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UNIVERSITY OF CALIFORNIA SAN DIEGO

Better with friends: engineering consortia-based solutions to complex problems

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Alyssa Jasmine Chiang

Committee in charge:

Professor Jeff Hasty, Chair
Professor Adam Feist
Professor Joe Pogliano
Professor Lingyan Shi
Professor Karsten Zengler

2025

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The Dissertation of Alyssa Jasmine Chiang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2025

DEDICATION

To my loving and supportive family.

EPIGRAPH

Science and everyday life cannot
and should not be separated.

Rosalind Franklin

The feeling of awed wonder that science can give us is
one of the highest experiences of which
the human psyche is capable.

Richard Dawkins

We don't succeed or fail because of fortune or luck. We succeed because we understand the way
the world works and what we have to do.

Viet Thanh Nguyen, The Sympathizer

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ACKNOWLEDGEMENTS

Six years ago, I started my Ph.D. in the Hasty Lab, bright-eyed and seeking something called "fulfillment". At the time, it was merely a vague notion that included scientific achievement, unwavering grit, and personal development. Full of big ideas and dreams for how I wanted the prime of my 20s to be both world- and life-changing, I thought, "5-6 years is plenty of time". Indeed, the journey has asked a lot in time and energy, but the six years has flown by and humbled me to the challenges (and, oftentimes, art) of scientific advancement and the volatility of what it really means to push the "cutting-edge".

At the sunset of this mentally and emotionally taxing hike, I've found that "fulfillment" has encompassed much more than I set out searching for. And for that, I acknowledge the village that has supported and pushed me to grow in countless ways. Closest to my soul are my family, who built the core of who I am. My parents, who taught me that ample self-motivation, heaping spoonfuls of discipline, sprinkles of persistence, a touch of resourcefulness, and a dash of preparation can cook up a feast. Mom has now been a rock for not just one, but two doctoral students (Dad and me), and Dad always insisted, to my teenage chagrin, on teaching me how to fish at the expense of feeding me for a day—to ensure that I could eat for my lifetime. Both have made me a better, stronger person. And my brother—best friend and sworn enemy in one—showed me the ends of my patience with one hand but offered infinite compassion and kindness with the other. And, of course, to the doctors and nurses who ensured, through these last tumultuous months of graduate school, that both my parents could be present with me today: thank you.

The journey would also have been incomplete without my chosen family—friends from various stages of my evolution. Veronica, who has grown together with me from high school, and our high school friend group who always reminds me that there's a crowd at home cheering me on. Alexis, who has shown me what authenticity and accountability in a friendship look like. Janette, a reliable friend I can always count on for some solid advice. And to my Lotus Magazine family: you have colored in the creative dimensions of my world with vibrance and joy.

When students considering graduate school ask for advice, I tell them to choose their

advisor carefully. Dr. Jeff Hasty, the chair of my committee, has provided invaluable guidance that has challenged me in pushing my work towards publication and opened the right doors for my career development. Learning to think about problems in new ways rather than brute-forcing through the most straightforward path showed me there is always a way if you give yourself the space to think hard enough. Jeff's nose for curating a community of quirky, unique personalities who mesh together has made the lab a sandbox for learning and growing alongside people I know will be lifelong friends. Some parts of graduate school I will miss dearly are the small moments between waiting for centrifuge spin steps, for cells to grow for a microfluidic experiment, or for someone to Nanodrop their sample so that you can laugh along when the concentration turns out low. I'll always remember singing Radiohead's *Creep* as a dissonant chorus, the stimulating (and controversial) lunch table conversations that challenged my preexisting ideas, happy hours with admittedly too much beer, cycles of shared cynicism about life, Art of Espresso with fellow early-birds (you know who you are), or the occasional midday snack rant with some "piping hot tea". My labmates have become my mentors, colleagues, friends, and travel buddies. And I guess these acknowledgements would be incomplete without a nod to my partner (and labmate), Austin, whose presence and support have kept me grounded when it felt like things were falling apart on every circle of Hell—we really had one hell of a year, didn't we? Graduate school has been memorable, productive, and, ultimately, fulfilling in ways that were better than I could ever have anticipated. Couldn't have written the plot twists myself.

Chapter 2, in part, is a reprint of the material as it appears in *Current Opinion in Microbiology* as 'Design of synthetic bacterial biosensors' (2024). Alyssa Jasmine Chiang and Jeff Hasty. The dissertation author was the primary author of this material.

Chapter 2, in part, and Chapter 3, in full, are currently under review at *Cell Systems* as 'A diversified consortium of engineered biosensors enables multi-target classification'. Alyssa Jasmine Chiang, Nicholas Csicsery, Richard O'Laughlin, Leo Baumgart, Elizabeth Stasiowski, Phuc Nguyen, Austin Doughty, Myra Ashraf, Raegan Mink, Elina C. Olson, Michael Ferry, Adam M. Feist, Nan Hao, Karsten Zengler, and Jeff Hasty. The dissertation author was the

primary investigator and author of this material.

Chapter 4 was co-authored with Shalni Kumar, Niklas van der Wagt, Lev Tsimring, and Jeff Hasty. The dissertation author was one the primary authors and researchers of this material.

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FIELDS OF STUDY

Major Field: Bioengineering

Studies in Synthetic Biology
Professors Jeff Hasty

ABSTRACT OF THE DISSERTATION

Better with friends: engineering consortia-based solutions to complex problems

by

Alyssa Jasmine Chiang

Doctor of Philosophy in Bioengineering

University of California San Diego, 2025

Professor Jeff Hasty, Chair

Engineering multi-strain systems has expanded the potential for synthetic biology as the tools and techniques for building cellular functionalities continue to approach systems-level understanding. Current work on multi-strain systems has primarily focused on developing stable ecologies with carefully tuned strain-to-strain interactions. In this work, I explore alternative multi-strain paradigms for two persistent biological problems: 1) bacterial biosensors for complex samples and 2) multi-strain population stability.

Whole-cell biosensors (WCBs) offer rapid, cost-effective monitoring of environmental contamination and human disease. Current WCB efforts to optimize detection of single target analytes under laboratory conditions have achieved vastly improved performance, setting the

stage for WCB deployment in complex environments. In the first part of this work, I propose a framework that leverages the cross-specificity of single-target WCBs to quantify multiple targets using supervised machine learning. Specifically, six sensors for heavy metal contaminants in laboratory *E. coli* were engineered. The laboratory strain was then evolved to generate five chassis with improved growth in seawater conditions. I transformed these chassis with the sensors, and characterized the variant dynamic behaviors with microfluidics, revealing significant phenotypic diversity. Leveraging this diversity, I employed supervised machine learning to combinatorically quantify multiple analytes using a consortium of signals from the diversified set of WCBs. Outperforming single-target biosensors in over 90% of test samples, the results form a generalizable framework that facilitates WCB translation toward settings beyond the laboratory.

In a different exploration of multi-strain paradigms, I work on building a single bacterial strain that circumvents population instability by differentiating into two spatiotemporally distinct phenotypes. Natural populations genetically encode the capability for numerous tasks and excel at functional diversification. However, engineering synthetic populations with higher complexity faces challenges of population instability due to burden and unwanted cross-talk. A single-strain “ecology” carrying two phenotypes on coupled orthogonal quorum-sensing systems that each drive their own lysis enables maintenance of both phenotypes. The strain’s continuous self-correction enhances the longevity of this system over an analogous two-strain ecology that suffers from inter-strain competition. This single-strain, multi-phenotype system can enable complex tasks such as multiple therapeutic delivery or metabolic division of labor.

Chapter 1

Introduction

1.1 Personal Perspective

As human beings, I find that we are often afraid of what we cannot see or understand within the "rules" we've established. Outer space, deep ocean, the brain...there is comfort in a world we can readily process and make sense of. Microscale biology lay on the outskirts of this comfort zone until the invention of the microscope and its first usage as a scientific instrument in the 17th century by Antonj van Leeuwenhoek and Robert Hooke¹. Since then, the field of biology has broadened in breadth and depth and has traveled a long way, with this newfound ability to see what we previously could not with our naked eyes. During my undergraduate studies in bioengineering, working in a lab for the first time exposed me to a sliver of the intricate micro-world that coexists right alongside us. Watching bacteria thrive under a microscope with a life of their own firsthand, I was filled with awe. Beneath the surface of everything we see and experience in our day-to-day lives, a whole complex ecosystem lives and functions on its own set of rules.

College coursework opened my eyes to the deterministic nature of this microscale biological ecosystem, governed by the same elegantly conserved genetic code that dictates a collection of traits and behaviors that support an organism's survival. In the first year of graduate school, my mentor lent me his copy of *The Selfish Gene* by Richard Dawkins. Published in the 1970s, it was influential in challenging conventional thought about evolution. He argued that

the gene—not the organism—was the primary unit driving evolution. Pivotal to the way I still think about biology and evolution (and certainly the seed for many fun and engaging lunchtable conversations), I joined Dr. Jeff Hasty’s synthetic biology lab, where I explored and challenged what was possible with this basic unit of biology. Not only would I delve into the rules that govern biological systems (as we currently understand them), but I also had the opportunity to stand at the cutting edge of the development and application of tools to alter them. In the grand scheme of things, my personal time in graduate school represents only a sliver of what there is to learn, but the humbling journey (and the support of my advisor) has given me the space to explore creative applications of the field.

1.2 A Brief History of Synthetic Biology

DNA, the beautiful blueprint to biology, lies at the heart of synthetic biology. The helical molecular structure of DNA, discovered by British chemist and X-ray crystallographer, Rosalind Franklin, was famously publicized by James Watson and Francis Crick^{2,3}. Subsequently, in the 60s, François Jacob and Jacques Monod detailed gene regulation in response to environmental cues by studying the lac operon in *E. coli*⁴. Inevitably, scientists around the world began to uncover the secrets of DNA regulation and, in the decade following, scientists began to manipulate DNA and recombine fragments. Polymerase chain reaction (PCR) in the late 80s facilitated DNA amplification, opening doors to DNA mutagenesis and novel methods for fragment assembly to modify or create new functions^{5–8}. The dawn of engineering synthetic biological circuits arose amidst these advancements in DNA manipulation techniques, with the genetic toggle switch and the repressilator, both published in *Nature* consecutively at the turn of the century^{9,10}. These became the foundations that kickstarted the young field of synthetic biology.

Only a quarter-century from those early circuits, synthetic biologists have designed and built creative constructs and complex networks, including oscillators, logic gates, and

population-level coordination¹¹. Furthermore, with the growth of big data capabilities that have accelerated our understanding of biology, the field of systems biology, a top-down approach to understanding biological systems and their interactions, will likely eventually converge with synthetic biology and connect knowledge with engineering¹². Especially with recent advancements in DNA sequencing¹³, DNA synthesis¹⁴, high-throughput methodologies, and data analysis techniques^{15,16}, the pace of development will not be slowing anytime soon.

1.3 The Cutting Edge

The frontier of synthetic biology continues to be a moving target, but some of the current spaces of exploration relevant to this body of work are described here.

1.3.1 Rational engineering and evolution

Biology already does so much on its own in nature that we can learn from. Operating on a set of rules which prescribe cellular functions and interactions, intercellular signaling, and metabolic pathways, biology contains an abundance of tools we can draw from. Rational engineering approaches allow us to deterministically apply what we know about cellular behaviors to engineer desired outcomes or even reveal novel information about the cell^{11,17,18}. On the other side of the same coin, evolutionary approaches harness nature's ability to sort out its own solutions, which, in turn, can reveal *de novo* information that can be useful to rational engineering approaches¹⁹.

1.3.2 Dynamic behavior and microfluidics

High-resolution imaging has paved the way for examining and tuning spatiotemporal behaviors of cells. Furthermore, microfluidic devices, which allow for continuous culture in a microenvironment, have facilitated design of dynamic behaviors in synthetic circuitry²⁰. Taken together, these tools and technologies can be harnessed to engineer continuous behaviors over time²¹, fine-tuned spatiotemporal control²², and adaptive strategies for survival in particular

environments²³.

1.3.3 Machine learning

Supervised machine learning approaches for mining large datasets to build predictive capabilities have risen in recent years. Particularly, there is ample potential for machine learning in the biosensor space, with the limited number of known transcription factors available and cross-reactivity between sensors²⁴. Attention has recently turned towards differential sensing, which, inspired by a mammal's olfactory sense, incorporates cross-reactive sensors to create a unique fingerprint for analyte detection²⁵. This can be useful especially for sensing challenging analytes for which no specific "lock-and-key" sensor has yet been identified^{26–28}. Coupled with machine learning strategies, multiplexed detection and quantification of multiple analytes in a single mixture using existing transcription factor-based biosensors are more feasible than ever.

1.4 Consortia: applications and possibilities

The tools described above are almost like modern science fiction, and one can imagine the countless novel interesting applications of synthetic biology ranging from human health to plant biology to environmental remediation to bioproduction. In fact, these are all current areas of exploration in synthetic biology. Recently, the field has developed an interest in engineering multistrain consortia to carry out increasingly complex tasks in which single-strain engineering is insufficient^{29–32}. Even in nature, microbes exist in delicate, fine-tuned ecosystems. Multistrain consortia are able to accomplish more complex functions and store more information, but the issue of stability in an engineered consortia continues to plague synthetic biologists. In this body of work, I explore alternative ways to take advantage of the benefits consortia offer while circumventing the issues of stable maintenance.

1.4.1 Biosensor signal consortium

In Chapters 2-3, I expand on the current notions of a bacterial transcription-based “biosensor”, which, in most applications in literature, consist of a single strain capable of sensing an isolated target under controlled laboratory environments. However, these single-target sensors often suffer from cross-reactivity and are limited by transcription-factor based targets. In my work, I sought to utilize multiple biosensor signals to generate a “fingerprint” readout to classify a combination of heavy metals simultaneously from a seawater sample. To this end, I generated a diversity of bacterial heavy metal biosensors and reduced the consortium of sensors to extract only their signals. This takes advantage of the ability of multiple strains to confer more information to carry out a complex task, while bypassing the need for engineering their stable coexistence. Using supervised machine learning, I disentangled these signals to accurately and efficiently characterize a sample. Though this work focuses on multiplexed heavy metal quantification in a seawater sample, this workflow for multiplexed differential sensing is transposable to a myriad of biosensing applications that require quantification of multiple targets in a biological sample.

1.4.2 Single-strain “consortium”

Chapter 4 challenges the typical design of a synthetic bacterial consortium by harnessing plasmids with coupled origins of replication to design a single strain capable of adaptably diversifying into multiple phenotypes in an environment-dependent way. By engineering heterogeneity into a single strain, cells have a tuning fork to facilitate its own relative numbers of two distinct phenotypes using copy number control so that they behave like a self-adjusting two-strain ecology. A single-strain consortium could facilitate bioproduction, sense-and-respond, and payload delivery schemes that could benefit from two-strain systems, while bypassing the issue of engineering an actual stably maintained two-strain synthetic consortia.

Chapter 2

Biosensor design and characterization

2.1 Introduction

Bacterial biosensors can provide real-time information in dynamic environments^{33–37}. Bacteria’s natural ability to withstand and adjust to frequently stressful conditions makes them strong candidates for applications such as human health diagnostics or pollution detection in terrestrial and marine environments. Furthermore, bacterial biosensors offer a low-cost, compact, environmentally sustainable, and robust option for continuous detection of specific analytes³⁸. Despite progress in engineering precise and sensitive biosensors, there are still challenges to overcome before they can be widely deployed in medical or environmental settings.

A whole-cell bacterial biosensor is typically composed of a sensing module—frequently a transcription factor or sensor kinase—that regulates a transducing module, such as a promoter, which drives a reporting module^{38,39}(Figure 2.1A). Many canonical biosensors have been based on transcriptional repressor topologies, which commonly exist in nature⁴⁰. Characteristics that define a bacterial biosensor’s efficacy are its specificity, sensitivity, and dynamic and operational ranges, which are often characterized by a Hill dose-response function⁴¹ (Figure 2.1B). A biosensor typically must be capable of sensing a desired target analyte in a dose-dependent manner with minimal, if any, cross-talk or antagonistic interaction with other structurally similar analytes or analytes that share transcriptional regulators or promoter constructs. Optimal dynamic and operational ranges characterize a biosensor’s ability to detect biologically relevant concentrations

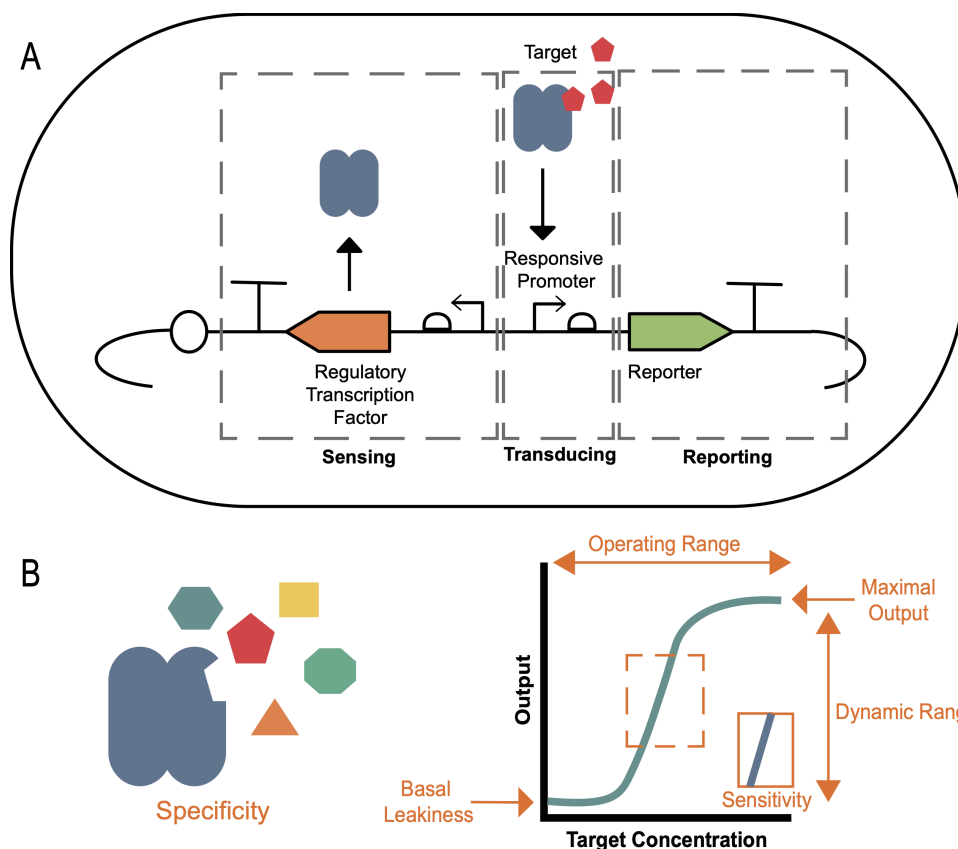


Figure 2.1. Transcription factor-based biosensor. (A) General structure of a transcription factor-based biosensor, with the transcription factor sensing element, its corresponding promoter(s) as the transducing element, and an output-reporting element. (B) Qualities and metrics in characterizations of biosensors, including specificity and dynamic and operating ranges.

of the target analyte and exhibit a distinguishable response gradient. Furthermore, absence of target analyte should yield minimal “leaky” expression, in which the reporter experiences basal expression in the presence of no target analyte.

Designing whole-cell bacterial biosensors requires consideration of the context of their application, as well as identification and characterization of suitable sensing components for the target analyte(s). While rational forward engineering approaches have dominated strategies for optimizing bacterial biosensor genetic elements, numerous recent studies have turned toward directed evolutionary tactics⁴². Recent work on bacterial biosensor design and characterization have focused on moving towards deployment. Specifically, the field has focused on 1) the development of biosensors that can withstand and sense under contextually relevant

conditions, 2) uncovering uncharacterized biosensing modules for new targets, 3) optimization of existing biosensors, 4) adjusting for spatiotemporally heterogeneous environments, and 5) innovative integration of output. This review focuses mainly on design of transcription factor based whole cell bacterial biosensors for small molecule ligands, but it should be noted that nucleotide-based biosensors, such as RNA-based biosensors have also achieved significant recent improvement^{39,41,43}.

2.2 Design and characterization of synthetic bacterial biosensors

2.2.1 Exploring novel host chassis

For deployment of whole-cell bacterial biosensors, the environment of their intended application must be considered throughout the design process. Different host chassis offer expanded functionalities and robustness in challenging environments that standard laboratory strains may not. More importantly, biosensor constructs characterized and tuned in model organisms may not be easily portable or exhibit the same behaviors in new organisms or environments⁴⁴.

Important considerations in selecting host chassis outside well-characterized model organisms, such as *Escherichia coli*, include biosafety, fitness in stressful or spatiotemporally heterogeneous biosensing environments, and genetic tractability⁴⁵. In a recent biosensing system, Cooper et al. harnessed horizontal gene transfer capabilities of *Acinetobacter baylyi* to detect tumor DNA, demonstrating the potential benefits of mining the utilities offered by new host chassis to develop unique biosensing solutions⁴⁶. To facilitate exploration of new host chassis, new toolkits and approaches for different host chassis are currently being studied and compiled⁴⁷. Broad-host range and portable expression tools, such as the Universal Bacterial Expression Resource (UBER), which decouples biosensing machinery from host expression by producing T7 polymerase to drive expression of the biosensing components on the plasmid, have also seen

recent development^{48,49}.

Standardization of genetic components and assembly of new toolboxes for non-model chassis have given rise to automated computational rational design of complex synthetic circuits⁵⁰. Cello was designed for researchers to specify logic gate circuit modules using Verilog with a user constraint file (UCF) that incorporates user-defined host organism, strain, and context as design specifications^{38,51}. Recently, Taketani et al. demonstrated the capacity of Cello for designing multi-input and -output biosensors by integrating bile acid and aTc sensing promoters in *Bacteroides thetaiotaomicron* to distinguish three distinct states (either aTc or bile acid, or neither) with a luminescence reporter³⁷.

Considering dynamic and transient contexts in characterization and optimization of biosensors is crucial, as many biosensor phenotypes are not conserved across diverse environments or hosts. Even altered growth rates can influence the dynamic range of well-characterized biosensors⁵². Simulating the environment of intended biosensor deployment, such as tuning oxygen availability or using gut models can be informative^{37,53,54}. Depending on the assay, modifying real samples to facilitate detection of small molecules can also aid application-centered biosensor design⁵⁵.

Beyond broad host-range engineering strategies for rational design and considering the environment of deployment, the continued foray into native microbial characterization will no doubt open further insights into biosensor engineering for specific environments.

2.2.2 Sensing new analytes

One of the largest concerns impeding the route to practical deployable biosensors is the lag in characterization of specific biological sensing elements behind the growth of target analytes of interest. To this end, high-throughput identification of potential transcriptional regulators and structure-guided mutagenesis have been immensely illuminating⁵⁶. Furthermore, new approaches in differential sensing and computational capabilities have opened doors towards unique ways of unlocking detection of analytes that have historically been challenging²⁵.

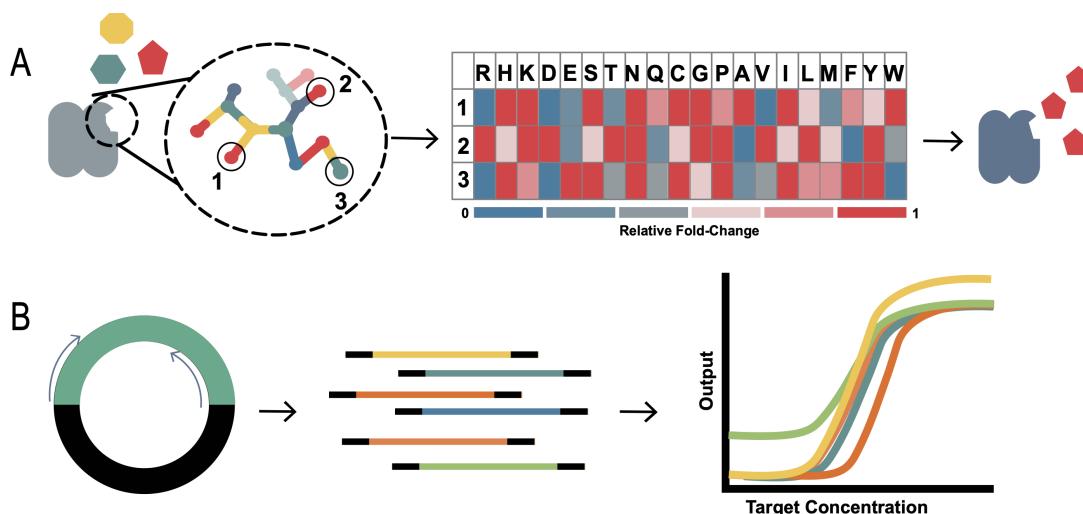


Figure 2.2. Directed evolution strategies for improvement of biosensors. (A) Structure-guided amino acid saturation mutagenesis entails identifying key residues of a transcriptional sensor either computationally with predictive software or X-ray crystallography. Key residues are swapped with all other possible amino acids to select variants with desired responses (i.e. greatest dynamic range). (B) Error-prone PCR harnesses error-prone polymerases to generate a mutant library of a specific sequence of interest (i.e. operator sites, RBS, etc.), so variants with desired responses can be selected in high-throughput.

