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Enhancing chemical bioproduction with rational control of bacterial post-translational modifications

September 2025

Joshua R Elmore

U.S. DEPARTMENT of ENERGY

Prepared for the U.S. Department of Energy
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Abstract

Efficient conversion of inexpensive feedstocks to valuable chemicals by microbes is critical for a robust bioeconomy, but the ability to rationally design bacteria is hampered by insufficient knowledge of how post translational modifications (PTMs) control bacterial protein function and thus bioproduction phenotypes. Our study will focus on the lysine acetylation, a ubiquitous bacterial PTM that can affect the function of enzymes in central metabolism that are often critical for bioproduction processes, disrupt transcriptional regulation, and reduce translation. However, most lysine acetylation data is observational, which means that we do not know when, how, and what specific acetylated residues affect protein function and bacterial physiology. For our model host, we will use a Pseudomonas putida strain that we previously engineered to convert lignocellulosic feedstocks into chemicals such as itaconic acid (ITA). With this strain, we use a dynamic two-stage bioproduction process in which ITA is produced during a non-growth associated production phase. Production is highest during growth stages when lysine acetylation is low in other organisms (early stationary phase) and stalls in conditions where acetylation is highest (late stationary phase). The switch from high to stalled ITA production is also correlated with an unexpected increase in acetate levels – the precursor to non-enzymatic lysine acetylation. As such, we predict that lysine acetylation plays a substantial role in regulating the metabolic pathways required for ITA production. We will develop a generalizable approach that combines high-throughput genetic screens and cutting-edge genome engineering with state-of-the-art proteomics, metabolomics, and genetic code expansion methods to identify and modulate lysine acetylation patterns in bacteria. Ultimately, these strategies aim to manipulate protein expression and acetylation patterns to enhance bioproduction phenotypes (e.g., sustained ITA production in late stationary phase).

Summary

Efficient conversion of feedstocks to valuable chemicals by microbes is critical for a robust bioeconomy, but the ability to rationally design bacteria is hampered by insufficient knowledge of how post translational modifications (PTMs) control bacterial protein function and thus bioproduction phenotypes. Our study will focus on lysine acetylation, a ubiquitous bacterial PTM that can deactivate the activity of the enzymes in central metabolism that are critical for bioproduction processes, disrupt transcriptional regulation, and reduce protein translation. Control of these processes is necessary for chemical production, but our limited knowledge of when, how, and what specific lysine acetylation affects protein function and bacterial physiology greatly restricts the ability to predict what genetic interventions will enable control over these processes. This work project aims to revolutionize the field of bacterial engineering by demonstrating how PTMs govern bioproduction phenotypes and by developing strategies to rationally control PTMs for desirable phenotypes.

Current gaps in understanding of lysine acetylation that this project aims to address include: (1) the identity of protein lysine (de)acetylases whose functions control bioproduction phenotypes, (2) the identity of individual proteins whose acetylation is associated with bioproduction phenotypes, (3) understanding of how to enhance bioproduction phenotypes with targeted, acetylation data-driven genetic interventions. This technical report covers efforts to develop and apply tools to identify protein lysine (de)acetylases whose functions control conversion of lignocellulosic sugars into itaconic acid by engineered *Pseudomonas putida*. For this, we onboarded and apply an inducible CRISPR interference system to evaluate the influence of lysine acetylase expression on itaconic acid production. Additionally, we utilized a barcode sequencing method to identify and characterize synthetic and native transcriptional promoters that enable consistent gene expression across multiple stages of bacterial growth. These promoters, unlike most commonly used promoters, will enable efforts to continuously maintain expression of protein lysine deacetylases across multiple distinct bacterial growth phases. This expression will be essential for understanding the influence of these deacetylases on both production of industrial biochemicals and other fundamental biological processes.

Acknowledgments

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Acronyms and Abbreviations

ATP	adenosine triphosphate
BONCAT	bioorthogonal non-canonical amino acid tagging
CRISPRi	clustered regularly interspaced short palindromic repeats interference
CV	crystal violet
ITA	itaconic acid
MSP	multi-stage bioproduction process
PTM	post-translational modification
SAGE	serine recombinase-assisted genome engineering
sfGFP	super folder green fluorescent protein

Contents

1.0 Introduction

The economic feasibility and environmental sustainability of biorefineries, which are the foundation of the bioeconomy, rely on advancements in both the carbon efficiency and the rate of lignocellulosic feedstock bioconversion. Multi-stage bioproduction processes (MSPs) — where growth and production are separated — can offer notable efficiency benefits compared to single-stage processes[1]. These MSPs also enable the production of chemicals that would otherwise inhibit microbial growth during single-stage systems[2]. Yet, a common and significant challenge in MSP design is the sharp decline in substrate conversion during the non-growth production phase, which dramatically reduces volumetric productivity (i.e., the rate of production). This drop in productivity hinders the economic competitiveness of MSPs.

