are of particular interest for bioprocess optimization [26, 30], where controlling their activity could mitigate growth arrest and reduce the metabolic burden associated with their activation [31, 32]. Under standard laboratory-scale bioreactor conditions, the Segregostat consistently reduced phenotypic heterogeneity, reflecting cell-to-cell variability in stress response, in both *E. coli* and *S. cerevisiae*. The magnitude of this reduction scaled proportionally with the metabolic burden imposed by activation of the target gene circuit, in agreement with previous observations [22]. We next implemented a two-compartment scale-down (bio)reactor (SDR) to reproduce the concentration gradients characteristic of poorly mixed large-scale bioreactors [103, 104]. Even under these highly heterogeneous conditions, the Segregostat maintained robust population-level control, demonstrating resilience to severe mixing limitations.

3. Segregostat reduces cell-to-cell heterogeneity (entropy) in stress response at laboratory scale

A variety of cell-machine interfaces are now available to enable direct communication with targeted cell populations and to monitor and/or control their physiological states [140, 145, 151, 155, 157, 158, 265, 266]. Among them, we utilized the Segregostat, a platform that combines AFC with a feedback control loop, to regulate the general stress response in bacterial and yeast populations (Figure 4.1 A). Under chemostat conditions, nutrient limitation triggers the transition to stress states. This transition

can be counteracted by timely glucose pulses. In our setup, glucose was automatically pulsed into the bioreactor whenever single-cell fluorescence measurements indicated that more than 50 % of the population exceeded a defined stress threshold (Figure 4.1 A-B). This approach created alternating phases of feast (glucose pulsing, promoting growth) and famine (no pulsing, promoting nutrient limitation and stress responses), ultimately driving coordinated and synchronized gene expression within the population [22].

Previously, we demonstrated that the degree of cell-to-cell heterogeneity in stress-related gene expression, quantified here using Shannon entropy (Figure 4.1 C; see Material and methods), is primarily driven by the fitness or switching cost, defined as the reduction in growth rate experienced by cells that activate stress responses [22]. By exploiting the alternation of stress induction and relaxation phases generated by the Segregostat, we were able to quantify the trade-off between growth and the expression of a stress-related gene (Figure 4.1 B) [212]. Interestingly, this fitness cost differs significantly between *E. coli* and *S. cerevisiae* (Figure 4.1 B) [212]. In *S. cerevisiae*, the fitness cost is considerable (i.e., highly stressed cells have been shown to stop growing [212]) and leads to a highly heterogeneous activation of the general stress response under chemostat conditions, as reflected by elevated entropy levels (Figure 4.1 D). Functionally, this elevated entropy enables the population to maintain an average growth rate above or equal to the dilution rate, even though individual cells may incur significant growth penalties. This population-level resolution of the trade-off between growth and gene expression has been previously described as the fitness-entropy compensation (FEC) effect [212]. This effect is particularly pronounced when monitoring the $P_{glc3}::\text{GFP}$ transcriptional reporter at low dilution rates, where stress responses are robustly induced [22, 212] (Figure 4.1 D, Supp. Note B.1). By contrast, *E. coli* exhibits a different diversification pattern, characterized by lower entropy under chemostat conditions (Figure 4.1 G). This is because the activation of stress-related genes in *E. coli*, monitored here using a $P_{ydcS}::\text{GFP}$ transcriptional reporter as a proxy for RpoS-dependent stress response, does not entail a significant switching cost [22, 212]. Beyond its analytical capabilities, the Segregostat also enables active control of population dynamics. Indeed, despite the mentioned species-specific differences, it successfully synchronized stress gene expression and reduced overall population heterogeneity in both organisms, compared to chemostat cultures operated at similar dilution rates (Figure 4.1 D-I, Supp. Note B.1).

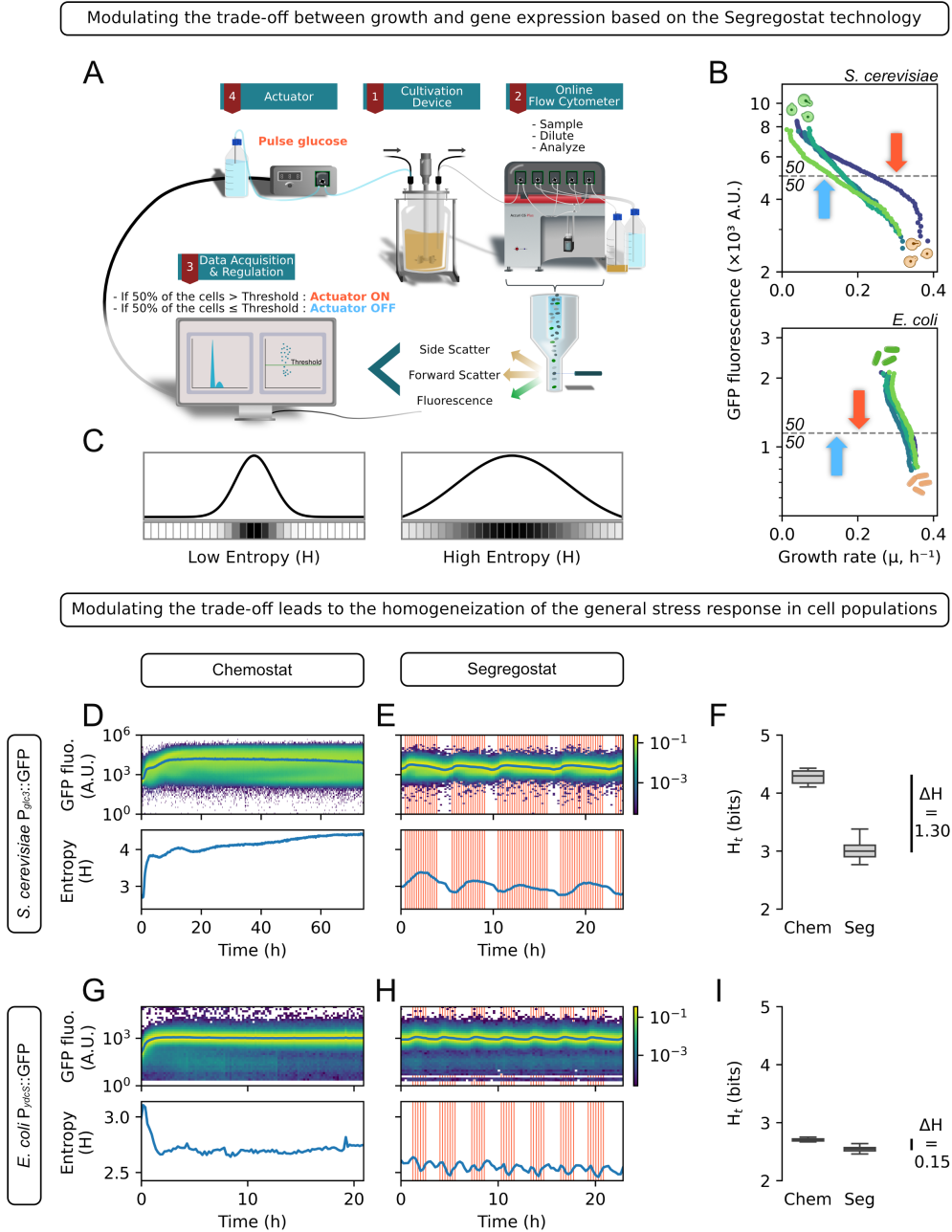


Figure 4.1: Synchronization and homogenization of stress-related genes based on the Segregostat technology. **A** Schematic representation of the Segregostat setup. **B** Control loop of the Segregostat and illustration of the trade-off between growth and stress-related gene expression in *E. coli* and *S. cerevisiae*. The horizontal gray dashed line indicates the fluorescence threshold. Red arrow indicates activation of glucose pulse, while blue once indicates its inactivation. The colored dots indicate different replicates for computing the trade-off (see Material and methods). (adapted from Delvenne et al. [212])

Figure 4.1: C Schematic explanation of population heterogeneity quantification by Shannon entropy (H). This measure comes from information theory and is computed from the distribution of the population across discrete bins. **D-E** Fluorescence time-scatter plots and corresponding entropy dynamics of *S. cerevisiae* $P_{glc3}::GFP$ in continuous chemostat cultivation at $D = 0.1 \text{ h}^{-1}$ (D) and under Segregostat control (Threshold = 3000 A.U., 0.4 g glucose/pulse) (E). Glucose pulses are marked by red vertical lines. **G-H** Fluorescence time-scatter plots and corresponding entropy dynamics of *E. coli* $P_{ydcS}::GFP$ in continuous chemostat cultivation at $D = 0.3 \text{ h}^{-1}$ (G) and under Segregostat control (Threshold = 1150 A.U., 1 g glucose/pulse) (H). Glucose pulses are marked by red vertical lines. **F-I** Basal entropy of *S. cerevisiae* (F) and *E. coli* (I), calculated from the fluorescence distribution of the population over time, represented as boxplots. For chemostat conditions, entropy is computed from 3 retention times onward until the end of cultivation ($n = 312$ for *S. cerevisiae*; $n = 58$ for *E. coli*). For Segregostat conditions, only the last 3-4 oscillations are considered ($n = 98$ for *S. cerevisiae*; $n = 91$ for *E. coli*).

However, all data collected so far has been generated using lab-scale bioreactors. It is well established that scale-up introduces significant challenges, particularly the reduction in global mixing efficiency, which leads to the formation of spatial concentration gradients that can impact cellular physiology. In our context, such gradients are expected to result in zones of glucose excess and starvation, which could limit the effectiveness of the Segregostat technology in regulating the general stress response at the population level by shadowing the environmental changes it is designed to impose. To explore this, we will assess the performance of the Segregostat in two-compartment SDRs, which are designed to mimic the heterogeneities encountered in large-scale industrial systems [92, 93].

4. Scaled-down conditions do not lead to an increase of population entropy

To assess the robustness of the Segregostat control under environmental perturbations, we designed a two-compartment SDR consisting of a standard lab-scale stirred-tank (bio)reactor (STR) connected to a recirculation loop (Figure 4.2 A-B). In this configuration, substrate is typically injected into the recirculation loop. Combined with cellular consumption along the loop, this generates concentration gradients that result in the formation of distinct zones of nutrient excess (red) and starvation (blue) (Figure 4.2 A-B; note the colors are a qualitative illustration, and the local manifestation of excess/starvation conditions may differ between *E. coli* and *S. cerevisiae* due to specific uptake rates). This setup mimics large-scale bioreactor heterogeneities, where subpopulations of cells are stochastically exposed to varying substrate concentrations.

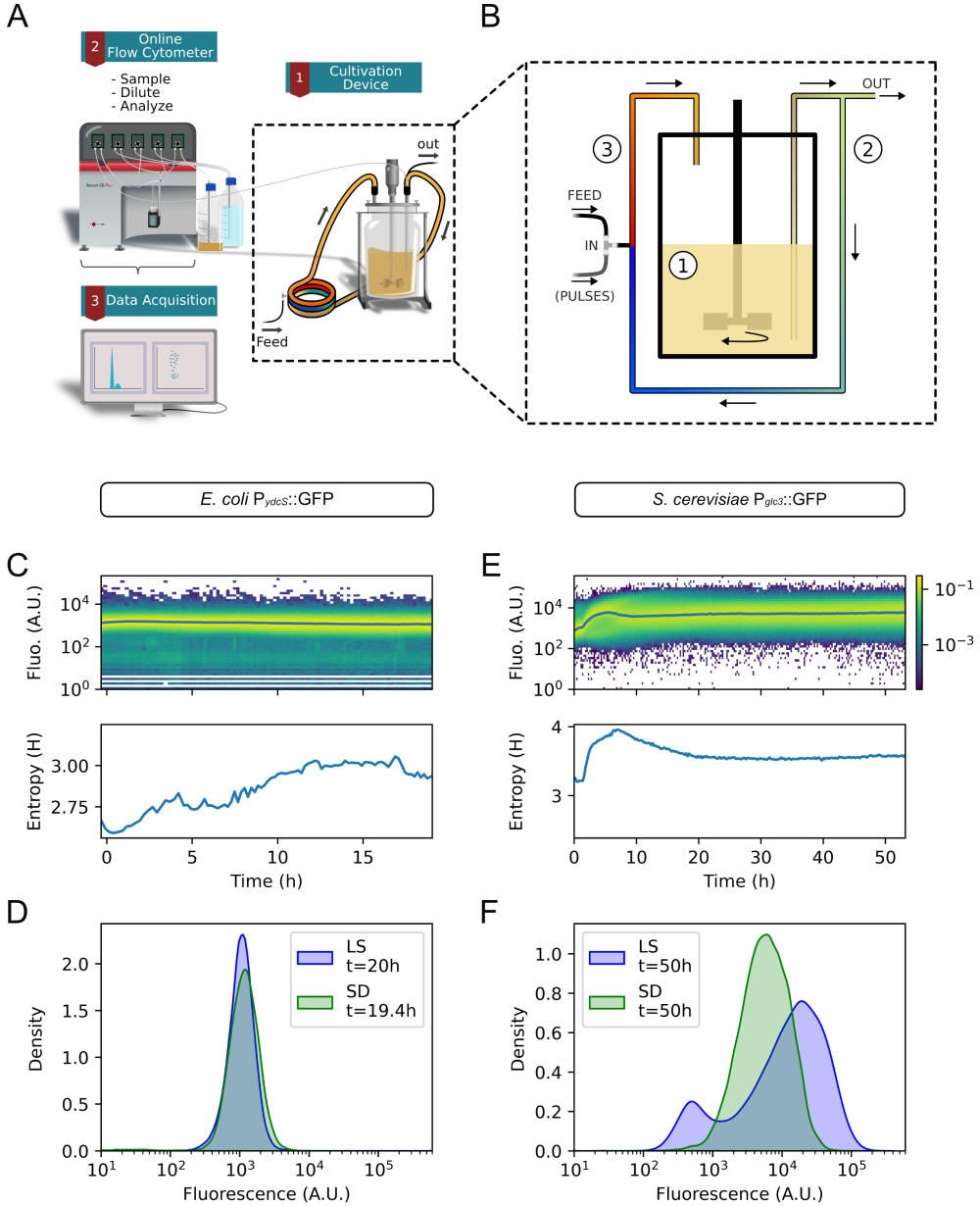


Figure 4.2: Scale-down effects on population dynamics in the absence of Segregostat regulation. **A** Schematic representation of the online flow cytometry (FC) platform for monitoring the scale-down reactor. **B** Simplified Scale-Down design composed by a STR (zone 1) ($V_1 = 0.8$ L) with a recirculation loop (zones 2 and 3) ($V_{2+3} = 0.2$ L, $V_2 = 0.067$ L, $V_3 = 0.133$ L). Feeding, and pulses when present, are performed at 2/3 of the recirculation loop (between zone 2 and 3). **C** & **E** Fluorescence time-scatter plots and corresponding entropy dynamics from continuous chemostat scale-down cultivation ($Tr_{Loop} = 8$ min) of *E. coli* $P_{ydcS}::GFP$ ($D = 0.3$ h⁻¹) (**C**) and *S. cerevisiae* $P_{gls3}::GFP$ ($D = 0.1$ h⁻¹) (**E**).

Figure 4.2: D & F Comparison of fluorescence distribution between Lab-Scale (LS) and Scale-Down (SD) chemostats conditions at steady state at $D = 0.3 \text{ h}^{-1}$ for *E. coli* (D) and $D = 0.1 \text{ h}^{-1}$ for *S. cerevisiae* (F). The time indicated corresponds to the duration spent in the condition prior to the measurement.

As a control, we first cultivated *S. cerevisiae* and *E. coli* in the SDR without applying the Segregostat control procedure (Figure 4.2 A-B). For *E. coli*, two different residence times in the loop (Tr_{Loop}) of 2 and 8 minutes were tested. Cultivations under these conditions did not result in any noticeable changes in the population profile, as assessed by AFC, the entropy level being close to the one recorded for lab-scale cultivations (Figure 4.2 C-D and Figure 4.3 C). In contrast, for *S. cerevisiae*, cultivation in the SDR with a Tr_{Loop} of 8 min resulted in a reduction in the overall entropy of the population (Figure 4.2 E-F and Figure 4.3 E). This outcome can be attributed to the yeast stress response being naturally stimulated by alternating feast-to-famine transitions [5, 267], mechanistically similar to the strategy employed by the Segregostat (Figure 4.1). In our configuration, cells experience such transitions since they follow a single path through the different conditions, with a stochastic residence time in the STR. Such dynamic environmental conditions have been shown to exert a stabilizing effect on the physiological and proteomic state of cells [268]. Interestingly, it seems that the residence time in the recirculation loop or the frequencies of the perturbations impact the heterogeneity of the population (Supp. Note B.2) [106]. Longer residence times or less frequent perturbations likely promote uniform adaptation and synchronized behavior, whereas shorter residence times lead to more frequent perturbations, eliciting diverse individual responses and increasing population heterogeneity [106]. In line with this hypothesis, chemostat cultivation of yeast in the SDR with a 4 minute Tr_{Loop} did not result in population homogenization (Figure 4.3 E).

These observations highlight the importance of conducting more detailed analyses of cell population behavior under simulated large-scale conditions, notably because, in some cases, environmental heterogeneity can lead to a more homogeneous population-level response.

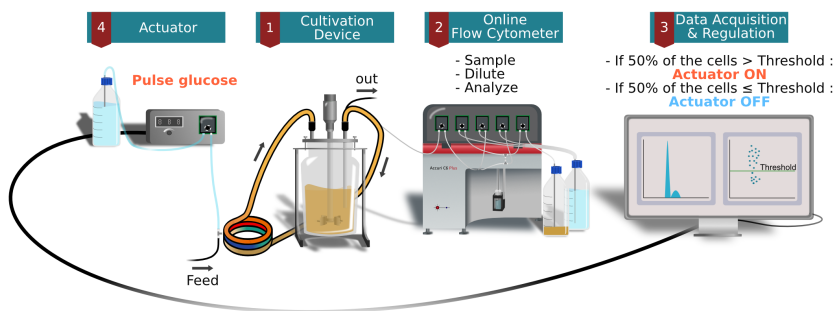
5. Segregostat control leads to robust population profile in scaled-down conditions

We then implemented the Segregostat technology to control gene expression in the SDR. To do so, the Segregostat actuator (i.e., glucose pulsing) was applied in the recirculation loop at the same point as the feed input (Figure 4.3 A and Figure 4.2 B). This setup introduces a key distinction from lab-scale conditions, as glucose pulses now interact with the concentration gradients naturally formed within the SDR. Consequently, the effectiveness of Segregostat-based control is expected to be influenced by these spatial nutrient variations.

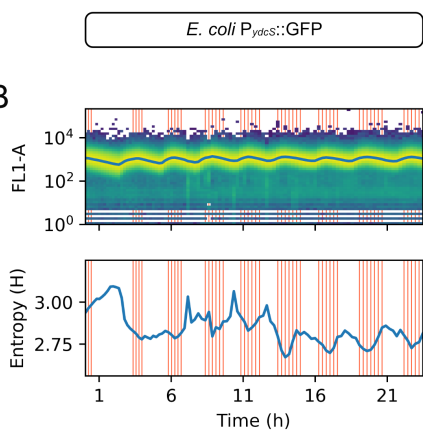
Despite this added complexity, Segregostat control consistently led to a reduction in population entropy compared to the corresponding chemostat cultures in the SDR, across all Tr_{Loop} tested (Figure 4.3 B-E, Supp. Note B.3). This demonstrates that, even under heterogeneous environmental conditions, the Segregostat can successfully control population dynamics. It is worth noting, however, that the reduction in entropy was less pronounced for *E. coli* than for *S. cerevisiae*. This difference reflects the lower heterogeneity of *E. coli* populations in chemostat conditions, due to their smaller switching cost [22, 212]. The environmental forcing imposed by the Segregostat appears to dominate over the perturbations introduced by spatial heterogeneities in the reactor.

Nonetheless, the effectiveness of glucose pulsing in modulating population behavior must be interpreted considering the system's spatial and temporal dynamics. For instance, monitoring dissolved oxygen levels in the STR during *E. coli* cultivations, both in lab scale bioreactor and in the SDR, revealed that successive glucose pulses likely overlap (Supp. Note B.4). Specifically, dissolved oxygen levels do not return to their pre-pulse maximum before the next pulse occurs, suggesting that glucose from the previous pulse has not been fully consumed. In addition, for yeast, the delayed rise in oxygen concentration following a series of pulses suggests a metabolic shift toward ethanol consumption. These results highlight how the pulsing strategy influences cellular physiology, a point discussed in greater detail in the following sections.