Multiple methods have been explored to hack sequence-based knowledge of transcriptional regulator topologies to find sensors for molecules with no characterized transcriptional regulators. Hanko et al. screened GenBank annotations for the common conserved LysR-type transcriptional regulator (LTTR) divergent pattern and cross-referenced The Comprehensive Enzyme Information System to systematically uncover 16 previously uncharacterized inducible sensors⁵⁶. Screening gene clusters upregulated in response to progesterone using RNA-seq, followed by validation with *in vitro* ChIP-seq and biolayer interferometry with predicted binding sequences, also resulted in the first documented and characterized progesterone transcriptional bacterial biosensor⁵⁷.

Other methods include tapping into existing characterized allosteric transcription factors to develop sensors for adjacent molecules. Some researchers have combined structure-guided mutagenesis and directed evolution approaches with fluorescence activated cell sorting (FACS) to select for desired biosensor phenotypes^{36,58}. Taylor et al. used LacI as a scaffold to assemble

mutational libraries with a combination of Rosetta-aided protein variant designs, amino acid saturation mutagenesis, and error-prone PCR (Figure 2.2A-B). Assembling these variants to drive GFP output upon activation, variants specifically responsive to fucose, lactitol, sucralose, and gentabiose and less promiscuous for the original ligand, IPTG, were selected for using FACS⁵⁸. Similar studies with transcriptional regulator scaffolds have yielded a caffeine-inducible system using error-prone PCR with FACS, and a selective sensor for aromatics ethylbenzene and m-xylene using structure-guided mutagenesis^{36,59}. Not only have these mutational library studies yielded specific biosensors for new target analytes, but analysis of the mutations across variants exhibiting desired behavior also elucidated previously uncharacterized structural functions.

To facilitate building new sensing modules from existing sensing pathways using a rational engineering approach, Chang et al. developed a modular receptor scaffold called Engineered Modularized Receptors Activated via Ligand-induced Dimerization (EMeRALD) to repurpose the sensing component of a two-component sensing system from *Vibrio* species to a standard DNA binding domain in *E. coli* that drives expression of a fluorescent reporter gene to build a sensor for bile salts⁶⁰.

Notably, RNA-based biosensors, such as riboswitches and toehold switches, particularly in the identification of aptamers for new target analytes without known biosensors, have also seen significant recent developments^{39,61,62}. High-throughput pipelines for RNA biosensor libraries have been developed and continuously improved, such as the *de novo* rapid *in vitro* evolution of RNA biosensors (DRIVER), which uses a self-cleaving ribozyme system to distinguish aptamers with ligand-binding affinity^{61,63}.

Lastly, inspired by olfactory and taste receptors found in nature, which process multiple inputs to identify smells and flavors, some groups have turned towards "differential sensing" or "combinatorial sensing"²⁵. Although this concept has yet to become more widely adopted in the bacterial biosensor space, it has been employed in various artificial olfaction and sensing applications, including e-tongue and e-nose development^{64,65}. Recently, Kim et al. has ventured into this space with bacterial biosensors with a fingerprinting technique using multiple mutant

transcription factors and machine learning-assisted analysis to improve limit of detection of targets of interest²⁶. With the rise in computational techniques, differential sensing is a promising direction that could build on the extensive work in transcription factor-based bacterial biosensor development for sensing new analytes or improving limits of detection.

2.2.3 Characterizing and optimizing biosensors

Rational, semi-rational, and evolutionary strategies have been employed to optimize bacterial biosensor parameters, including specificity, sensitivity, dynamic range, and basal expression⁴². Multiple tuning forks for biosensors exist, including the transcriptional regulator that binds the target and the genetic elements responsible for its expression (i.e. promoter, RBS, etc.), or the transducing element regulated by the transcription activator/repressor.

Commonly, assembling a mutational library can help rapidly fine-tune a biosensor with desired characteristics for a particular application, similarly to finding new transcriptional biosensors in “Sensing New Analytes”. Tuning sensitivity or narrowing specificity of a promiscuous transcription factor can be achieved by identifying key residues of a transcriptional sensor to create a structure-guided variant mutational library for directed evolution^{34,54,60}(Figure 2.2A). Snoek et al. recently generated a variant library with error-prone PCR on the effector binding domain of BenM, a transcriptional activator that binds *cis-cis* muconic acid, to obtain biosensors exhibiting greater specificity, sensitivity, and inversion of function in high-throughput (Figure 2.2B). Variants with desired characteristics, including one with more than 1000x increase in maximum fold-change induction, were selected using FACS⁶⁶. Instead of FACS, Rottinghaus et al. used antibiotic resistance and sucrose counterselection as positive and negative reporters, respectively, to screen responses of a variant library of promiscuous transcription factors TyrR and FeaR to phenylalanine, tyrosine, phenylethylamine, and tyramine. These selective markers allowed selection of variants that respond to the desired amine and not to others³⁶. Furthermore, high-throughput microfluidic technology can be harnessed for screening biosensors’ spatiotemporal dynamic characteristics, which can offer output tuning beyond single-timepoint

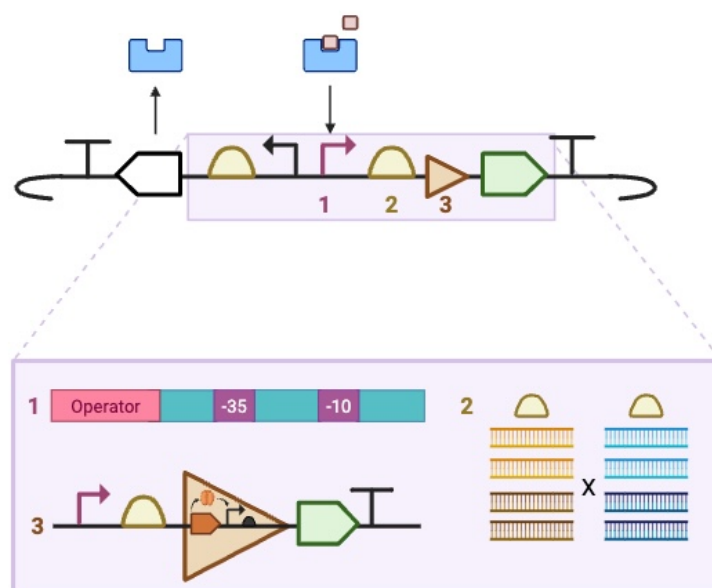


Figure 2.3. Rational strategies for optimizing biosensor output. Colored box highlights genetic parts frequently tuned in adjusting biosensor output. (1) Denotes the responsive promoter, which includes transcriptional activator-binding site (operator), the -35 and -10 RNAP-binding sites, and the spacers in-between these parts. Repositioning operator and RNAP-binding site positions and varying their nucleotide sequences can influence biosensor output. In (2), varying combinations of the RBSs of the transcriptional regulator and the output can produce varied dynamic range. (3) Conceptually shows the mechanism of added amplifier elements upstream of the reporting element to improve output amplitude and detection sensitivity. (Created with BioRender.com)

approaches⁶⁷.

Origin of replication, promoter, ribosomal binding site (RBS), and protein degradation tags are frequently involved in initial tuning of a biosensor's sensitivity and dynamic range^{53,68–70} (Figure 2.3). Hybrid promoters can be engineered by integrating operator sequences into a promoter sequence responsive to a desired regulator, as Zhang et al. did with *hrtO*, a promoter regulated by the heme-responsive transcription factor HrtR³⁴. Repositioning and altering promoter components on the sensing construct can alter biosensor behaviors, as shown with the arsenic-responsive P_{arsR} promoter, among others^{33,34,71} (Figure 2.3).

New methods and information for predictive tuning have recently emerged. Effects of

operator site spacing, operator and RNA polymerase (RNAP) binding sequence combinations, and alternative promoter architectures on fold-change response and leakiness were recently evaluated⁷². RNAP binding site offers another tuning fork for predictively building hybrid multi-input promoters⁷³. Another study used a design of experiments statistical method to analyze reporter expression as a function of expression strength of the transcription factor and the regulated promoter and RBS, resulting in “rules” for transcription factor-based biosensor design⁷⁴. For example, a weaker promoter driving the transcriptional regulator generally yields a greater dynamic range, likely due to steric interference, which is supported by several other studies^{54,74,75}. A library of cross-RBSs ($\text{RBS}_{\text{regulator}} \times \text{RBS}_{\text{output}}$) was also recently evaluated for glucarate transcription factor-based biosensor *cdaR* and sfGFP to increase dynamic range and develop a deep learning algorithm for predictively tuning regulator and output translation (Figure 2.3)⁷⁶. Taken together, these computational and experimental studies set the stage for rational engineering approaches toward sensor optimization.

Efforts toward standardization and modularization have expedited biosensor tuning⁵⁰. For example, characterized promoters can be easily swapped to improve fold-change response and limit of detection⁵⁴. Notably, Meyer et al. developed a set of optimized “Marionette” strains using an elegant dual selection directed evolution scheme to achieve biosensor transcription factors and their corresponding responsive promoters that exhibit low basal leakiness, improved sensitivity and dynamic range, and high specificity⁷⁷. These standardized parts allow for systematic, rapid tuning of genetic constructs, while showcasing the ability to optimize individual biosensor transcription factors and their corresponding promoters through directed evolution.

Amplification elements have also been explored to boost existing biosensor output. Wan et al. added amplifier elements to the previously characterized *arsR*- P_{ars} arsenic biosensor, drastically improving output and sensitivity⁷⁵ (Figure 2.3). Sensor-driven expression of T7 RNAP, with the T7 promoter driving fluorescence output, opens another promising avenue for biosensor amplification, as demonstrated with previously characterized biosensors for copper and glucose⁷⁸. More recently, RepL was integrated into biosensor response to increase copy

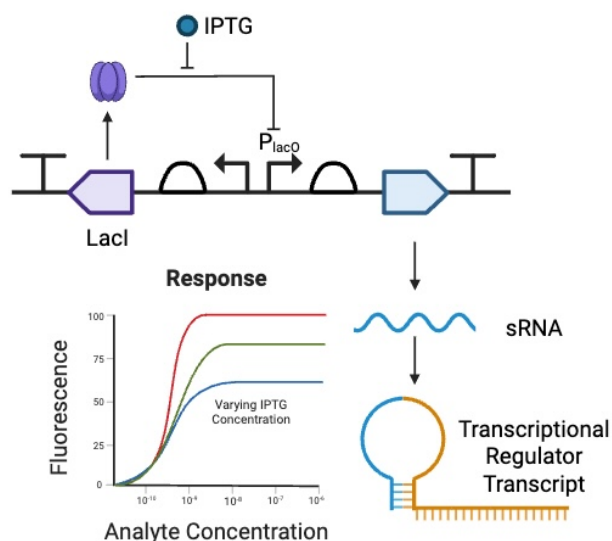


Figure 2.4. Post-construct tuner for biosensor output. Tuner mechanism for a biosensor that uses a toehold switch regulated by an external inducer, so a single strand of RNA is produced that binds to the biosensor transcriptional regulator transcript to modulate its translation rate. This allows for post-construction modulation of biosensor dynamic range. (Created with BioRender.com)

number to amplify biosensor output⁷⁹. In some cases, the reporting segment can even be manipulated to reduce basal leakage and improve dynamic range. For instance, splitting the large and transcriptionally inefficient *lux* luminescence cassette so that *luxABDE* was integrated into the *E. coli* Nissle genome and *luxC* was expressed on the plasmid in response to heme resulted in improved limit of detection and dynamic range^{80,81}.

Though most efforts at optimizing biosensors have focused on the biosensor itself or its regulatory genetic components, modulating the intracellular concentration of the target ligand can also serve as an innovative tuning fork by regulating its diffusion out of or uptake into the cell^{70,74}. For example, Miller et al. improved a sensor for erythromycin A (EryA) by expressing a macrolide resistance protein to phosphorylate EryA and keep it from diffusing out of the cell, improving the sensor's sensitivity⁷⁰.

Finally, Bartoli et al. proposed a method for tuning a biosensor post-construction with a toehold switch regulated by an external inducer to modulate the translation rate of the

transcriptional biosensor. Although the expanded range of detection limits came at the expense of fold-change in the output driven by the regulated promoter, this poses a promising approach for adjusting biosensors to various sensing environments or host chassis post-construction or for accommodating existing biosensors to different detection range needs⁶⁸ (Figure 2.4).

2.2.4 Incorporating features for spatiotemporally dynamic environments

Other features of existing biosensor constructs that preclude deployment include dynamic regulation in a transient environment, memory of independent sensing events, biocontainment, and long-term stability⁸².

Dynamic regulation is essential for the constantly fluxing conditions many applications of biosensing call for. In many recent studies, researchers have linked CRISPRi to biosensor response to regulate known metabolic pathways^{33,34,83} (Figure 2.5). For instance, one study coupled a transcription factor-based biosensor for GlcN6P to expression of sgRNAs that blocked transcription of proteins integral to pathways competitive to GlcNAc to optimize GlcNAc synthesis³³. The capacity for low-burden, orthogonal, and predictable regulation offered by CRISPRi has been demonstrated as Santos-Moreno, J et al. rebuilt some of the hallmark circuit designs of synthetic biology using CRISPRi: the bistable toggle switch and the repressilator^{9,10,83}. CRISPRi offers a potentially powerful tool for biosensor engineering.

Due to the transient nature of many biosensing contexts, genetic recording and memory have also become an important area of research. Previously, recombinase-based memory circuits were commonly used as a memory feature, but recently, Zou et al. integrated a base editor enzyme along with sfGFP into the output of the arsenic biosensor construct $\text{arsR-P}_{\text{ars}}$, with an interference sgRNA that edited the sfGFP. Not only was this a heritable genomic memory mechanism, but it also was erasable when coupled with a selective marker, so that recording cells could be “reset”^{84,85} (Figure 2.6). This was employed in a two-component biosensor that coupled detection of thiosulfate to a colorimetric reporter and regulated delivery of immunomodulator AvCystatin⁶⁹. In another memory circuit, Riglar, D; et.al. coupled a Cro/CI-based transcriptional

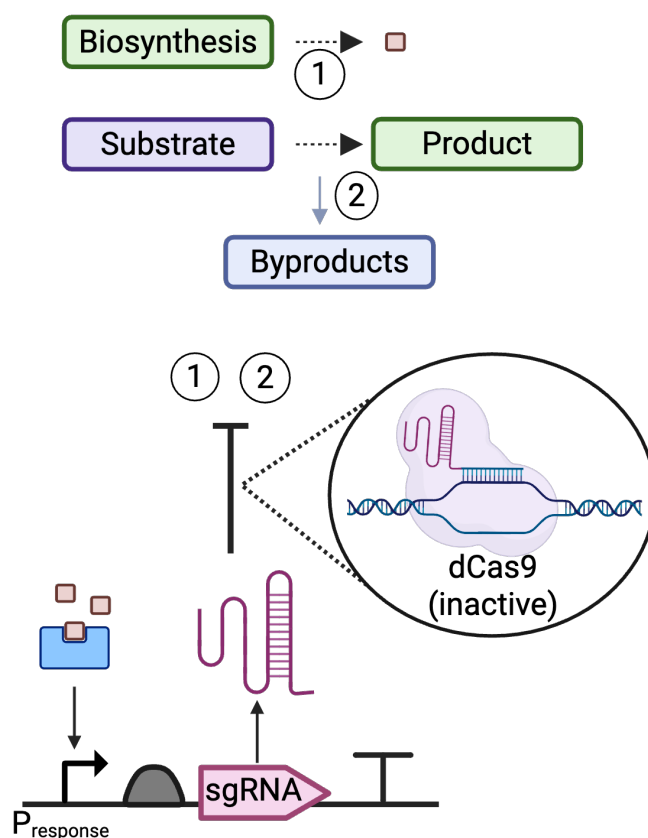


Figure 2.5. CRISPRi-mediated dynamic regulation. dCas9 protein is inactivated, so the sgRNA can be used to interfere with transcription of enzymes along (1) biosynthetic or (2) metabolic pathways. In the former, CRISPRi can be integrated into a biosensor to regulate production of the target ligand and maintain it at a threshold level. In the latter, it can be used to optimize production of an end product by inhibiting pathways for byproducts. (Created with BioRender.com).

switch memory system to a TtrR/TtrS two-component system to record tetrathionate sensing events, and evaluated the construct's long-term stability *in vivo*, establishing its potential as a living diagnostic^{86,87}. Stirling et al. subsequently applied the same switch to implement a pulse counter⁸⁸.

Finally, including built-in biosafety features is important in ensuring that biosensors can be safely deployed without jeopardizing their environment of deployment. This has been addressed in recent efforts to couple the biosensor to essential metabolic functions, as Chien et

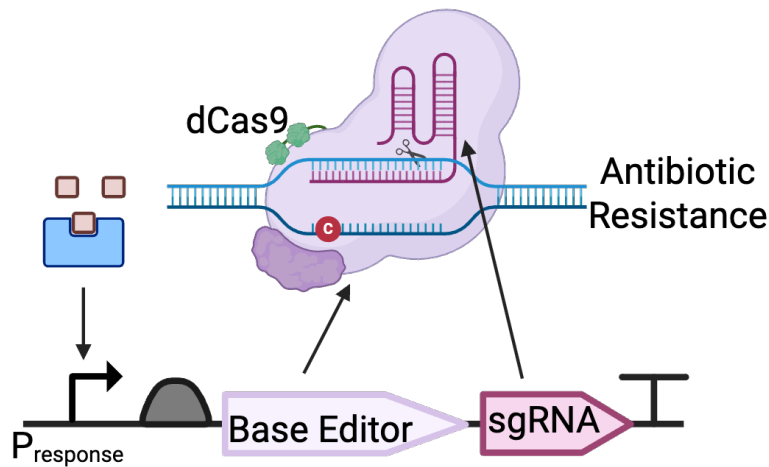


Figure 2.6. CRISPR-based memory mechanism. CRISPR-based memory mechanism via base editing on the genome allows for recording of sensing events. This is erasable when coupled with a selective marker, such as kanamycin resistance, so edited cells lose resistance and can be ‘reset’ by applying selective pressure. (Created with BioRender.com).

al. did to promote localization of bacterial biosensors to tumor microenvironments which exhibit a variety of distinct physicochemical gradients, including pH, oxygen, and lactate⁵³. Another strategy for biocontainment is using kill-switches, which terminate the bacterial sensor under specifically engineered cues, such as certain molecules or physicochemical properties. The persistent challenge for kill-switches is maintaining genetic stability due to their strong selective pressure. Stirling et al. employed a pH-sensitive switch using a toxin-antitoxin system, in which the toxin is produced under acid-shock conditions, thus permitting the strain to exist only under neutral pH⁸⁸ (Figure 2.7). More recently, a CRISPR-based switch was developed, which causes double-stranded genomic cleavage in response to specified stimuli. Genomic redundancy of the kill-switch cassette improved efficiency, and coupling the plasmid to an essential gene enhanced its stability⁸⁹. Acoustic and mechanical stimuli, such as magnetic waves, also offer innovative methods for localization⁹⁰.

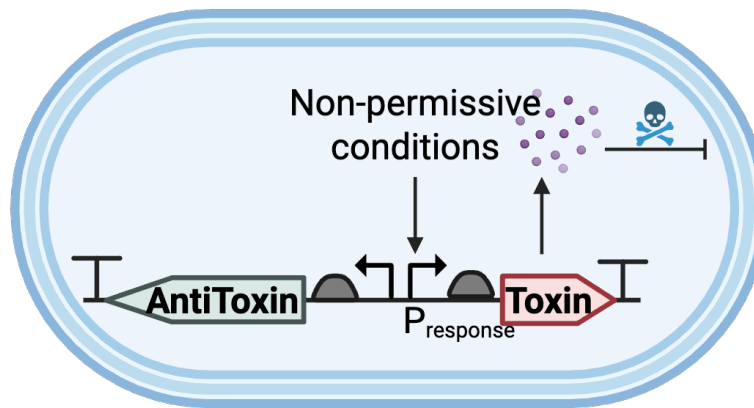


Figure 2.7. Toxin–antitoxin switch for biocontainment. Switch terminates the bacterial sensor under specific cues. The antitoxin is expressed constitutively at low levels to counter basal expression of the toxin under permissive conditions, but nonpermissive conditions increase expression of the toxin, leading to cell death. (Created with BioRender.com).

2.2.5 Integrating output for deployment

For translation into practical applications, recent work has been done in exploring unique output integration that facilitates practical use of bacterial biosensors. For example, Din et al. paired an arsenic biosensor with the seminal synchronized lysis circuit (SLC) to couple electric signal admittance with the release of charged ions from sensor strain population oscillations^{21,91}. Single photon-avalanche photodiode (SPAD) technology has also been coupled to biosensors for a low-cost, portable sensing platform⁸⁰. Some studies have used colorimetric outputs as diagnostic devices and microfluidic platforms for fluorescent readouts^{71,75}. Furthermore, Me et al. recently built an ingestible capsule for wireless biosensing, demonstrating its efficacy in a murine and porcine gut⁹².

2.2.6 Summary and outlook

Numerous advancements have been made in designing whole-cell transcription factor-based bacterial biosensors. Bacterial biosensors offer an affordable, dynamic, and environmentally friendly solution for detection, optimization of biosynthesis, and regulation of metabolic pathways. However, safe and effective deployment still calls for many developments. Most

bacterial biosensors have been engineered and characterized within laboratory conditions to single targets in isolation, making them unsuitable for the frequently stressful heterogeneous conditions of applications and subject to off-target sensing. New host chassis can serve as more robust sensors; notable recent developments have included toolkits and portable methods for exploring less characterized bacterial species. Furthermore, many analytes of interest still lack characterized sensors. To this end, researchers have developed techniques for mining genomic sequences to uncover new sensors, evolving existing biosensors for new target ligands, and rewiring known two-component systems for molecules that cannot diffuse into the cell. Many existing sensors must also be optimized, especially when considering deployment environments, which can result in a noisy signal. Their dynamic range, limit of detection, and specificity need to be tuned to biologically relevant levels. Standardization of genetic parts continue to be essential in initial rational tuning. Directed evolution approaches have also risen in importance for optimizing sensors. Some researchers have added additional genetic components for signal amplification or post-construction tuning with external inducers. Furthermore, differential fingerprint sensing approaches are a promising field for building upon the body of work on bacterial biosensors to target new analytes or improve detection limits. A largely untapped area, dynamic features of bacterial biosensors have yet to be mined, particularly with the increasingly mature development of microfluidic platforms which enable long-term survival of biosensors. Other future prospects of biosensor engineering include dynamic regulation, heritable recording mechanisms, and fail-safe approaches to biocontainment. CRISPRi can be employed in dynamic regulation to minimize byproducts of biosynthetic pathways or to regulate the production of target ligand by using an inactivated dCas9 protein with a single guide RNA to interfere with the transcription of enzymes producing undesired byproducts or the target ligand to limit its production, respectively. CRISPR technology can also be harnessed for its base editing capabilities in recording sensing events. Towards biocontainment, kill-switches can be developed so that bacteria will only exist under permissive conditions, but will self-destruct under non-permissive ones, promoting its localization. Finally, creative methods for output integration into the final intended contexts,

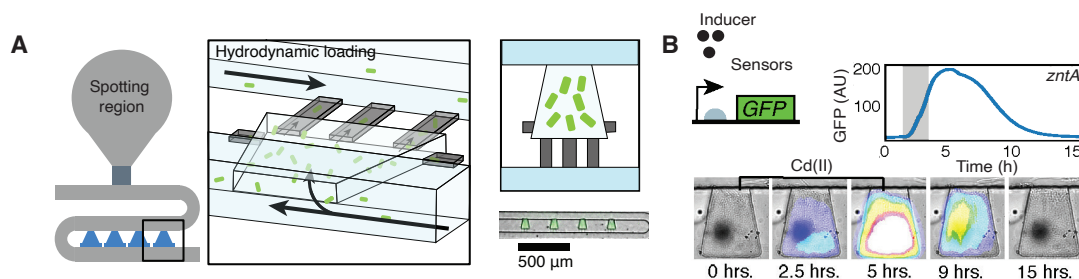


Figure 2.8. A versatile, multiplexed chemostat array. (A) A drawing of an individual strain's spotting and trapping region. Each growth region is comprised of a spotting region where cells are deposited, after which hydrodynamic loading enables media flow to carry cells into shallower empty traps for imaging. Upon filling the traps, media flow proceeds through the main channel, perpendicular to the traps. (B) Inducible fluorescence reporting system (GFP expression) tested with our microfluidic layout. The *zntA* WCB driving GFP is exposed to a two-hour exposure of 1 μ M Cd (gray bar).

such as ingestible capsules or portable microfluidics, will be crucial in ensuring safe deployment of bacterial biosensors.