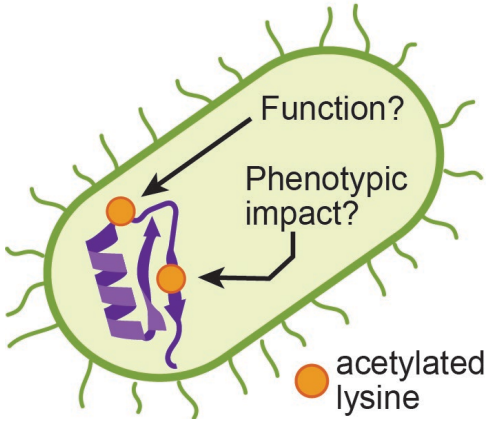

Figure 1. Increasing evidence suggests that the ubiquitous and rapidly reversible bacterial PTM lysine acetylation plays a key role in regulating pathways, regulatory networks, and key metabolic nodes that are critical for both switching between metabolic states and robust biochemical production. However, *the current understanding of how lysine acetylation influences protein function, and phenotypes in vivo is insufficient to rationally manipulate lysine acetylation in biosystems design of bioenergy-relevant microbes*.

Various strategies have been employed to sustain the metabolic activity needed for high productivity, such as inducing ATP wasting cycles[3], with mixed levels of success. However, these strategies are often narrowly tailored to specific microbes or target chemicals and lack generalizability. Generalizable approaches are essential due to the broad diversity of microbes and engineered metabolic pathways required to produce the wide array of chemicals and materials demanded by industry. The current boutique strain engineering practices fail to scale effectively to meet these needs.

One promising approach to address this issue is modulating post-translational modifications (PTMs) to influence metabolic activity and sustain productivity during MSP non-growth phases. Lysine acetylation, a highly prevalent PTM in bacteria, affects multiple aspects of bacterial physiology[4-5] and modulating the PTM could provide a universal strategy for enhancing bioprdouction performance. Conditions favoring lysine acetylation often overlap with those used in the production phases of MSPs, making acetylation control particularly relevant. However, our understanding of lysine acetylation remains largely observational — meaning we lack crucial insights into when, how, and which specific lysine residue acetylations impact protein functionality *in vivo* and bacterial physiology. These gaps in knowledge must be filled to enable rational strain engineering.

To address this, we integrate multiple cutting-edge approaches to measure and manipulate lysine acetylation, aiming to significantly enhance microbial chemical production. These approaches include rapid genetic screens and functional assays to assess PTM machinery's role in bioproduction, high-throughput methods to identify transcriptional promoters independent of growth phase, and efficient genome engineering tools to streamline the construction of required strains. Specifically, in this pilot project we used:

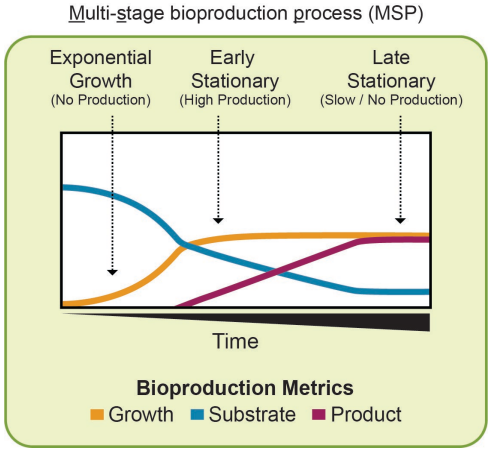

Figure 2. Multiple-stage bioproduction processes contain three distinct phases as shown above. Extending the highly productive second stage is critical for processes to efficiently utilize feedstocks and be economically viable.

1. Multiplex CRISPR interference (CRISPRi): This technology helps evaluate how reduced expression of PTM machinery affects bioproduction.