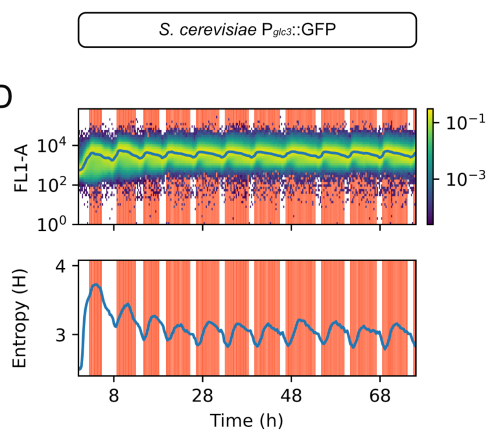
A



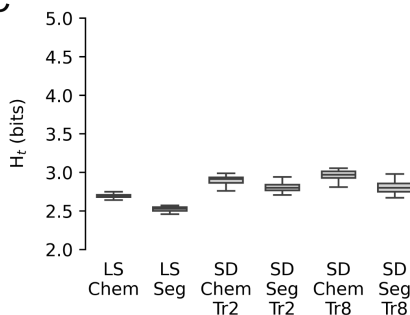
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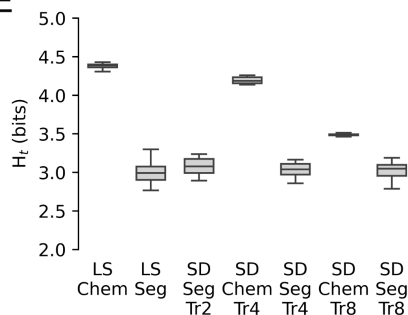


Figure 4.3: Segregostat exhibits robust control under scale-down conditions. **A** Schematic representation of the Segregostat platform for controlling the scale-down reactor. **B & D** Fluorescence time-scatter plots and corresponding entropy dynamics from continuous Segregostat scale-down cultivation ($Tr_{Loop} = 8$ min) of *E. coli* $P_{ydcS}::GFP$ ($D = 0.3 \text{ h}^{-1}$) (B) and *S. cerevisiae* $P_{glc3}::GFP$ ($D = 0.1 \text{ h}^{-1}$) (D). **C & E** Basal entropy of *E. coli* (C) and *S. cerevisiae* (E), calculated from the fluorescence distribution of the population over time, represented as boxplots, for different culture conditions. For all conditions shown in (C) & (E), data from the last 12 hours (*E. coli*) or 20 hours (*S. cerevisiae*) were used, corresponding to (quasi) steady state under chemostat operation. Therefore, sample sizes per condition range from $n = 44$ to $n = 64$ for *E. coli*, and from $n = 80$ to $n = 141$ for *S. cerevisiae*. Detailed sample sizes for each specific condition are provided in Supp. Note B.3.

6. Segregostat control induces overflow metabolism and diauxic shifts, but uniformize growth rate distributions

The Segregostat does not abolish SD effects; instead, it modulates population physiology by synchronizing the expression of target genes. Unlike the stochastic fluctuations characteristic of large-scale bioreactors, typically mimicked in SDRs, the environmental perturbations imposed by the Segregostat are deliberate and coordinated. In our system, glucose pulsing drives overflow metabolism and diauxic shifts, as reflected in dissolved oxygen traces and base addition profiles (e.g., for both organisms the rate of base addition in SD with $Tr_{Loop} = 8$ min was 3.5 times higher in Segregostat than in chemostat) (Supp. Note B.4). These transitions resemble those observed in spatially heterogeneous industrial fermenters, where their random and unpredictable nature often compromises process efficiency [32, 87]. By contrast, when the timing and magnitude of environmental changes are actively controlled, the detrimental impact of such metabolic shifts can be substantially mitigated. In yeast, for example, glucose pulses induce the Crabtree effect, leading to ethanol excretion; under Segregostat operation, the ethanol produced during the feast phase is fully re-consumed in the subsequent famine phase (Supp. Note B.4). This prevents the accumulation of inhibitory by-products and, crucially, reduces the likelihood of a subpopulation entering a persistently stressed state. Such long-term stress responses, often accompanied by growth arrest and metabolic slowdown [22, 110, 212], are far more damaging to culture performance than transient excursions into suboptimal metabolic states.

Beyond stress mitigation, the Segregostat also promotes a more homogeneous population compared to chemostat cultures operated at similar dilution rates (Figure 4.3 C & E, Supp. Note B.3). This phenotypic alignment likely results from the strong, population-wide coordination imposed by the pulsing regime, which reduces the emergence of divergent subpopulations despite underlying spatial heterogeneities in the reactor. This feature is particularly relevant for yeast, where the strong trade-off between growth and gene expression leads to pronounced cell-to-cell heterogeneity in both gene expression and growth rate, depending on the environmental conditions and the mode of control (i.e., either simple chemostat or Segregostat) (Figure 4.4). Indeed, homogenous populations, both in terms of stress response and growth rate (Figure 4.4 B & D) are obtained when the Segregostat is used. This homogenization effect likely arise from the specific environmental perturbation profiles (e.g., in terms of frequency and amplitude) applied in each case. This aspect is further explored in the following section using a simplified cybernetic model.

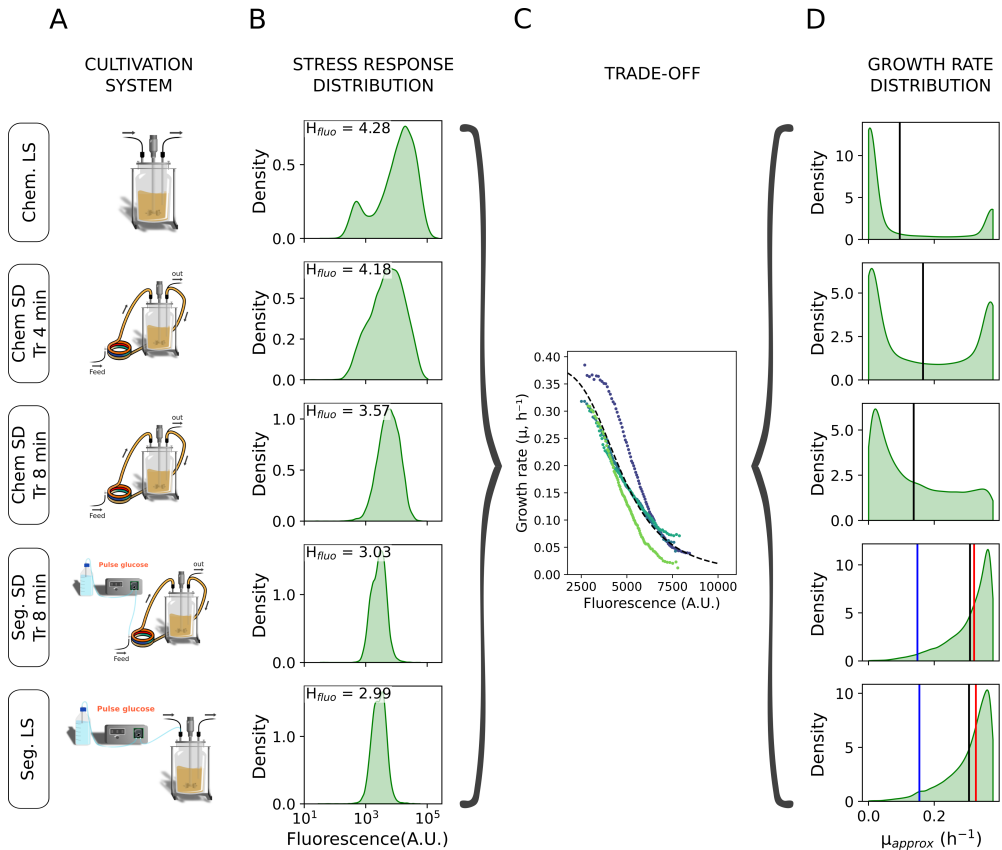


Figure 4.4: Impact of bioreactor operating mode on yeast population stress response and growth rate distribution. **A** Schemes of the different cultivation devices. **B** Distributions of GFP fluorescence related to stress response at steady state. **C** Trade-off curve obtained from Segregostat experiment linking single cell growth rate to the level of stress response. **D** Growth rate distributions inferred from the GFP distribution and the trade-off curve. The vertical black lines mark the mean expected growth rate. Under Segregostat conditions, both the mean growth rate and entropy vary over time; the sample shown was selected because its entropy is close to the median across all samples from the corresponding Segregostat experiment. Blue and red vertical lines indicate the minimum and maximum expected growth rates, respectively.

7. Segregostat-imposed environmental perturbations dominate over mixing-induced heterogeneities and drive population entrainment

In large-scale bioreactors, cells experience irregular perturbations of relatively high frequency and low amplitude due to mixing imperfections (Figure 4.5 A, Supp. Note B.6) [91, 92]. Such dynamic heterogeneity in glucose concentration was mimicked in our scale-down reactor (Figure 4.5 C). In contrast, the Segregostat, through real-time monitoring and control of stress expression, generates feast-to-famine transitions (by alternating between pulse series and pulse-free phases) that align with host-circuit dynamics (Figure 4.5 B & D). These regular perturbations can be viewed as a quasi-square-wave signal characterized by relatively low frequency and high amplitude (Figure 4.5 D). The step time of this signal, and thus the duration of each phase (feast, when glucose concentration is not limiting, and famine, when cells experience stress), is automatically determined by the Segregostat, allowing the population to respond homogeneously (Figure 4.3 D & E).

When both effects are superimposed (Figure 4.5 E), Segregostat-imposed fluctuations dominate, regardless of the residence time in the loop, and the quasi-square-wave signal is robustly transmitted to the cells. In other words, SD-induced environmental perturbations do not disrupt the Segregostat signal. Analysis of the population-averaged glucose concentrations perceived over time further highlights that the Segregostat perturbations are coherently perceived by all the cells, whereas SD perturbations are randomly perceived in the loop (Figure 4.5 C-F). In this sense, the Segregostat can be regarded as a “noise-canceller” for the cells.

These observations are consistent with well-established principles in systems and synthetic biology. Both modeling [7, 94, 95] and experimental [96–98] studies have shown that cellular systems evolve two modes of phenotypic switching depending on the nature of environmental perturbations to enhance population fitness. When fluctuations are irregular in amplitude and timing and occur at high frequency, stochastic switching between gene circuit states is favored, thereby increasing cell-to-cell heterogeneity. Conversely, when fluctuations are more regular, deterministic switching strategies prevail, leading to more homogeneous populations. Our data align with these predictions (Figure 4.4 and Figure 4.6): population entropy is higher when perturbations from cell-environment interactions dominate (as in large-scale bioreactors) (Figure 4.5 A and Figure 4.3 E), while entropy is lower under Segregostat control, where fluctuations are primarily governed by cell-gene circuit interactions (Figure 4.5 B and Figure 4.3 E).

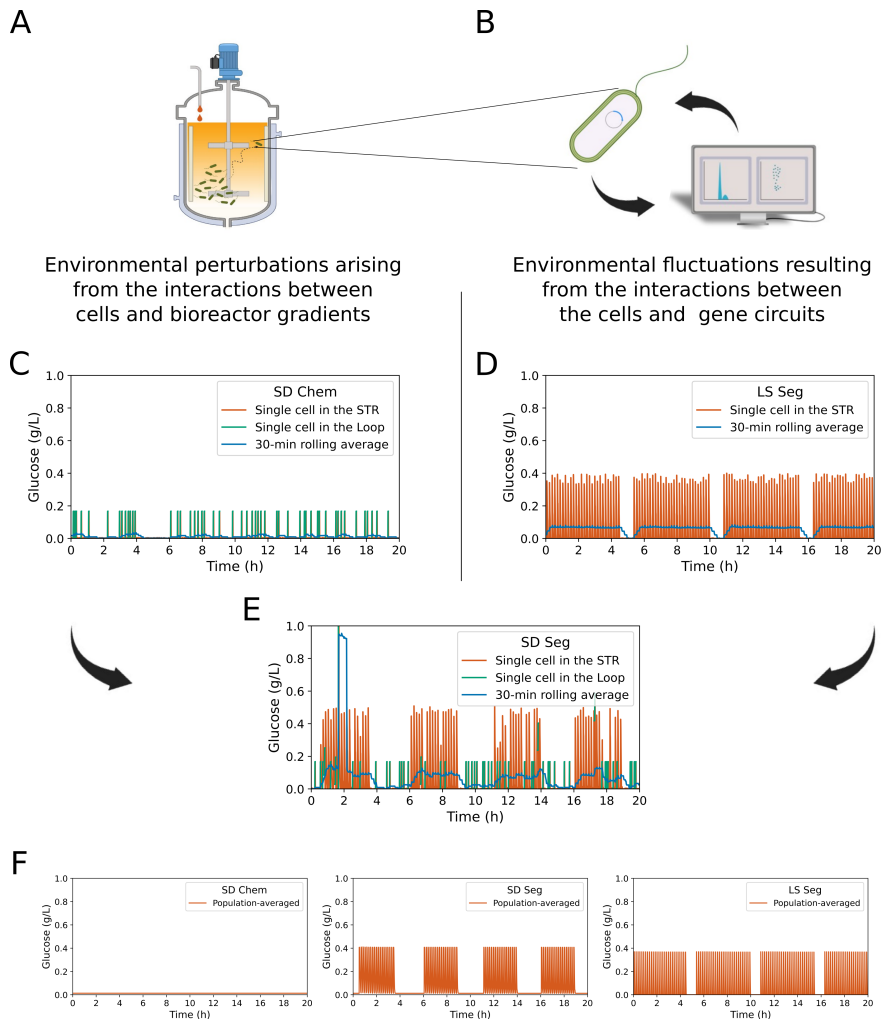


Figure 4.5: Different types of interactions drive different kinds of environmental fluctuations. **A** The interactions between cells and bioreactors lead to stochastic cell lifeline, characterized by low amplitudes and high frequencies. **B** The interactions between the gene circuit and the cellular host, enforced by the Segregostat, leads to environmental fluctuations with high amplitude and low frequency. **C-E** Simulated single-cell lifelines in terms of glucose concentrations perceived in continuous cultures. Lines are orange when the cell is in the STR and green when it is in the recirculation loop. Blue lines indicate a 30-min rolling average. (C) In SD conditions ($Tr_{Loop} = 4\text{min}$) without control, where interactions between cells and bioreactors dominate, (D) In LS Segregostat conditions, where fluctuations following the interactions between the gene circuit and the cellular host dominate, and (E) In SD Segregostat ($Tr_{Loop} = 4\text{min}$) were both interactions are present. **F** The corresponding mean glucose concentration perceived by approx. 1600 cells over time, showing that regular perturbations of the Segregostat dominate even in scale-down conditions.

Interestingly, the homogenization of the population observed for longer residence times in the loop ($Tr_{Loop} = 8\text{min}$) could be explained by the same principles. With a longer residence time, cells are exposed to higher glucose concentrations for extended periods (Figure 4.6). Although these fluctuations remain random, their amplitude, frequency, and duration allow the cells to respond in a more similar manner, as discussed above.

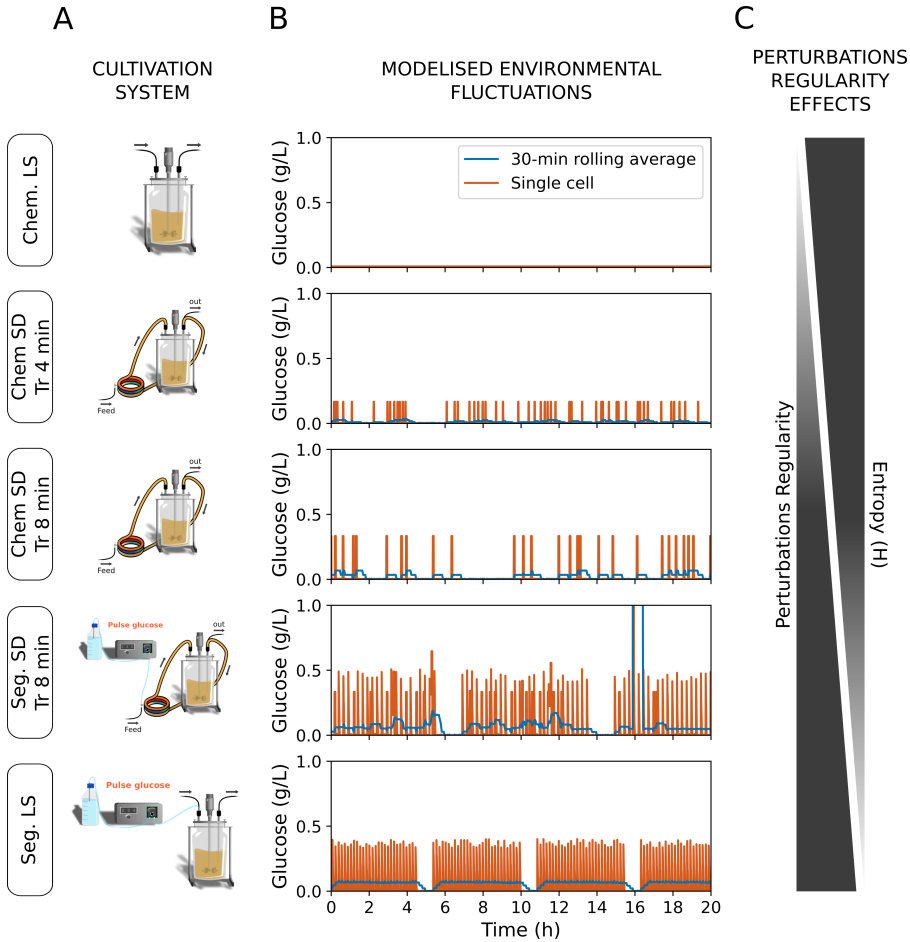


Figure 4.6: Impact of bioreactor operating mode and the corresponding environmental perturbations profiles on yeast population heterogeneity. **A** Schemes of the different cultivation devices. **B** Simulated single-cell lifelines in terms of glucose concentrations perceived in corresponding culture conditions. **C** Consistency of environmental perturbations (i.e., suitable frequencies, amplitudes and step-times) and heterogeneity of the population in corresponding culture conditions. In this representation, we considered the perturbations arising in a chemostat at lab-scale as extremely frequent and of very small amplitude.

8. Discussion

Controlling cell population dynamics and phenotypic diversification is a central challenge in systems and synthetic biology, where engineered gene circuits are expected to elicit uniform responses upon stimulation [159]. Traditionally, this problem has been addressed through a variety of control strategies, most notably cell-machine interfaces. Such interfaces modulate complex host-circuit interactions by triggering feedback, for instance based on the expression level of a target gene, thereby promoting more homogeneous gene expression across single cells within a population [135, 155].

In this work, we introduce an additional component by considering environmental perturbations, such as those typically encountered by cell populations in heterogeneous bioreactor environments [87]. Specifically, we focused on the control of the general stress response (GSR) in two industrially relevant hosts, *S. cerevisiae* and *E. coli*, and performed SD cultivation experiments to evaluate the control performance of a previously developed cell-machine interface known as the Segregostat [22, 145]. This system employs AFC to continuously monitor cellular physiology in real time and autonomously initiates feast-to-famine transitions, thereby coordinating stress responses across the population. In contrast to many previously reported control strategies, which often rely on optogenetics and microscopy-based setups [151, 155, 157, 158], the Segregostat is directly compatible with bioreactors and can be applied to native physiological regulatory circuits rather than being restricted to optogenetic implementations.

Remarkably, the Segregostat demonstrated robust control performance for all SD conditions tested. This robustness likely arises because the Segregostat imposes and amplifies perturbations that align with host-circuit dynamics and that are not disrupted by those introduced by bioreactor constraints, thereby preserving control performance. By directly monitoring gene circuit activation, the Segregostat captures intrinsic dynamics [149] that, like other components of cellular regulatory networks, have evolved under selective pressures imposed by environmental fluctuations and their timing [27, 94, 96, 167]. As a result, the environmental perturbations imposed by the Segregostat synchronize with the intrinsic rhythms emerging from the interplay between the cellular system and its stress-responsive gene circuit. These homogeneous, periodic, and precisely defined fluctuations promote responsive switching and coordinated gene expression across the population. This entails a reduction in entropy, more pronounced in *S. cerevisiae* than in *E. coli*, reflecting the lower switching cost and associated baseline heterogeneity of *E. coli* populations [22, 212].