2.3 Microfluidics facilitates screening dynamic behavior in real-time

Dynamic features in bacterial biosensors are an underexplored space, with single-timepoint approaches dominating the field, often masking dynamic behaviors which can carry more information on WCB response over time^{60,93–97}. Microfluidic technology offers a promising approach to characterization of dynamic features, by providing a stable microenvironment to facilitate live-cell imaging. To this end, we developed a versatile, cost-effective microfluidic screening system with modular design elements to enable simultaneous testing of multiple microbial strains as well as varied conditions in parallel.

2.3.1 Hydrodynamic traps

Central to the platform are the cell trapping regions, which are easily interchangeable with different architectures, depending on end user needs using our microfabrication process (Figure 2.8)^{20,98}. Hydrodynamically trapping cells in a spatially isolated location from the

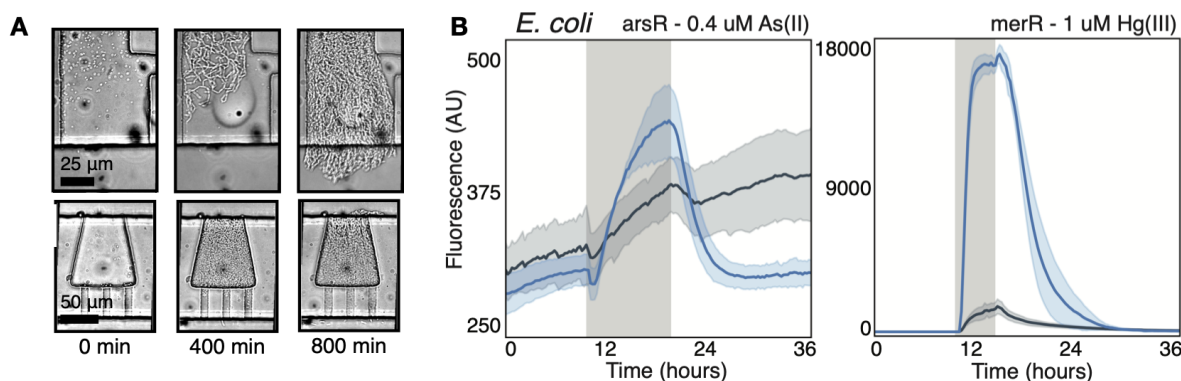


Figure 2.9. Microfluidic device scalability and flexibility for cell transfer. (A) HD traps fill within 8 hours of loading. (B) Direct comparison of fluorescence traces extracted from the spotting region (dark gray) and HD traps (blue). Each strain was grown in the absence of the inducing metal, and exposed for a several hour induction window (gray bars).

original cell deposit spot, the trapping regions feature narrow, shallow $0.5\ \mu\text{m}$ back channels that allow for even flow of nutrients to cells through the trap (Figure 2.8A)⁶⁷. Upon device setup, growth media flows in $50\ \mu\text{m}$ channels through and perpendicular to the spotting region features, carrying spotted cells to the downstream cell traps. Downstream cell traps are loaded as cells flowing through the channels are caught in a shallower $4\ \mu\text{m}$ layer for imaging. Traps hold up to 50,000-100,000 cells. Testing a sample whole-cell biosensor, with cadmium-responsive promoter P_{zntA} driving green fluorescent protein (GFP) as a reporter, we see a fast strong readout when induced (Figure 2.8B).

Within roughly 8 hours, cell traps reach confluence (Figure 2.9A). Past iterations of our microfluidics platforms forewent the spatially isolated hydrodynamic (HD) traps, instead opting for a single trap directly connected to the cell-spotting region⁹⁹. While such an implementation generated useful data from the cultured strains, the separation of cell spotting and imaging regions in this hydrodynamic design generate clearer, more pronounced dynamics (Figure 2.9B).

2.3.2 Design modularity

The hydrodynamic trap geometry is easily adaptable to various microfluidic architectures to accommodate diverse end-user needs.

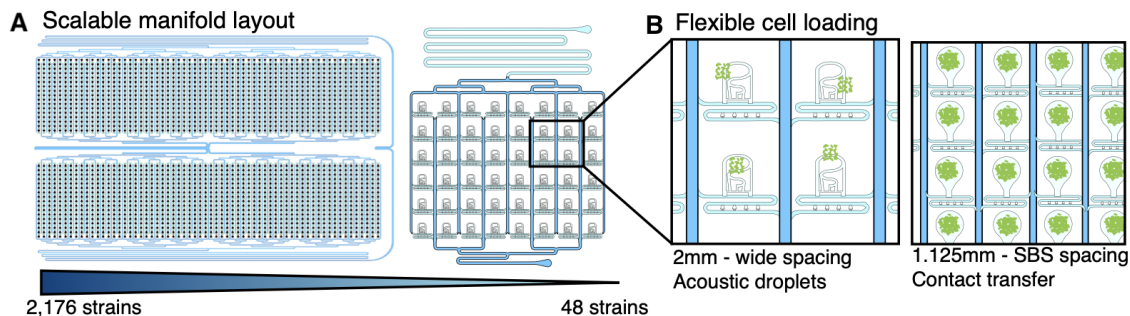


Figure 2.10. Multiple strains, single condition (A) Using $160\ \mu\text{m}$ branched, parallel fluidic manifolds, multiplexed arrays can be scaled to house between 48 and 2,176 individual strains^{99,100}. (B) Numerous cell loading methods are compatible with the manifold design, including acoustic droplet ejection (2 mm between strains) of liquid cultures and contact transfer using pin pads of colonies from solid agar plates (SBS-spaced, 1.125 mm between strains). Spotting region geometry and spacing can be adjusted to best fit the spotting method.

Multiple strains, single condition

To evaluate responses of multiple strains to a single condition in parallel, we designed a scalable manifold array design capable of housing 48 to 2,176 strains simultaneously, based on our previous multiplexed designs^{67,99,101}, enabling high-throughput assessment of large numbers of strains (Figure 2.10A). The simple 48-strain array maintains a single-layer and single inlet-outlet. Prior designs required cells to be loaded onto the chip either by fluidic lines^{102,103} or deposited directly onto microfluidic components before bonding^{104–106}. However, direct deposition of cells onto our platform circumvents the need for multiple fluidic ports and cell loading lines typically required to inoculate multi-strain devices without cross-contamination, greatly simplifying the experimental setup of these devices with manual loading capability. Easily scalable, the manifold design allows for modifications that suit different cell deposition methods (Figure 2.10B), including acoustic liquid droplet handling and SBS pin pad contact transfer methods.

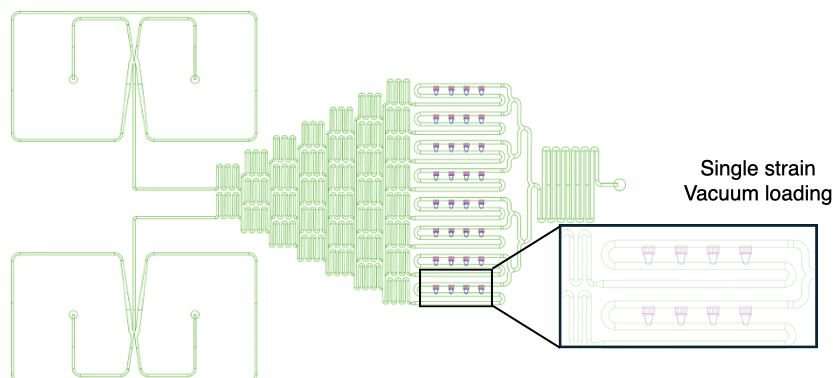


Figure 2.11. Gradient generator with HD traps. Microfluidic device containing HD traps configured to generate an inducer gradient for single-strain characterization across multiple conditions in parallel, based on a previous gradient design¹⁰⁷. Cells can be rapidly vacuum-loaded and the gradient is automatically generated using the branched channel network.

Single strain, multiple conditions

To evaluate a single strain's response to multiple conditions simultaneously, we transposed our modular HD traps into a single-strain gradient generator device for efficient screening across multiple conditions in parallel, based on a previously designed branched mixing array upstream of the cell culture region¹⁰⁷. A discrete gradient of conditions is generated with distinct mixing ratios of two inlet media (Figure 2.11). This design forewent the cell spotting regions altogether, instead opting for vacuum-loading of cells through the outlet during the final step of the experimental setup, allowing devices to be plasma-bonded to glass slides ahead of time, prior to cell deposition (See 'Materials and Methods').

2.4 Application: Transcription factor-based biosensors for heavy metal detection

Human industrial and agricultural activities have resulted in considerable waste and toxic byproducts that have left marine environments with increasing levels of pollutants such as heavy metals^{108–110}. As they accumulate, heavy metals become toxic, harming biodiversity^{108,111} and presenting public health risks for local populations, particularly in developing countries through

consumption and exposure^{112,113}. This can lead to devastating neurological and physiological damage on human health¹¹¹. As a result, cost-effective biomonitoring solutions that are robust to high salinity conditions while capable of frequent and rapid monitoring are essential to facilitating pollutant mitigation and reduction efforts.

Whole-cell biosensors (WCBs) have gained recent traction in detection of target analytes, informing biological solutions in human or environmental health^{60,93,99,114}, agriculture¹¹⁵, or food safety¹¹⁶. Though chemical or physical detection methods exist for many analytes—such as mass spectrometry or high-performance liquid chromatography¹¹⁷—these assays can be expensive, time-consuming, and salinity-sensitive, while also requiring experienced personnel or complex equipment^{94,118,119}. Furthermore, effective decision-making and remedial action cannot be taken in response to real-time information. Whole-cell biosensors, on the other hand, not only offer affordable, versatile, rapid, and simple readouts to address these needs, but can also be directly linked to remedial actions such as therapeutic payload release^{21,53} or toxin neutralization¹²⁰, especially with the rise of greater precision in synthetic biology and genetic engineering^{35,95,121}.

2.4.1 Biosensor design

Transcription factor-based biosensor constructs were engineered to monitor five heavy metals relevant to marine environments^{108,110}: arsenic, mercury, copper, lead, and cadmium. To this end, six sensor constructs were engineered (Figure 2.12A, Table 2.1). The sensing element consisted of a transcription factor that interacts with the target, which was placed either on the engineered construct or is endogenously expressed on the host genome. This regulates the transducing element, an inducible promoter, that drives the reporting element, GFP expression. Each construct was designed to be specific to a certain metal and to produce a fluorescent output corresponding to the metal concentration present.

All sensor constructs were placed on a medium-copy p15A origin plasmid with a kanamycin resistance gene. Individual sensor parts were identified through literature. The

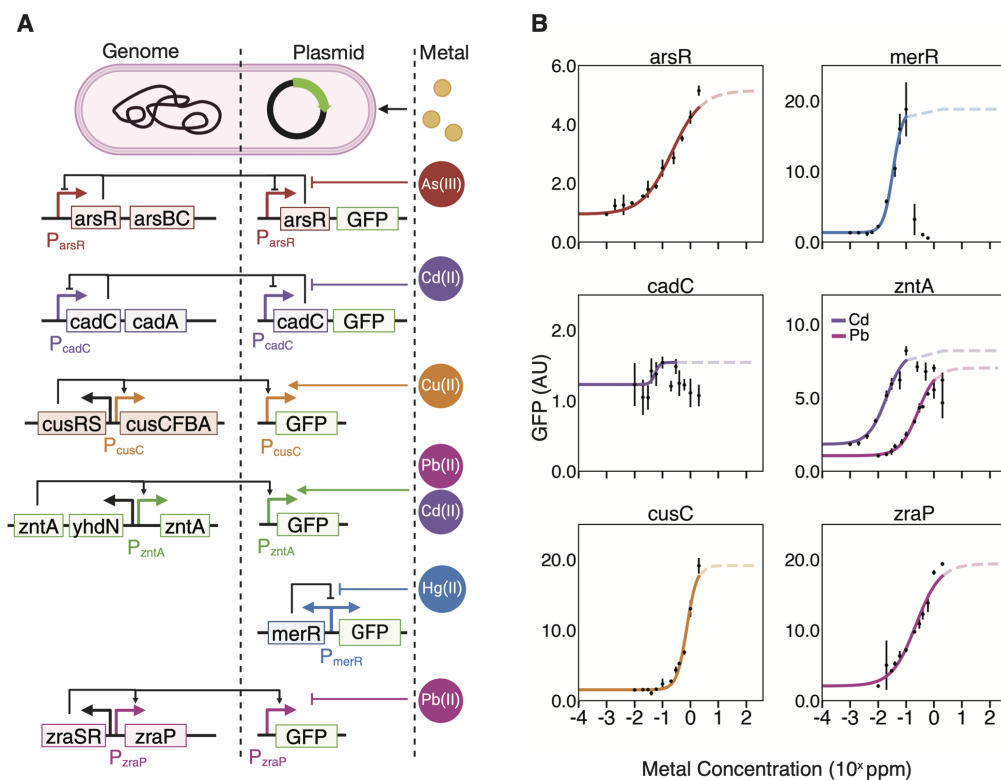


Figure 2.12. Development and characterization of real-time transcription factor-based biosensors for heavy metal quantification. (A) Six plasmids were engineered to respond to Cu(II), As(III), Pb(II), Cd(II), and Hg(II) (rightmost column) in the *E. coli* K-12 MG1655 strain. The center column indicates the engineered circuit elements that are encoded on the plasmid. In some cases, these engineered elements interact with transcription factors encoded by the host MG1655 genome, as indicated by the leftmost column. Created with BioRender.com. (B) Metal-fluorescence dose-response was measured for each of the synthetic fluorescent biosensor strains. Concentrations indicated by the dashed line affect cell growth and were excluded from the dose-response curve fit. Data are *mean* $\pm$ *SD*, with N=3 independent replicates.

native *E. coli* P_{arsR} promoter was selected for arsenic detection. This promoter has previously been characterized and shown to respond to arsenic, through the action of the transcriptional repressor protein ArsR, which binds to the promoter region and inhibits transcription in the absence of arsenic¹²². Similarly, the native *E. coli* P_{cadC} promoter was selected for sensing cadmium, as it has previously been characterized to behave similarly to the *arsR* system, via the transcriptional repressor protein CadC¹²³. For detection of mercury, we selected the well-characterized MerR transcriptional activator with its corresponding bidirectional promoter. The *merR* system is naturally found in a variety of Gram-negative bacteria and plasmids on the transposon Tn21¹²⁴;

Table 2.1. Biosensor plasmid construct parts. Each biosensor plasmid is constructed of a heavy metal-responsive promoter, which is regulated by a transcription factor. In P_{arsR} , P_{cadC} , and P_{merR} , the regulatory transcription factors are included in the plasmid, because it is borrowed from a different host strain. In P_{cusC} , P_{zntA} , and P_{zraP} , the regulator transcription factors the plasmid interacts are endogenously expressed in the *E. coli* K-12 MG1655 host strain.

Promoter	Transcription Factor	Source Strain
P_{arsR}	arsR	<i>E. coli</i> R773
P_{cadC}	cadC	<i>S. Aureus</i> pI258
P_{merR}	merR	Tn21
P_{cusC}	n/a	<i>E. coli</i> MG1655
P_{zntA}	n/a	<i>E. coli</i> MG1655
P_{zraP}	n/a	<i>E. coli</i> MG1655

while native to some species of *E. coli*, it is not naturally found in the MG1655 strain. In addition, the promoters P_{cusC} , which is sensitive to copper¹²⁵, and P_{zraP} , which is sensitive to lead¹²⁶, are natively present on the MG1655 genome and were previously found to be responsive without overexpression of additional transcriptional factors. Promoter P_{zntA} , responsive to cadmium and lead¹²⁷, was also included, given its well-documented sensor performance.

Sensor construct performance was initially verified in batch in wild-type *E. coli* K-12 MG1655 in minimal media background, with dose-response curves measured for each strain using the Hill equation (Figure 2.12B). Due to the cellular toxicity of heavy metals, several sensors arrest growth before the GFP-toxin response is saturated; however, a detectable level of fluorescence was measured at non-toxic concentrations for all strains, which exhibited various

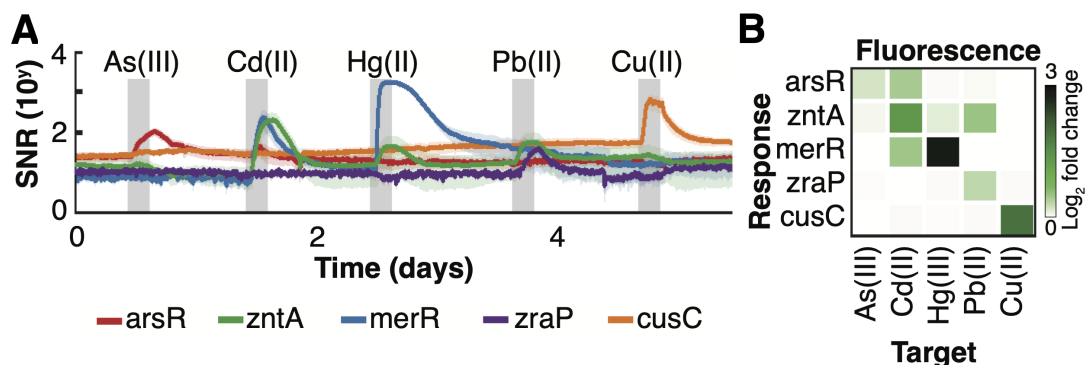


Figure 2.13. Dynamic characterization of biosensors in multistrain microfluidics. (A) A plot of signal-to-noise ratio of the sensor strains subjected to temporal exposures of the five metals, as represented by the gray shaded regions. Fluorescence traces were extracted over time, and signal-to-noise ratio calculated. Solid lines represent mean of N=12 HD traps in the microfluidic experiment and shaded region represents standard deviation. (B) A heatmap of the fold-change for each fluorescent sensor in response to each heavy metal in the experiment described in panel A. Metal concentrations were: 1 μM As(III), 0.5 μM Cd(II), 0.5 μM Hg(II), 5 μM Pb(II), and 2 μM Cu(II).

dynamic and operational ranges of fluorescence and working concentrations, respectively. The *zntA* sensor responded to both cadmium and lead as intended, but its cross-specificity for the two metals revealed a potential weakness of WCBs manufactured to detect isolated target analytes: crosstalk across multiple analytes can lead to confounding signals in mixed samples.

2.4.2 Biosensor characterization

Dynamic characterization

The heavy metal WCBs characterized in batch culture were screened on our multiplexed 48-strain microfluidic platform (See 'Microfluidics facilitates screening dynamic behavior in real-time') to analyze their dynamic behavior when subjected to temporal exposures of each of the five metals (Figure 2.13A). When exposed to four-hour pulses of each metal, sensors exhibited a typical first-order-like response, increasing fluorescence upon metal introduction, with exponential relaxation upon metal removal. Fluorescence returned to baseline within 24 hours of metal removal. The parallel culture of multiple strains on the same device enabled the specific distinction of each metal and the determination of crosstalk during each metal exposure

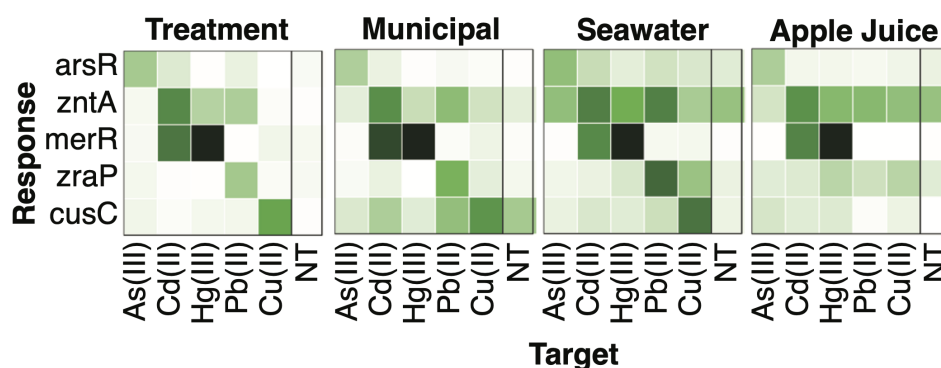


Figure 2.14. Fluorescence response on different water supplies. The fluorescent strains were tested on four environmental water sources: water from a local water treatment plant, municipal potable water, seawater, and commercial apple juice. Metal concentrations were: 1 μM As(III), 0.5 μM Cd(II), 0.5 μM Hg(II), 5 μM Pb(II), and 2 μM Cu(II). No toxin (NT) represents an exposure to the water sample with no additional dissolved metals.

(Figure 2.13). Interestingly, many of the sensors exhibited off-target responses to other heavy metals outside of their intended targets, potentially jeopardizing their efficacy and reliability as single-target WCBs (Figure 2.13B).

Water supply assays

Biosensor response was evaluated on four different water supplies to further evaluate their real-world deployability: water from the Alvarado Water Treatment Plant in San Diego, California which provides drinking water locally, municipal potable tap water, seawater, and commercial apple juice (Figure 2.14). The strains were subjected to five consecutive exposures of metals in these four different water supplies, spiked with the same heavy metal concentrations as in Figure 2.13. On water from the treatment facility, the activation profile for all fluorescent sensors was very similar to the results from lab water. However, on municipal potable tap water, sensors experienced more crosstalk, especially the *cusC* and *zntA* sensors. Apple juice proved to be a difficult sensing background, especially with its high autofluorescence masking the sensor response. The *zntA* sensor was continuously activated by apple juice, likely due to the high amount of trace metals, particularly zinc which is known to interact with the *zntR* regulator¹²⁷. Furthermore, copper and lead were not detected on this background media by *zraP* and *cusC*. This

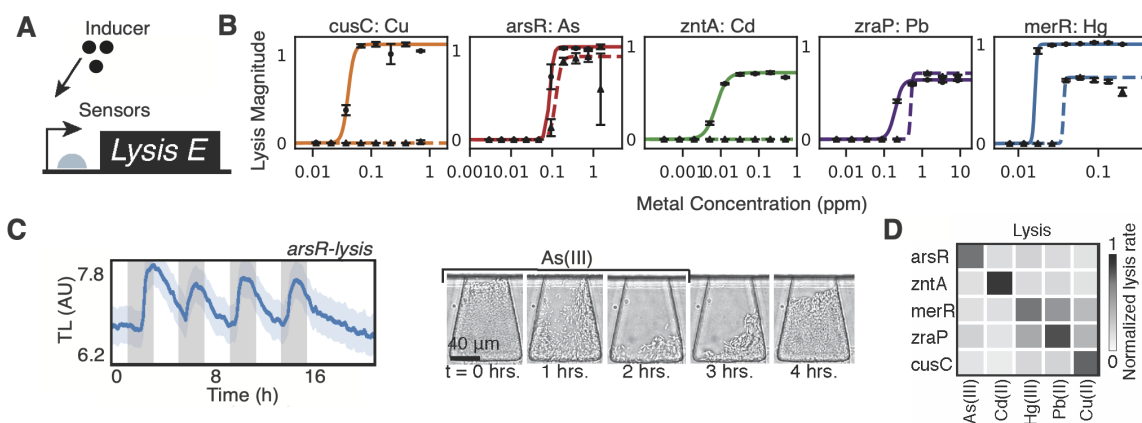


Figure 2.15. Biosensor constructs with lysis reporting. (A) A lysis-based inducible reporting system using transmitted light changes (E lysis protein from phage ϕ X174). (B) Lysis magnitude is measured as the ratio of the change in OD due to lysis over the change in OD due to growth before toxin exposure. Lysis magnitude is measured at 1 hour (solid line, black circles) and 8 hours after toxin exposure (dashed line, black triangles). Error-bars represent standard deviation of triplicate samples. (C) Lysis-based circuit responding to periodic 2-hour pulses of 75 ppb As(III) (gray bars). (D) Fold change of each lysis sensor when run on microfluidics are shown in response to each heavy metal.

may have been attributed to near-toxic levels of trace copper already present in apple juice, the potential presence of chelating agents, or high background fluorescence that obscured the signal. This inconsistency in response in a new background highlighted the weakness of relying on laboratory-characterized single-target sensors. Furthermore, seawater induced an unconventional response in the sensors, likely due to osmolar stress in the high-salinity environment; most laboratory strains of *E. coli* used for biosensor development struggle to survive and function under high salinity environments, suffering hyperosmotic shock and subsequent limitations in population growth¹²⁸. In particular, *zntA* was activated in the absence of any added metal, despite low levels of trace cadmium and lead in the seawater (Figure 2.14). Taken together, the observed inconsistency in sensor responses across various non-laboratory background water supply revealed that the conventional methods of engineering biosensors for specific analytes under laboratory conditions falter when faced with different and often stressful deployment conditions. Furthermore, disentangling individual signals among combinations of analytes becomes particularly challenging in biosensor applications in the presence of crosstalk.

Lysis reporting mechanism

Swapping the fluorescent protein for expression of a cell lysis gene, we also demonstrated changes in transmitted light (TL) as another mechanism of dynamic response that is compatible with our microfluidic platform but otherwise inaccessible through single-timepoint measurements (Figure 2.15A). Lysis magnitude was measured as a function of TL, which served as a proxy for optical density (Figure 2.15B). An example of the oscillations produced with arsenic-responsive promoter P_{arsR} driving the lysis protein is shown (Figure 2.15C). Similarly to the GFP readouts, upon heavy metal induction, strains lyse, causing a drop in the population before it recovers upon heavy metal removal. Like the GFP reporter versions of the biosensors, varying levels of crosstalk can also be seen across the engineered sensors when exposed to the different heavy metals (Figure 2.15D).