2. Host-agnostic genome engineering technology (SAGE): SAGE minimizes the time and resources necessary for strain engineering.

3. Differential relative transcriptional assays: Through pooled barcode sequencing, we can monitor engineered promoter activity across diverse conditions and time points to identify promoters that maintain expression of deacetylases and CRISPRi machinery in all growth phases.

Our work utilizes *Pseudomonas putida* as a model organism to study the role of lysine acetylation in bioproduction. This bacterium is highly suitable as an industrial chassis due to its rapid growth, tolerance to xenobiotics, robust metabolism, and exceptional genetic tractability. Additionally, *P. putida* features advanced genetic tools and has demonstrated the ability to produce a wide range of bioproducts. Crucially, we have previously developed a model MSP where an engineered *P. putida* strain converts lignocellulosic feedstocks into itaconic acid (ITA) with high yields. Our existing tools — CRISPRi[6], SAGE[7], and others — are already well-optimized for this organism.

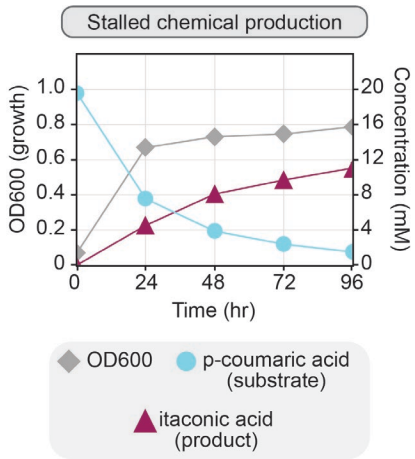

Figure 3. Above is an example of productivity slowing after a burst of efficient itaconic acid production during early stationary phase.

This MSP employs a dynamic, two-stage bioproduction process. ITA is produced during a non-growth production phase, with peak production occurring during conditions typically associated with low lysine acetylation (e.g., early stationary phase). Conversely, production stalls during conditions with high acetylation levels (e.g., late stationary phase). Thus, we hypothesize that lysine acetylation plays a role in deactivating metabolic pathways essential for ITA production. By focusing on understanding and manipulating lysine acetylation, we aim to overcome the limitations of MSPs and enable more economically viable and sustainable biorefineries.

2.0 Results

A key objective for FY 2025 was to develop robust gene regulatory systems in *Pseudomonas putida* that could maintain either consistent gene expression or sustained gene repression throughout all stages of a multi-stage bioproduction process. The existing bacterial gene expression systems, along with their complementary gene repression mechanisms, typically rely on genetic elements that ensure reliable transcription during specific growth phases — most commonly, either the exponential phase or early stationary phase. However, these systems are usually not designed to function reliably across all growth phases, particularly late stationary phase. This limitation poses a challenge for consistent expression or repression during the full range of an MSP, which spans multiple growth stages.

To address this challenge, our efforts focused on identifying and developing genetic elements and transcriptional repression systems capable of delivering stable and predictable expression of target enzymes across all bacterial growth stages. These enzymes are critical for achieving project goals related to sustaining metabolic activity and optimizing bioproduction performance.

By overcoming the growth phase-specific constraints of current expression and repression systems, this approach would enable better control of gene functions throughout the entire MSP, which is essential for engineering more efficient and reliable bioproduction processes.

2.1 Development of a CRISPR interference system to reliably control gene expression across growth phases

Exploring Inducible Gene Regulation for Broad-Host CRISPR Interference Systems

Genetic screening is a key tool for uncovering biological processes and understanding protein functions. Among the most efficient and versatile genetic study methods is CRISPR interference (CRISPRi)[6,8], which leverages catalytically inactive Cas proteins, such as dCas9[8] or dCas12[6], paired with short guide RNAs to selectively repress genes in a programmable, sequence-specific manner.

Development of a Broad-Host CRISPRi Toolkit

In recent work, we developed a broad-host range CRISPRi toolkit capable of simultaneously repressing multiple genes in phylogenetically diverse bacteria[6]. Unlike plasmid-based systems, which are limited in host range and often challenging to replicate across different species, our system integrates CRISPRi machinery directly into bacterial chromosomes. This integration ensures compatibility across a wide variety of bacterial hosts. To achieve this, we employed serine recombinase-assisted genome engineering (SAGE), a host-agnostic tool that facilitates efficient chromosomal integration[7]. While our original CRISPRi system was effective for other applications, it lacked inducibility and was always active. For this project, we aimed to create a system with external controllability, allowing precise gene regulation — critical when targeting essential proteins.