Dynamic environmental conditions have been reported to stabilize gene expression [106], the physiological and proteomic states of cells [268], and even the structure of microbial communities [249, 269, 270]. In some cases, such conditions were predicted

by models, while in others they arose unintentionally from uncontrolled fluctuations rather than deliberate control. We also observed a comparable homogenizing effect in chemostat experiments under SD conditions with extended residence times in the recirculation loop. Importantly, the temporal features of these fluctuations are a critical determinant of the outcome [106, 271]. This raises the question of whether fixed perturbations, applied at frequencies matching the time constants of gene expression, could replicate the effects achieved with Segregostat control. Crucially, however, the Segregostat goes beyond such chance occurrences: its closed-loop control autonomously identifies the relevant time constants and imposes fluctuations precisely matched to host-circuit dynamics, thereby ensuring robust population-level control.

The control strategy applied in this study was intentionally simple, relying on a single threshold for one monitored gene, but the framework is readily extensible. For example, cell size has already been used as a control variable to discriminate between yeast and bacteria in co-culture systems [272]. A natural next step would be to couple this approach to circuits directly linked to a product of interest, or to synthetic gene networks engineered for bioproduction. Incorporating multi-reporter strains would further allow simultaneous monitoring of multiple phenotypic traits, paving the way for more sophisticated, multi-parametric control strategies [106]. Ultimately, the pulse sequences identified by the Segregostat in SD conditions could be transferred in open-loop mode to large-scale bioreactors, for instance using sequential batch operation, and validated under industrially relevant conditions.

In summary, the Segregostat emerges as a promising tool to stabilize long-term cultures by ensuring an optimal trade-off between growth and stress response. In the future, this principle could also be applied to bioproduction system by finding the optimal trade-off between growth and product formation, thereby preventing the selection of less productive phenotypes [21, 268]. By aligning environmental perturbations with the intrinsic dynamics of regulatory networks, this strategy offers a powerful means to achieve robust, coordinated gene expression at scale. Looking forward, we believe that future bioprocess design should adopt a more cell-centric perspective: beyond optimizing physical parameters of the reactor, such as mixing or aeration, it is crucial to integrate the physiology and regulatory logic of the host as central elements of the system. Cells should be regarded not merely as passive entities subjected to environmental perturbations, but as active partners whose intrinsic dynamics can be harnessed for robust and productive processes. Our work contributes to building this cell-centric mindset by strengthening the integration between systems biology, synthetic biology, and bioprocess engineering, ultimately fostering the design of next-generation bioprocesses that are both efficient and resilient.

9. Material and methods

9.1. Code and data availability

Scripts used for data analysis, figure generation, and for the cybernetic model are available at <https://gitlab.uliege.be/mipi/published-software/2025-scaledownsegregostat>. // The raw data have been deposited in Zenodo under DOI: <https://doi.org/10.5281/zenodo.17453611>.

9.2. Microbial strains

The bacterial model used was the *Escherichia coli* MG1655 $P_{ydcS}::GFPmut2$ strain from the Zaslaver collection [214]. This strain carries a plasmid containing a transcriptional reporter for P_{ydcS} and a kanamycin resistance marker.

The yeast used in this work was the *Saccharomyces cerevisiae* CEN.PK 113-7D strain with the chromosomal integration of a reporter cassette $P_{glc3}::eGFP$ [111, 258].

The rationale for selecting P_{ydcS} and P_{glc3} as reporters of the general stress response in *E. coli* and *S. cerevisiae* has been described previously [212].

9.3. Growth media

Unless otherwise specified, the following media were used.

E. coli has been cultivated in a mineral medium containing (in g/l), K_2HPO_4 : 14.6, $NaH_2PO_4 \cdot 2H_2O$: 3.6, Na_2SO_4 : 2, $(NH_4)_2SO_4$: 2.47, NH_4Cl : 0.5, $(NH_4)_2H$ -citrate: 1, glucose: 5, thiamine: 0.01. The medium is supplemented with a trace element solution totaling 11 ml/l assembled from the following solutions (in g/l), 3/11 of $FeCl_3 \cdot 6H_2O$: 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 g/L): $CaCl_2 \cdot 2H_2O$: 0.74, $ZnSO_4 \cdot 7H_2O$: 0.18, $MnSO_4 \cdot H_2O$: 0.1, $CuSO_4 \cdot 5H_2O$: 0.1, $CoSO_4 \cdot 7H_2O$: 0.21. For plasmid maintenance, it has been supplemented with kanamycin (50 mg/l). The trace element solution, the thiamine and the antibiotic were filter-sterilized (0.22 μm) before supplementation of the other component that were heat-sterilized (at 120°C).

S. cerevisiae has been cultivated in a mineral medium based on the recipe of Verduyn et al. [259]. It contains, per liter, $(NH_4)_2SO_4$: 5 g, KH_2PO_4 : 3 g, $MgSO_4 \cdot 7H_2O$: 0.5 g, EDTA: 95.55 mg, $ZnSO_4 \cdot 7H_2O$: 22.5 mg, $MnCl_2 \cdot 4H_2O$: 5 mg, $CoCl_2 \cdot 6H_2O$: 1.5 mg, $CuSO_4 \cdot 5H_2O$: 1.5 mg, $Na_2MoO_4 \cdot 2H_2O$: 2 mg, $CaCl_2 \cdot 2H_2O$: 22.5 mg, $FeSO_4 \cdot 7H_2O$: 15 mg, H_3BO_3 : 5 mg, KI: 0.5 mg and glucose: 7.5 g. After heat sterilization (at 120°C) of those components, the filter-sterilized (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.

9.4. Continuous cultivations operations

The operating parameters of continuous cultures are provided in the Supp. Tables in appendix B.

To prevent unwanted reporter activation, strains were cultivated in two successive precultures. Both steps were carried out in 500 ml or 1 L flasks containing 50 or 100 ml of medium, respectively, incubated at 37 °C for *E. coli* or 30 °C for *S. cerevisiae*, with agitation at 150 rpm. The first preculture was started either from a glycerol stock or from a single colony grown on agar plate (LB supplemented with 50 mg/L kanamycin for *E. coli*, YPD for *S. cerevisiae*) and incubated overnight. The second preculture was inoculated with this culture to reach an initial $\text{OD}_{600} \approx 0.5$ and incubated until sufficient biomass was obtained for bioreactor inoculation (approximately 3 h for *E. coli* and 6 h for *S. cerevisiae*). Batch phases were initiated at an OD_{600} of about 0.1 (or 0.2 for experiments performed in the Biostat B-Twin bioreactor).

Continuous cultivations were conducted in either Biostat B-Twin (Sartorius, Göttingen, Allemagne) or in Bionet F1 bioreactor (Bionet, Murcia, Spain), with a working volume of 1 L, an agitation speed of 1200 rpm for *E. coli* or 1000 rpm for *S. cerevisiae*, and an aeration rate of 1 VVM. Temperature was maintained at 37 C for *E. coli* and 30 C for *S. cerevisiae*, while pH was regulated at 7 and 5, respectively. Each cultivation began with a batch phase that continued until the carbon source was fully depleted (as indicated by a rise in dissolved oxygen) or, for yeast, after approximately 15 h. Chemostat conditions were applied at $D = 0.3 \text{ h}^{-1}$ for *E. coli* and $D = 0.1 \text{ h}^{-1}$ for *S. cerevisiae*, with each condition maintained for at least five residence times. These dilution rates were selected to operate under nutrient-limited conditions and to promote activation of the general stress response. In chemostat operation, the dilution rate sets the specific growth rate and is correlated with residual substrate concentrations, while being anti-correlated with the expression of stress-related genes [62, 216]. In Segregostat experiments, a feedback control algorithm (custom MATLAB script based on online FC data, see below) actuated a pump to deliver glucose pulses (1 g per pulse for *E. coli* and 0.4 g per pulse for *S. cerevisiae*). These pulse magnitudes were selected based on preliminary exploratory experiments and previous Segregostat studies. For *S. cerevisiae*, an intermediate pulse magnitude was chosen, as control could be achieved over a range of concentrations tested between 0.2 and 0.8 g per pulse (data not shown). For *E. coli*, the lowest pulse magnitude enabling reliable population control was selected. As a result of glucose pulsing, the effective dilution rate temporarily exceeded the nominal chemostat values (up to 0.33 h^{-1} for *E. coli* and 0.125 h^{-1} for *S. cerevisiae*). Pulses were triggered when more than 50 % of the population exceeded a predefined fluorescence threshold (see Supp. Tables).

The 50/50 partition was chosen because it corresponds to the median of the population distribution, providing a robust and intuitive criterion to determine whether most cells are above or below the fluorescence threshold. This choice reduces sensitivity to outliers and has been previously used in population-level feedback control strategies.

The simplified scale-down bioreactor consisted of a STR (working volume 0.8 L) coupled with a recirculation loop (working volume 0.2 L) (Figure 4.2 B). The recirculation loop was composed of a 10 m long, 0.5 cm diameter silicone tube. Feeding and pulse additions were applied at two-thirds of the tube length, i.e. one-third before returning to the STR. Chemostat and Segregostat cultures were performed at different residence times in the recirculation loop (see Supp. Tables for the list of tested conditions). All other operating parameters were identical to those used in the lab-scale bioreactors described above. No limitations other than the carbon source were observed (Supp. Note B5).

9.5. Continuous cultivations monitoring and data treatment

Dissolved oxygen and pH were monitored in all experiments. For experiments performed in Bionet F1 bioreactor, biomass was monitored with Hamilton Dencytte RS485 probes. Single-cell dynamics were monitored throughout the cultures using an AFC setup. This system combined a custom-built sampling device (integrated in the Segregostat platform [22, 145]) with a benchtop flow cytometer (BD Accuri C6 or BD Accuri C6+). Every approximately 12 minutes, a sample was withdrawn from the bioreactor, diluted in phosphate-buffered saline (PBS), and subsequently analyzed.

For measurements obtained with the BD Accuri C6, the cytometer was operated with a custom fluidics setting (24 $\mu\text{L}/\text{min}$, 8 μm core), and the FSC-H threshold was adjusted to 20,000 for *E. coli* and 80,000 for *S. cerevisiae*. When using the BD Accuri C6+, the flow rate was set to “medium” (35 $\mu\text{L}/\text{min}$, 16 μm core), and the threshold was set to 15,000 for *E. coli* and 80,000 for *S. cerevisiae*. Under these configurations, roughly 40,000 cells were acquired per sample. GFP fluorescence was recorded in the FL1-A channel (excitation at 488 nm and emission filter 533/30 nm).

To enable direct comparison between replicates measured on different flow cytometers, data from the BD Accuri C6 were transformed as previously described [212]. Flow cytometry datasets were pre-processed by discarding zero values and doublets.

To quantify population-level heterogeneity in gene circuit activation, we used Shannon entropy, a metric derived from information theory. Compared to traditional metrics such as the coefficient of variation or the Fano factor, entropy is particularly well suited to capture heterogeneity in multimodal distributions and is less sensitive to changes in average expression levels [14, 144]. Entropy (H) was calculated from the distribution of single-cell fluorescence values across discrete bins using the formula below, where m is the number of bins and p_i the probability of a data point falling into

bin i . In our case, the fluorescence distribution was discretized into 50 logarithmically spaced bins spanning from 1 to 10^6 a.u.. A schematic illustration of the entropy computation is provided in Figure 4.1 C. As illustrative extreme cases, entropy is maximal when the population is evenly distributed across all bins and minimal ($H = 0$) when the entire population occupies a single bin.

$$H = - \sum_{i=1}^m p_i \cdot \log_2(p_i) \quad (\text{Eq. 4.1})$$

The functions linking fluorescence and growth rate (i.e., trade-off curve), used to infer single-cell growth rates, were determined by exploiting the fluorescence relaxation phases in Segregostat experiments. The underlying assumptions and validation of this approach are presented in detail in Delvenne et al. [212]. Briefly, the relation was inferred from the fluorescence relaxation phases occurring between successive inductions. During these phases, fluorescence production is halted and its decrease is dominated by dilution due to cell growth, as protein degradation is negligible over the considered time scale. Assuming that growth rate remains approximately constant over short relaxation intervals and that growth rate is a monotonically decreasing function of fluorescence, the decay of fluorescence was tracked for different quantiles of the population over five successive flow cytometry measurements (≈ 1 h). For each relaxation phase, growth rates were estimated from the fluorescence decay of multiple percentiles spanning the distribution (5-95 %). These estimates were combined across relaxation phases and biological replicates to infer a functional relationship between fluorescence and growth rate, which was then used to reconstruct growth rate distributions from fluorescence measurements.

9.6. Cybernetic modelling of continuous cultivations

Models for the different continuous cultivations, with and without plug flow (bio)reactor (PFR) loops, were performed using a simplified version of a toolbox published in a previous work (MONKS) [249]. The toolbox and data files used for the calculation of each model as well as the jupyter notebooks with each of the performed models can be found in the GitLab repository referenced above.

The main difference are the modifications to adapt a PFR to the main MONKS modeling toolbox. To this end, the model was set on creating volumetric cells with a fraction of the total volume of the STR and PFR volumes. Initial Biomass is then divided into each volumetric cell. These volumetric cells then virtually exist inside the STR until randomly selected to enter the PFR. The selection is performed every delta time and made with a uniform probability density function for the remaining volumetric cells inside the STR. The number of volumetric cells selected to enter the PFR is calculated considering the delta volume equal to the fraction of the volume needed to enter PFR according to the flow rate for each experiment (calculated from

the retention time in the PFR and the total volume of the PFR). Once in the PFR the volumetric cells pass through it as sections of volumes and only updated in their concentrations until arriving the section of the feed and pulse ports (2/3 of the total PFR volume). Feed is continuously updated at this point, and pulses are performed according to the experimental files. Once cells exit the PFR the remaining mass of the feeds and pulses are used to update the concentration in all the cells of the STR. For all simulations between 1500 and 2000 volumetric cells were used to simulate different cell experimental lifelines as each volumetric cell contains the biomass information and the environmental substrates and products available, at this point and for each delta time MONKS cybernetic modeling approach is used to calculate the biomass growth, substrate consumption and product formation within each volumetric cell. Finally, in the STR the effect of each cell is distributed for all cells as environmental information, while in the PFR the environmental information is not distributed and therefore the biomass can only use substrate directly in their own volumetric cell.

5

Chapter 5: General discussion and conclusions

1. Contrasting general stress response strategies in *S. cerevisiae* and *E. coli* populations

In this thesis, phenotypic diversification and its control were investigated through the lens of the general stress response (GSR), given its relevance to both ecological and bioprocess contexts. Based on previous studies (see chapter 1 sections 1 & 2), the GSR was expected to display two key features that favor phenotypic diversification: intrinsically noisy gene expression and a high fitness cost resulting from the reallocation of cellular resources. These expectations were fully met in *Saccharomyces cerevisiae*, but were less evident in *Escherichia coli*, regardless of the experimental scale investigated (i.e., from microfluidics to scale-down bioreactors). Our results rather emphasize that the GSR of *S. cerevisiae* and *E. coli* differ in their impact on growth rate.

This difference likely originates from the distinct physiological architectures and stress-response logics of the two organisms (see chapter 1 section 2). In both cases, activation of the GSR involves a broad metabolic reprogramming that redirects energy and resources toward protection, at the expense of growth. In *S. cerevisiae*, this reduction can even lead to a complete growth arrest, with cells entering a quiescent state. This trade-off between stress resistance and growth potential manifests at the population level through the emergence of stressed and non-stressed subpopulations. It likely underlies the bet-hedging strategy observed for *glc3* expression, as well as the fitness-entropy compensation (FEC) mechanism observed in our continuous cultures. In contrast, the *E. coli* GSR, largely coordinated by the sigma factor RpoS, operates in a more graded and dynamic manner. While RpoS activation represses functions associated with rapid proliferation, it does not fully inhibit growth. Rather, it enables the expression of stress-adaptive genes that support survival and limited metabolic activity. *E. coli* thus exhibits a mutual regulation between growth rate and stress response, allowing cells to dynamically adjust their physiology; upregulating protective genes while maintaining a reduced but still relatively high growth rate under stress. For instance, the homogeneous activation of *ydcS* observed in our continuous cultures suggests that the maximum growth rate of stress-induced cells remains above the imposed dilution rate, allowing cells to maintain a stable proliferation. As a result, the fitness cost associated with stress activation in *E. coli* appears moderate, and population heterogeneity remains primarily driven by biological noise rather than by major physiological shifts.

As mentioned, activation of the GSR in yeast can ultimately lead to quiescence, a reversible dormant state that preserves cell viability under prolonged starvation. In *E. coli*, entry into a quiescent-like state under nutrient stress is less documented and was not observed under the conditions tested in this work. Investigating the impact of more severe stresses (such as complete carbon source deprivation, strong acid stress, or antibiotic treatment) on the trade-off mediated by RpoS may provide further insight

into the entry into a dormant protective state. Notably, tolerance and persistence have been more extensively characterized in *E. coli* [28, 273]. Tolerance refers to a transient growth arrest that increases the minimum duration required for killing (MDK) in the presence of antibiotic concentrations above the minimal inhibitory concentration (MIC), whereas persistence denotes tolerance restricted to a subpopulation of isogenic cells [28, 273]. Although not exactly equivalent to quiescence in yeast, since the associated phenotypes typically arise under more severe stress conditions, these mechanisms also involve a temporary cessation of growth. Interestingly, some studies have shown that deletion of *rpoS* [28] or of *rpoS*-regulated genes such as *elaB* [274] increases persistence in *E. coli*. This observation suggests that RpoS activity may actually prevent the formation of growth-arrested persister cells. However, evidence regarding the influence of RpoS on tolerance and persistence remains inconsistent, with some studies reporting opposite conclusions depending on the experimental setup, the antibiotic tested, or the research group involved [28].

Beyond the physiological and regulatory aspects discussed above, another possible explanation for the low fitness cost and the absence of a clear trade-off observed in our experiments may lie in strain domestication. Indeed, due to its central role in coordinating the balance between growth and stress survival, RpoS is actually a key evolutionary target [4, 24]. Because different strains inhabit environments where stresses vary in frequency and intensity, their GSR systems evolve accordingly. Depending on the ecological niche, mutations and regulatory adjustments can fine-tune the threshold and amplitude of GSR activation. For instance, some may prioritize growth at the expense of stress resistance, while others, such as pathogens, maintain high RpoS levels to support the expression of virulence genes [4, 24]. Interestingly, mutations that attenuate RpoS activity tend to arise under conditions of prolonged nutrient limitation without additional severe stress, such as those encountered in many laboratory settings (e.g., stationary phase or continuous culture under low nutrient supply) [4, 56]. In such cases, mutations reducing RpoS activity effectively readjust the threshold at which cells switch to the stress survival state to even lower nutrient concentrations, thereby improving growth under such conditions [4]. It is also important to note that this evolutionary tuning of the trade-off does not rely exclusively on mutations within *rpoS* itself, but can also involve changes in its upstream regulatory regions, modulating RpoS activity, or in downstream genes that adjust their sensitivity to RpoS-dependent regulation [215]. Testing this domestication hypothesis therefore goes far beyond a simple comparison of the *rpoS* sequence between our strain and a more ancestral isolate, but rather requires a much broader investigation, reflecting the complexity of the RpoS regulatory network itself.

Comparing our observations with studies conducted on the same strains provides valuable context for interpreting our results. However, such comparisons are often challenging due to differences in experimental design, growth conditions, and data acquisition methods. These variations can lead to different interpretations of GSR

outcomes, even when similar phenomena are being investigated. For instance, most of the stress-related reporters used in similar previous studies on *E. coli* are known to be co-regulated by other sigma factors (e.g., σ^{70}), while *ydcS* is primarily controlled by RpoS, with no other transcription factors currently known to initiate its expression, suggesting a much tighter dependence. Moreover, most of them were conducted under non-limiting conditions with exponentially growing cells, either in batch cultures or in mother-machine microfluidic devices [14, 64, 65]. Nonetheless, their results were generally consistent across setups regarding reporter noise [65] and, in agreement with our observations, Patange et al. [64] and Sampaio et al. [65] reported correlated increases in stress expression and growth reduction, though without complete growth arrest. In contrast, under microfluidic single-cell cultivation (MSCC), *ydcS* was almost not expressed in our non-limiting conditions, likely due to its strong dependence on RpoS activity, and its distribution under nutrient limitation appeared relatively heterogeneous, resembling that of reporters with broader regulatory inputs under non-limiting conditions [65]. Even when using the same reporter, discrepancies can arise between microfluidic and continuous-culture observations. In our case, *ydcS* activation appeared more heterogeneous in microfluidic experiments than in chemostat cultures, where the population response was largely homogeneous at all tested dilution rates. Beyond the differences in culture regimes, this may also reflect how fluorescence data are acquired and processed. Comparisons between setups require careful calibration, even between flow cytometers of the same model (appendix A.2). Consequently, metrics (e.g., entropy) can only be quantitatively compared when data are acquired under identical conditions or properly calibrated post-acquisition. These considerations highlight the importance of transparent data sharing and standardized formats that facilitate cross-study analysis. Making raw and calibrated datasets publicly available would allow results to be reanalyzed or combined under new perspectives.