2.5 Materials and Methods

Biosensor plasmid construction

Biosensing plasmids were constructed using PCR amplification of host strain components listed in Table 2.1, followed by Gibson Assembly. The green fluorescent protein-containing backbones were obtained from existing plasmids in our lab. Plasmid sequences were confirmed with Sanger sequencing (Integrated DNA Technologies, San Diego, CA) and transformed into chemically competent *E. coli* K-12 MG1655 cells.

Dose-response experiments

Overnight cultures grown in LB media were reseeded into HM9 minimal media at 1:1000 dilution and grown until they reached an OD_{600} of about 0.3-0.4. The HM9 minimal media composition, containing minimal traces of metal, was based on prior studies^{99,129} and optimized for microfluidic *E. coli* growth. For fluorescence dose-response curves, 96-well plates were prepared with media and indicated heavy metal concentrations. Cultures were diluted

to OD₆₀₀ of about 0.1 on the plate. Fluorescence and OD₆₀₀ values were measured using an Infinite® M200 PRO Multimode Microplate Reader. Fluorescence measurements were taken at maximum induction, which occurred around 4 hours into the experiment. This was normalized by fluorescence of a promotorless strain to correct for cell autofluorescence and by OD₆₀₀.

Microfluidic device development and fabrication

Microfabrication techniques in patterning SU-8 photoresist onto a silicon wafer to create a mold for microfluidic devices have previously been described^{20,98,101}. Polydimethylsiloxane (PDMS) microfluidic devices were made with a mixture of 10:1 Sylgard 184 to curing agent, which was poured over the wafer centered on a glass petri dish and degassed to remove bubbles in the PDMS. The wafer with the PDMS was cured for one hour at 95°C on a level surface.

Microfluidic device loading and bonding

PDMS devices and glass slides were cleaned with MilliQ water and 70% Ethanol. Adhesive tape was used to remove any remaining fibers on the device. Overnight cultures were reseeded at 1:100 to an OD₆₀₀ of approximately 0.3-0.4. For multi-strain devices, 1mL of the overnight cultures was centrifuged, the supernatant was discarded, and cell pellets were resuspended in 20 µL fresh LB to make highly concentrated cell cultures. The devices and slides were exposed to oxygen plasma, after which 23ga syringe tips were used to transfer cells from the concentrated suspension to the devices by lightly contacting the cell traps on the PDMS. The device and glass slide were then bonded and cured at 37°C for two hours. To load the gradient devices, they were instead bonded and cured prior to vacuum loading using a previously described protocol¹⁰⁷.

Multi-strain microfluidic experimental protocol

Microfluidic experiments were performed on a Nikon Eclipse Ti epifluorescent inverted microscope (Nikon Instruments Inc., Tokyo, Japan). The inlet and outlet ports were connected to 50mL syringes with Tygon tubing. The inlet syringes were filled with LB with Kanamycin

and 0.075% Tween-20. Tween-20 is a surfactant to help prevent biofilm formation that can clog microfluidic channels and reduce the longevity of experiments. This has previously been shown to have no adverse effect on *E. coli* in many microfluidic experiments^{20,130}. The waste outlet was filled with approximately 5 mL of MilliQ water, to provide negative pressure. The inlet and outlet syringes were separated by a 10" height difference. Strains were grown to confluence in the cell traps after at least 8 hours, and switched to HM9 minimal media to maintain confluence without biofilm buildup. For inductions, a predetermined heavy metal concentration was pipetted into the inlet syringe. After a 4-hour induction period, any remaining inducer solution was pipetted out and replaced with fresh HM9. Fluorescence time series were extracted and normalized to remove device background fluorescence and strain background fluorescence.

Source water testing

Sensor strains were tested on four different water sources: (1) water from the Alvarado Water Treatment Plant in San Diego, CA, (2) municipal potable tap water from San Diego, CA, (3) Seawater from Blacks Beach in La Jolla, CA (4) and apple juice (Trader Joe's Fresh Pressed Apple Juice SKU#88463). Once a day, each device was exposed to a mixture of a single toxin dissolved in HM9 made from the respective water source for 4 hours. Signal-to-noise ratio was determined as the fluorescence value at each time point divided by the standard deviation of fluorescence 7.5 hours before induction. Fold-change was calculated from the signal-to-noise ratio for four biological replicates of each sensor.

2.6 Discussion

Building upon the advancements made in the whole-cell transcription factor-based bacterial biosensor space, we designed and characterized six biosensor constructs for detecting five heavy metals of interest in seawater. Using these WCBs, we demonstrate the utility of microfluidics in collecting dynamic features of biosensors. A modular microfluidic platform featuring spatially isolated hydrodynamic (HD) cell traps was built, which could be modified for

screening multiple strains in parallel to a single condition or a single strain to multiple conditions in parallel. Microfluidic devices offer insight into biosensor behavior over time, laying the groundwork for integration of time-based metrics in the biosensing space. Characterization of the engineered constructs revealed significant crosstalk due to off-target detection, limiting their practical utility. Furthermore, the stressors of various sensing environments, such as the high salinity of seawater, can cause autofluorescence, making the biosensors potentially unreliable in new stressful environments. To move towards deployment, we need to engineer methods in biosensing that are robust to crosstalk and to stressful environments. Future work should integrate dynamic features and consider combinatorial detection for practical deployment, to overcome the pervasive issue of non-specific biosensors. Chapter 3 ('Generating diversity for machine learning-assisted combinatorial biosensing') presents an exploration of some of these ideas.

2.7 Acknowledgements

This chapter contains material currently under review at *Cell Systems* as 'A diversified consortium of engineered biosensors enables multi-target classification'. Alyssa Jasmine Chiang, Nicholas Csicsery, Richard O'Laughlin, Leo Baumgart, Elizabeth Stasiowski, Phuc Nguyen, Austin Doughty, Myra Ashraf, Raegan Mink, Elina C. Olson, Michael Ferry, Adam M. Feist, Nan Hao, Karsten Zengler, and Jeff Hasty. The dissertation author was one of the primary authors and researchers of this material. This chapter also contains material reproduced from *Current Opinion in Microbiology* as 'Design of synthetic bacterial biosensors'. Alyssa Jasmine Chiang and Jeff Hasty. The dissertation author was the primary author of this material.

Chapter 3

Generating diversity for machine learning-assisted combinatorial biosensing

3.1 Introduction

Whole-cell biosensor development has focused largely on engineering and tuning strains for specificity and sensitivity in detecting single targets within a controlled laboratory environment¹³¹. These efforts have resulted in sensors for the detection of compounds including heavy metals^{93,94,99,132,133}, caffeine¹³⁴, aromatics^{36,59}, and waterborne pathogens^{96,135}. WCBs have employed a variety of sensing approaches, including DNA-^{46,63}, RNA-^{61,63}, transcription factor-based^{43,58,93,99,134,136}, and optical approaches^{137,138}, and more recently, evolutionary and rational engineering approaches have expanded the repertoire of possible targets for WCBs^{36,58,59,61,66}. While these advances have vastly improved WCB capabilities, their focus on single-target sensing in well-characterized and controlled environments limits their deployability in complex environments, leaving a gap in realizing WCBs with field potential^{139,140}. In fact, single-target biosensors often suffer from crosstalk, preventing the simultaneous detection of multiple biomarkers in a sample reliably^{135,141}. Furthermore, WCBs characterized under laboratory settings face difficulties in survival, long-term stability, and signal interference^{82,121} under environmental stressors, including temperature extremes, pH, and osmolarity, as well as exposure to toxins and oxidative compounds¹⁴².

Here, instead of focusing on optimizing single-target biosensors, we shift our attention to

sensing multiple target analytes in a mixed sample under high salinity seawater conditions. We harness adaptive laboratory evolution (ALE) with the well-characterized *E. coli* K-12 MG1655 laboratory strain to efficiently generate a library of diverse biosensor signals to five target heavy metals of concern in polluted seawater samples^{108,110}, circumventing the problem of environmental fitness defects and the need to characterize new chassis altogether, without painstaking rational engineering that would require *de novo* information about microbe stress tolerance. To more fully capture the phenotypic diversity of the evolved library, we use our microfluidic screening platform developed in Chapter 2 to elucidate WCB dynamic behavior in continuous culture with high temporal resolution^{99,102,143}. Our modular microfluidic design allows parallel screening of multiple strains or conditions in a single experiment. Exploiting our WCB diversity, we train a simple supervised learning regression model to quantify a combination of heavy metals by disentangling the consortium of signals generated by an array of individual strains exposed to approximately 500 distinct combinations of the five heavy metals in seawater. Analogous to a fingerprint or barcode, using a consortium of responses instead of relying on single-target WCB responses yields estimations of heavy metal concentrations that outperform those of single-target biosensors, which are confounded by signal crosstalk.

A microbial consortium paradigm of WCBs opens a path toward field deployability, by shifting the engineering approach from optimization of single-target WCBs toward maximization of data collected from WCBs. The consortium-based biosensor can be transposed to a myriad of diagnostic applications that require quantification of multiple biomarkers under stressful environmental conditions, where single-target WCBs may fall short.

3.2 Generating diversity

We aimed to integrate multiple biosensor signals to enhance sensor performance under deployment conditions where combinations of analytes are present. Although novel chassis using native bacteria to circumnavigate the pressure of withstanding environmental stressors are

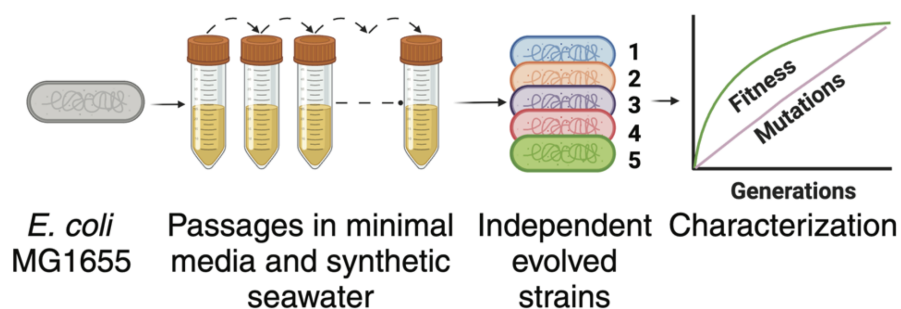


Figure 3.1. Adaptive laboratory evolution of laboratory *E. coli* K-12 MG1655 to tolerate seawater conditions. The adaptive laboratory evolution process. Five independent replicates of *E. coli* MG1655 were serially propagated in flasks in equal parts minimal media and synthetic seawater (HM9/seawater) using an automated platform. Evolved populations were cryopreserved with every marked increase in growth rate. Final evolved strains were isolated at approximately 150 passages, plated on agar. Created with BioRender.com.

an active area of research, genetic tractability and engineered circuit behavior portability remain challenging and hamper rapid engineering for biological applications^{45,48,49}. On the other hand, adaptive laboratory evolution (ALE) enables a well-characterized and engineerable strain to efficiently acquire mutations favorable to its fitness through prolonged propagations in selected conditions, such as high salinity, extreme pH, or high temperatures^{144–147}.

3.2.1 Adaptive laboratory evolution

We aimed to harness the high-throughput, automated capability of ALE to not only enhance the fitness of the *E. coli* MG1655 laboratory strain in seawater, but also to generate genotypically distinct variants with different phenotypic behaviors without compromising strain tractability or requiring extensive characterization and tuning.

The laboratory *E. coli* K-12 MG1655 strain has been well-characterized and engineered for many purposes, including increased tolerance against toxins¹⁴⁸, improved metabolic function¹⁴⁹, and enhanced detection of pathogens of interest¹⁵⁰. Previously, MG1655 strains have been successfully evolved to grow rapidly on the non-native substrate sucrose¹⁴⁷ and in minimal media¹⁴⁶. As such, we evolved the wild-type MG1655 laboratory strain in synthetic seawater to improve its fitness under high salinity conditions. Using a previously developed automated ALE

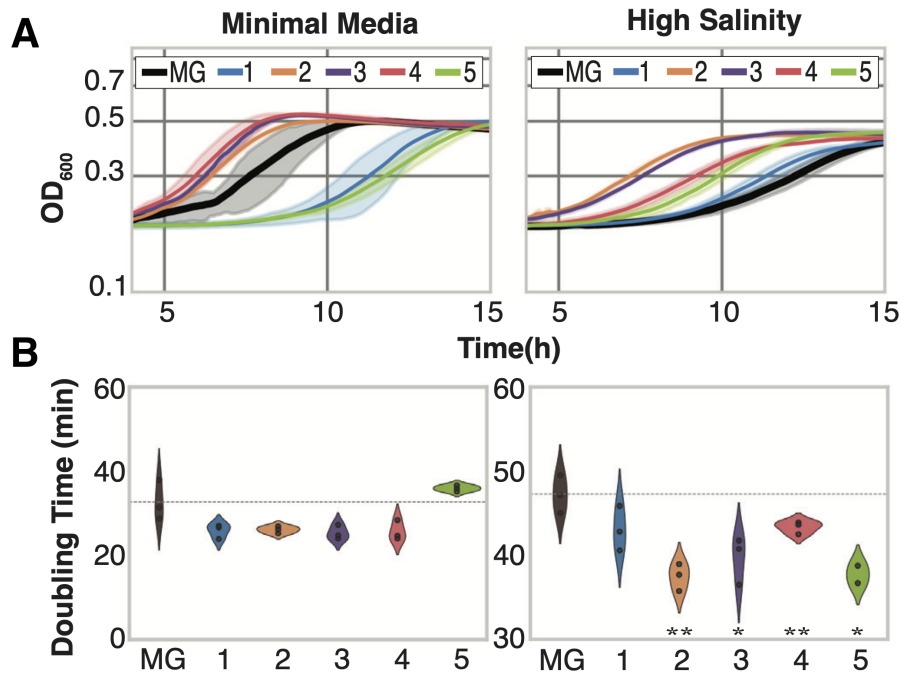


Figure 3.2. Improved growth of evolved strains in high salinity. (A) Plots of optical density as a proxy for growth for each of the evolved strains in comparison to the starting strain. Solid lines represent mean of N=3 independent wells, and shaded region represents standard deviation. The black line represents the wild-type *E. coli* MG1655 strain. (B) Doubling times in synthetic seawater are reduced for evolved strains, in comparison to the wild-type strain. *P = 0.05 and **P = 0.01.

platform^{146,147}, five independent replicates of the MG1655 strain were, in parallel, successively propagated in batch in equal parts minimal media and synthetic seawater (HM9/seawater) to accumulate and select for mutations corresponding to increased fitness in high salinity conditions. The resulting evolved strains, labeled ALE1-ALE5, were isolated from the final populations and whole-genome sequenced for comparison (Figure 3.1).

3.2.2 Improved growth

The final evolved strains exhibited a statistically significant improvement in doubling time in seawater, with at least 10% reduction below the starting wild-type strain (Figure 3.2), suggesting improved fitness to the high salinity environments presented by seawater. Evolved strains' growth rates and doubling times in HM9/seawater were estimated using Curveball, an

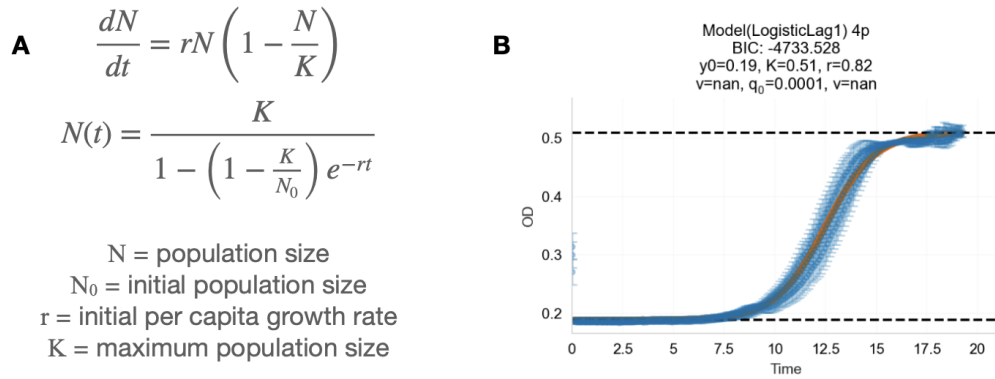


Figure 3.3. Growth rate calculation using Curveball. (A) Logistic model Curveball uses to estimate the growth rate. (B) Example output from Curveball logistic model.

open source software used for growth curve analysis from Tecan plate reader measurements¹⁵¹ (Figure 3.3). This marked improvement in growth of the evolved strains over the wild-type strain in high salinity was further validated with NaCl supplementation to minimal media (Figure 3.4), as a control. Furthermore, recent studies have indicated lag phase duration as an organized and adaptive process for bacteria, which can vary upon presence of stressors¹⁵². Under high salinity conditions, the wild-type strain presented a lag phase that extended almost two hours beyond its evolved counterparts, suggesting its sensitivity to higher osmolarity (Figure 3.2A).

3.2.3 Genotypic diversity

Sequence analysis of the evolved strains pointed to various independent mutations that distinguish evolved strains from the wild-type (Figure 3.5, Table 3.6). The *pyrE* and *rph* mutations were commonly associated with ALE growth rate selection in minimal media. *E. coli* K-12 wild-types have a frameshift mutation in *rph* causing pyrimidine starvation, which is typically remedied through the ALE process in minimal media^{146,153}. Apart from these, mutations varied among the independently evolved replicates. Some evolved strains exhibited *phoE* and *ompC* mutations; these are outer membrane porins, which regulate uptake of small molecules^{154,155}. To mitigate high salinity stresses, *E. coli* typically employ a strategy of uptake

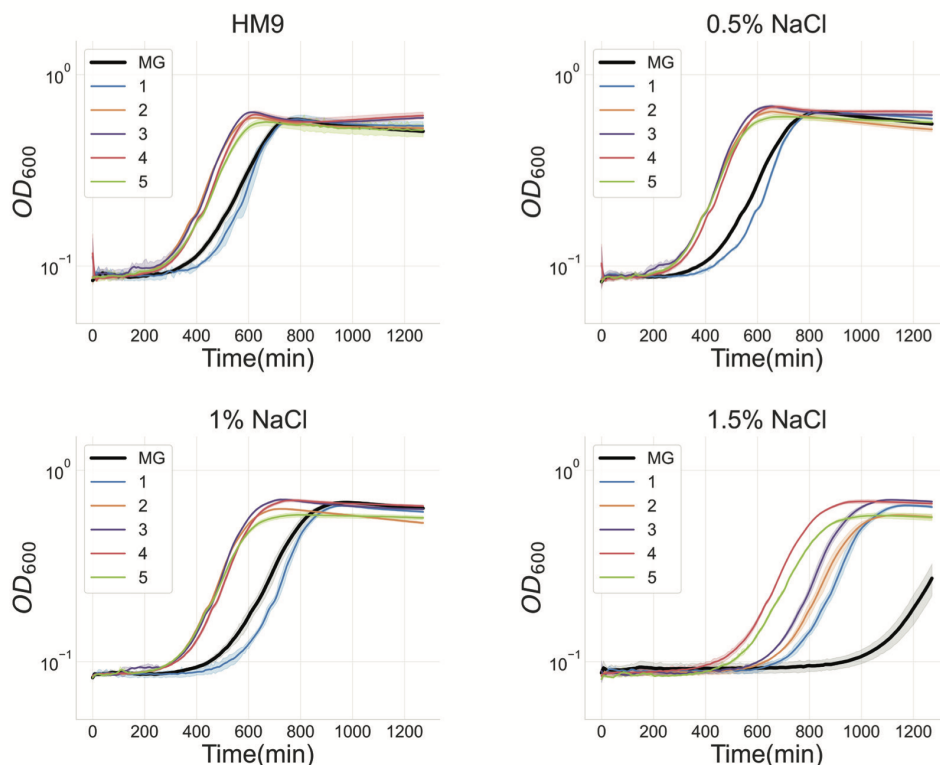


Figure 3.4. Growth curve controls with added NaCl. Growth curves of each evolved strain at different concentrations of NaCl. Solid lines represent mean of N=3 independent wells, and shaded region represents standard deviation. The black line represents the wild-type *E. coli* MG1655 strain.

or biosynthesis of biocompatible solutes, such as trehalose or L-glutamate to counterbalance the flood of potassium ions under high osmolarity conditions¹⁵⁶. As a result, these mutations could be related to biocompatible solute uptake. Studies in the past decade have strongly suggested that adaptation to stressful environments can often occur through loss of function, by rewiring the cell's metabolism without the need to refine enzymatic activities or acquire gain-of-function mutations^{157,158}. As such, observed mutations such as those in *nagA* and *nagC* may potentially be knockouts that confer stress resistance, as suggested in another recent ALE study with osmotic stress¹⁵⁹. Other mutations between strains were diverse and their independent or additive effects were unclear from sequence analysis alone. These data on the strains' mutations could serve as useful jumping points for future work on bacterial studies for high-salinity environments. The

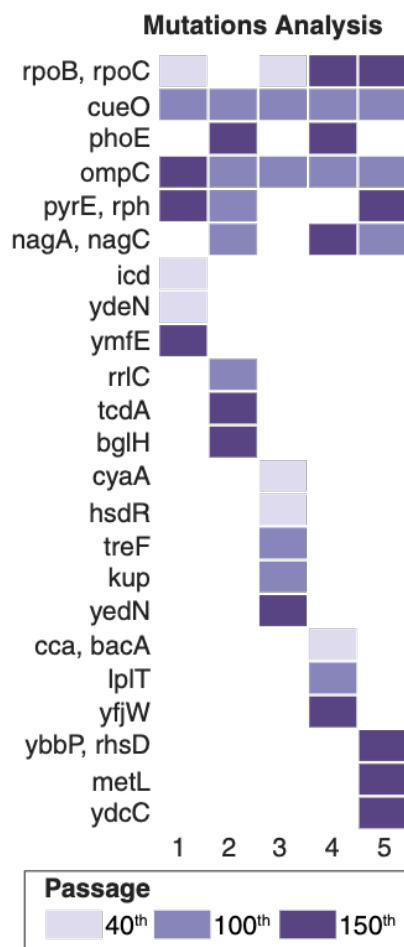


Figure 3.5. Genomic changes for each strain. Analysis of genomic changes across different timepoints of ALE process for each strain. Shaded boxes represent presence of mutation in identified gene, and color gradient represents flask number according to the color key.

mutations could be causally linked to their effects through knock-ins and knock-outs, which re-create the fine-grained sequencing analysis results. Transcriptomics studies could also further illuminate the functional effects of the mutations in the evolved strains. Finally, comparisons between the final evolved strains and previous strains in their respective lineages could offer some insight into the mutations' effects within the context of their evolutionary pathway. However, this study focused on the phenotypic diversity adaptive laboratory evolution produced and its applicability for a differential biosensing approach. We postulated that the evolved strains' genotypic diversity gives rise to phenotypic diversity.

Position	Mutation Type	Sequence Char	Gene	Details
Strain 1				
1,196,232	SNP	C->T	icd	T370T (ACC->ACT)
1,196,220	SNP	C->T	icd	H366H (CAC->CAT)
1,196,245	SNP	T->C	icd	L375M (TTA->CTG)
1,196,247	SNP	A->G	icd	L375M (TTA->CTG)
1,581,258	SNP	G->A	ydeN	P423S (CCC->TCC)
4,182,809	SNP	C->A	rpoB	S522Y (TCT->TAT)
4,183,814	SNP	T->A	rpoB	V857E (GTG->GAG)
138,616	MOB	IS5 (+) +4 bp	cueO	coding (1534-1537/1551 nt)
1,198,505	INS	(AATGATGA)6->	ymfE, lit	intergenic (-268/-190)
2,312,011	INS	(+)T	ompC	coding (739/1104 nt)
3,815,810	DEL	Δ 1 bp	pyrE, rph	intergenic (-42/+53)
Strain 2				
702,331	DEL	Δ 1 bp	nagA	coding (421/1149 nt)
138,575	SNP	C->A	cueO	A498E (GCG->GAG)
3,815,810	SNP	G->T	pyrE, rph	intergenic (-42/+53)
3,945,587	SNP	G->T	rrlC	noncoding (1884/2904 nt)
2,312,333	MOB	IS2 (-) +5 bp	ompC	coding (413-417/1104 nt)
260,264	MOB	IS5 (-) +4 bp	phoE, proB	intergenic (-164/-121)
700,858	SNP	C->A	nagC	G246V (GGC->GTC)
3,901,200	SNP	A->T	bglH	W341R (TGG->AGG)
2,945,057	DEL	Δ 384 bp	tcdA	coding (403-786/807 nt)
Strain 3				
3,440,459	SNP	G->A	rpoA	R191C (CGT->TGT)
3,993,490	DEL	Δ 1 bp	cyaA	coding (2338/2547 nt)
4,586,594	DEL	(G)6->5	hsdR	coding (168/3513 nt)
3,931,615	SNP	A->T	kup	S100S (TCA->TCT)
2,312,239	SNP	G->A	ompC	Q171* (CAG->TAG)
3,669,839	MOB	IS5 (-) +4 bp	treF	coding (248-251/1650 nt)
138,611	MOB	Δ 3 bp :: IS186 (-)	cueO	coding (1529-1535/1551 nt)
4,186,605	SNP	A->C	rpoC	H419P (CAC->CCC)
2,012,123	SNP	C->A	yedN	pseudogene (229/1129 nt)
4,584,395	SNP	G->T	hsdR	I789I (ATC->ATA)
Strain 4				
3,203,158	DEL	Δ 91 bp	cca, bacA	intergenic (+29/+62)
2,973,393	SNP	A->C	lplT	V157G (GTG->GGG)
137,918	SNP	A->G	cueO	E279G (GAG->GGG)
2,312,023	DEL	Δ 633 bp	ompC	coding (95-727/1104 nt)
4,186,532	SNP	A->G	rpoC	K395E (AAA->GAA)
259,748	MOB	IS1 (+) +9 bp	phoE	coding (345-353/1056 nt)
2,774,047	SNP	A->G	yfjW	T244A (ACA->GCA)
701,623	INS	(TTGAGTTACGA)	nagA	coding (1129/1149 nt)
Strain 5				
702,276	SNP	A->C	nagA	L159R (CTG->CGG)
137,093	SNP	G->T	cueO	R4L (CGT->CTT)
523,224	SNP	C->T	ybbP, rhsD	intergenic (+394/-37)
4,132,235	SNP	T->G	metL	S801A (TCG->GCG)
3,815,811	SNP	C->T	pyrE, rph	intergenic (-43/+52)
702,244	SNP	T->G	nagA	T170P (ACC->CCC)
2,312,740	DEL	Δ 1 bp	ompC	coding (10/1104 nt)
137,092	SNP	C->T	cueO	R4C (CGT->TGT)
4,183,826	SNP	C->A	rpoB	A861E (GCG->GAG)
1,532,261	SNP	C->A	ydcC	S149Y (TCT->TAT)

Figure 3.6. Specific mutations from ALE. Extended mutation information from ALE process, broken down by strain.

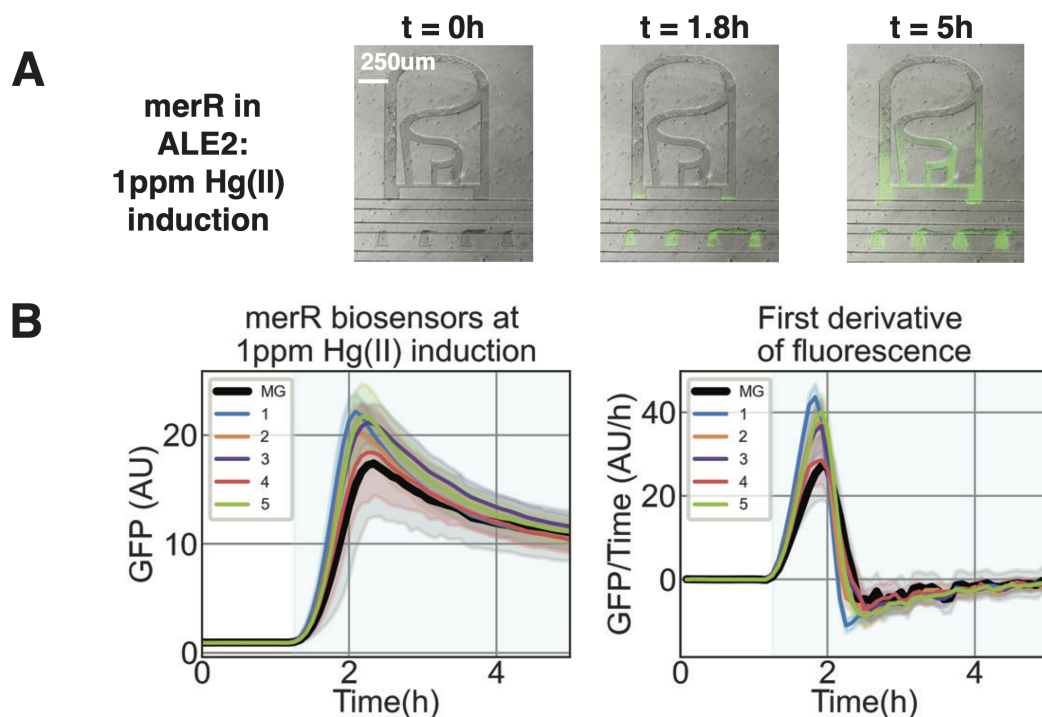


Figure 3.7. Sample experiment with merR variant phenotypic characterization. (A) A 48-strain multiplexed microchemostat array was employed to simultaneously induce each evolved strain carrying the same heavy metal-sensing plasmid in HM9/seawater. Images of merR-ALE2 induced with 1ppm Hg(II) in multi-strain device are displayed. (B) Fluorescence time traces of each merR biosensor variant, in comparison to merR-MG1655. Solid lines represent mean fluorescence (N=8 HD traps) measured from HD trap regions for each strain, while shading around lines represents standard deviation. Dark line represents the merR-MG1655, while all other lines represent evolved WCBs. Shading indicates 4-hour induction period.

3.3 Characterizing phenotypic diversity

The six engineered plasmids were transferred into the five newly evolved bacterial chassis variants, producing a library of 30 strains. To evaluate the WCBs' diversity, they were characterized in our microfluidic screening devices to evaluate temporal sensor performance diversity across multiple chassis in HM9/seawater media. Representative temporal fluorescence data for merR WCBs collected using the 48-strain microfluidic array are displayed (Figure 3.7). Similarly to the wild-type WCBs, four-hour pulses of each metal yielded first-order-like fluorescence responses, before relaxing upon metal removal. Microfluidic characterization of the strains allowed analysis of temporal metrics beyond the fluorescence intensity, thereby revealing rich phenotypic

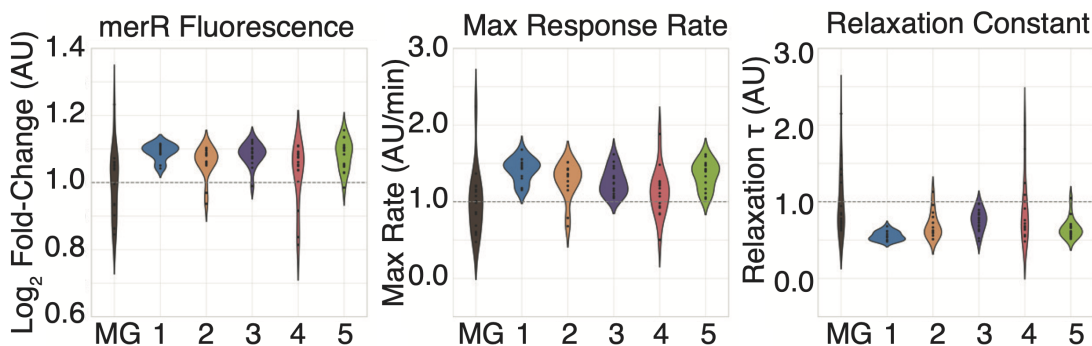


Figure 3.8. Temporal metrics of merR WCBs from microfluidic experiments. Maximum fluorescence fold-change of each merR biosensor in comparison to merR-MG1655, (left), maximum response rate for each evolved merR biosensor (center), and relaxation time constant for each evolved merR biosensor (right). merR-MG1655 is normalized to 1 for all metrics.

diversity across the evolved strains. In fact, the merR WCBs exhibited diverse dynamic response phenotypes in several critical parameters, such as the maximum fluorescence response, maximum response rate, and rate of relaxation (Figure 3.8). The maximum response rate was computed as the maximum of the first derivative of the fluorescence time series. The response relaxation was analogous to the voltage relaxation in resistor-capacitor (RC) circuit charge pulse¹⁶⁰. As such, the characteristic time constant, τ , was computed as a proxy for the response rate of relaxation. Screening multiple strains simultaneously allowed for rapid evaluation of response diversity between evolved strains, with the availability of a variety of informative metrics from each experiment (Figure 3.9A). This opens the door to efficient biosensor variant selection for particular biosensing applications. For instance, if rapid response rates are required, ALE2 may be desirable, given the relatively faster response rates of the *zntA*, *arsR*, and *cadC* variants. Individual temporal data for the other biosensor variants in are available in Figure 3.9B. The diversity in SNR, response rates, and relaxation constants highlighted the phenotypic diversity achieved through the ALE process across evolved variants.

3.3.1 Single-strain characterization in microfluidics

We further demonstrated our microfluidic system capabilities in single-strain characterization by using our single-strain gradient generator device to efficiently screen individual biosensor

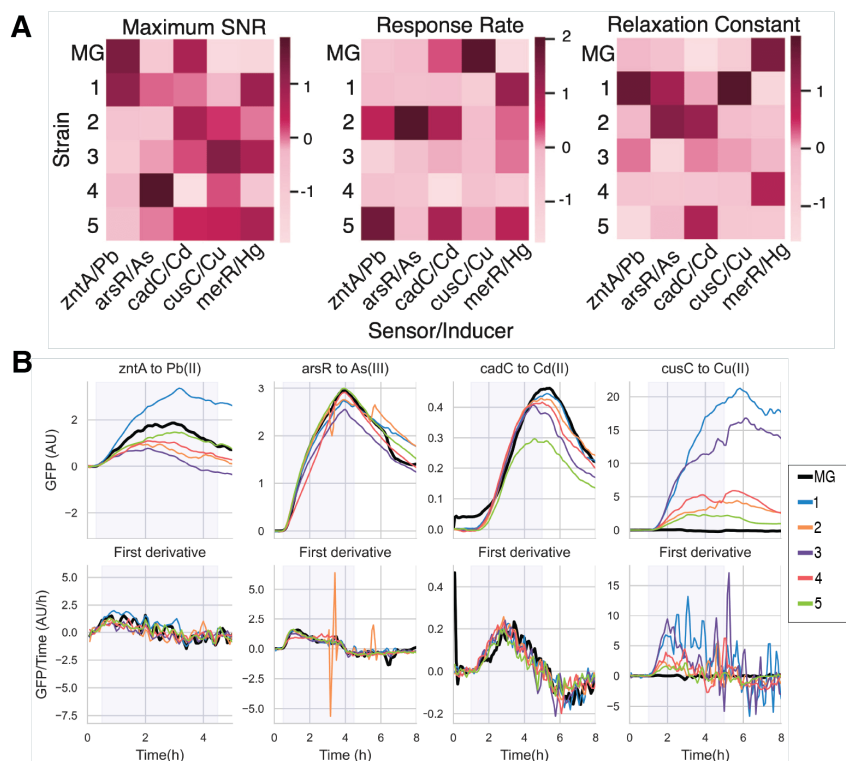


Figure 3.9. Evolved strains exhibit diverse dynamic behaviors, including fluorescence, response ramp rate, and response relaxation rate in the presence of seawater. (A) Maximum signal-to-noise ratio, maximum response ramp rate, and relaxation time constant for each biosensor strain in the presence of the indicated heavy metal. Respective units of measure are the same as in Figure 3.8. (B) Top row displays time traces of zntA, arsR, cadC, and cusC WCBs, in comparison to the MG1655 WCB. Solid lines represent mean fluorescence measured from N=8 HD traps for each strain. Black line represents the MG1655 WCB, while all other lines represent evolved WCBs. Heavy metal inductions were 1 ppm for all. Bottom row displays first derivative of fluorescence time traces from top row. Purple shaded region indicates the 4-hour induction period.

responses to multiple conditions in parallel. This device is easily vacuum-loaded and allows a single strain to be exposed to a discrete gradient of inducer concentrations. We employed this device to examine the responses of the arsR-MG1655 and arsR-ALE2 biosensors to multiple metal concentrations simultaneously (Figure 3.10). The sensors were subjected to a gradient of arsenic concentrations automatically generated in the microfluidic device. Sulforhodamine dye, an inert dye that does not affect cellular response¹⁶¹, was used to visually indicate the gradient across the device as the channels mix, as shown in sample experiments (Figure 3.10A). At a

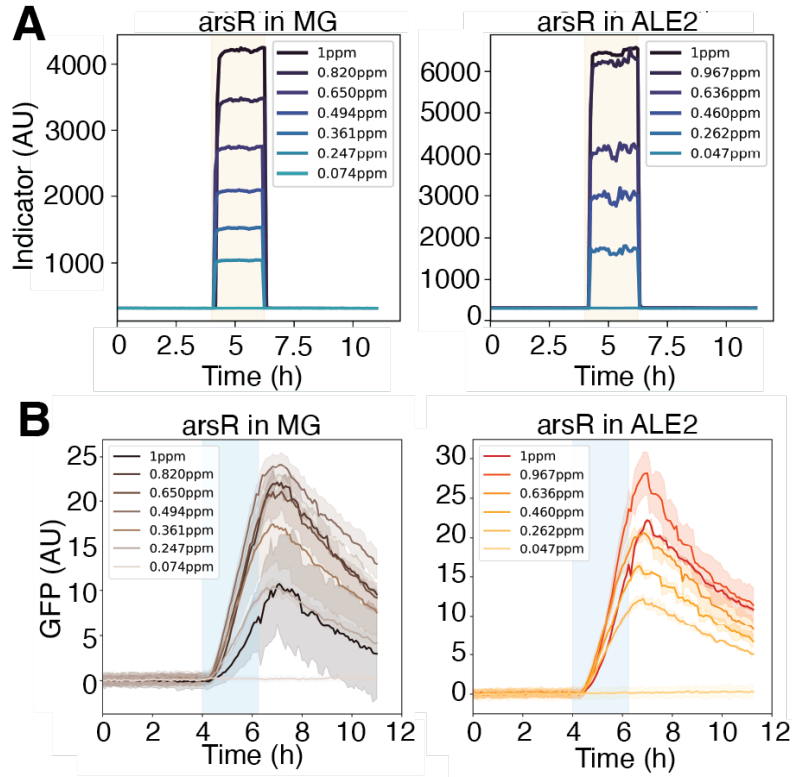


Figure 3.10. arsR-ALE2 v. arsR-MG1655 in single-strain gradient generator. (A) Gradient in concentrations as measured by sulforhodamine dye, simultaneously generated by the microfluidic device. Shaded area indicates the period of induction, and color gradient from light to dark mirrors the increase in concentration of As(III). Solid lines represent mean fluorescence (N=4 HD traps) measured from HD trap regions for each strain. (B) MG1655 and ALE2 carrying arsR biosensor, exposed to simultaneous gradient of As(III) concentrations. The blue shaded box indicates when heavy metal is introduced. Solid lines represent mean fluorescence (N=4 HD traps) measured from HD trap regions for each strain, while shading around lines represents standard deviation.

maximum concentration of 1ppm arsenic, the sulforhodamine dye indicated the concentration gradient generated as the channels mixed the dyed 1ppm-arsenic inducer media with plain HM9/seawater media. The temporal GFP responses of the two strains are displayed for each concentration generated by the device (Figure 3.10B). A gradient of fluorescence intensities can be seen corresponding to the gradient of arsenic concentrations generated, with the strains exhibiting the same first-order behavior, followed by a signal relaxation upon inducer removal.

Representative images from the experiment are shown in Figure 3.11A. Using the maximum fold-change responses from the temporal data, a Hill function was fitted to the responses

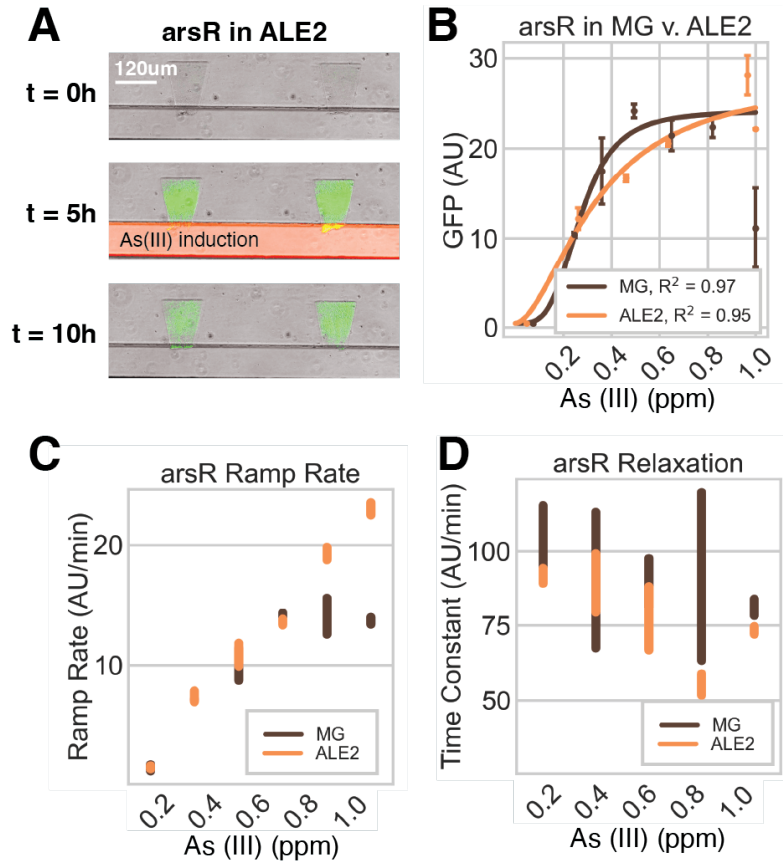


Figure 3.11. *arsR*-ALE2 v. *arsR*-MG1655 dynamic characterization. (A) Images from microfluidic experiments before, during, and after induction of ALE2 carrying *arsR*. Sulforhodamine dye (red) was used as a visual indicator for the presence of heavy metal upon induction. (B) Hill-curve fit of *arsR*-MG1655 and *arsR*-ALE2. (C) Maximum response ramp rate of *arsR*-MG1655 and *arsR*-ALE2 strains at each As(III) concentration. (D) Relaxation time constant of *arsR*-MG1655 and *arsR*-ALE2 strains at each As(III) concentration.

of *arsR*-MG1655 and *arsR*-ALE2 (Figure 3.11B). The Hill curves revealed that fluorescence response monotonically increased in correspondence with heavy metal inducer concentrations for a wider concentration range in *arsR*-ALE2 than in the wild-type strain, with *arsR*-MG1655 reaching its maximum at approximately 0.494 ppm and *arsR*-ALE2 at 0.967 ppm, nearly double. The concentrations where a drop-off in fluorescence occurred were omitted from the Hill curve fit, as the responses were no longer reflective of increasing metal concentration. By using the single-strain gradient device, this difference in monotonic response range was rapidly illuminated.

Beyond GFP fold-change, temporal characteristics, including response ramp rate and relaxation rate, were observed, similarly to the multi-strain device (Figure 3.11C-D). For relaxation rate, concentrations yielding no significant response (defined as below 1 AU) were excluded. The response ramp rates and relaxation rates, which varied with induction concentrations, provide an additional metric for biosensing that is unavailable to single-timepoint approaches. For example, the response ramp rate increased monotonically for the wild-type strain up to approximately 0.6 ppm, despite its fluorescence fold-change capping at approximately 0.5 ppm; therefore, the ramp rate provided an additional metric that could improve the predictive accuracy of a biosensor beyond single-timepoint measurements. Taken together, the multiplexed dynamic characterizations of the WCBs with high temporal resolution demonstrated the response diversity across the evolved strains, achieved with no additional rational engineering or need for *de novo* information about stress resistance. This response diversity could provide more information in the presence of a combination of analytes in deployment conditions than single-target laboratory-characterized WCBs.

3.4 Combinatorics

A longstanding weakness of WCBs is their difficulty with combinatorial detection, as most samples contain multiple targets, causing biosensor crosstalk and complicating accurate detection¹⁴¹. As such, a biosensor that could sense a combination of analytes in parallel would be economical and efficient. In nature, olfaction and taste are a result of an input of multiple receptor signals, which are interpreted by the brain as smells or flavors²⁵. Rather than perfecting single-target biosensors, taking utilizing a combination of them as a collective input could yield more accurate outputs. Supervised learning classification approaches have recently been purposed for interpreting biological data^{162–165}. Recent studies have explored this idea of sensing using multiple signals, in "differential" or "combinatorial" approaches, such as electronic noses or tongues^{25,64,65}. The bacterial biosensor space has yet to more extensively incorporate this

concept, but some groups have begun to explore its potential for historically challenging sensing problems^{26,166}. The rich diversity of response phenotypes observed in microfluidics across sensor strains generated through ALE offered a promising method for a differential sensing approach. To this end, we combined the diverse response phenotypes of the WCBs to develop a consortium-based model for biosensing using a supervised learning classifier, benchmarking the model's predictive accuracy against that of single-target WCBs, particularly in classifying a combination of all five heavy metals simultaneously.

As a proof of concept, we used batch culture Tecan plate reader experiments to characterize features of the evolved strains in high-throughput. Though these did not provide dynamic features or insight that microfluidics would, they were insightful in demonstrating combinatorial capabilities. As such, the evolved WCBs were exposed to different concentrations of each heavy metal to evaluate their dose-dependent responses in HM9/seawater (Figure 3.12, Figure 3.13A). The response profiles differed between evolved strains, offering a selection of sensors that can be chosen depending on the desired detection ranges or sensitivities for particular biosensing applications. Across the evolved strains, there is a diversity in maximum fluorescence intensity, autofluorescence baselines, and ranges of fluorescence or sensitivity.

Furthermore, we examined the evolved WCBs' fold-change responses to each heavy metal of interest to evaluate cross-specificity in HM9/seawater (Figure 3.13B). Interestingly, the evolved strains, even when carrying the same plasmids, did not display the same levels of specificity or cross-specific interactions. In fact, some of the sensor strains exhibited greater responses to heavy metals they were not designed for. For example, *merR* and *arsR* in ALE1 become responsive to the other metals. Most of the *zntA* biosensors displayed promiscuity, similarly to the wild-type *zntA* biosensor, likely because it is a P-type ATPase that works as a pump for multiple divalent heavy metals, including lead, cadmium, and zinc^{167,168}. Notably, *cadC* exhibited a response to cadmium with weak specificity, but some of the evolved strains carrying *cadC*, such as *cadC*-ALE5, responded strongly to other metal inducers, such as mercury and arsenic. The *zraP* biosensors showed strong specificity to mercury, and high promiscuity to

other heavy metal inducers, though it was initially designed for detecting lead.

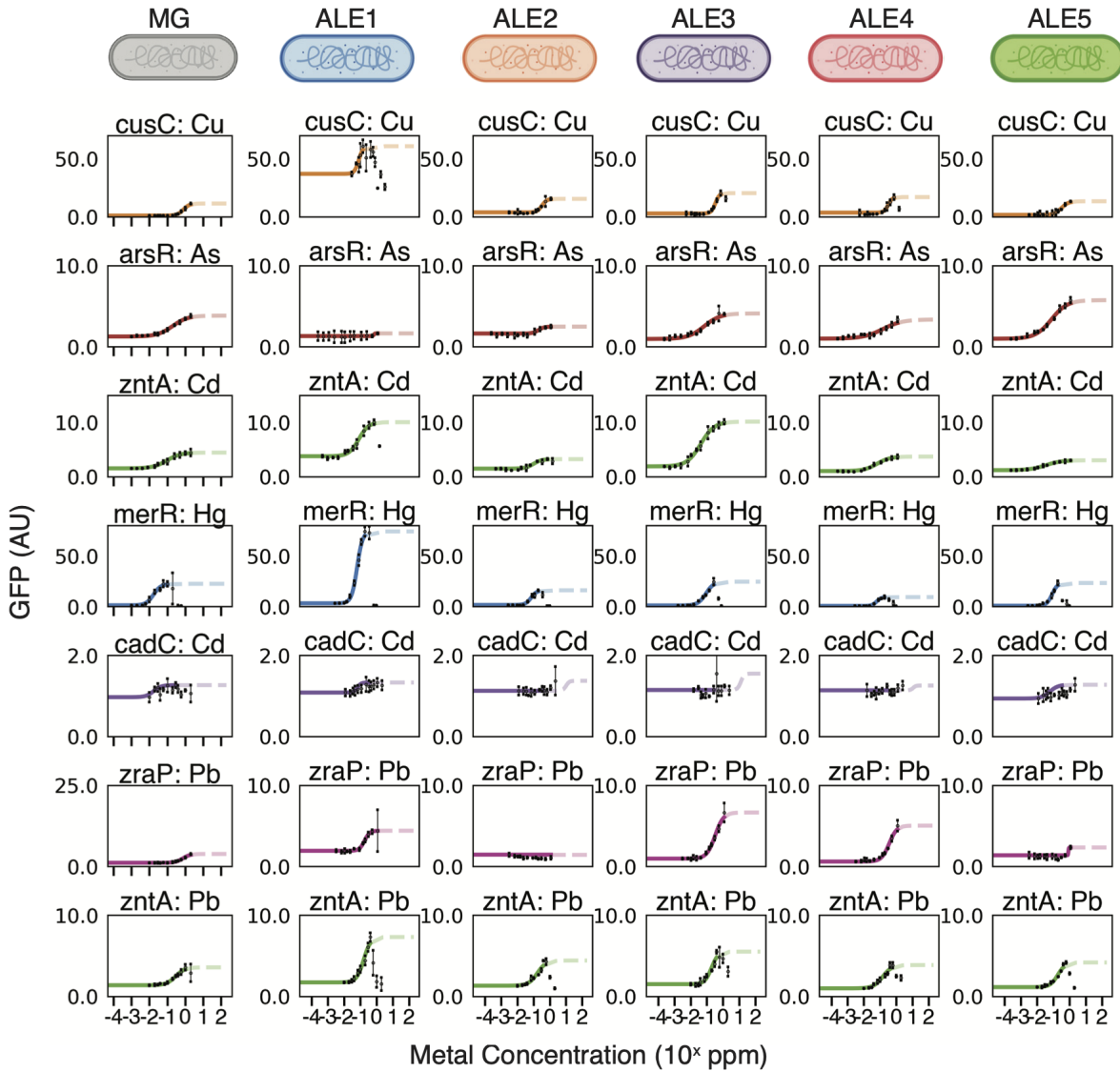


Figure 3.12. Dose-response curves of evolved strains in high salinity conditions. Batch experiment in Tecan fluorescence plate reader to compare dose-response behaviors of MG1655 WCBs to evolved WCBs in background of HM9/seawater. Concentrations indicated by dashed line affect cell growth and were not used to fit the dose-response curve. Error-bars represent standard deviation of triplicate samples.