Overall, these differences also emphasize that bioprocesses cannot be optimized through a one-size-fits-all strategy; rather, they require an understanding of how cells perceive and respond to their environments. This perspective forms the basis for a more cell-centric approach to bioprocess design, which will be discussed in the following section.

2. Toward a cell-centric approach for the future of bioprocess design

This thesis was guided by two overarching objectives: to elucidate the functional basis and mechanisms of phenotypic diversification in continuous cultures, and to assess how such heterogeneity can be controlled under dynamic environments mimicking large-scale bioreactors. Together, these objectives aimed to bridge the gap between fundamental microbial population dynamics and applied bioprocess control.

Regarding the first objective, we confirmed the initial hypothesis that an increase in entropy under continuous culture, specifically in systems with high fitness costs, correlates with the overall fitness of the population. We identified an underlying mechanism, which we termed FEC, whereby, when the dominant induced phenotype is maladapted to the culture conditions, a minority of cells that escape this state, via variability in gene expression or repression, can proliferate and support the survival of the entire population. This is a competitive dynamic that prevents the establishment of a single exclusive phenotype. Instead, it promotes a dynamic equilibrium among phenotypes, resulting in increased heterogeneity which is quantitatively captured by an increase in population entropy. The analysis of the FEC mechanism was made possible by quantifying the trade-off between growth rate and gene expression. Our approach relied on automated flow cytometry (AFC) measurements collected during the relaxation phase under Segregostat control, during which fluorescence levels decrease as a result of protein dilution caused by cell growth and division. In contrast to microfluidics, which is the main alternative for mapping fitness to phenotype, our method provides a more direct and efficient means of assessing growth rates within bioreactor environments. We propose that this technique constitutes a critical step toward a more robust understanding of bacterial population dynamics. An important aspect of the FEC mechanism is that the emergence of alternative phenotypes is facilitated by an environmental escape process. In the case of the partial relaxation of yeast cells from the stressed to the non-stressed state in low dilution rate chemostats, this phenomenon can be explained by the reduced metabolic activity of stressed cells, which tend to shift toward a more quiescent state and therefore consume less substrate. Fully stressed cells may even stop growing and cease nutrient uptake altogether. As a consequence, substrate depletion slows down, creating a more favorable environment for cells with lower stress-related gene expression. These cells can then escape the stressed state through a such environmental feedback mechanism. Contrary to the idealized view of chemostats as homogeneous environments, our results reveal that microbial populations exert a feedback on their surroundings creating transient nutrient fluctuations that sustain the FEC mechanism. This finding reminds us that continuous cultures are interactive systems shaped by both the environment and the cells themselves.

Beyond its analytical capabilities mentioned above, the Segregostat also enables active control of population dynamics. Its effectiveness has been demonstrated across multiple systems [22]. Notably, it successfully synchronized stress gene expression and reduced population heterogeneity compared with chemostats operated at similar dilution rates. However, all these experiments were conducted in laboratory-scale bioreactors, which provide well-mixed environments. In this work, we introduced an additional layer of complexity by considering environmental perturbations representative of those encountered by cell populations in large-scale industrial bioreactors. Such heterogeneities, reproduced here using a two-compartment scale-

down (bio)reactor (SDR), can strongly influence cellular physiology and potentially interfere with our closed-loop control strategy. After assessing the impact of the scale-down (SD) settings on population heterogeneity (see chapter 4 sections 4 & 8), the Segregostat was implemented to test its robustness under these heterogeneous conditions. Remarkably, the Segregostat maintained strong control performance across all tested scenarios. This robustness likely arises because the Segregostat imposes and amplifies perturbations that align with host-circuit dynamics and that are not disrupted by those introduced by bioreactor constraints. Indeed, by continuously monitoring gene circuit activation, the Segregostat identifies the natural dynamics of the targeted regulatory system and autonomously imposes fluctuations (characterized by specific frequency, amplitude, and phase durations) that precisely match them. These controlled perturbations promote coordinated responsive gene expression and therefore reduce phenotypic heterogeneity. However, these conclusions regarding the robustness of the Segregostat under SD conditions should be interpreted with caution to avoid overstatements, as they are based on a limited number of model systems. Notably, the particularly effective control obtained with *S. cerevisiae* may be partly attributed to its Crabtree-positive metabolism, which provides a strong physiological lever for dynamic regulation (see chapter 4 sections 6). Whether such a strategy remains as efficient in Crabtree-negative strains or when using non-fermentable carbon sources remains to be determined. Furthermore, it is important to acknowledge other experimental differences that may influence the comparison between our two models, such as the use of a plasmid-based reporter in *E. coli* versus a chromosomal integration in *S. cerevisiae*, as well as the distinct impact of replicative aging in yeast. Consequently, while our results are highly promising, further investigations using a broader range of metabolic backgrounds, and specifically industrial production strains, are required to confirm the universality of this approach. Nonetheless, these findings suggest that such population-level control could be achieved even in industrial-scale conditions.

Overall, our findings highlight that conventional chemostats, though designed as homogeneous systems, are in fact dynamic environments shaped by continuous feedback between cells and their surroundings. Such feedback influences the stability and diversification of microbial populations. By contrast, applying environmental perturbations that align with the intrinsic dynamics of the targeted regulatory networks maintains system responsiveness and offers a powerful means to achieve more homogeneous gene expression. Looking forward, we believe that future bioprocess design should adopt a more cell-centric perspective. More robust, predictable, and productive processes could be achieved by better integrating the physiology, regulatory logic, and adaptive dynamics of the host into process design and control strategies. Altogether, this work contributes to fostering this cell-centric mindset, where systems biology, synthetic biology, and bioprocess engineering converge toward the development of next-generation bioprocesses. Implementing such a paradigm will

require advanced tools capable of monitoring and controlling cellular behavior in real time. In this context, recent technological developments, including the Segregostat, open promising perspectives for achieving dynamic, feedback-based regulation of microbial populations.

3. A critical assessment of cell population controllability

Before turning to perspectives on single-cell monitoring and population control, it is important to first highlight the key aspects of Segregostat operation identified throughout this work.

By directly monitoring gene circuit activation, the Segregostat captures the intrinsic dynamics of the system and accordingly generates regular environmental changes. In other words, these environmental perturbations are timed to match the characteristic response time of the cells. Although individual cells may respond with variable delays due to biological noise, this periodicity allows most of them to eventually reach a similar expression level or phenotypic state. In practice, not all cells necessarily achieve the same target phenotype; for instance, the control threshold is typically set such that 50 % of the population exceeds a predefined fluorescence level, ensuring that at least half of the cells reach this level of expression. Nonetheless, the more synchronous response of the entire population enabled by these environmental changes can result in overall homogenization.

Contrasting with other control strategies, such as internal genetic controllers or optogenetic systems, this method offers several advantages. Internal controllers often require complex *de novo* genetic engineering of the host, a tedious and iterative process [201] to develop controllers that can remain difficult to tune and may fail to perform as intended under the heterogeneous environmental conditions typical of large-scale bioreactors [275]. Optogenetic systems, while offering high temporal resolution, are frequently limited by light penetration issues in dense cultures and often rely on microscopy-based setups [155, 158]. In contrast, the Segregostat is directly compatible with standard bioreactor environments and is more versatile as it enables the control of both synthetic and natural circuits [149], as exemplified in chapter 4 by GSR-related genes. However, the question arises as to how universal this strategy truly is, in particular, whether it can be applied to different types of genes and regulatory circuits under various continuous culture conditions.

Beyond stress-related gene regulation, recent and ongoing research highlights the broad potential of this approach in terms of controllable gene circuits. The Segregostat has, for instance, been successfully applied in laboratory-scale bioreactors to control genes involved in the utilization of alternative carbon sources, sporulation, and the activation of T7-based expression systems [22]. However, we observed that control

is not always beneficial. While the control of systems with high fitness cost, that are heterogeneously expressed under chemostat conditions, results in an homogenization of the expression, controlling systems with low fitness cost resulted in little decrease or even an increase in population heterogeneity [22].

It is important to remember that Segregostat control is obtained under specific technical parameters influencing gene expression, such as the dilution rate, the selected setpoint (i.e., the fluorescence threshold), the feedback frequency (which depends on the time between two measurements), and the amplitude of the feedback signal (e.g., the amount of glucose pulsed). Depending on the system investigated, these parameters may require more or less adjustment. For instance, in *E. coli*, unlike in *S. cerevisiae*, activation of the GSR does not substantially reduce the growth rate; the maximum growth rate of stressed cells remains higher than the imposed dilution rate (cf. chapter 3). As a result, Segregostat control at low dilution rates (e.g., $D = 0.1 \text{ h}^{-1}$) can fail: repeated glucose pulses lead to continuous biomass accumulation, thereby decreasing the effect of subsequent pulses and preventing the reporter signal from being sufficiently diluted to fall below the activation threshold. This creates a feedback loop where pulses are never interrupted, biomass continues to increase, and the maximum glucose input (from feed and pulses) becomes limiting at the single-cell level, ultimately leading to a population-wide stress response.

These observations highlight a critical aspect of Segregostat operation: while the system is fully automated and closed-loop, its success hinges on (i) the properties of the biological system under study (e.g., host physiology as well as basal expression, dynamic range, fitness cost, heterogeneity, and regulation of the reported gene), (ii) the culture conditions (e.g., dilution rate), and (iii) the design of the control algorithm (e.g., setpoint selection, pulse amplitude, and minimum time between pulses). Achieving stable control and synchronized responses thus requires careful consideration, tailored to the metabolic and regulatory landscape of the organism in use.

4. Perspectives on single-cell monitoring and population control

Recent advances, including those presented in this thesis, point toward promising developments in single-cell monitoring and population-level control. Understanding and steering phenotypic heterogeneity remains a key challenge in microbial bioprocessing, where predictability, stability, and productivity depend on how phenotypes are distributed within the population. This final section highlights some limitations, potential improvements, and future directions that could further expand the applicability of cell-machine interfaces such as the Segregostat.

In this work, the population was monitored at the single-cell level using a single fluorescent reporter, capturing only one phenotypic trait. While this approach provided

valuable insights into the dynamics of the GSR, it necessarily offers a partial view of the population's phenotypic landscape. Cells may differ substantially in other traits not monitored here, such as metabolic activity or transporter expression, which could also influence diversification dynamics. Furthermore, we showed that genes belonging to the same regulon do not always diversify in a similar manner across different levels of induction or activation (see chapter 2 section 5.3). It therefore appears that the selection of a representative reporter is crucial, and that conclusions drawn from its monitoring must be interpreted cautiously to avoid biased generalizations. Gaining a more comprehensive view of phenotypic diversification will thus require multiplex strategies capable of monitoring several traits simultaneously. Single-cell omics technologies could provide a comprehensive snapshot of cellular states, revealing transcriptional, translational, or metabolic heterogeneity that remains invisible to fluorescence-based measurements. However, such methods are difficult to implement and remain unsuitable for real-time applications in continuous cultures. As an alternative, omics analyses performed on subpopulations isolated according to the expression level of a monitored gene (e.g., via fluorescence-activated cell sorting (FACS)) could already offer valuable insights, including on co-regulated pathways. A more accessible strategy, better suited for real-time monitoring, would be to track several fluorescent reporters simultaneously, thereby following a limited number of key physiological processes in parallel. Such multiplex approaches have already proven effective for monitoring up to three processes [117, 233], including, for instance, product formation, growth, and oxygen limitation [106]. Nevertheless, the number of reporters is constrained by fluorophore properties, such as maturation time, spectral overlap, and the physiological burden associated with their simultaneous expression. Careful selection of reporters and fluorophores thus remains crucial.

Beyond monitoring, the purpose of cell-machine interfaces such as the Segregostat is to actively control microbial population behavior. In this work, the control algorithm was intentionally kept simple, relying on a single threshold applied to one monitored gene. Despite its simplicity, this approach proved effective in coordinating stress responses and reducing population heterogeneity. However, the framework is readily extensible toward more sophisticated regulation schemes. Future implementations could incorporate additional control variables, such as cell size or the expression of multiple reporters, allowing for multi-parametric feedback strategies. Expanding the dimensionality of the control system would not only enable work with more complex systems, such as multi-species communities, but also allow finer tuning of population behavior and open new opportunities for adaptive, context-specific bioprocess optimization. By dynamically modulating environmental cues according to the population state, such systems could balance resource allocation between different cellular functions, such as biomass formation and product synthesis [276], thereby enhancing overall process efficiency.

Regarding its practical implementation, the transition of the Segregostat from

laboratory-scale proof-of-concept to industrial settings represents a key perspective for bioprocess scaling up. Moving toward industrial application, the Segregostat can be envisioned as a powerful process development tool in addition to its established role as a versatile laboratory interface. Although the real-time closed-loop monitoring of a large-scale fermentor remains technically challenging (e.g., due to sterilized sampling requirements, the need for multiple sampling points to account for spatial heterogeneity, and the practical constraints of using fluorescent reporters in production strains), the optimal pulse sequences identified at the laboratory scale under representative SD conditions could be transferred in an open-loop mode to large-scale bioreactors. For instance, this could be practically implemented using sequential batch operations or timed feeding strategies that mimic the successful control patterns previously determined. Such a transfer would bypass the need for fluorescence-based sensing at scale, while still leveraging the host's regulatory dynamics to maintain population homogeneity and productivity under industrially relevant conditions. For this purpose, future research should focus on applying this workflow to industrial production strains and validating the open-loop transfer at large-scale.

Altogether, these advances illustrate how combining single-cell monitoring with dynamic feedback control can strengthen the integration between microbial physiology and bioprocess engineering. By integrating cellular behavior as a core element of process design, such cell-centric strategies hold great promise for developing more robust, predictable, and adaptive bioprocesses.

A

Supplementary materials for the Chapter 3

This appendix is an adapted version of the Supplementary Material of the preprint referenced below, which corresponds to the chapter 3 of this thesis.

Delvenne, M., Vandenbroucke, V., Henrion, L., Sehr, M., Martinez, J. A., Sloodts, A., Telek, S., Zicler, A., and Delvigne, F. *A Fitness Entropy Compensation Effect Is Set by the Trade-off between Growth and Gene Expression in Cell Populations*. 2025. DOI: 10.1101/2025.07.05.663304. URL: <https://www.biorxiv.org/content/10.1101/2025.07.05.663304>. Pre-published

1. Supplementary Note 1. A theoretical analysis of the Fitness-Entropy compensation mechanism

In this note, 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 [277]). Then, starting in section 1.1, we go into the details of the measurement and interpretation of entropy in the context of biological measurements, why entropy should increase mechanically as a result of the competition in simplified and idealised cases (section 1.3), and finish with a more detailed reasoning that can be applied to realistic cases such as the ones we measured (section 1.4).

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

The Shannon entropy (H , measured in bits) is as a measure of how heterogeneous a probability distribution is in a given interval of measurements. Figure A.1 illustrates how we can expect entropy to behave for different distributions.

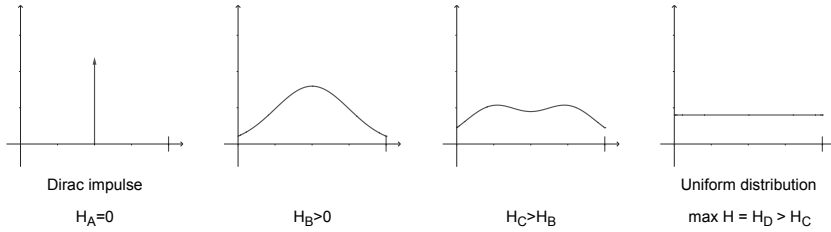


Figure A.1: Illustration of Shannon entropy H for different distributions in the same interval. A Dirac impulse (leftmost plot) has an entropy of 0, as all the probability is concentrated in a single point and there is no heterogeneity. A uniform distribution (rightmost plot) is the most heterogeneous 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 $H(\chi) = -\sum_{x \in \chi} p(x) \log_2 p(x)$, 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 paper we used a set of constant bins equally spaced in log space to compare different distributions.

For flow cytometry (FC) 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 A.2.

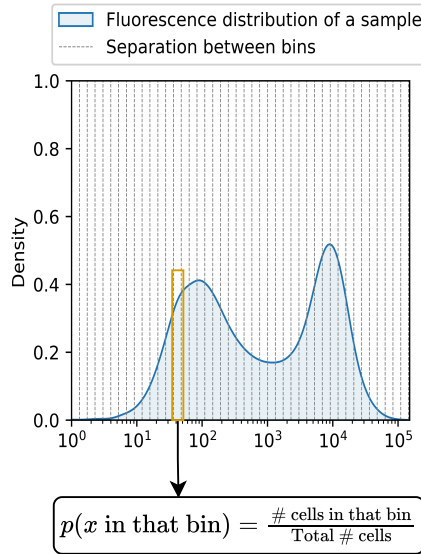


Figure A.2: Example of the binning strategy used for the entropy measurements. The bins are equally spaced in log space, and cover the range of the measurements. The number of bins is much larger than the number of phenotypes expected (2), but much smaller than the number of cells measured (>10,000).

1.2. 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:

$$H(k \cdot \chi_1 + (1 - k) \cdot \chi_2) \geq k \cdot H(\chi_1) + (1 - k) \cdot H(\chi_2) \quad (\text{Eq. A.1})$$

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:

$$H(k \cdot \chi_1 + (1 - k) \cdot \chi_2) = k \cdot H(\chi_1) + (1 - k) \cdot H(\chi_2) - k \cdot \log_2(k) - (1 - k) \cdot \log_2(1 - k) \quad (\text{Eq. A.2})$$

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 A.3. 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 A.2, and the resulting entropy is shown with the blue curve.

The distributions of interest for this paper 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.

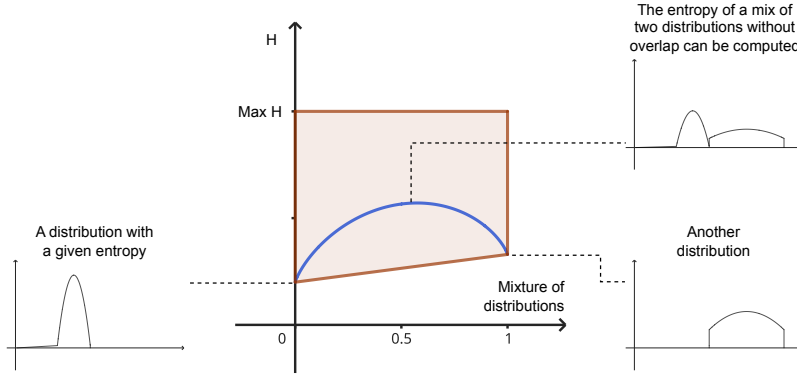


Figure A.3: Entropy of the mixture of two different distributions; the x-axis represents the proportion of each population in the mixture, with 0 being 100% of the first population and 1 being 100% of the second one, and the y-axis shows the entropy. The quadrilateral on the entropy plot represents the 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.

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

Let us now investigate the Fitness-Entropy compensation effect. To do so, we can start with a simplified case in a chemostat, where the growth rate of the cells is the only factor determining their fitness. To showcase the effect, it is sufficient to consider two cases:

- 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 A.4 A & 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 abundance of the induced phenotype in the population, as shown in Figure A.4 C & D.

When looking at 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 A.4 D would normally be the tail end of the distribution in Figure A.4 B, and are the noise of the distribution.