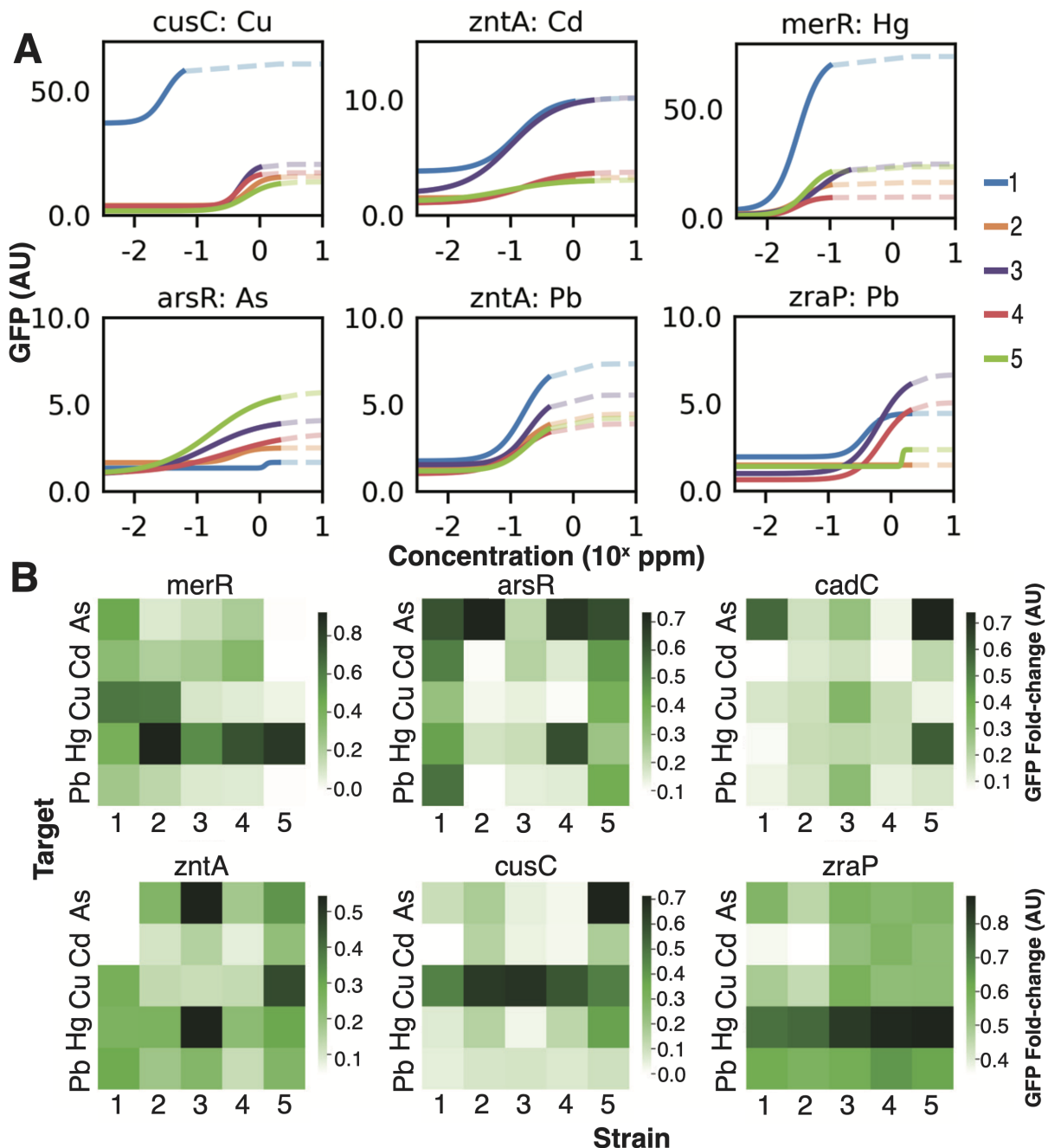


Figure 3.13. Response diversity across evolved strains. (A) Dose-response curves of each evolved biosensor strain in HM9/seawater when exposed to the heavy metal it was engineered to detect, showcasing the strains' diversity. Data shown are mean, with $N=3$ independent replicates, and solid and dashed lines are as indicated in Figure 2.12B. (B) Characterization of specificity across evolved strains indicated potential for machine learning classification due to their diversity. Each strain, MG1655 and evolved versions, carrying each sensor plasmid, was exposed to each heavy metal. The color gradient corresponds to each sensor variant's fold-change response to the corresponding metal. Heavy metal induction concentrations are the same as in Figure 2.13.

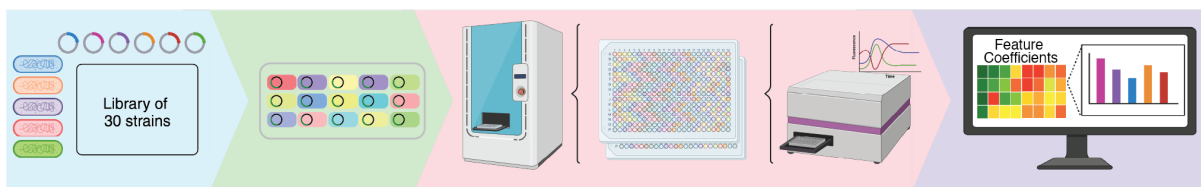


Figure 3.14. Machine learning training. Schematic illustrating development of a training set for a supervised learning classification model. A collection of 15 strains was selected and exposed to approximately 500 heavy metal combinations, resulting in a rich training set for developing a machine-learning model. Created with BioRender.com.

Instead of treating multi-target specificity as a hindrance as in conventional single-target biosensors^{141,169}, we sought to use the combined responses of multiple strains in combinatorial biosensing to combat crosstalk effects and produce more robust predictions. By examining the combined phenotypic behaviors of selected sensor strains in parallel, we could generate a distinctive response signature, similar to a barcode or a fingerprint, to quantify individual metals.

3.4.1 Training supervised machine learning models

As such, a more compact set of 15 WCBs was selected from the full set of biosensors available to test a combinatorial prediction of heavy metals in a sample using a consortium of strains (Figure 3.14). Three variants for each heavy metal with varied specificity and sensitivity were randomly selected to generate a collective response. Strains *cadC-ALE1*, *cadC-ALE3*, and *cadC-ALE5* were included, since they exhibited varied but clear specificity to heavy metals outside of cadmium, though cadmium was their intended target. The *zraP* WCBs were excluded due to their strong responses to mercury and high promiscuity, since most of the *merR* WCBs already exhibited strong specificity for mercury.

Using an ECHO automated acoustic droplet handler, a total of approximately 500 heavy metal combinations, each of which consisted of different concentrations of each of the five heavy metals, was introduced to each of the 15 biosensors independently to collect a training dataset in high-throughput (Figure 3.14). Features were extracted from the batch data for proof-of-concept, including fluorescent fold-change response maxima and minima, number of

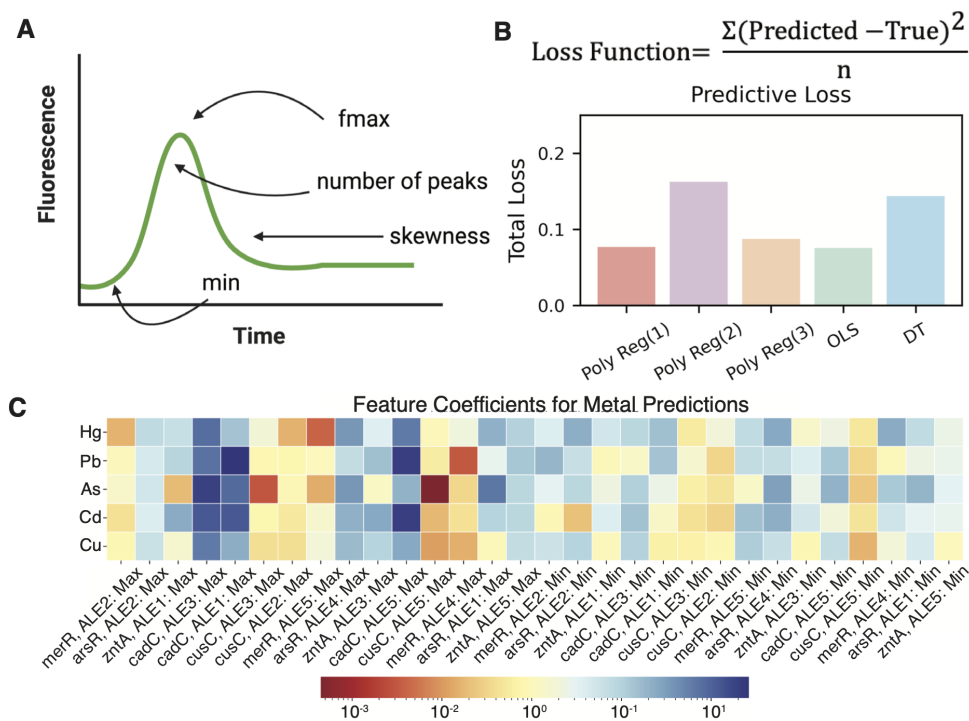


Figure 3.15. Feature engineering and supervised learning model selection. (A) The maximum, minimum, number of peaks, and skewness were among common time-series features that were evaluated. These 4 exhibited the widest distribution, indicating potential to contain information useful to classification. (B) A loss function was applied as a standard metric for evaluating predictive loss in models. Total predictive loss of first, second, and third degree polynomial regression models, ordinary least squares, and decision tree models is shown. (C) Feature coefficients for the max and min features in the ordinary least squares classification are shown to illustrate how the model makes decisions.

peaks, and curve skewness, to generate a distinct response signature of each of the 15 WCBs to each combination (Figure 3.15A). These 15 response signatures were concatenated to create a single distinct "barcode" for each heavy metal combination. Using these "barcodes", supervised machine learning regression models were trained on these response signatures to quantify the concentrations of the five heavy metals present. Multiple supervised learning regression models were applied and compared, including polynomial regressions, decision trees, and ordinary least squares regression, using the scikit-learn Python packages¹⁷⁰. Using a k-fold cross-validation strategy to prevent model bias and overfitting, the training set of combinations was divided into k=6 segments, each of which served as a validation set while the model was trained on the

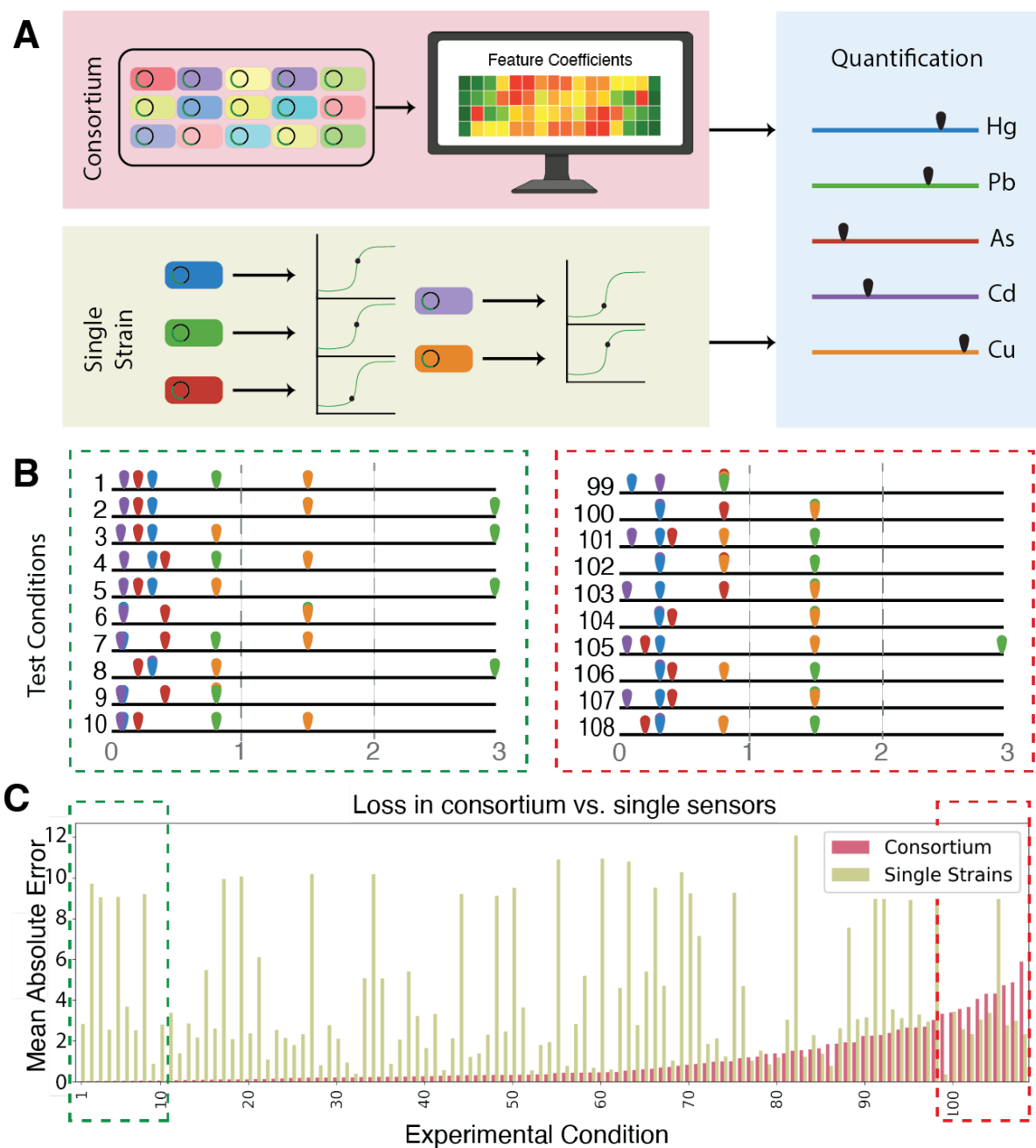


Figure 3.16. Consortium outperforms single-target biosensors in predictions of heavy metal combinations. (A) Schematic illustrating response from consortium vs. conventional single strain responses. (B) Sample test combinations, including the conditions in which the model performed the best and the worst. (C) Comparison of total loss across all heavy metal predictions according to loss function between consortium and single-target biosensor predictions, organized in order of increasing loss in consortium predictions. Heavy metal combinations of the consortium's best and worst predictions are specified in the tables.

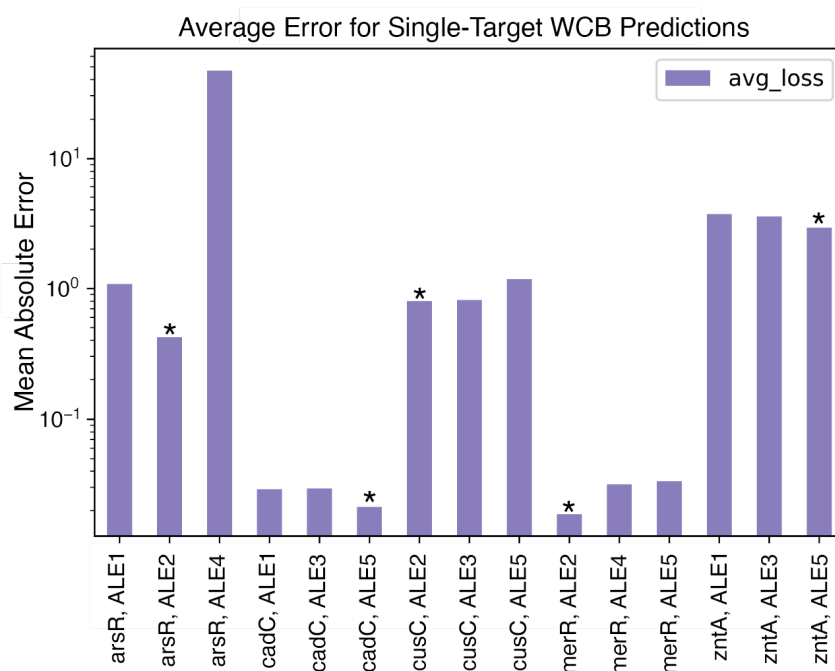


Figure 3.17. Selection of single-target WCBs with optimal performance. Each WCB’s response to its intended target in each test combination was measured using its corresponding dose-response curve. The average loss of each strain across all test combinations is displayed. Each heavy metal sensor’s strain exhibiting the lowest average loss was taken as the “best” single-target biosensor to compare with the consortium biosensor output, marked with ”*“.

other five segments. This process was repeated, with each of the six segments serving as the validation set. The mean absolute error (MAE) between the predicted and true concentrations of each heavy metal was computed. The sum of the MAE for each metal was totaled across all the conditions in each validation set, and this total error was averaged across the six validation sets. This average total MAE was used as a metric to compare the predictive strengths of each model (Figure 3.15B). The ordinary least squares model proved sufficient. One major disadvantage and critique of complex machine learning models is that they tend to be a “black box”, masking how the model makes decisions for the user¹⁷¹. However, the ordinary least squares model provides information about its decision-making process by revealing the magnitude of the coefficients it uses, elucidating its pathway to identifying each heavy metal concentration (Figure 3.15C).

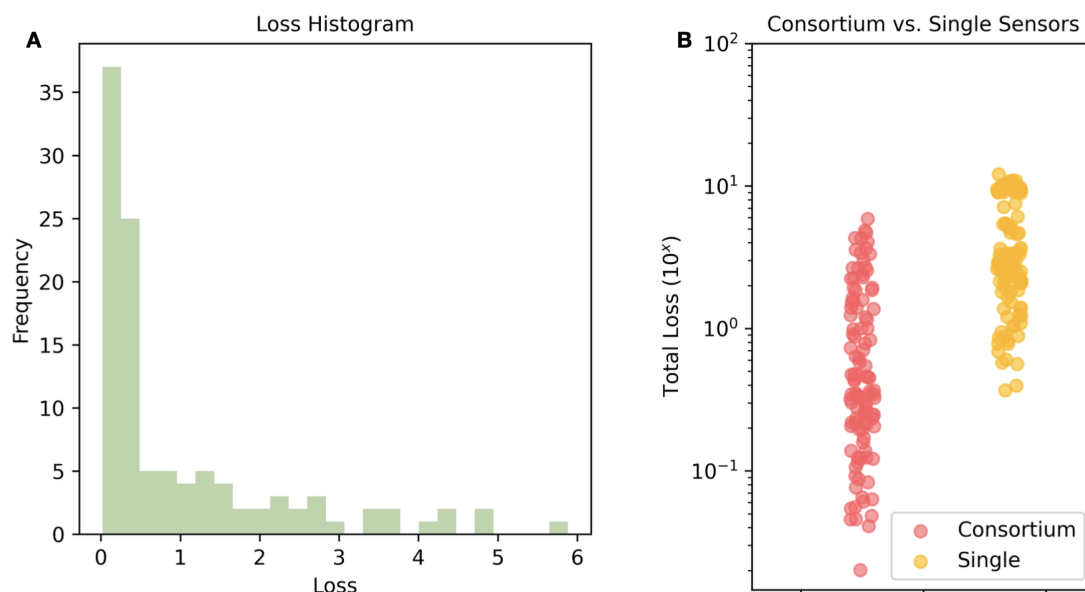


Figure 3.18. Consortium performance exceeds that of single-target sensors. (A) Loss histogram of consortium responses to test combinations. (B) Loss density plot comparing consortium and single-target biosensor paradigms.

Feature selection and extraction

The fluorescent fold-change maxima and minima, number of peaks, and curve skewness were features selected for analysis. The maxima and minima were extracted directly from the fluorescence time traces. The number of peaks and curve skewness were extracted alongside 163 other time-series features from the TSFELs package¹⁷², including temporal, statistical, and spectral features. To evaluate which features may offer the most information, the features for all the time-series for all 15 strains in response to approximately 500 heavy metal combinations were extracted and each feature's measurements were min-max normalized. The standard deviation for each feature's normalized measurements was computed. Those with the greatest standard deviation, number of peaks and skewness, were selected. However, ultimately, when observing the coefficients output by the ordinary least squares regression model, the number of peaks and curve skewness caused minimal effect, with maxima and minima offering the greatest effects.

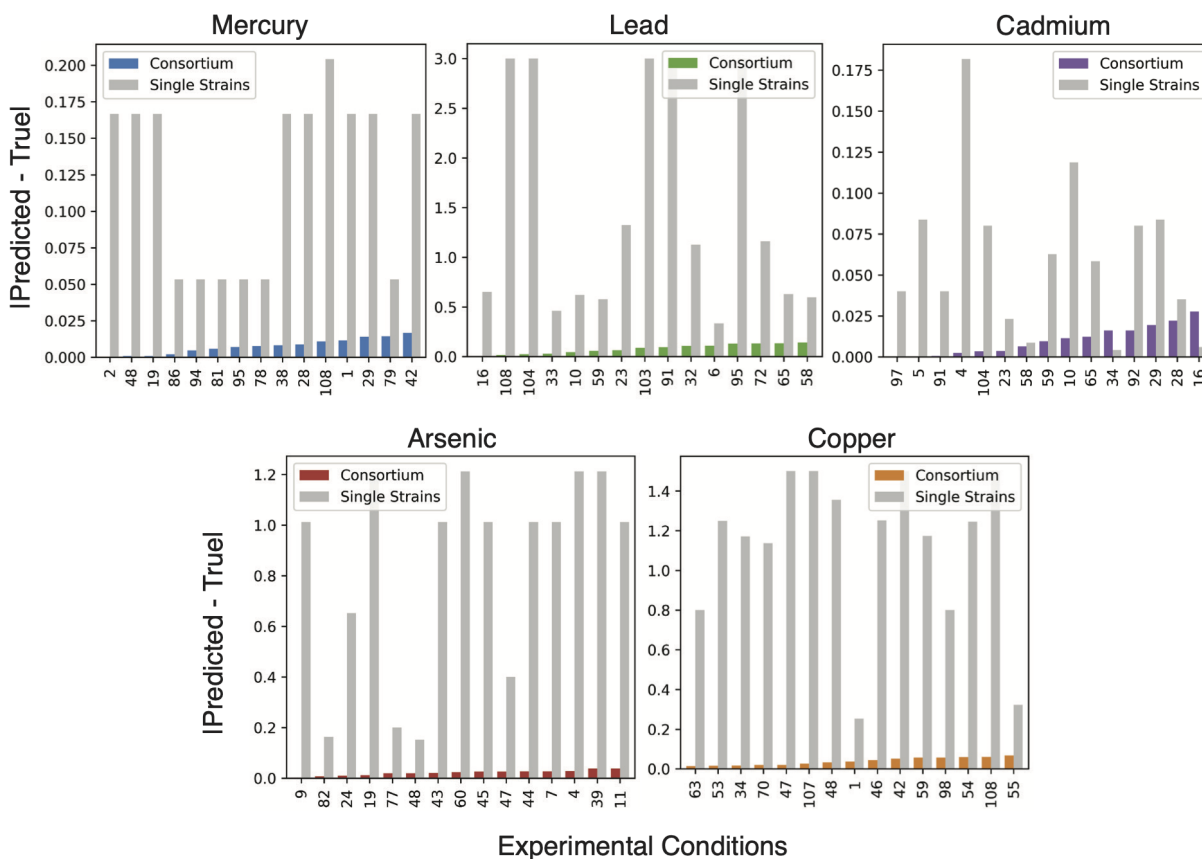


Figure 3.19. Consortium v. single-target biosensor performance, by heavy metal. Absolute difference for the top 15 consortium predictions and their corresponding single-target predictions, organized by heavy metal. Experimental condition labels correspond to those in Figure 3.16C. The proportion of experimental conditions in which the consortium outperforms the single-target predictions for each heavy metal is as follows: 68% for mercury, 78% for lead, 90% for arsenic, 46% for cadmium, and 86% for copper.

3.4.2 Testing the model

To evaluate the predictive capabilities of the model, a test dataset was collected with more than 100 new heavy metal combinations previously unseen to the model, and the model built using the training set was applied on this new test set. The consortium model's performance was compared to the conventional single-target biosensor paradigm (Figure 3.16A). Each individual WCB's predictions for its intended heavy metal target was computed using the respective biosensor's dose-response data. From these predictions, the WCB that output the lowest mean absolute error for each heavy metal was selected as the most optimal single-target WCB for that

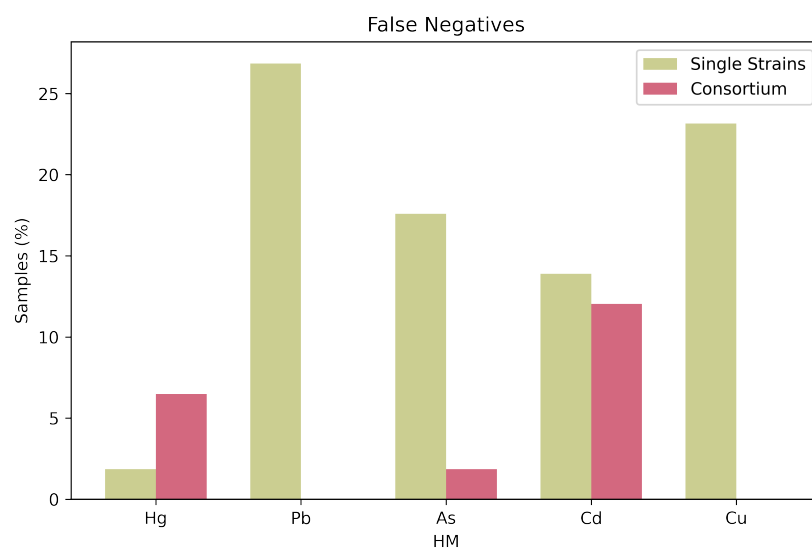


Figure 3.20. Consortium v. single-target biosensor false negatives. Count of false negatives output by consortium v. single-target biosensor predictions, by heavy metal. False negatives are defined as outputs 0 and below for consortium, and readouts below the lower limit of the biosensor’s Hill function for single-target biosensors.

heavy metal (Figure 3.17).

The mean absolute error of the consortium-based predictions were benchmarked against those of the collection of optimal single-target WCBs. Examples of test conditions are displayed in Figure 3.16B. The consortium-based predictions outperformed the single-target predictions in 93% of the test combinations, exhibiting significantly reduced total error in using the consortium’s responses (Figure 3.16B-C, Figure 3.18). We further analyzed the consortium-based predictive performance against those of the single-target sensors, broken down for each of the heavy metal constituents (Figure 3.19). For most of the heavy metals, with the exception of cadmium, the consortium outperformed the predictions of the single-target sensors in a majority of the sample combinations: 68% for mercury, 78% for lead, 90% for arsenic, 46% for cadmium, and 86% for copper.

Finally, false negatives can be more deleterious than false positives, when identifying whether a heavy metal is present at all. Generally, in biosensing applications, more conservative

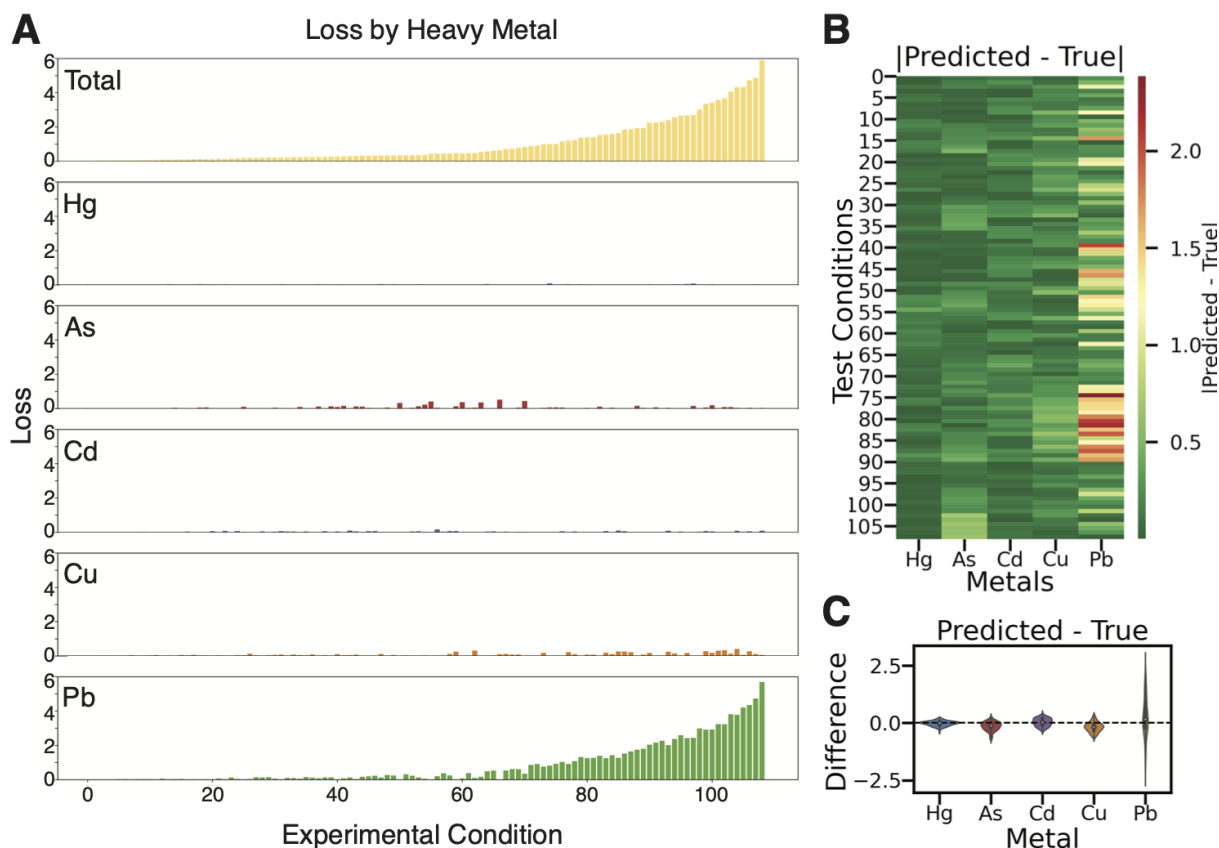


Figure 3.21. Analysis of loss identifies where consortium sensor needs improvement. (A) Total loss of consortium predictions, disaggregated into loss in each heavy metal individually. Experimental conditions are the same as in Figure 3.16B-C. (B) Heatmap of absolute difference between predicted and true heavy metal concentrations across experimental conditions, for each heavy metal. (C) Violin plots, by heavy metal, of difference between predicted and true values.

predictions are preferred. As such, the proportion of false negatives generated by the consortium-based and single-target sensors for each heavy metal was computed. Outside of mercury, the consortium-based model predictions reduced the false negative rate for each heavy metal constituent (Figure 3.20).

3.4.3 Model improvements

To further evaluate the consortium predictions, we separated the total mean absolute error into its individual heavy metal constituents to analyze each metal's error contributions (Figure 3.21A). This deconvolution revealed that mis-predictions in lead concentrations consistently

contributed a majority of the error in the conditions where the model faltered and produced greater error. This error is further exemplified in the absolute differences between the predicted and true concentrations across the experimental conditions (Figure 3.21B-C). With the exception of lead, the consortium predictions were mostly within 0.5 ppm of the true concentrations. Mercury and cadmium were the most closely predicted, with arsenic and copper consistently slightly underestimated. Lead predictions were vastly varied, which is likely due to the promiscuity of *zntA*, the primary lead sensor in the consortium. Further improvement of the consortium model could potentially be achieved by inclusion of more WCBs with diverse responses to lead, such as the excluded *zraP* biosensors, or tuning the lead sensor plasmid construct itself to produce more specific responses to lead. Analysis of the model provides useful information as to how to improve its performance.

3.4.4 Future model refinement

Although a subset of 15 strains was selected for this study as a proof-of-concept, this consortium could see further refinement in future extensions of the study. Using the training data, starting with a smaller combination of, say, 3 or 4 strains, we could repeat the study of training models and testing their performance using the same regression analysis and data collected. The performance would likely be poor at this point, but upon adding strains to the top few performing combinations, eventually we should expect the performance to level out with diminishing returns as more strains are added in. If so, this would also help pinpoint a narrower set of essential strains that are most useful in the decision-making and perhaps even highlight strains that are doing more harm than good to the heavy metal quantification. If not, then it can be expected that the model should be able to see further refinement with greater than 15 strains. Furthermore, if there exists a narrower set of essential strains, analyzing the features and their contributions to the model can be informative in terms of the diversity of behaviors we want to strive for, in biosensor design.

3.5 Materials and Methods

Adaptive laboratory evolution

Adaptive laboratory evolution (ALE) was carried out by Adam Feist using a previously described method^{146,147}. Briefly, five biological replicates from *E. coli* strain K-12 MG1655 were grown overnight in 1:1 HM9 to synthetic seawater (HM9/seawater). The HM9 minimal media composition, containing minimal traces of metal, was based on prior studies^{99,129} and optimized for microfluidic *E. coli* growth. Using an automated liquid-handler, this culture was serially passaged during exponential phase over approximately 140 passages into fresh HM9/seawater. Final strains used in experiments were isolated at the end of the evolution process and whole-genome sequenced.

Batch growth assays

Overnight cultures were grown in LB media at 37°C with antibiotics for 16 hours and reseeded into 1:100 diluted HM9/seawater media. Once an OD₆₀₀ of approximately 0.3-0.4 was reached as measured by the Spectrophotometer, they were diluted to OD₆₀₀ of approximately 0.01. Growth was then monitored by measuring OD₆₀₀ were taken every 5 minutes for 12-16 hours.

Plate reader experiments

Overnight cultures grown in LB media were reseeded into HM9/seawater at 1:1000 dilution and grown until they reached an OD₆₀₀ of about 0.3-0.4. For fluorescence dose-response curves, 96-well plates were prepared with media and varying heavy metal concentrations. Cultures were diluted to OD₆₀₀ of about 0.1 on the plate. Fluorescence values were measured using an Infinite® M200 PRO Multimode Microplate Reader. For high-throughput experiments, 384-well plates were used, and inducer was transferred to the plates using the Echo Acoustic Liquid Handler. For all plate reader experiments, fluorescence measurements were taken at maximum induction, which occurred around 4 hours into the experiment. This was normalized

by fluorescence of a promotorless strain to correct for cell autofluorescence and by OD₆₀₀.

Multi-strain microfluidic experimental protocol

See 'Chapter 2 Materials and Methods.'

Gradient single-strain microfluidic experimental protocol

Microfluidic experiments were performed on a Nikon Eclipse Ti epifluorescent inverted microscope (Nikon Instruments Inc., Tokyo, Japan). Two inlet ports and one outlet port were connected to 5mL syringes with Tygon tubing. One inlet contained HM9/seawater with Kanamycin and 0.075% Tween-20, and the other contained the same with a predetermined heavy metal concentration added, along with sulforhodamine dye for imaging. The waste outlet was filled with approximately 5 mL of MilliQ water, to provide negative pressure. The inlet and outlet syringes were separated by a 5" height difference. Initially, a tube slide clamp was used for the induction syringe to allow strains to grow to confluence in the cell traps prior to induction. After at least 8 hours, the clamp was switched over to the inlet syringe containing only media with no inducer, allowing the media and inducer to flow through the device. After a 2-hour induction period, the clamp was switched back to the induction syringe. Fluorescence time series were extracted and normalized to remove device background fluorescence and strain background fluorescence.

Supervised learning classification

The scikit-learn machine learning package in Python was used for training and testing the different classifier models, including polynomial regression, ordinary least squares, and decision trees. GFP fluorescence of each strain was normalized by GFP fluorescence with no inducer added¹⁷⁰. Features were extracted from each time series, including the maximum fluorescence, minimum fluorescence, time at which the fluorescence reaches half its maximum, number of peaks, and skewness of curve¹⁷². These features were standardized to prevent feature bias.

Quantification and statistical analysis

To calculate the normalized fluorescence of multi-strain microfluidic experiments, the mean fluorescence pixel value (GFP) for each trap was extracted across the 10X microscope image stack using ImageJ. All GFP values were background-normalized to the mean GFP pixel value in a selection of equal size in a part of the chip with no cells (GFP_{BG}). Background-normalized GFP values were normalized to the inverse of similarly background-normalized transmitted light ($\frac{TL}{TL_{BG}}$) pixel values, as a proxy for cell density. The following formula was used:

$$\text{Normalized Fluorescence} = \frac{GFP}{GFP_{BG}} \times \frac{TL_{BG}}{TL}$$

3.6 Discussion

As whole-cell bacterial biosensor development has focused on detecting targets in isolation under controlled laboratory conditions, we engineered a differential biosensor paradigm to combinatorially quantify multiple heavy metals in seawater using a consortium of signals. We employed adaptive laboratory evolution to obtain a diverse range of *E. coli* strains that are equivalently optimized for high-salinity seawater environments while bypassing the need for additional rational engineering. Housing transcription factor-based biosensing constructs for the detection of five heavy metals relevant to seawater in these new variants, the final WCBs were screened using a high-throughput, multiplexed, modular microfluidic platform for continuous culture. This measurement of dynamic temporal behavior highlighted the diversity achieved through the ALE process. Using the diversity in sensor behaviors and cross-interference collectively as a “fingerprint” readout, we used supervised machine learning to quantify combinations of heavy metals in seawater conditions, outperforming single-target sensor performance.

Toward environmental monitoring, the tools developed in this work tackled some of the pressing issues in biosensing, including design for stressful environments, capture of dynamic output, and disentanglement of convoluted signals as a result of crosstalk. Whole-cell biosensors,

notable for their ability to sense a wide range of compounds, have been previously developed for the detection of harmful compounds for environmental safety^{75,104,130,173–177}, metabolites relevant to bioproduction^{178–182}, or biomarkers in *in vivo* medical diagnostics^{87,183–185}. However, engineering for real-world deployability and bioremedial uses calls for strains robust to the harsh environments of their application¹²¹ and capable of distinguishing multiple targets despite crosstalk. Adaptive laboratory evolution provides a rapid method for achieving resilient variants^{144–147}. Future work on developing strains for extreme temperatures, pH differences, or hypoxic conditions could utilize ALE to generate deployment-robust strains. Recent work has also delved into the resulting strains' genomic mutations, opening the door to probing the characteristics essential to fitness in specific environments, informing future rational engineering approaches for applications such as bioremediation^{144–146}. The whole-genome sequencing analyses are informative to eventual reverse-engineering of strains well-suited to particular environments upon characterization of the specific mutations through transcriptomic studies or gene knock-ins or knock-outs.

Furthermore, well-established analytical techniques such as atomic absorption spectroscopy (AAS) or inductively coupled plasma mass spectrometry (ICP-MS) are highly sensitive, but are limited not only by the number of detectable compounds^{175–177} but also by measurement of a single sample at a single timepoint^{186,187}. Multiplexed microfluidic platforms fully utilize the diverse sensing target capabilities of WCBs, enabling screening of multiple engineered strains to multiple conditions while collecting their dynamic data in real-time^{103,104}. We believe the accessibility, throughput, dynamics, and week-long time scales achievable with our microfluidics screening tool can push the frontier of the biosensing landscape. More broadly, the microfluidics platform can serve as a useful tool to adjust genetic circuit output with parallel screening for a desired phenotype, a persistent challenge in synthetic biology design, as our generation of genotypic diversity currently exceeds our ability to screen complex phenotypes¹⁸⁸. Microfluidics has served as powerful tool for approximating complex real-world environments in the past, simulating environments spanning soil to human organs^{189,190}. While not a perfect recreation of

these complex environments, tuning environmental and time-dependent parameters achievable with microfluidics serves an important role in the prototyping and scale-up of all classes of gene circuits. Despite limited means for dynamic phenotype screening, canonical gene circuit motifs, including oscillators, logic gates, and feedback controllers have been increasingly deployed in time-dependent applications spanning metabolic engineering to therapeutic delivery^{21,191–194}. Multiplexed microfluidics, such as ours, can aid in the development of circuits like these.

Typically a hindrance rather than an asset¹⁴¹, the crosstalk generated by the diverse strains was incorporated into training a supervised learning classification model on test data from the biosensors to quantify multiple heavy metal inputs. Outperforming the single-target biosensor predictions, the consortium biosensor's promising performance advances the ability to combinatorially detect multiple targets in real-world samples and builds upon the large body of work on biosensor development. Further work in harvesting the feature coefficient information could help identify features and strains most useful to the model, which could narrow down a minimal set of strains needed for a biosensing application or identify the most essential features for particular biosensing needs. Gathering more data from single-target sensors will also further refine our understanding of the robustness, limitations, and potential of the consortium model approach to biosensing, with regards to the number of strains necessary, the amount and nature of the training data, effects of background media, and feature extraction and engineering. Future approaches that could also further enrich the field of biosensing and advance their deployment include integrating dynamic features, such as frequency measurements, response rate, and relaxation rate, such as those collected in our microfluidics platform.

Lastly, future approaches may consider other machine learning models. Other pattern recognition machine learning models that have been employed in similar array-based or combinatorial approaches to detect a unique fingerprint output include k-nearest neighbors, random forests, k-means clustering, and gradient-boosted trees^{25,26,166}. These have been used in artificial nose and tongue applications, which, inspired by natural olfactory senses, similarly aim to disentangle multiple inputs into a single output. Linear discriminant analysis (LDA), principal

component analysis (PCA), support vector machine (SVM) regression, and hierarchical agglomerative clustering (HAC) are great tools for pattern recognition which can be incorporated into the framework of biosensor engineering and design²⁵. Considering output of diverse signals with the intention to generate distinguishable patterns that work well with these machine learning algorithms may create more degrees of freedom for future biosensor design and engineering than limiting ourselves simply to increasing sensitivity and specificity.

3.7 Acknowledgements

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Chapter 4

Coupled oscillators differentiate single strain into stable distinct phenotypes

4.1 Introduction

Natural populations genetically encode the capability for numerous tasks and excel at functional diversification. However, engineering synthetic populations with higher complexity faces challenges of population instability due to burden and unwanted cross-talk. Here, we build a single bacterial strain that circumvents these challenges through phenotypic switching via coupled orthogonal quorum-sensing systems that each drive their own lysis. We use agent-based modeling that accounts for dynamic allocation of plasmid to observe the theoretical dynamics of this single strain. Experimental testing of this system in microfluidics yields dual lysis cycles that promote dual phenotypic maintenance, which is qualitatively mirrored in our model. Finally, we show that continuous self-correction by our single-strain “ecology” enhances the longevity of this system over an analogous two-strain ecology that suffers from inter-strain competition. Our single-strain, multi-phenotype system can enable complex tasks which require stable maintenance of multiple behaviors, such as multiple therapeutic delivery or metabolic division of labor.

4.1.1 Synchronized Lysis

In 2016, Din et al. engineered a bacterium to exhibit synchronized lysis cycles on a population-level, allowing for coordinated release of a payload²¹. The synchronized lysis circuit (SLC) relies on a small, membrane-diffusible autoinducer molecule (AHL) to generate population-level behavior. Once bound to LuxR, the complex transcriptionally activates the P_{lux} promoter, which drives the LuxI gene responsible for more AHL production¹³⁰. The bacteriophage lysis gene (ϕ X174 E) is also placed under the P_{lux} promoter, functioning as a negative feedback mechanism, as it causes cell lysis upon buildup¹⁹⁵. Coupling these positive and negative feedback mechanisms, the SLC allows a bacterial population to reach a quorum threshold with the buildup of AHL, before synchronously releasing bacterial contents and desired payload^{130,196}. A small number of surviving cells re-seeds the population, generating oscillatory dynamics, which can be useful for continuous, dose-dependent delivery of therapeutics or other payloads¹⁹⁷.

4.1.2 Plasmid Coupling

Heterogeneity typically is a source of noise in synthetic circuit engineering. However, in nature, microbial populations benefit from phenotypic heterogeneity, using it as a mechanism for adaptation in unpredictable environments. Kumar, et al. showed that using copy number to couple distinct plasmids artificially creates a mechanism for heterogeneity that bacterial populations can exploit to adapt to their environment¹⁹⁸. These two-plasmid systems can also be stably maintained, providing engineered cells with a mode of autonomous self-optimization while performing desired tasks.

In recent work, plasmid copy number has been probed as a tuning fork, with variant libraries¹⁹⁹, inducible copy numbers^{200–202}, and even a copy number oscillator²⁰³ being developed. Copy number is typically regulated with a negative feedback system that limits replication to a set point; the ColE1 origin of replication uses an antisense RNA as its inhibitory mech-

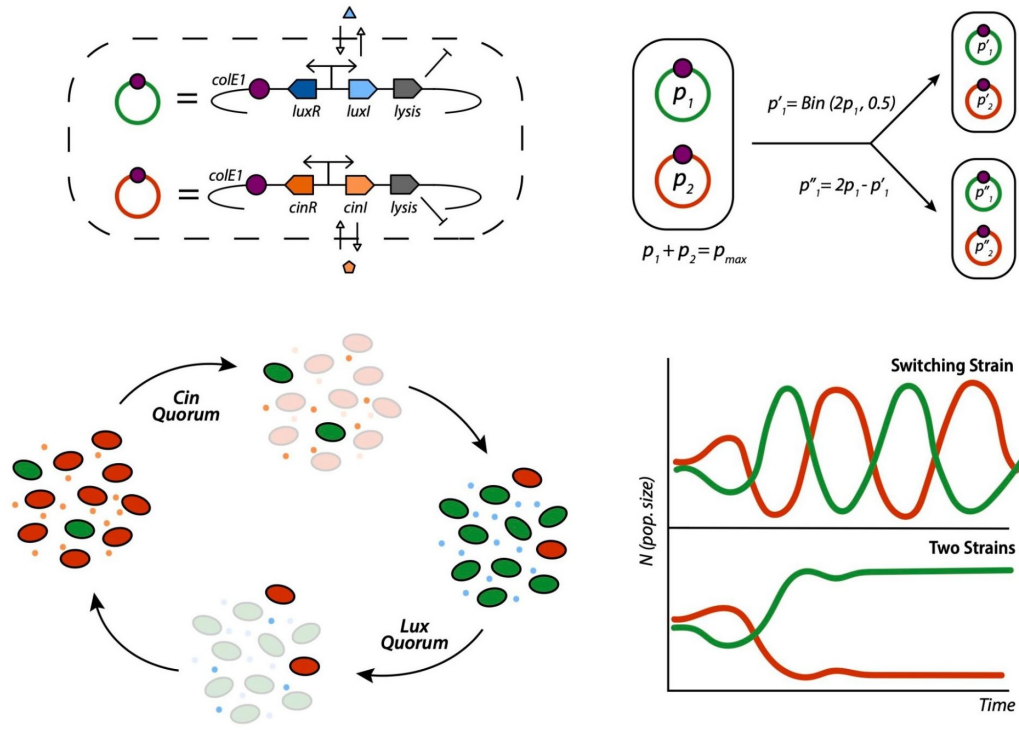


Figure 4.1. Coupled oscillators drive dual phenotypic maintenance. Orthogonal synchronized lysis circuits on the same plasmid origin, coupled by copy number in a single cell, promotes maintenance of both release schemes in a single cell. Modeling of coupled plasmid system supports stable phenotypic switching.

anism to maintain its set point²⁰⁴. Plasmids sharing the same origin of replication also share their regulation strategy, which is generally accepted to lead to plasmid instability. However, more recent publications have challenged this idea^{205,206}. In Kumar et al.'s work, they find that coupling origins of replication actually gives cells some autonomy in regulating the two different plasmid copy numbers, revealing that cells are able to self-adjust their relative copy numbers in response to their environment. Instead of the presumed instability, shared origins of replication actually mimics phenotypic heterogeneity in nature, by providing a mechanism for adaptation to stressful environments or burdensome synthetic payloads. We expand on this idea to maintain two separate synchronized lysis circuits in a single cell to generate two stable and distinct release schemes.