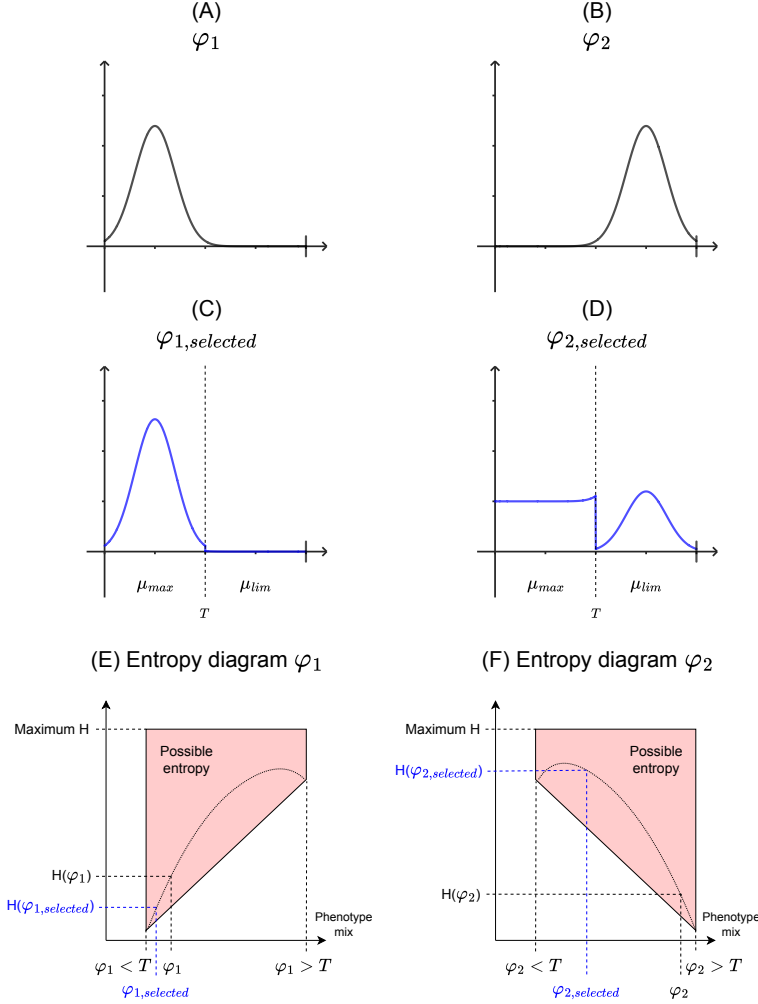


Figure A.4: (A,B) Example probability distributions (not to scale) of fluorescence for two well-defined phenotypes, where the phenotype is not affecting fitness. (C,D) The same distributions, but where the phenotype is affecting fitness. Since they are well defined phenotype, we can expect most of the 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 $\mu_{lim} < \mu_{max}$. (E,F) The entropy of the distributions compared to the entropies of the distribution split between the induced and uninduced regions. The entropy of the noisy part is much larger than the entropy of the main phenotype (by virtue of being noise).

Let us now look at the effect of this selection on the entropy of the 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 A.2, since we are considering no overlap. In practice, we can expect 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.

In Figure A.4 E, it can be easily seen 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 A.4 F, we can see that, by contrast, selecting the noise instead of the induced phenotype naturally leads to an increase in entropy instead. This is the Fitness-Entropy Compensation mechanism and explain how entropy can be a measure of how much a phenotype affects fitness.

1.4. 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.

1.4.1. 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 A.5.

To determine what is the entropy of such a distribution, Equation A.2 can be generalised to any number of phenotypes, as shown in Equation A.3.

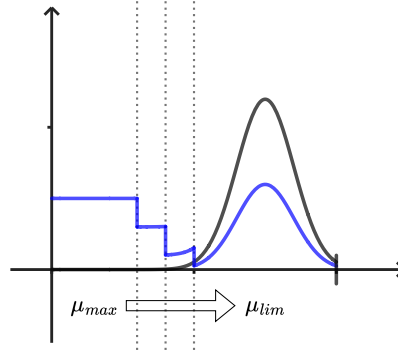


Figure A.5: Entropy of a distribution with several phenotypes. In black the phenotype before selection. In blue, the phenotype after selection, where each successive region from left to right has a lower growth rate than the previous one.

$$\begin{aligned}
 H\left(\sum_{i=1}^n k_i \cdot \chi_i\right) &= \sum_{i=1}^n k_i \cdot H(\chi_i) - \sum_{i=1}^n k_i \cdot \log_2(k_i) \quad , \text{ where } \sum_{i=1}^n k_i = 1 \\
 &= \text{mean}(H(\chi_i)) - \sum_{i=1}^n k_i \cdot \log_2(k_i) \quad (\text{Eq. A.3})
 \end{aligned}$$

This cannot be represented graphically as in Figure A.4, 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.

1.4.2. 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 traveling 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:

$$\frac{d\varphi_1}{dt} = \mu_1\varphi_1 - r_1\varphi_1 - D\varphi_1 + r_2\varphi_2 = 0 \text{ at steady state} \quad (\text{Eq. A.4})$$

$$\frac{d\varphi_2}{dt} = \mu_{lim}\varphi_2 - r_2\varphi_2 - D\varphi_2 + r_1\varphi_1 = 0 \text{ at steady state} \quad (\text{Eq. A.5})$$

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, then $r_1 + D > \mu_{max}$ leads to the first phenotype being washed out, and the second follows, making the Fitness-Entropy compensation impossible when there is little noise and strong, fast induction. This explains why the Fitness-Entropy compensation effect was not observed for the toggle-switch, for example.

This model does not consider the environmental feedback that was observed conjointly with the Fitness-Entropy compensation effect, that would amplify any switching and ensure both populations can survive.

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

We've seen 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, *ycdS*, and *glc3* systems were measured in different conditions to assess the validity of the effect more quantitatively. The results are presented in Figure A.6.

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

For the *ycdS* 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 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

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.

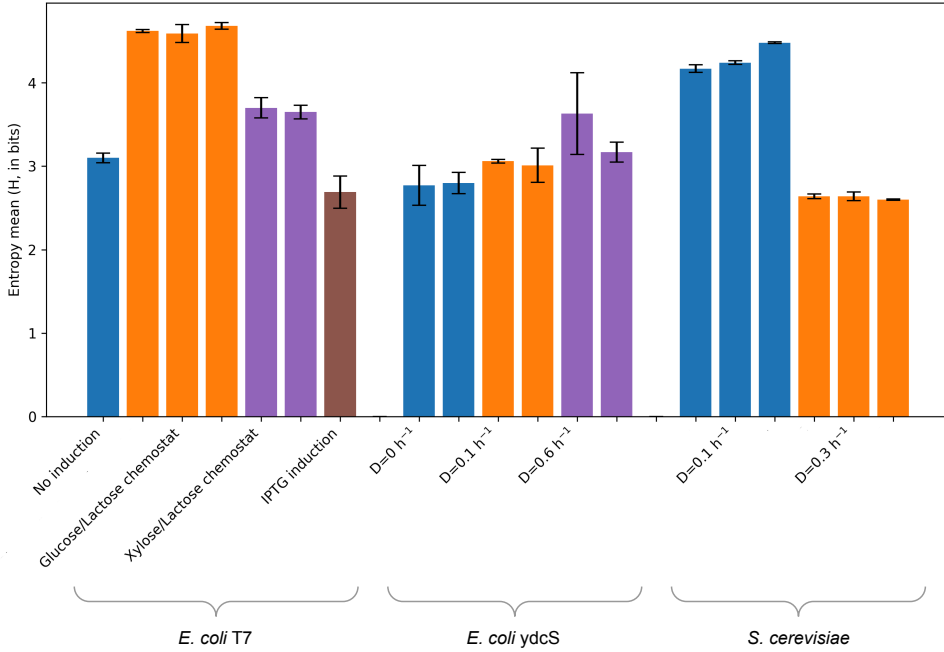


Figure A.6: Entropy of the different systems measured in different conditions. Error bars are three standard deviations on 6 samples (technical replicates), except for the Uninduced and IPTG induced samples for the T7 system, which only have 2 samples each and come from flasks instead of bioreactors. Biological replicates are juxtaposed and in the same color. 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}).

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 FC 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.

2. Supplementary Note 2. Calibration of the automated flow cytometry

Three instruments were used for the automated flow cytometry (AFC) 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 pre-cultures 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 A.7 A), but for the T7 system the relationship was not linear at all (Figure A.7 B). 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 prioritized 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 A.7 C & D). 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 A.6).

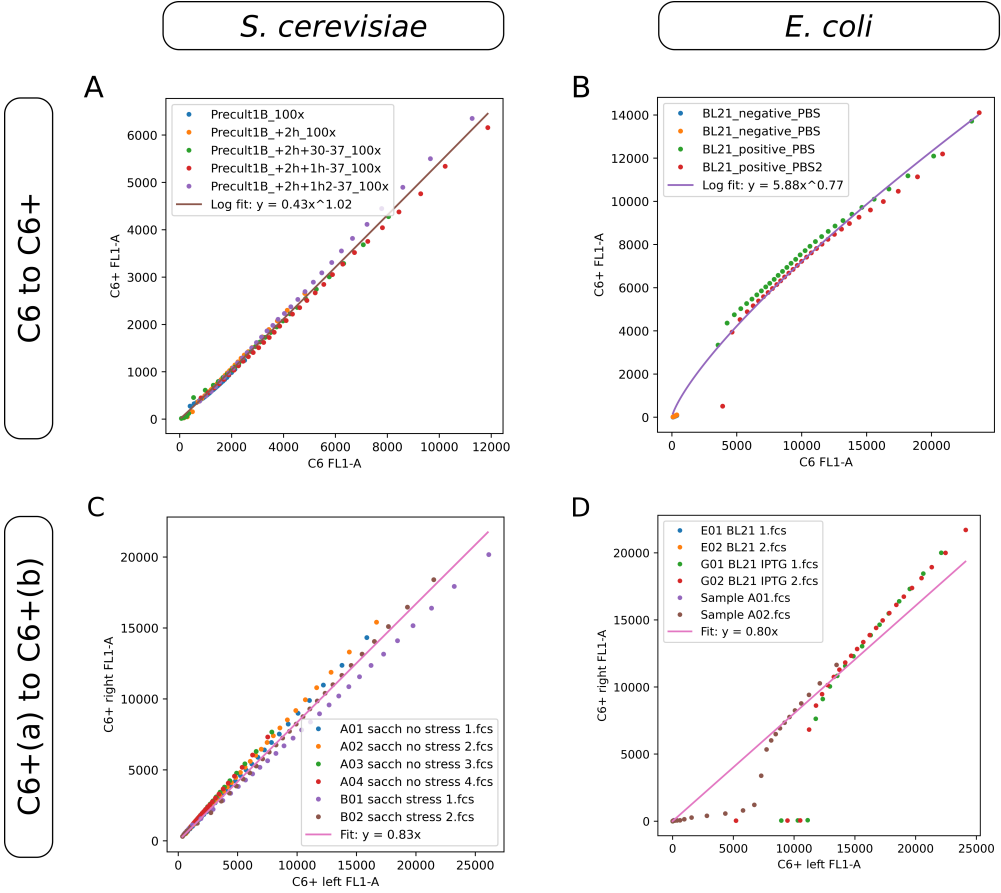


Figure A.7: Calibration of the Accuri C6 and Accuri C6+ cytometers. The data points are the percentiles of the measurements, and the curves are the fitted correlations. (A-B) Calibration of the Accuri C6 cytometer with the Accuri C6+ cytometer for (A) *S. cerevisiae* and (B) *E. coli*. (C-D) Calibration of the Accuri C6+ cytometer with another Accuri C6+ cytometer for (C) *S. cerevisiae* and (D) *E. coli*.

3. Supplementary Note 3. Determination of the trade-off curves based on Segregostat experiments

3.1. Basic principles of the method

To determine fitness, we needed to determine the growth rate as a function of the measured phenotype. To do so, we made use of Segregostat measurements, where induction is periodic instead of constant. Thanks to the periodicity, after each induction is a relaxation phase where no fluorescence is produced (see Figure A.8 A). 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, and calculating it is equivalent to calculating the growth rate. This is pertinent because protein degradation is negligible compared to growth in most cases [240].
- 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 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 A.8 B).

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 AFC 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 (representing the inhibition of growth by fluorescence) that would correctly approximate the growth rate as a function of fluorescence (Figure A.8 C 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.

The code for this procedure 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 various errors as described below.

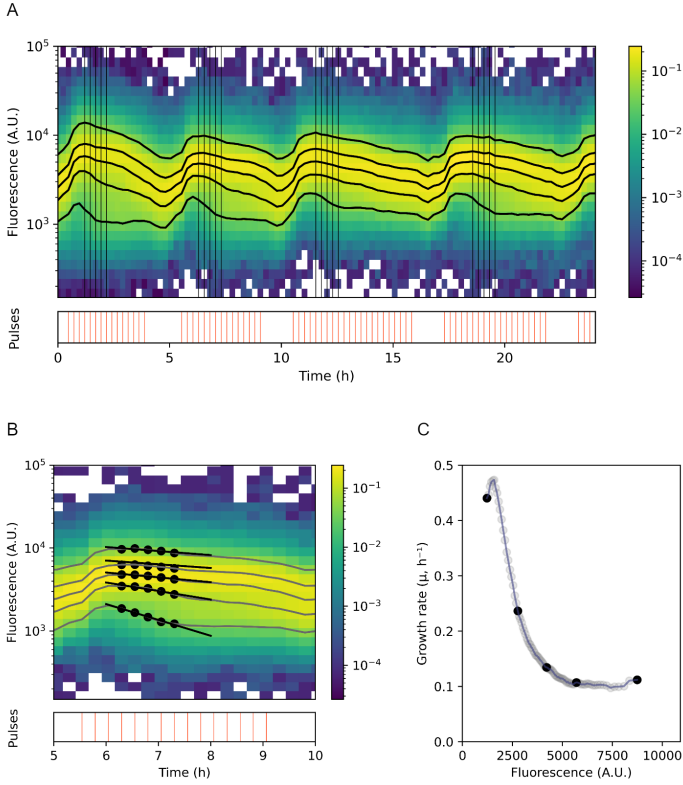


Figure A.8: A. The time scatter of a segregostat experiment for the yeast, showing the measured fluorescence over time, lighter colors indicate more cells at that time and fluorescence. The lines in the ‘pulses’ rectangle indicate when glucose was pulsed to reduce the stress levels. The black vertical lines on the scatter plot indicate the ample 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.

- **Cells that cease growth for reasons other than the studied circuit's logic** may generate a bias in our estimations. If a subset of cells were to stop growing for reasons unrelated to the induction (e.g., stochastic stress), they could accumulate fluorescence more rapidly during induction and/or fail to dilute it during relaxation, thereby undermining the assumptions of the method. This could bias the estimation of the fitness cost for high-fluorescence phenotypes, or simply disturb the relaxation phase and bias the percentile-based estimation. However, the consistency of our decay curves and the predictable shift of percentiles suggest that such non-specific growth arrest remained negligible in our experiments.
- **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 [278], 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 A.9). Rather, they follow a decaying exponential shifted up towards the auto-fluorescence ($Fluo = Fluo_0 \cdot e^{-\mu t} + Fluo_{auto}$). 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.

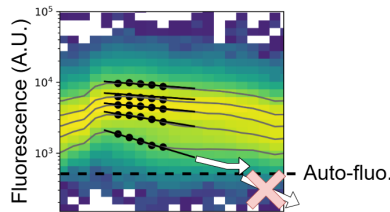


Figure A.9: When fitting a simple decaying exponential, we assume the fluorescence is decaying towards zero. However, the fluorescence measurement is a sum of fluorescence due to GFP and auto-fluorescence.

Figure A.9: 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%.

- **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 A.10). 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, then 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* 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, with dilution rates of 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.

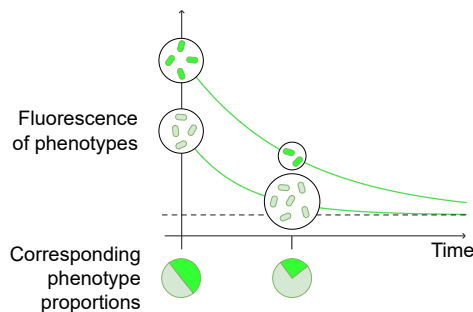


Figure A.10: As the fluorescence decreases, faster-growing phenotypes also increase in proportion in the population. Based on the estimated growth rates, this change in proportion is estimated, and taken into account to correct the growth rates.

- **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, and an increase in growth rate is often measured instead (as can be observed in Figure A.8 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 anyways 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 2. 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 growth rate/dilution rate’. 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 A.11). If the first are underestimated and the last are overestimated, then fitting the Hill function on all the points provides the best possible estimate. The adapted procedure to find a function fit is available in the file `Approx_mu_fFLUO_Seg_Ecoli_T7.py`.

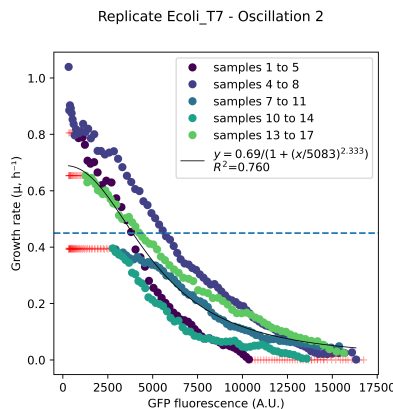


Figure A.11: One relaxation phase of the *T7* system in *E. coli* BL21. Each color corresponds to one set of samples. The fitted Hill function was fitted on not all the relaxation phases of the replicate, though only one is shown here. The earlier measurements are under-estimated, the later ones are 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 *ara* in *E. coli* are examples where the trade-off between growth and fluorescence should be small or inexistent. The results are shown in Figure A.12. Interestingly, for these two cases, correcting the final hill parameters by the dilution rate did not change the results much.

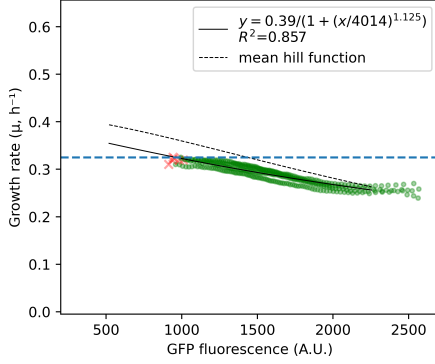
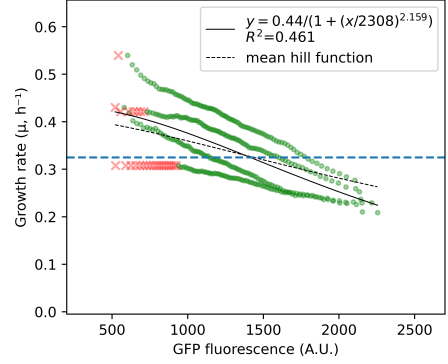
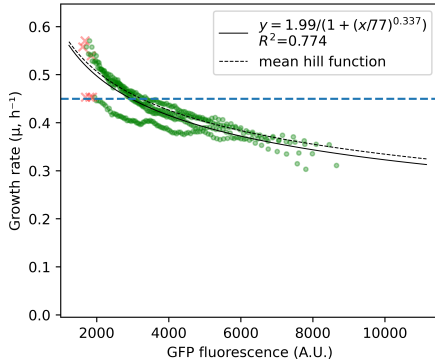
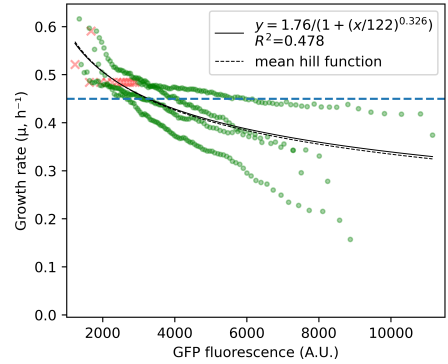
A. *ycdS*

 B. *ycdS* replicate

 C. *araB*

 D. *araB* replicate


Figure A.12: Growth rates measured for the *ycdS* 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 replicates then averaged (dashed line). The horizontal dotted line is the dilution rates of 0.325 h^{-1} for *ycdS* and 0.45^{-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 *ycdS* and 0.44 and 0.46^{-1} for *araB*. The scaling factor mentioned in the previous section is already applied in these figures.

For the *ycdS* system, the measurements in chemostat show that the fluorescence can be much higher than what is obtained at 0.325 h^{-1} when the dilution rate is lower (see Figure 3.6 A). Therefore, given the homogeneity of the population, an approximation of the trade-off function was obtained using chemostat data acquired

at various dilution rates (Figure A.13). The adapted procedure is available in the file `Approx_mu_fFLUO_varD_fitfct.py`. Only one replicate of chemostat at $D = 0.3 \text{ h}^{-1}$ have been performed in Bionet. Nonetheless, the values at $D = 0.3 \text{ h}^{-1}$ are close to the ones observed in segregostat. Furthermore, cultivations at the 3 considered growth rates have also been performed in Dasgip and provides similar results (Figure A.18). In addition, microfluidics measurements show only a slight reduction in growth rate with fluorescence. This is confirmed here, as we measure a growth rate that never gets below even 0.2 h^{-1} .

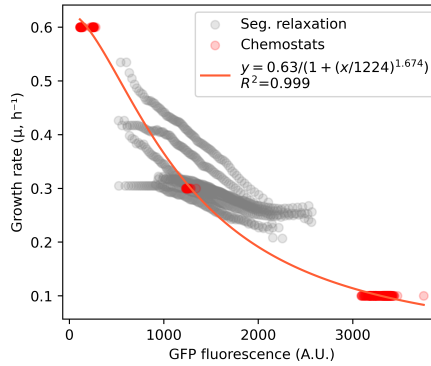


Figure A.13: Trade-off function of the *ydcS* system fitted on median fluorescence values obtained during chemostat cultivations at different dilution rates (see ‘`Approx_mu_fFLUO_varD_fitfct.py`’). The points obtained with the method exploiting fluorescence relaxation in segregostat are indicated for comparison.