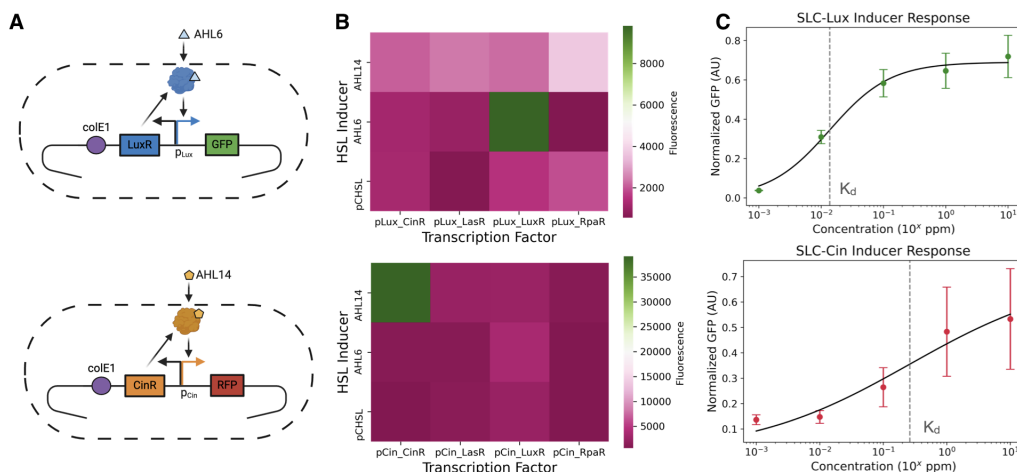


Figure 4.2. Orthogonal quorum sensing systems. (A) Lux- (top) and Cin- (bottom) based quorum sensing system test circuits, engineered to express GFP and RFP, respectively, in response to their corresponding N-acylhomoserine lactones (AHLs). (B) Both quorum sensing systems respond to their own respective AHL, but the Cin system requires more than an order of magnitude more of its AHL to produce its half-maximal response. (C) Quorum sensing systems were evaluated for inducer- or transcription factor-level crosstalk, and both systems exhibited minimal crosstalk with one another.

4.2 Engineering circuitry for stable dual-SLC behavior

In order to create a single microbial strain capable of population-level phenotypic switching, we use two orthogonal quorum sensing systems with coupled origins, allowing diversification into two predominant subpopulations with distinct phenotypes. Using the synchronized lysis circuit (SLC) developed and characterized in Din, et al., we first sought to assemble an analogous circuit using a quorum sensing system orthogonal to the Lux system. In the Lux SLC system, lysis protein is expressed after sufficient accumulation of a Lux-specific membrane-diffusile N-Acyl homoserine lactone (AHL), a quorum sensing molecule.

We identified three alternative quorum sensing systems from literature outside the Lux system used by Din et al., including the Cin, Rpa, and Las systems, and assembled control strains to evaluate their crosstalk interactions Figure 4.2A²¹. These control strains consisted of the constitutively expressed R-protein and the quorum sensing promoter driving a fluorescent protein.

Separating the R-protein and the promoter components allowed assaying both inducer-level and transcription factor-level orthogonality efficiently. The Cin quorum sensing system exhibited both inducer-level and transcription factor-level orthogonality to the Lux-specific AHL and LuxR, respectively Figure 4.2B.

As a result, we used batch culture experiments to determine the fluorescence response to increasing concentrations of AHL. The Lux SLC system exhibited a dissociation constant approximately an order of magnitude below that of the Cin SLC system, 0.02 and 0.26, respectively, indicating that a greater concentration of the Cin-specific AHL is necessary to reach quorum Figure 4.2C.

4.2.1 Modeling

To predict the dynamics of the coupled SLCs and inform our experimental design, we first modeled the system using an agent-based approach (Figure 4.3). Briefly, cells undergo growth at a constant rate and division upon reaching a set size. At division, plasmids within each cell are doubled in number and then binomially partitioned between daughter cells. The total copy number is always maintained by only partitioning one plasmid type and then setting the second plasmid type to the difference between total copy number and the copy number allocated to the first plasmid type. Cell lysis occurs when the local concentration of AHL multiplied by the corresponding plasmid copy number is above a set threshold. In other words, if AHL1 is locally high and an individual cell has enough copies of the plasmid1 to reach threshold, this cell will lyse. We found that simulations of this system show alternating population subtype dominance where as one subtype takes over the population, it triggers its own specific lysis allowing space for subsequent takeover of the other subtype (Figure 4.3A). Quantitatively, this antiphase behavior can be seen both in overall plasmid ratio across the entire population and in subtype cell density (Figure 4.3B).

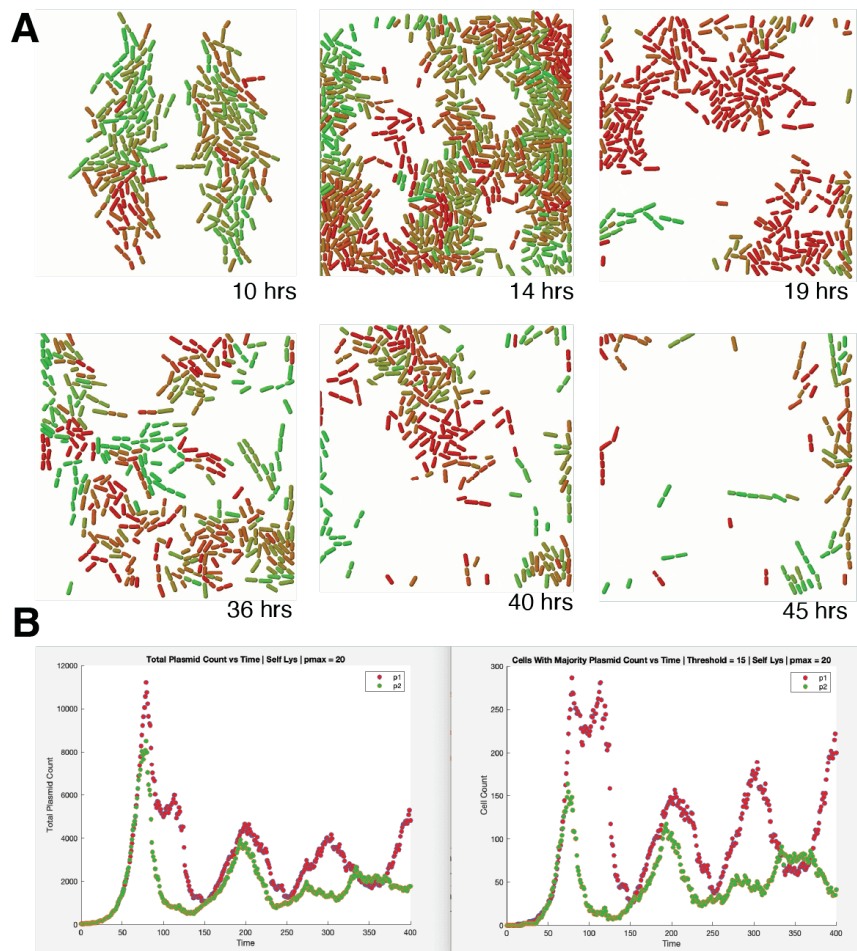


Figure 4.3. Agent-based modeling of dual plasmid design. (A) Agent-based approach, in which plasmid copy number is binomially distributed between daughter cells. As each SLC's respective AHL reaches a local threshold, this triggers lysis, allowing both phenotypes to be maintained. (B) Model quantification, which demonstrates an antiphase behavior between the dual plasmids.

4.3 Coexistence

To experimentally test the model, we constructed an analogous SLC plasmid using the Cin quorum sensing system, which exhibited both sensitivity to its own AHL and minimal cross-reactivity with the Lux quorum sensing system. We co-transformed these two analogous plasmids into *E. coli* K-12 MG1655 and used microfluidic devices to observe the dual strain's dynamic behavior while carrying both plasmids. To visualize population dynamics, we cultured this strain using a microfluidic device containing small, monolayer cell traps (Figure 4.4A). Each

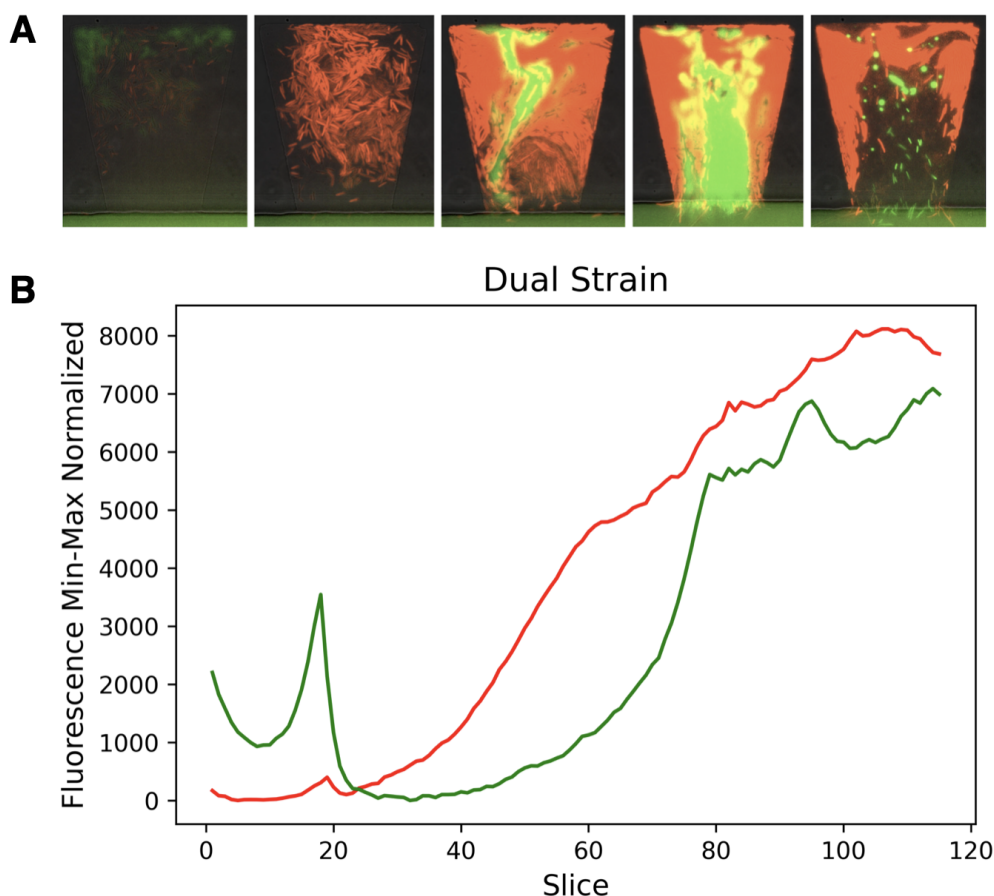


Figure 4.4. Dual-SLC strain in monolayer device. (A) Images from one of the traps of the monolayer microfluidic device of the dual-SLC strain. (B) GFP and RFP min-max normalized over time in the cell trap. Each slice is taken 10 minutes apart.

trap was $50\ \mu\text{m}$ wide and $4\ \mu\text{m}$ deep. Grown in the presence of antibiotics and glucose, which has been shown to repress the more quorum-sensitive Lux system, the strains were loaded into the microfluidic device according to the “Materials and Methods” prior to reaching quorum density. They were imaged every 10 minutes with fluorescence microscopy for high temporal resolution.

In almost all the traps, we observed a mixture of both green and red cells, indicating a mixture of cells containing a majority of Lux-SLC and Cin-SLC plasmids, respectively. The green phenotype consistently reached quorum first, inducing lysis, allowing for the red phenotype to take over (Figure 4.4B). However, the red phenotype tended to elongate and adhere to the

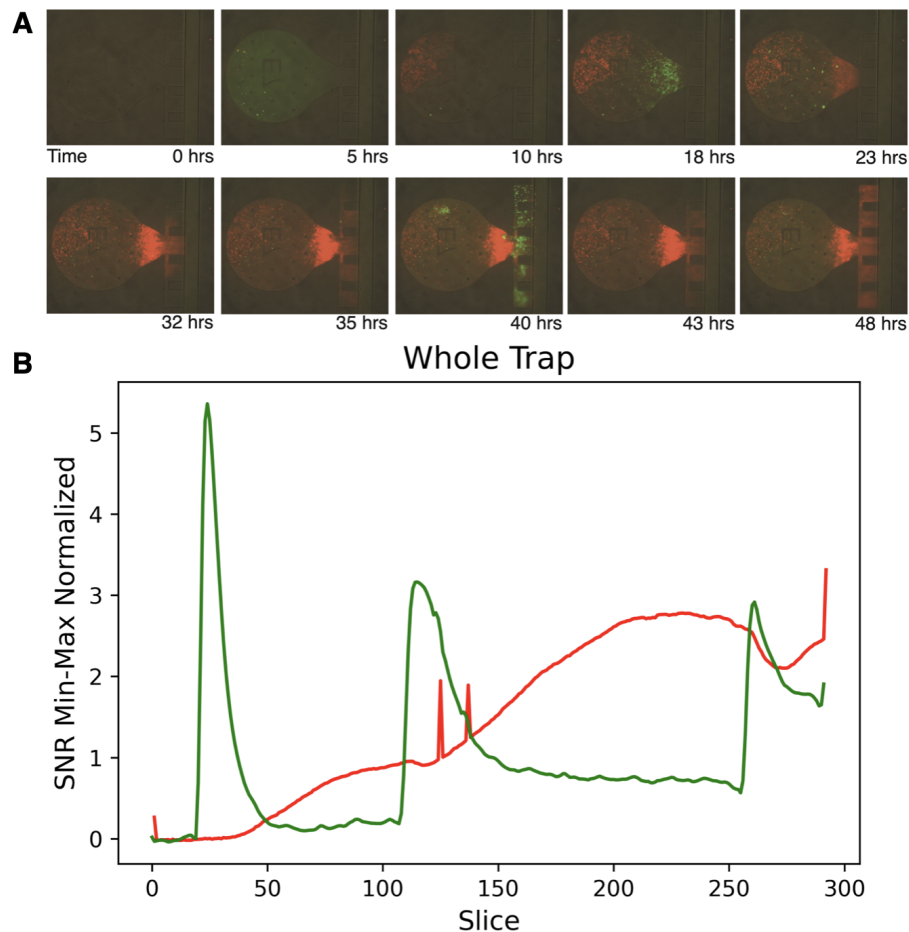


Figure 4.5. Dual-SLC strain in large cell trap with reduced flow. (A) Images from the microfluidic device over the course of 48 hours. (B) Alternating GFP/RFP lysis events across the trap. Each slice is taken 10 minutes apart.

edges of the trap while undergoing incomplete quorum-induced lysis, suggesting that the cells were becoming unhealthy before they could reach quorum. Despite the incomplete synchronized lysis, the green phenotype returned and underwent a second lysis event in some of the traps.

These results demonstrated that our strain could diversify into two dominant phenotypes and maintain spatial and temporal separation in a microfluidic trap. Furthermore, the phenotypic switch from green to red following the first green lysis event supports our design and model simulations for phenotype switching. To achieve bidirectional switching, a red lysis event must be followed by a buildup of the green phenotype. However, we hypothesized that the red phenotype does not exhibit a clean synchronous lyse due to the Cin-SLC system's low cooperativity or high

density threshold, which reduces its oscillatory behavior when co-transformed with its analogous Lux-SLC counterpart.

As result, we opted to reduce the flow rate across the cell chamber to allow more Cin-specific AHL buildup to occur, pushing the red phenotype to synchronized lysis. High flow can quickly dilute AHL, keeping the population from reaching its quorum threshold. To this end, we used a monolayer microfluidic device that delivers flow tangentially to the cell traps, rather than through them (Figure 4.5A). This design results in lower flow at the back of the spotting region relative to the mouth of the chamber. As cells travel towards the mouth of the chamber, we see the temporal switching of population phenotype noticeable as spatial bands that alternate between green and red fluorescence for three complete cycles (Figure 4.5B). In the experiment movie, the red lysis event can be seen driving movement of the cells in the back of the chamber towards the mouth, as well as clearance in the small traps adjacent to the spotting region. However, unhealthy red cells are still present in the aftermath of the lysis event, potentially due to gradual or leaky accumulation of lysis protein.

To demonstrate the utility of our single self-diversifying strain, we constructed a two-strain ecology, separating the Cin-SLC and Lux-SLC systems into two distinct strains altogether to observe the ecology's dynamics (Figure 4.6). We mixed the cultures at a 1:1 OD ratio, prior to reaching quorum. The mixture was vacuum-loaded into a monolayer microfluidic device with a longer trap, similar to prior experiments. These monolayer traps allow visualization of strain competition along the x dimension while lineages are maintained in the y dimension, providing a middle ground between a spatially larger trap and the smaller traps which likely inhibit sufficient buildup of the Cin-specific AHL to trigger synchronized lysis.

Initially, the two-strain ecology seeded with both red and green cells, followed by expansion and lysis of the green strain, similar to the initial experiments with the dual-SLC system. However, after this first lysis event, one of two fates followed. In some traps, the green phenotype remained for the duration of the experiment with loss of the red phenotype altogether. In others, the red phenotype either fully or partially took over after lysis of the green phenotype,

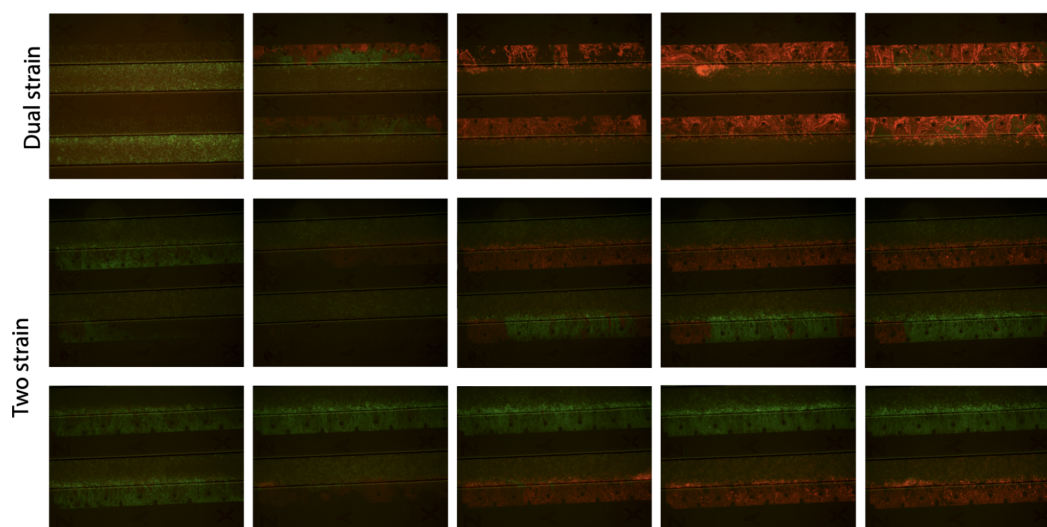


Figure 4.6. Dual-SLC strain maintains both GFP and RFP phenotypes better than 2-strain system. Images from microfluidic device showing dual-SLC strain (top) and 2-strain control ecology (bottom).

resulting in loss of the green phenotype instead or maintenance of small pockets of the green phenotype.

The dual-SLC strain, in comparison, resulted in coexistence of both phenotypes following periods of green and red dominance. This result highlights that our diversifying single strain is capable of longer-term maintenance of two functional phenotypes than a comparative ecology due to regeneration of each phenotype from the opposing subpopulation. The longer-term stable maintenance is useful for complex tasks such as division of labor or cyclic therapeutic dosage. Finally, we cultured the strain in a larger three-dimensional microfluidic device, with 2 mm diameter cell chamber and 25 μm depth, to evaluate whether culturing a higher cell density in a microfluidic trap could reveal unique spatial dynamics over a larger space (Figure 4.7). The microfluidic device was observed for approximately 1.5 days. Upon seeding, the cells organized into phenotypic rings, with the more lysis-sensitive cells forming an outer ring and periodically undergoing pronounced coordinated lysis events. Upon the green lysis events, the red phenotype expanded, as the green phenotype experienced further lysis events in the outer ring, maintaining the same spatial pattern over time. Qualitatively, these results align with those

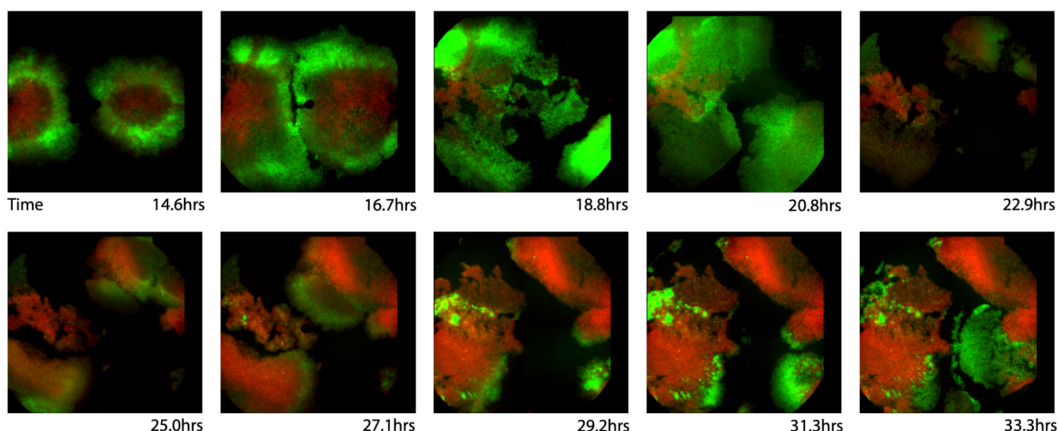


Figure 4.7. Dual-SLC strain in continuous flow chamber. Three-dimensional microfluidic device with continuous flow images, over the course of more than 30 hours.

of the previous, smaller microfluidic devices, with the diversification of our strain into two distinct and spatiotemporally separated phenotypes.

4.4 Materials and Methods

Strains and plasmids

We primarily used *E. coli* K-12 MG1655 laboratory strain for our experiments, and cultured them in LB media with antibiotics in a 37C shaking incubator. Cloning was done in NEB 5-alpha Competent *E. coli* for higher transformation efficiency. Plasmids were constructed using PCR and Gibson and Golden Gate Assembly, using parts used in previous work from our group or from the MoClo kit. Both plasmids contain identical ColE1 origins of replication and distinct antibiotic resistance genes (spectinomycin or chloramphenicol). For both quorum sensing systems (Lux and Cin), the quorum-sensitive promoter drives a fluorescent protein (GFP or RFP, respectively), the E lysis gene, and the “I” gene responsible for AHL production. The “R” transcriptional activator is constitutively expressed. These were co-transformed into the MG1655 strain for the dual-SLC strains. For the control strains, the antibiotics on both plasmids were the same, and they were transformed independently into the MG1655 strain.

Batch culture experiments

Strains were seeded from glycerol stocks and grown in LB with antibiotic and 0.2% glucose to repress lysis until they reached an OD₆₀₀ of about 0.1, prior to lysis. They were diluted 100-fold onto 96-well plates prepared with media and the noted concentrations of AHL. Fluorescence and OD₆₀₀ were measured using an Infinite M200 PRO Multimode Microplate Reader. Fluorescence measurements were min-max normalized for comparison.

Microfluidics devices

Polydimethylsiloxane (PDMS) microfluidic devices were made using a mixture of 10:1 Sylgard 184 to curing agent poured over a microfabricated wafer with the desired design. The PDMS was degassed to remove bubbles, and the wafer with PDMS was cured for one hour at 95°C on a level surface. After curing, PDMS devices and glass slides were cleaned with MilliQ and 70% Ethanol. Adhesive tape was used to remove any remaining fibers. The cleaned devices and slides were exposed to oxygen plasma and bonded and cured at 37°C for two hours.

Microfluidic experiments

Strains were seeded from glycerol stocks and grown in LB with antibiotic and 0.2% glucose to repress lysis until they reached an OD₆₀₀ of about 0.1, prior to lysis. 1mL of the culture was centrifuged at 6000g, and resuspended with 20uL LB containing antibiotic and 0.075% Tween-20. Tween-20 is a surfactant to help prevent biofilm formation that can clog microfluidic channels and reduce the longevity of experiments. This concentrated culture was then vacuum loaded using a previously described protocol¹⁰⁷. Microfluidic experiments were performed on a Nikon Ti2. The inlet and outlet ports were connected to 5mL syringes with Tygon tubing. Inlet syringes were filled with approximately 5mL LB media containing antibiotic and Tween-20 and inducer if applicable. The waste outlet was filled with 1mL MilliQ water, to provide negative pressure. The inlet and outlet syringes were separated by a 5" height difference. Strains were grown to confluence in the cell traps, and fluorescence time series were extracted

and normalized to remove device background fluorescence and strain background fluorescence.

4.5 Discussion

While heterogeneity is common in nature and promotes the successful survival of many species in the face of environmental pressures, exploration of engineering heterogeneity into synthetic systems has been limited. With advancements in microbial techniques and refinement of DNA manipulation, intentionally creating heterogeneity is now more feasible. Copy number has recently been explored as a tuning fork for synthetic biology engineering. With Kumar et al.'s recent work in harnessing copy number machinery to force a cell to maintain multiple distinct phenotypes, we have engineered a dual synchronized lysis strain¹⁹⁸. To this end, we incorporated an orthogonal version of Din et al.'s Lux-based synchronized lysis circuit that uses the Cin-based quorum sensing system, coupling the two on identical origins of replication²¹. Our single-strain system differentiates into two spatiotemporally distinct behaviors that are more stably maintained than an analogous two-strain ecology in which these two circuits are separated into two distinct strains. Future work could explore the translational applications of this dual phenotype, including metabolic division of labor or multiple payload delivery, by replacing the fluorescent proteins with actual desired functions.

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