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} [22]. Similarly to *ydcS*, we show no growth rate below 0.3 h^{-1} .

For both systems, even though there should be no trade-off, we still measure a difference in growth rate with fluorescence, although it is much smaller than the cases with trade-offs. This can be explained by two factors:

- On the one hand, while there might be no 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 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 no trade-off, we can consider the minimum growth rates obtained for these cases as the minima that need to be exceeded to ensure there is, actually, a trade-off between growth and gene expression. 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 A.14. 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 the chemostat experiments, we use the parameters of the first replicate as the trade-off curve in the main paper.

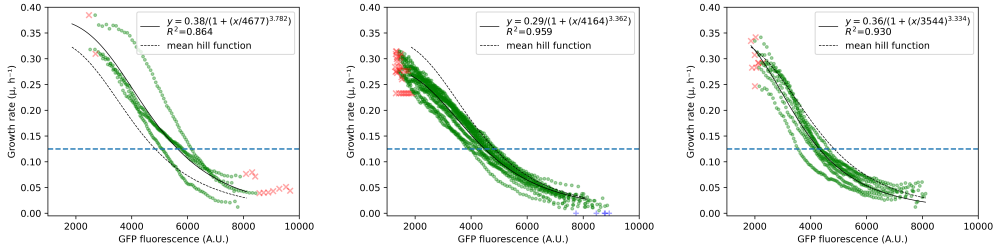


Figure A.14: Fitness determination results for the *glc3* system in *S. cerevisiae*. Each green point is a valid measurement of the growth rate as a function of fluorescence. Each blue '+' was measured as <0 and replaced by 0 for the correction of percentiles (subpopulation proportions) based on the 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.

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 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 (see file `Approx_mu_fFLU0_Seg_Ecoli_T7.py`). Nevertheless, we obtained a reasonable estimate of the trade-off curve, as shown in Figure A.11. 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. Supplementary Note 4. Characterization 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 characterized 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 ± 0.014 (std) h^{-1} , while the non-growing phenotype had a growth rate of 0.295 ± 0.010 (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 the main text, 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 the main paper (Figure 4), that of the negative control strain, which has no effect on growth, is presented below (Figure A.15).

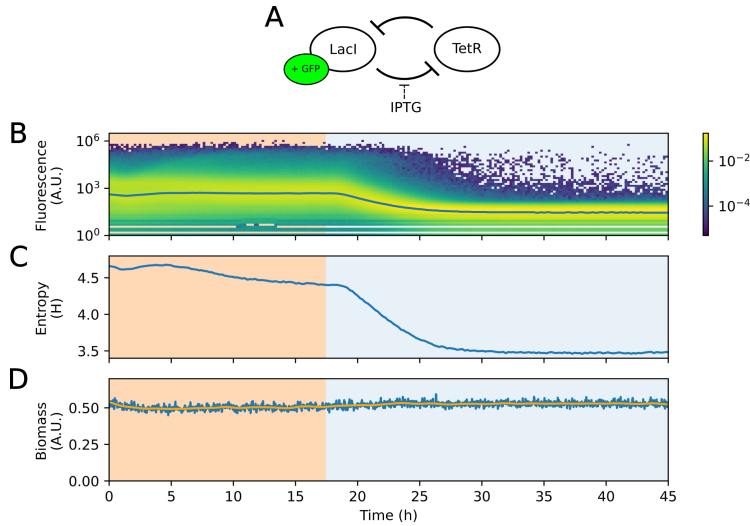


Figure A.15: Continuous cultivation of the negative control toggle switch (without serine auxotrophy), upon addition of IPTG in the feed (blue background), the production of GFP was inhibited. (A) Schematic representation of the toggle switch. (B) Single-cell fluorescence distributions over time, the 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.

4.1. Extended materials and methods

4.1.1. Characterization in Biolector platform

Pre-cultures in defined medium from glycerol stocks were first made without inducer. These pre-cultures were then used to inoculate flasks with either 150 ng/mL 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_{600} was then adjusted to inoculate a Biolector plate at an OD_{600} 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, as it had been found enough to induce the phenotype but below the minimal inhibitory concentration (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).

5. Supplementary Note 5. 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 [14], (ii) regulatory complexity (including σ^S dependence, co-regulation by other transcription factors, position in the regulatory network (regulator/regulated), (p)ppGpp dependency, etc.), (iii) sensitivity to RpoS concentrations [60], (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 A.16). Samples were taken during the exponential growth phase and late stationary phase for cytometry analysis and Shannon entropy computation (Figure A.17). 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 [62] (Figure A.18). From these experiments, we ultimately identified YdcS as the optimal reporter. YdcS is a putative transport protein associated with natural and chemical transformations [217] and carbon flux distribution [218]. Its transcription is specifically initiated by σ^S -containing RNA polymerase ($E\sigma^S$) [217]. Follow-up analysis using Microfluidic Single-Cell Cultivation (MSCC) confirmed that YdcS is a reliable reporter of the general stress response (section 6).

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 [4, 57, 214]. 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 [4]. These challenges necessitated the use of an alternative approach. An ideal reporter gene would be specifically regulated by $E\sigma^S$ and exhibit a simple regulatory system — criteria approached by *ycdS* based on current knowledge.

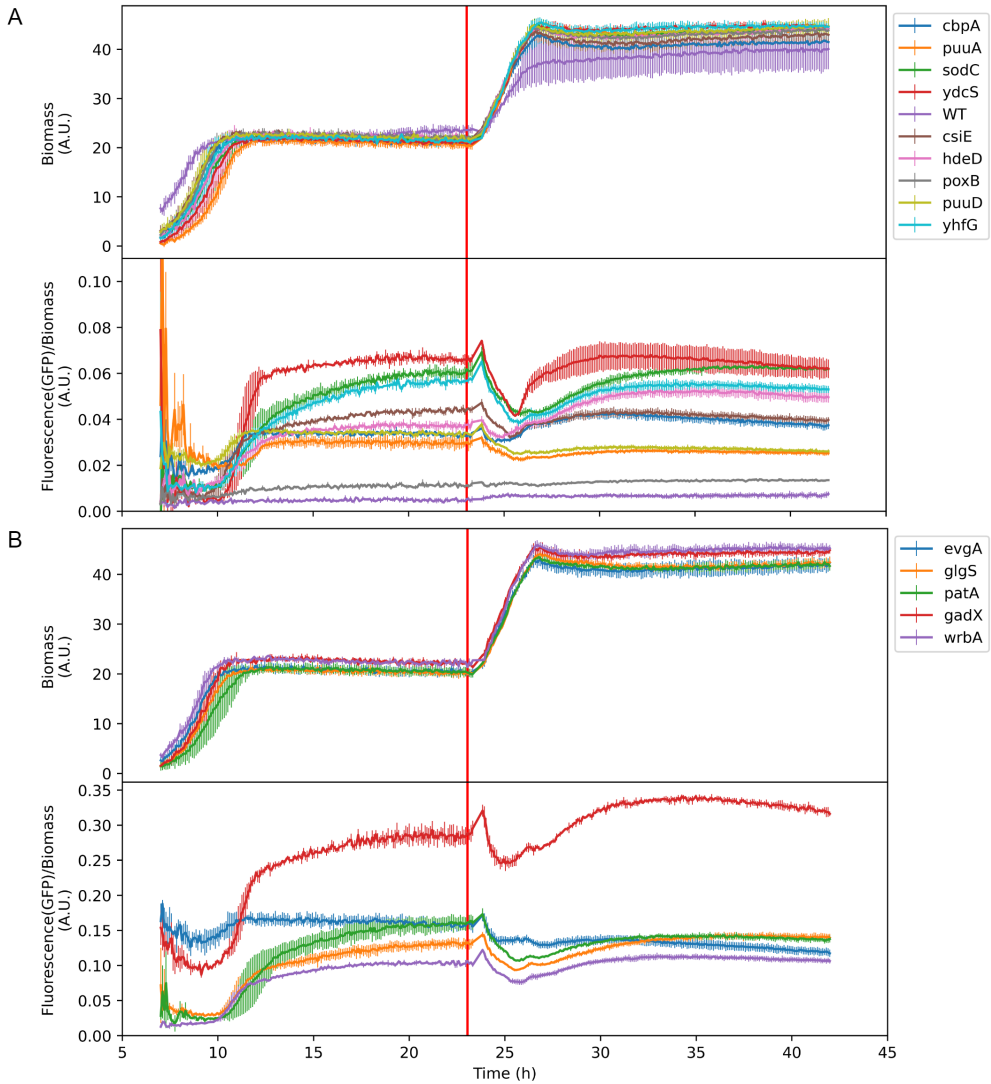


Figure A.16: Example of visualization of data from the Biolector screening for reporters with low to intermediate expression levels (A) and reporters with higher expression levels (B). The

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 monotonic 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.

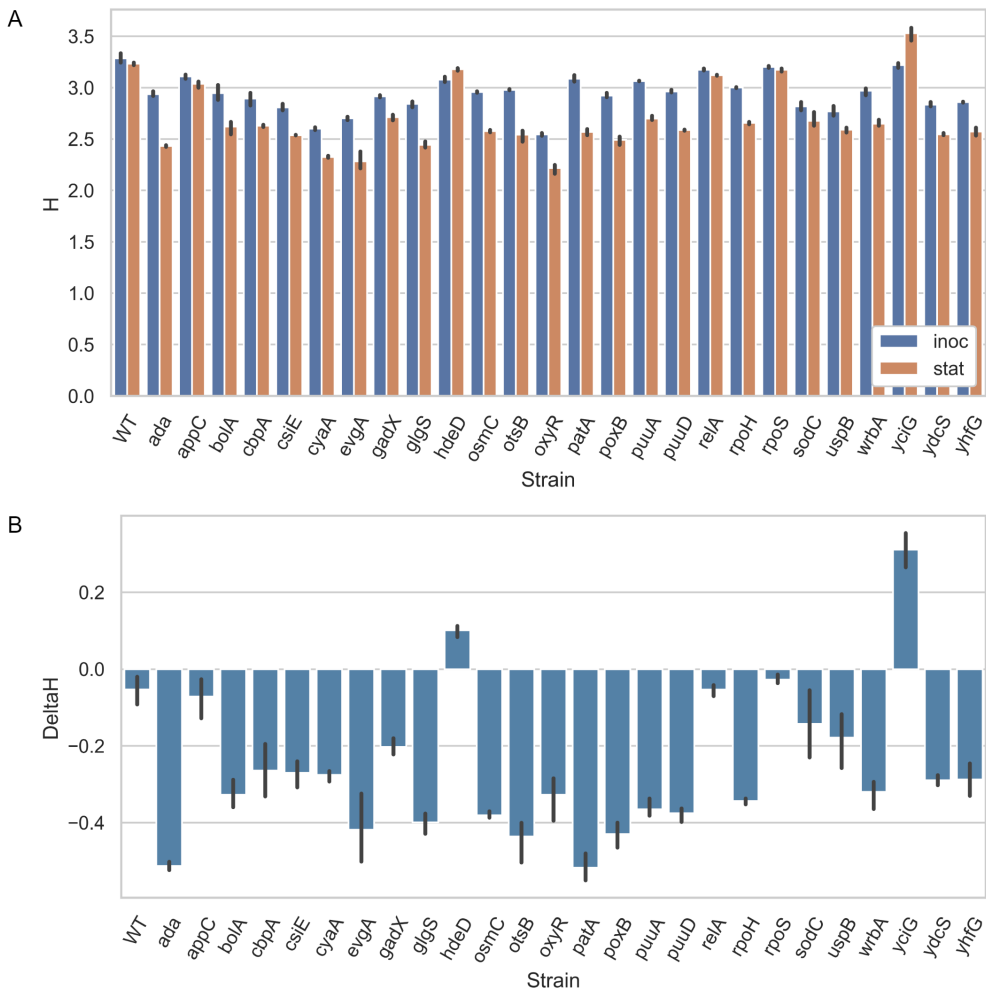


Figure A.17: Shannon entropies of different transcriptional reporters sampled in the Biolector platform during exponential growth and stationary phase ($n = 3$) (A), and the differences of entropies between these phases (B).

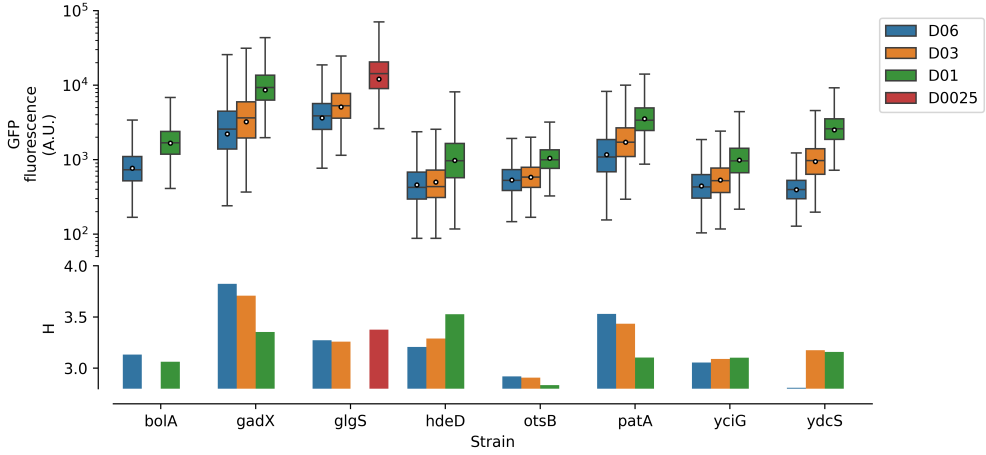


Figure A.18: Summary plot of continuous cultures at different dilution rates performed with a subset of transcriptional reporters. The top panel represents the distribution of fluorescence as boxplots, where each boxplot corresponds to a single cytometry file from the final measurement of each dilution rate (see Extended materials and methods). The mean is indicated as a white dot and whiskers are drawn to the farthest datapoint within $1.5 \times$ Interquartile range from the nearest hinge. The second panel represent the shannon entropies of corresponding samples.

To verify that *ydcS* is a good indirect reporter of $E\sigma S$ activity, we used a dual-reporter strain (*E. coli* MG1655 $P_{rpoS}::CyOFP1$ $P_{ydcS}::GFPmut2$). 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 Materials and Methods in the main manuscript). Using microfluidics, we demonstrated that *ydcS* (GFP) expression increases under stressful conditions and correlates well with *rpoS* (CyOFP) levels (Figure A.19). This finding confirms that *ydcS* is a reliable and convenient reporter for general stress response activation.

5.1. Extended materials and methods

5.1.1. Strains and plasmids

E. coli MG1655 strains from the Zaslaver collection [214] 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 analyzed 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 the main document.

5.1.2. Screening in Biolector platform

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 the main document, 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 pre-cultivated 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 flower-plates (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 correspondings wells of readable biolector flower-plate (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 analyzed 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.

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.

5.1.3. Screening in chemostat experiments at three different dilution rates

Cultivation in the Dasgip bioreactors: Precultures were performed in two steps, as described in the main document. 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".

Online cytometry analysis: For AFC analyses of 3 Dasgips reactors running in parallel, the “multi-stat” system was used. It is an adaptation of our in-house Segregostat [145] 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 phosphate-buffered saline (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 A.18 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.

6. Supplementary Note 6. 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 characterized using time-lapse microscopy in a Microfluidic Single Cell Cultivation (MSCC) device (Figures A.19-A.21).

As described in the main text, the trade-off between growth and gene expression of *S.cerevisiae* P_{glc3}::GFP was also monitored in this device (Figure A.22).

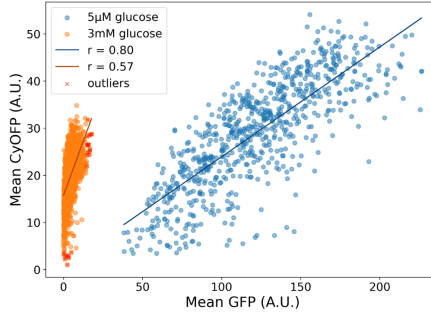


Figure A.19: Correlations between $P_{rpoS}::\text{CyOFP1}$ and $P_{ydcS}::\text{GFPmut2}$ expression in limiting (5 μM glucose, blue) and non-limiting (3 mM glucose, orange) conditions in a MSCC device. Each dot represents a single-cell (from a total of 5 cultivation chambers per glucose concentration condition). Outliers are datapoint with a $|z\text{-score}| > 3$.

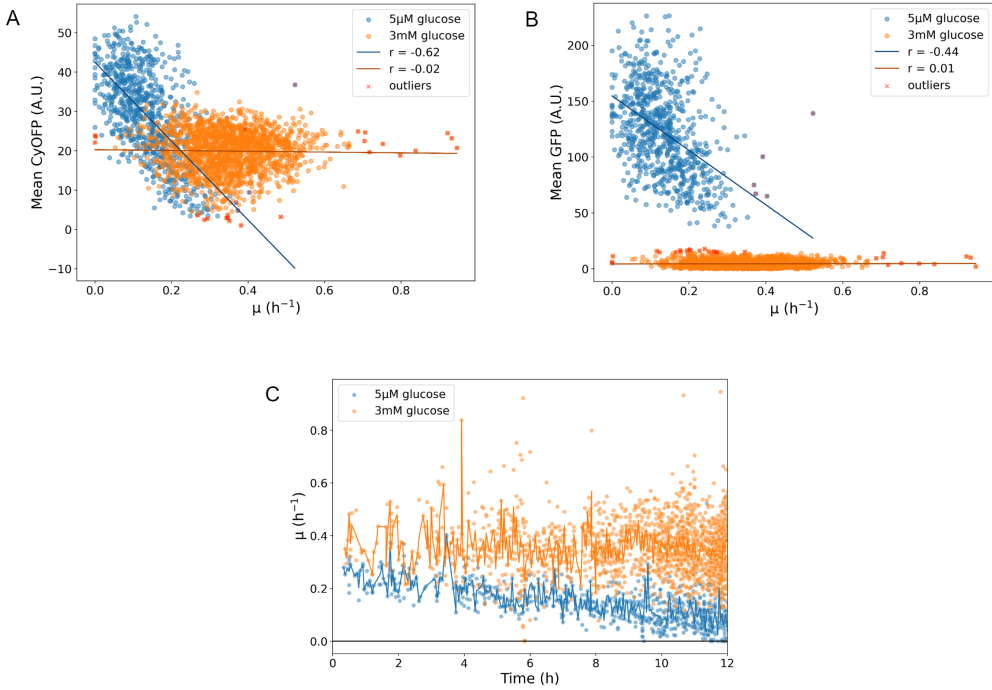


Figure A.20: Single-cell growth in limiting (5 μM glucose, blue) and non-limiting (3 mM glucose, orange) conditions in a MSCC device. Each dot represents a single-cell (from a total of 5 cultivation chambers per glucose concentration condition). Outliers are datapoint with a $|z\text{-score}| > 3$. (A) 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.

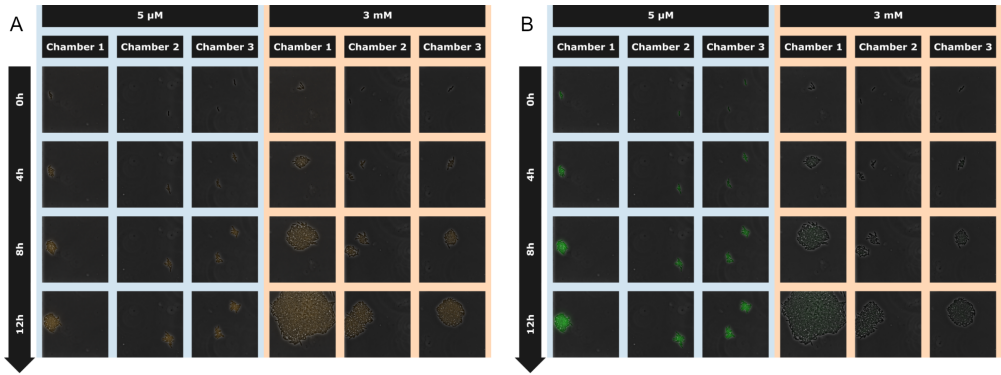


Figure A.21: Time-lapse microscopy pictures of *E. coli* $P_{rpoS}::\text{CyOFP1}$ $P_{ydcS}::\text{GFPmut2}$ cultivated in MSCC, three microfluidic chambers per condition. Stressful condition ($[\text{glu}] = 5 \mu\text{M}$) is indicated with blue background and non-stressful condition ($[\text{glu}] = 3 \text{ mM}$) is indicated with orange background. (A) CyOFP fluorescence. (B) GFP fluorescence.

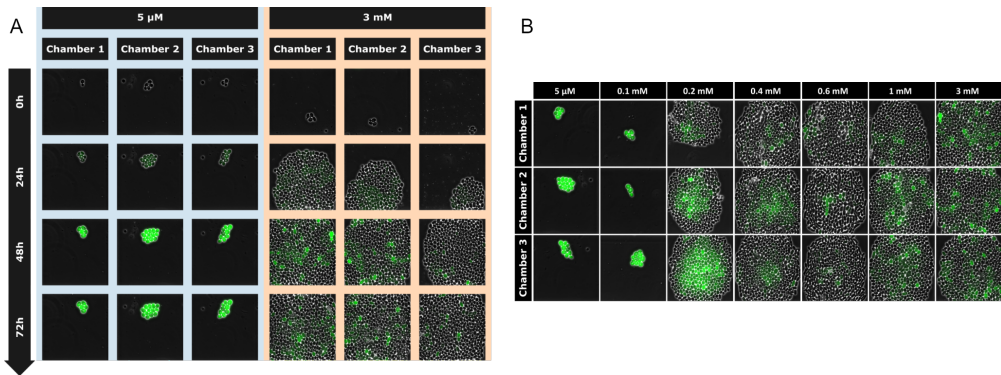


Figure A.22: Microscopy pictures of *S. cerevisiae* $P_{glc3}::\text{GFP}$ cultivated in MSCC, three microfluidic chambers per condition. (A) Time-lapse in stressfull condition ($[\text{glu}] = 5 \mu\text{M}$) indicated with blue background, and in non-stressful condition ($[\text{glu}] = 3 \text{ mM}$), indicated with orange background. (B) Microscopy pictures at different glucose 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. [249]).

7. Supplementary Note 7. Cultivations of *S. cerevisiae* $P_{glc3}::GFP$ at various dilution rates

Residual glucose concentrations at different dilution rates were obtained through changestat experiments [279]. 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.

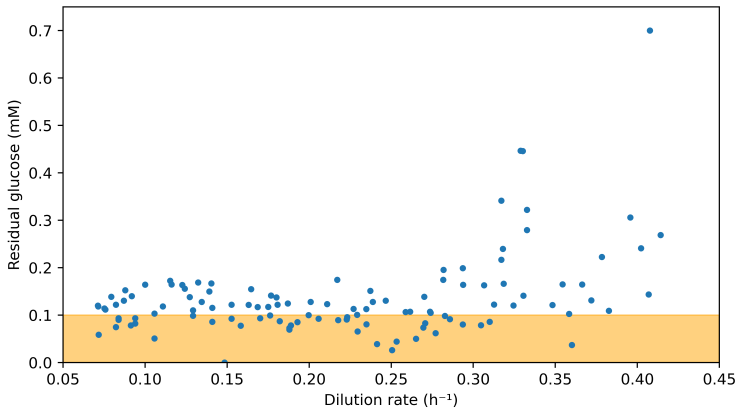


Figure A.23: Residual glucose concentrations at different dilution rates (data from Maximilian Sehart's work [279])

8. Supplementary Tables

8.1. Strains and plasmids

Table A.1: Primers used for the construction of the *E. coli* MG1655 P_{rpoS}::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} ::CyOFP1	Cassette construction	Fw Homo1	cgcagagcaactggataagcc
		Rv Homo1 + Tail-RBS	ctagtattctcctctttctctagattactcgcggaacagcgc
		Fw CyOFP1 + Tail-RBS	tctagagaaagaggagaaatactagatggtgagcaagggcgagg
		Rv CyOFP1	ttacttgtacagctcgccatgcc
		Fw Homo2 + tail-CyOFP1	ggacgagctgtacaagtaagtaagcatctgtcagaaagg
		Rv Homo2	agccgcatttattttcactgg
	Construction of pTarget	Fw pTarget-rpoS	{ agctagctcagtcctaggataataactagt GACAGATGCTTACTTACTCG gttttagagctagaaatagcaagttaaaa
		Rv pTarget	actagtattatacctaggactgagctagctg
		Fw verif_pTarget-rpoS	gacagatgcttacttactcg
		Rv verif_pTarget	ttgccgactaccttggtgat
		Fw verif_prpoS-CyOFP1	tggttactggtgctggagg
	Verification /Sequencing	Rv verif_prpoS-CyOFP1	ccgtactattcgtttgccg

Table A.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	cgggtattgtgggcatggt
		Rv Homo1_lacAYZI	gagtcattcagggtggtgaatattataaaaattgcctgatacgt-gcg
		Fw Homo2_lacAYZI	attcaccacctgaattgactc
		Rv Homo2_lacAYZI	ggtgatgactatcaactggcacgg
	Construction of pTarget	Fw pTarget-lacI	$\left\{ \begin{array}{l} \text{agctagctcagtcctaggataataactagt} \\ \text{AACGGTCTGATAAGAGACAC} \\ \text{gttttagagctagaaatagcaagttaaaa} \end{array} \right.$
		Rv pTarget	actagtattatacctaggactgagctagctg
		Fw verif_pTarget-lacI	AACGGTCTGATAAGAGACAC
		Rv verif_pTarget	ttgccgactaccttggtgat
	Verification /Sequencing	Fw verif_lacAYZI	tatggctcgccatcaggatcgg
		Rv verif_lacAYZI	ttatcctttcaccgggcaatggt
<i>E. coli</i> MG1655 Δ lacAYZ Δ lacI Δ serA	Cassette construction	Fw Homo1_serA	gtggtgaccgataatggcaactgat
		Rv Homo1_serA	ttaccaatcctgtcttttgaaatgttgtg
		Fw Homo2_serA	gacaggattgggtaattcccttctctgaaaaatcaacgggc
		Rv Homo2_serA	ctgcggtggttacaccgtcgaa
	Construction of pTarget	Fw pTarget-serA	$\left\{ \begin{array}{l} \text{agctagctcagtcctaggataataactagt} \\ \text{AGACAAGATTAAGTTTCTGC} \\ \text{gttttagagctagaaatagcaagttaaaa} \end{array} \right.$
		Rv pTarget	actagtattatacctaggactgagctagctg
		Fw verif_pTarget-serA	agacaagattaagtttctgc
		Rv verif_pTarget	ttgccgactaccttggtgat
	Verification /Sequencing	Fw verif_serA	gtattgcagacgctccaagcagg
		Rv verif_serA	ccgattagacgtgctgatcacacc

8.2. Lab-scale bioreactors cultivations

Table A.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 <i>P_{araB}::GFPmut2</i>	Bioreactor : Biostat B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) D = 0.45 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation = 1000 rpm Regulation threshold = 1000 A.F.U. [glucose] _{FEED} = 5 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 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation = 1000 rpm Regulation threshold = 1000 A.F.U. [glucose] _{FEED} = 5 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 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation = 1000 rpm [glucose] _{FEED} = 5 g/L [lactose] _{FEED} = 1 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 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation = 1000 rpm [glucose] _{FEED} = 5 g/L [lactose] _{FEED} = 1 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 <i>E. coli</i> BL21(DE3) pET28::GFP	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.45 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation = 1000 rpm [xylose] _{FEED} = 5 g/L [lactose] _{FEED} = 1 g/L	2025-05-05_ALS_Chem_Ecoli_BL21_xylose 2025-05-28_FDE_Chem_Ecoli_BL21_xylose	This paper

Table A.3: *Continued from previous page*

Culture type and strain	Operating conditions	Replicates	Publication
Chemostat <i>E. coli</i> MG1655 Δ lacAYZ Δ lacI pECJ3	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.5 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation = 1000 rpm	2024-02-14_VIV_ToggleControl1 2024-05-09_VIV_ToggleControl2	This paper
Chemostat <i>E. coli</i> MG1655 Δ lacAYZ Δ lacI Δ serA pECJ3 (PLtetO-1::serA::GFP)	[glucose] _{FEED} = 5 g/L 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 _{ydcS} ::GFPmut2	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.3 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation : 1000 & 1200 rpm [glucose] _{FEED} = 5 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 _{glc3} ::eGFP	Bioreactor : Biostat B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) D = 0.1 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 5 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 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 5 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

Table A.3: *Continued from previous page*

Culture type and strain	Operating conditions	Replicates	Publication
Chemostat <i>E. coli</i> MG1655 $P_{ydcS}::GFPmut2$	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.6 & 0.1 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation = 1000 rpm [glucose] _{FEED} = 5 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 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation : 1000 [glucose] _{FEED} = 5 g/L	2025-01-29_MaD_Chem_Ecoli_ydcS	This paper
	Bioreactor : Dasgip (Eppendorf, Germany) Cytometer : BD Accuri C6 (BD Biosciences) D = 0.6, 0.3 & 0.1 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation : 1000 [glucose] _{FEED} = 5 g/L	See the "Supp_EcoliStressReporterSelection" folder in provided data	This paper
Chemostat <i>S. cerevisiae</i> CEN.PK 113-7D $P_{glc3}::eGFP$	Bioreactor : Bionet F1 bioreactor (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.3 & 0.1 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 7.5 g/L	2024-07-04_MaD_Chem_Sacch 2024-08-19_MaD_Chem_Sacch	This paper

B

Supplementary materials for the Chapter 4

This appendix is an adapted version of the Supplementary Material of the article referenced below, which corresponds to the chapter 4 of this thesis.

Delvenne, M., Martinez, J. A., Haringa, C., Noorman, H., Minden, S., Takors, R., and Delvigne, F. “Overriding Bioprocess Perturbations With a Cell–Machine Interface for Reliable Microbial Stress-Response Control”. In: *Microbial Biotechnology* 19.4 (2026). doi: 10.1111/1751-7915.70329

1. Supplementary Note 1. Distribution of stress response activation in *S. cerevisiae* at different dilution rates

In changestat 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 [279]. This strategy was chosen to ensure a quasi steady state at each step.

Chemostat cultures of *S. cerevisiae* were performed at $D = 0.1 \text{ h}^{-1}$. However, with the addition of pulses in Segregostat, the dilution rate was slightly increased. We therefore compared the entropy values of Segregostat cultures with those of changestat cultures at similar dilution rates (Figure B.1).

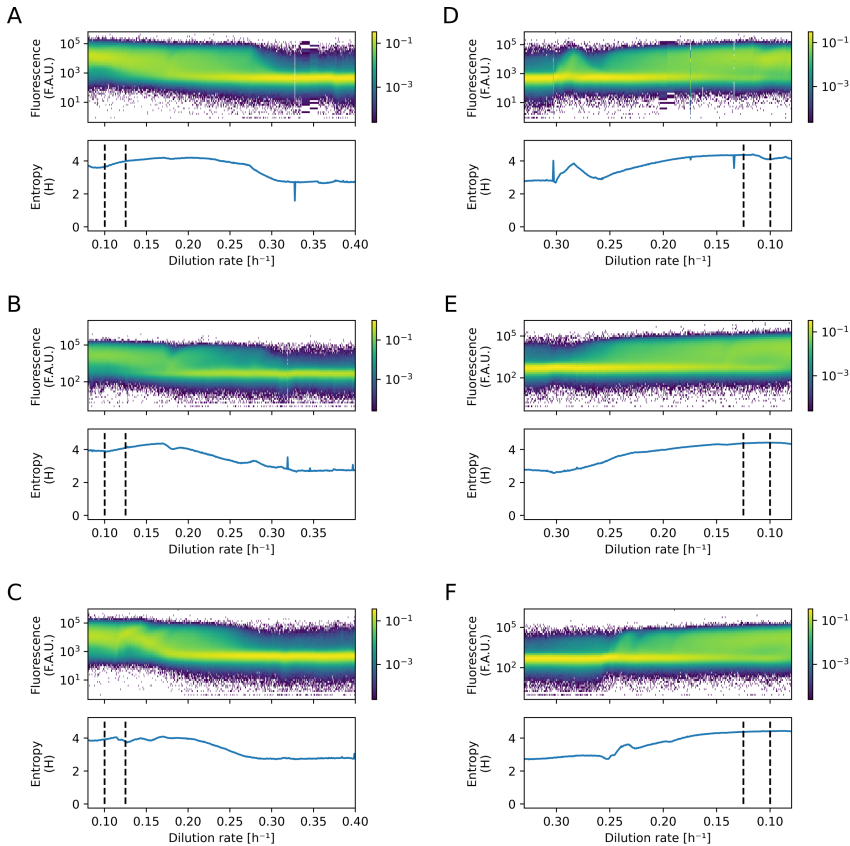


Figure B.1: Scatter plots of single-cell fluorescence of *S. cerevisiae* as a function of dilution rate and the corresponding entropy values, for accelerostats (A-C) and decelerostats (D-F) in triplicates. Black dotted vertical lines indicate the minimum and maximum dilution rates applied in Segregostat cultures of *S. cerevisiae*. Data from Maximilian Sehrt's work [279].

2. Supplementary Note 2. Impact of pulsed glucose feeding on the population heterogeneity

When glucose was supplied in pulses rather than continuously, lower pulse frequencies resulted in a more homogeneous population (Figure B.2).

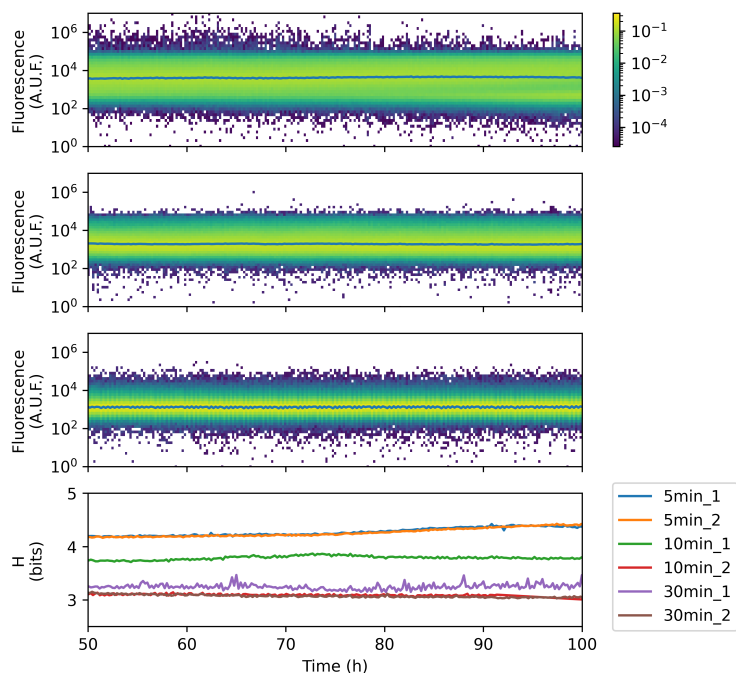


Figure B.2: Population heterogeneity of *S. cerevisiae* under pulsed glucose feeding at different frequencies. Single-cell fluorescence distributions are shown for pulsed feeding every 5 min (top), 10 min (middle), and 30 min (bottom). The lower panel shows the corresponding Shannon entropy values (H) over time for two biological replicates per condition. Lower pulse frequencies (e.g. 30 min) result in reduced entropy values, indicating more homogeneous populations.

2.1. Extended materials and methods

Continuous cultures with pulsed glucose feeding were performed in a Biostat B-Twin bioreactor (Sartorius, Göttingen, Germany), in the same way as the chemostat cultures at $D = 0.1 \text{ h}^{-1}$ (see Materials and Methods of the main document), except for the feeding strategy. In this case, medium without a carbon source was continuously supplied, while glucose was provided in pulses (from a 20% solution, delivered every 5, 10, or 30 min). The total amount of glucose supplied over time was the same as in the chemostat cultivation at $D = 0.1 \text{ h}^{-1}$.

3. Supplementary Note 3. Basal entropy values for all experiments and their replicates

Basal entropy values for all replicates are shown in Figure B.3 for *E. coli* and in Figure B.4 for *S. cerevisiae*.

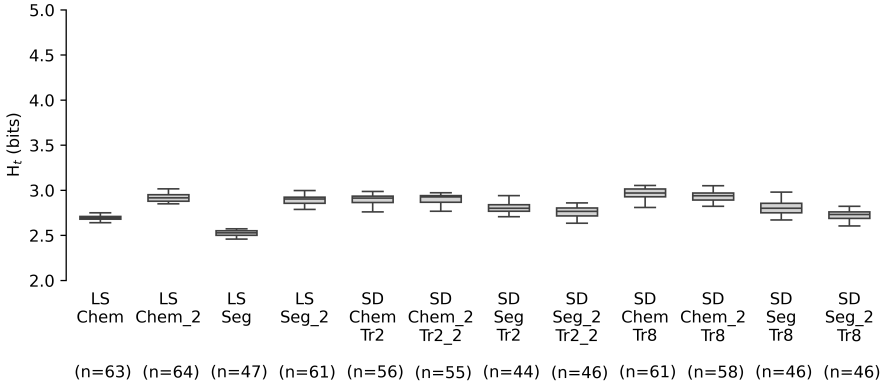


Figure B.3: Basal entropy of *E. coli*, calculated from the fluorescence distribution of the population over time, represented as boxplots, for different culture conditions. For all conditions shown, data from the last 12 hours of the condition were used, corresponding to (quasi) steady state under chemostat operation. Sample sizes (n) for each boxplot are indicated below the respective labels.

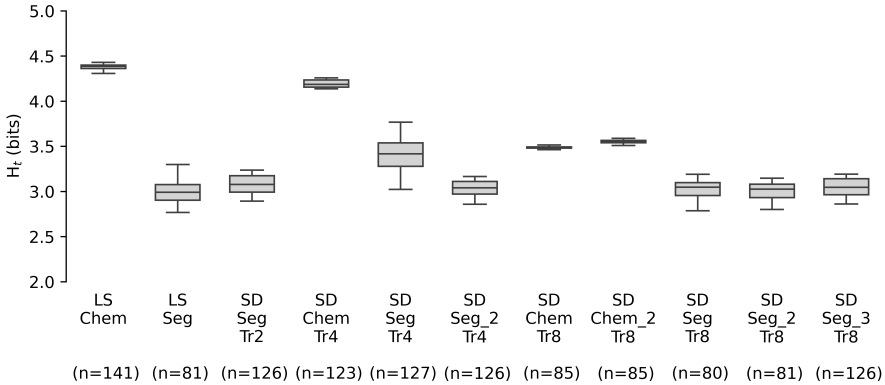


Figure B.4: Basal entropy of *S. cerevisiae*, calculated from the fluorescence distribution of the population over time, represented as boxplots, for different culture conditions. For all conditions shown, data from the last 20 hours of the condition were used, corresponding to (quasi) steady state under chemostat operation. Sample sizes (n) for each boxplot are indicated below the respective labels.

4. Supplementary Note 4. Examples of monitored reactor parameters indicating overflow metabolism

Differences in pH and dissolved oxygen between chemostat and segregostat cultures indicate overflow metabolism in the latter cultivation mode. Furthermore, in experiments where pH control was achieved solely through base addition (without acid supplementation), the base addition rate reflects the acidification of the medium. These variables are shown in Figure B.5, and approximated base addition rates are presented in Figure B.6.

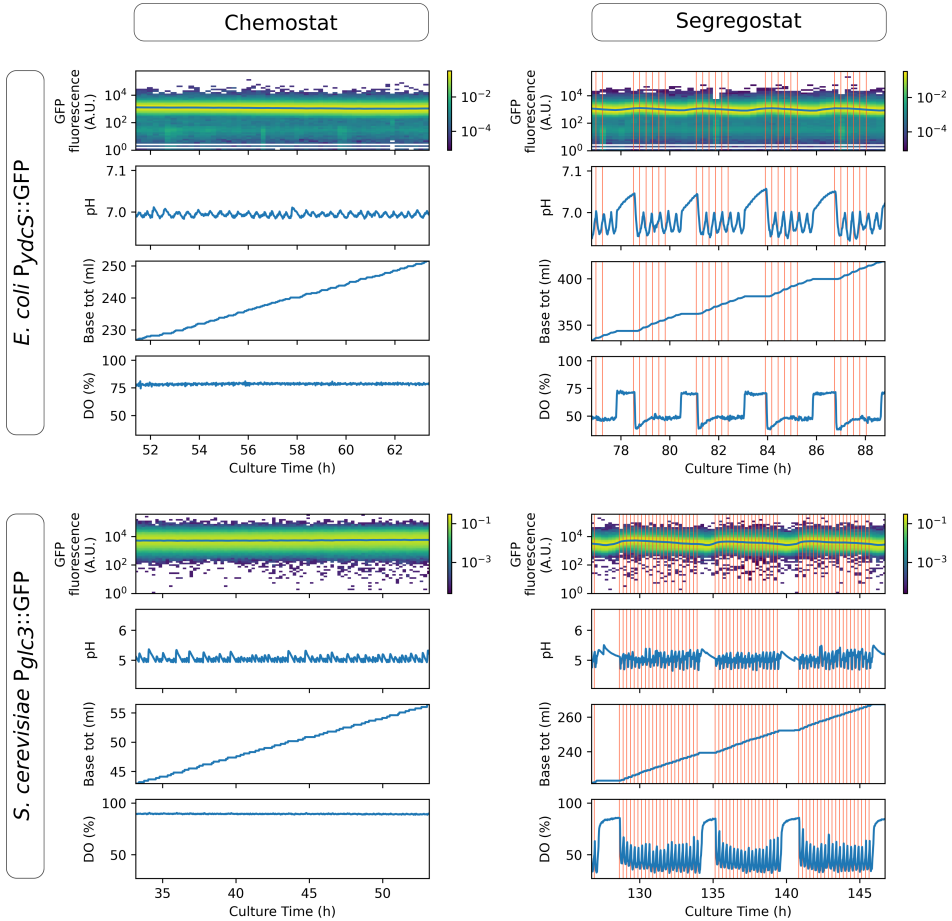


Figure B.5: Examples of monitored reactor parameters indicating overflow metabolism in representative cultures of *E. coli* and *S. cerevisiae* under chemostat and Segregostat scale-down ($Tr_{Loop} = 8$ min) conditions. For each culture, single-cell GFP fluorescence distributions, pH profiles, total base addition, and dissolved oxygen (DO) are shown over time. Red vertical lines in segregostat experiments indicate the timing of glucose pulses.

Figure B.5: Segregostat cultures exhibit higher base addition rates, stronger pH fluctuations, and marked oscillations in DO compared to chemostats, reflecting overflow metabolism induced by glucose pulses. For instance, in *S. cerevisiae* Segregostat cultures, the dissolved oxygen (DO) level does not return to its maximal value after a glucose pulse, suggesting that the following pulse occurs before carbon source availability becomes limiting. Even after the final pulse of a series, DO requires a delay before stabilizing, which suggests a subsequent re-consumption of ethanol.

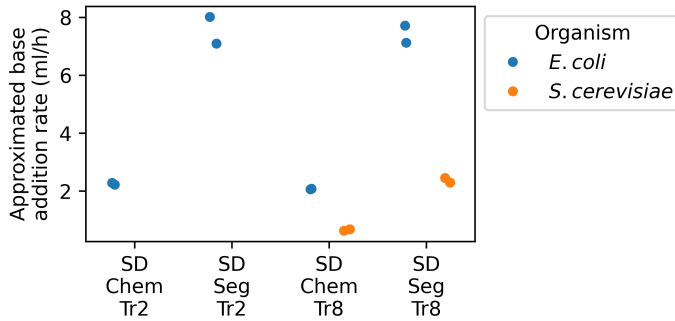


Figure B.6: Approximated base addition rates in different culture conditions. Scatter plot of the average base addition rates (ml/h) in cultures of *E. coli* (blue) and *S. cerevisiae* (orange) under scale-down (SD) chemostat and Segregostat experiments with different retention times in the recirculation loop (2 and 8 min). Each point corresponds to an individual replicate.

4.1. Extended materials and methods

The base addition rate was approximated from the difference in the cumulative base added over a fixed time window taken at the end of each culture. For *E. coli*, a 12 h window was used, whereas for *S. cerevisiae* a 20 h window was considered. This approximation is only valid for experiments in which pH control was performed exclusively through base addition, and not through combined acid and base titration.

5. Supplementary Note 5. Absence of oxygen limitation in the recirculation loop

The recirculation loop consisted of silicone tubing, which is permeable to oxygen. In addition, small air bubbles were entrained into the loop together with the culture broth. Oxygen limitation was therefore not expected. This assumption was verified by measuring dissolved oxygen concentrations at different positions along the recirculation loop. An example of these measurements is shown in Figure B.7.

It is worth noting that small temperature variations may have occurred in the recirculation loop, since this part of the system was not temperature-controlled; temperature regulation was applied only in the stirred tank.

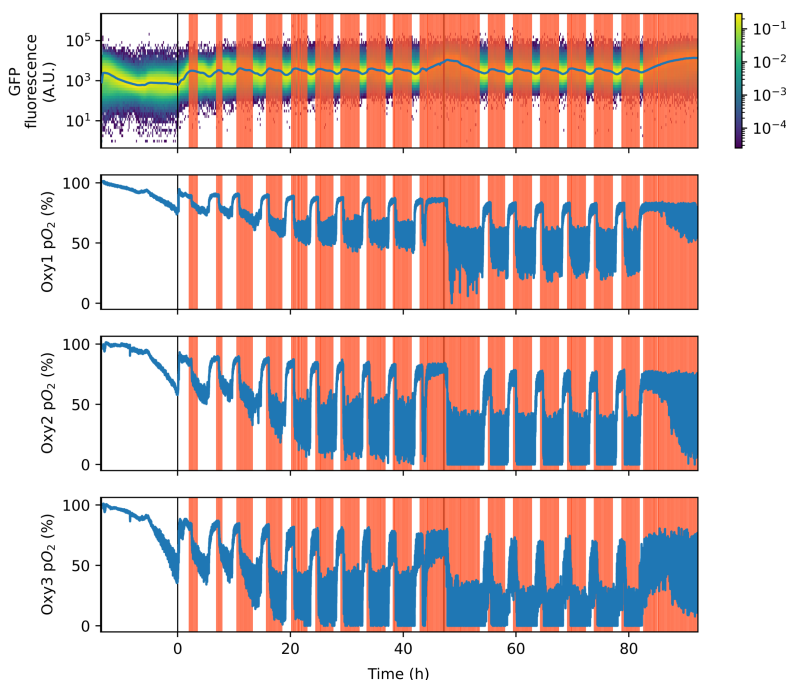


Figure B.7: Dissolved oxygen dynamics in the recirculation loop during experiment “2021-06-30_FDE_SD_Seg_Sacch_Tr4-8min.” The top panel shows single-cell GFP fluorescence distributions over time with the median indicated in blue. The three lower panels show dissolved oxygen concentrations (pO_2 , %) measured by three probes positioned along the recirculation loop (Oxy1: 1.25 m, Oxy2: 5 m, both upstream of the feeding point; Oxy3: 8.75 m downstream of the feeding point). The experiment was performed in segregostat mode with a residence time (Tr) of 4 min followed by 8 min. Transitions between conditions (batch phase, segregostat $Tr_{Loop} = 4$ min, segregostat $Tr_{Loop} = 8$ min) are indicated by vertical black lines. The final series of pulses in the $Tr_{Loop} = 4$ min condition does not correspond to true pulses due to depletion of the glucose pulse bottle.

5.1. Extended materials and methods

Measurements of dissolved oxygen in the recirculation loop were performed during scale-down experiments in B-Twin bioreactors (Sartorius) (see subsection 7.1. Bioreactors cultivations). Oxygen was monitored using OXY-4 mini probes (PreSens Precision Sensing GmbH, Regensburg, Germany). Two probes were positioned in the starvation zone (at 1.25 m and 5 m from the beginning of the recirculation loop), and one probe was positioned in the excess zone, downstream of the feeding point (at 8.75 m).

6. Supplementary Note 6. Computational fluid dynamics (CFD) simulation of industrial aerated fermentation of *S. cerevisiae*

Examples of cell lifelines from computational fluid dynamics (CFD) simulation of a 22m³ aerated fermentation of *S. cerevisiae* are displayed in Figure B.8.

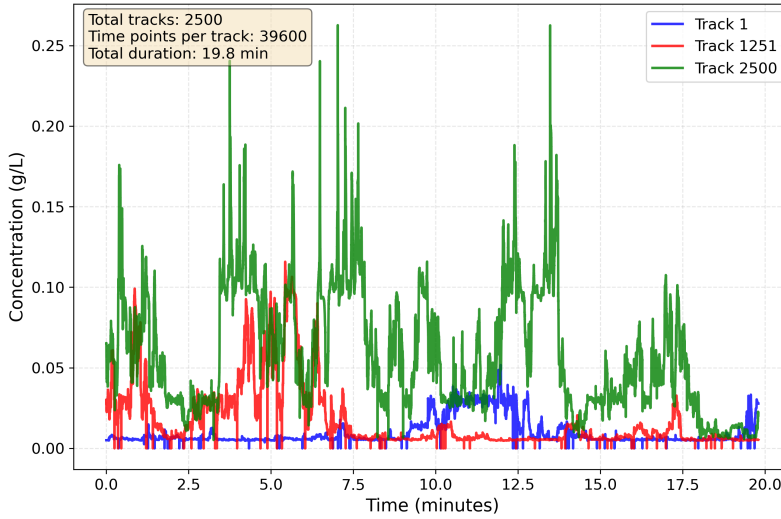


Figure B.8: Representative examples of three cell lifelines randomly sampled from the CFD simulation, shown as perceived glucose concentration over time. The 0.03 s timestep ensures proper resolution. Data from Haringa et al. [93].

7. Supplementary Tables

7.1. Bioreactors cultivations

Table B.1: Summary of specific operating conditions of the continuous cultures performed in bioreactors.

Culture type and strain	Operating conditions	Replicates	Publication
Chemostat (LS) <i>E. coli</i> MG1655 $P_{ydcS}::GFPmut2$	Bioreactor : Bionet F1 (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.3 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation : 1200 rpm [glucose] _{FEED} = 5 g/L	2025-01-13_MaD_Chem_Ecoli_ydcS 2025-01-29_MaD_Chem_Ecoli_ydcS	This paper Delvenne et al. (2025)
Segregostat (LS) <i>E. coli</i> MG1655 $P_{ydcS}::GFPmut2$	Bioreactor : Bionet F1 (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.3 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation : 1000 & 1200 rpm [glucose] _{FEED} = 5 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	Delvenne et al. (2025)
Chemostat (LS) <i>S. cerevisiae</i> CEN.PK 113-7D $P_{glc3}::cGFP$	Bioreactor : Bionet F1 (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.1 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 5 g/L	2025-02-10_MaD_Chem_Sacch Replicates*	This paper *

* Replicates not provided in this work. They can be found in Henrion et al. (2023) and in Delvenne et al. (2025).

Table B.1: *Continued from previous page*

Culture type and strain	Operating conditions	Replicates	Publication
Segregostat (LS) <i>S. cerevisiae</i> CEN.PK 113-7D $P_{glc3}::eGFP$	Bioreactor : Bionet F1 (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.1 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 5 g/L Regulation threshold = 3000 A.F.U. Regulation pulse = 0.4 g glucose (5.7 ml)	2025-02-10_MaD_Seg_Sacch Replicates*	Delvenne et al. (2025) *
Chemostat (SD - Tr = 2min) Segregostat (SD - Tr = 2min) Chemostat (SD - Tr = 8min) Segregostat (SD - Tr = 8min) <i>E. coli</i> MG1655 $P_{ydcS}::GFPmut2$	Bioreactor : Bionet F1 (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.3 h ⁻¹ pH = 7 T = 37 °C Aeration = 1 L/min Agitation : 1200 rpm [glucose] _{FEED} = 5 g/L Tresidence in the loop = 2 min and 8 min Regulation threshold (Seg.) = 1000 A.F.U. Regulation pulse (Seg.) = 1 g glucose (5.7 ml)	2025-01-06_MaD_SD_ChemSeg_Ecoli_Tr2-Tr8min 2025-01-13_MaD_SD_ChemSeg_Ecoli_Tr2-Tr8min	This paper

* Replicates not provided in this work. They can be found in Henrion et al. (2023) and in Delvenne et al. (2025).

Table B.1: *Continued from previous page*

Culture type and strain	Operating conditions	Replicates	Publication
Chemostat (SD - Tr = 8min) Segregostat (SD - Tr = 8min) <i>S. cerevisiae</i> CEN.PK 113-7D $P_{glc3}::eGFP$	Bioreactor : Bionet F1 (Bionet) Cytometer : BD Accuri C6+ (BD Biosciences) D = 0.1 h ⁻¹ (+Chem.) 0.3 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 5 g/L Tresidence in the loop = 8 min Regulation threshold (Seg.) = 3000 A.F.U. Regulation pulse (Seg.) = 0.4 g glucose (5.7 ml)	2025-02-19_MaD_SD_ChemSeg_Sacch_Tr8min 2025-03-03_MaD_SD_ChemSeg_Sacch_Tr8min	This paper
	Bioreactor : B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) D = 0.1 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 5 g/L Tresidence in the loop = 8 min Regulation threshold (Seg.) = 5000 A.F.U. Regulation pulse (Seg.) = 0.4 g glucose	2021-06-30_FDE_SD_Seg_Sacch_Tr4-8min	
Segregostat (SD - Tr = 2min) <i>S. cerevisiae</i> CEN.PK 113-7D $P_{glc3}::eGFP$	Bioreactor : B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) D = 0.1 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 5 g/L Tresidence in the loop = 2 min Regulation threshold (Seg.) = 5000 A.F.U. Regulation pulse (Seg.) = 0.4 g glucose	2021-06-20_FDE_SD_Seg_Sacch_Tr2min **	This paper

** No replicate available

Table B.1: *Continued from previous page*

Culture type and strain	Operating conditions	Replicates	Publication
Chemostat (SD - Tr = 4min) <i>S. cerevisiae</i> CEN.PK 113-7D $P_{glc3}::eGFP$	Bioreactor : B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) D = 0.1 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 5 g/L Tresidence in the loop = 4 min	2021-07-15_FDE_SD_Seg_Sacch_Tr4min **	This paper
Segregostat (SD - Tr = 4min) <i>S. cerevisiae</i> CEN.PK 113-7D $P_{glc3}::eGFP$	Bioreactor : B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) D = 0.1 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 5 g/L Tresidence in the loop = 4 min Regulation threshold (Seg.) = 5000 A.F.U. Regulation pulse (Seg.) = 0.4 g glucose	2021-06-30_FDE_SD_Seg_Sacch_Tr4-8min 2021-07-15_FDE_SD_Seg_Sacch_Tr4min	This paper
Chemostat (LS) with pulsed feed <i>S. cerevisiae</i> CEN.PK 113-7D $P_{glc3}::eGFP$	Bioreactor : B-Twin (Sartorius) Cytometer : BD Accuri C6 (BD Biosciences) D = 0.1 h ⁻¹ pH = 5 T = 30°C Aeration = 1 L/min Agitation : 1000 rpm [glucose] _{FEED} = 0 g/L Pulse glucose : every 5, 10 or 30 min, from a 20% solution. The total amount of glucose supplied over time was the same as in the chemostat cultivation at D = 0.1 h ⁻¹ .	2019-07-16_BoZ_Pulsed_Sacch_5min 2019-08-13_BoZ_Pulsed_Sacch_5min 2019-07-09_BoZ_Pulsed_Sacch_10min 2020-02-05_BoZ_Pulsed_Sacch_10min 2019-07-29_BoZ_Pulsed_Sacch_30min 2020-02-12_BoZ_Pulsed_Sacch_30min	This paper

** No replicate available

Table B.2: Chronological sequences of operating phases in multi-condition experiments.

Experiment	Conditions sequences
2025-01-13_MaD_Chem_Ecoli	Chem. (t = 97:46 , d = 25:49) (following 2025-01-13_MaD_SD_ChemSeg_Ecoli_Tr2-Tr8min)
2025-01-29_MaD_ChemSeg_Ecoli	Batch (t = 0 , d = 5:48) ; Chem. (t = 5:48 , d = 21:06) ; Seg. (t = 45:52 , d = 24:43)
2025-02-10_MaD_ChemSeg_Sacch	Batch (t = 0:0, d = 18:54); Chem. D=0.3 (t = 18:54, d = 27:25); Chem. D=0.1 (t = 46:19, d = 74:38); Seg. D=0.1 (t = 160:09, d = 29:1)
2025-01-06_MaD_SD_ChemSeg_Ecoli_Tr2-Tr8min	Batch (t = -2:35, d = 6:10) ; Chem. Tr=2 (t = 3:35, d = 17:19) ; Seg. Tr=2 (t = 20:54, d = 27:21) ; Chem. Tr=8 (t = 48:15, d = 19:23) ; Seg. Tr=8 (t = 67:38, d = 23:37)
2025-01-13_MaD_SD_ChemSeg_Ecoli_Tr2-Tr8min	Batch (t = 0, d = 5:27) ; Chem. Tr=2 (t = 5:27, d = 18:20) ; Seg. Tr=2 (t = 23:47 , d = 25:40) ; Chem. Tr=8 (t = 49:27, d = 19:29) ; Seg. Tr=8 (t = 68:56 , d = 28:50); + LS: 2025-01-13_MaD_Chem_Ecoli
2025-02-19_MaD_SD_ChemSeg_Sacch_Tr8min	Batch (t = 0, d = 18:5); Chem. D=0.1 Tr=8 (t = 18:5, d = 52:27); Chem. D=0.3 Tr=8 (t = 70:32, d = 19:34); Seg. D=0.1 Tr=8 (t = 90:06, d = 76:15)
2025-03-03_MaD_SD_ChemSeg_Sacch_Tr8min	Batch (t = 0, d = 18:32); Chem. D=0.1 Tr=8 (t = 18:32, d = 53:11); Chem. D=0.3 Tr=8 (t = 71:43, d = 22:05); Seg. D=0.1 Tr=8 (t = 93:48, d = 71:53)
2021-06-20_FDE_SD_Seg_Sacch_Tr2min	Batch (t = 0, d = 14:30); Seg. D=0.1 Tr=2 (t = 14:30, d = 85)
2021-07-15_FDE_SD_ChemSeg_Sacch_Tr4min	Batch (t = 0, d = 13:00); Chem. D=0.1 Tr=4 (t = 13:00, d = 46:30); Seg. D=0.1 Tr=4 (t = 59:30, d = 33:30)
2021-06-30_FDE_SD_Seg_Sacch_Tr4-8min	Batch (t = 0, d = 13:45); Seg. D=0.1 Tr=4 (t = 13:45, d = 47:00); Seg. D=0.1 Tr=8 (t = 60:45, d = 35:30)

t : start time (h), d : duration (h), LS : Lab-Scale, SD : Scale-Down, Chem. : chemostat, Seg. : Segregostat, D : dilution rate (h^{-1}), Tr : retention time in the recirculation loop (min)

Overview of Scientific Publications

Publications as first and co-first author

Published in international journals

Delvenne, M., Martinez, J. A., Haringa, C., Noorman, H., Minden, S., Takors, R., and Delvigne, F. “Overriding Bioprocess Perturbations With a Cell–Machine Interface for Reliable Microbial Stress-Response Control”. In: *Microbial Biotechnology* 19.4 (2026). DOI: 10.1111/1751-7915.70329

Martinez, J. A., Delvenne, M., Henrion, L., Moreno, F., Telek, S., Dusny, C., and Delvigne, F. “Controlling Microbial Co-Culture Based on Substrate Pulsing Can Lead to Stability through Differential Fitness Advantages”. In: *PLOS Computational Biology* 18.10 (2022). DOI: 10.1371/journal.pcbi.1010674

Henrion, L., Delvenne, M., Bajoul Kakahi, F., Moreno-Avitia, F., and Delvigne, F. “Exploiting Information and Control Theory for Directing Gene Expression in Cell Populations”. In: *Frontiers in Microbiology* 13 (2022). DOI: 10.3389/fmicb.2022.869509

Prepublished

Delvenne, M., Vandenbroucke, V., Henrion, L., Sehart, M., Martinez, J. A., Sloodts, A., Telek, S., Zicler, A., and Delvigne, F. *A Fitness Entropy Compensation Effect Is Set by the Trade-off between Growth and Gene Expression in Cell Populations*. 2025. DOI: 10.1101/2025.07.05.663304. URL: <https://www.biorxiv.org/content/10.1101/2025.07.05.663304>. Pre-published

Publications as co-author

Duperray, M., Delvenne, M., François, J. M., Delvigne, F., and Capp, J.-P. “Genomic and Metabolic Instability during Long-Term Fermentation of an Industrial *Saccharomyces cerevisiae* Strain Engineered for C5 Sugar Utilization”. In: *Frontiers in Bioengineering and Biotechnology* 12 (2024). DOI: 10.3389/fbioe.2024.1357671

Henrion, L., Martinez, J. A., Vandenbroucke, V., Delvenne, M., Telek, S., Zicler, A., Grünberger, A., and Delvigne, F. “Fitness Cost Associated with Cell Phenotypic Switching Drives Population Diversification Dynamics and Controllability”. In: *Nature Communications* 14.1 (2023). DOI: 10.1038/s41467-023-41917-z

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