Challenges and Solutions in Inducible Expression Systems

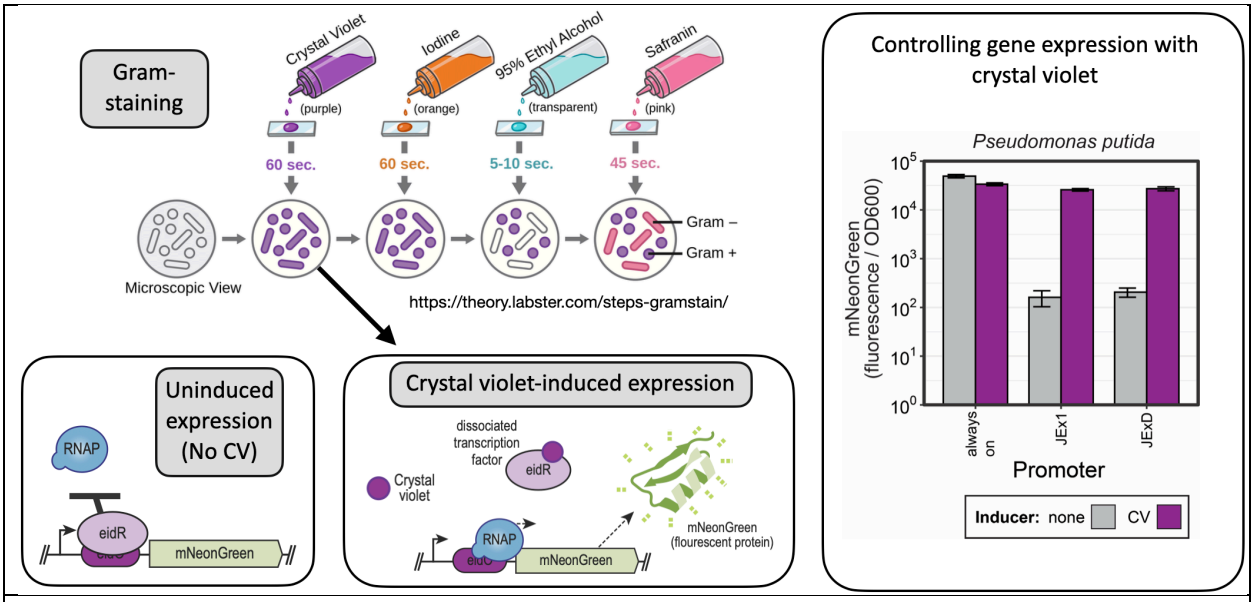

Figure 4. The Jungle Express system utilizes the eidR transcription factor to block transcription of specific promoter elements in the absence of crystal violet (CV). The bottom right compares mNeonGreen production using either a unregulated promoter, or two eidR-regulated promoters (JEx1, JExD). The production of mNeonGreen is measured using a multi-well fluorescent plate reader. Error bars represent the standard error in three biological replicates.

A major limitation of many inducible expression systems is their reliance on inducer compounds that cannot be imported or metabolized by some bacterial species. For example, L-arabinose is widely used due to its tight regulatory capabilities in many proteobacteria, but its effectiveness is restricted in species lacking the ability to consume or internalize this sugar. To address these limitations, we selected Jungle Express[9], an inducible system regulated by crystal violet (CV) — a compound that can passively diffuse through bacterial membranes and is not metabolized by most species.

Evaluation of Jungle Express in *Pseudomonas putida* chromosome

As a proof of concept, we tested the Jungle Express system by controlling the expression of a chromosomally integrated fluorescent reporter protein in *Pseudomonas putida* (Figure 4). Results showed tight regulation of expression alongside robust activation of the reporter protein, affirming the system's suitability for CRISPRi applications.

Inducibly Controlling CRISPRi in *Pseudomonads*

We next assessed the effectiveness of Jungle Express in regulating CRISPR interference across multiple *Pseudomonas* species. To do this, we measured the repression of a constitutively expressed superfolder GFP (sfGFP) reporter protein using a CRISPRi system. CRISPRi requires two components for gene repression: a guide RNA and a Cas protein (Figure 5). We tested systems in which both components were unregulated, only the Cas12 protein was regulated, and both components were regulated by Jungle Express.

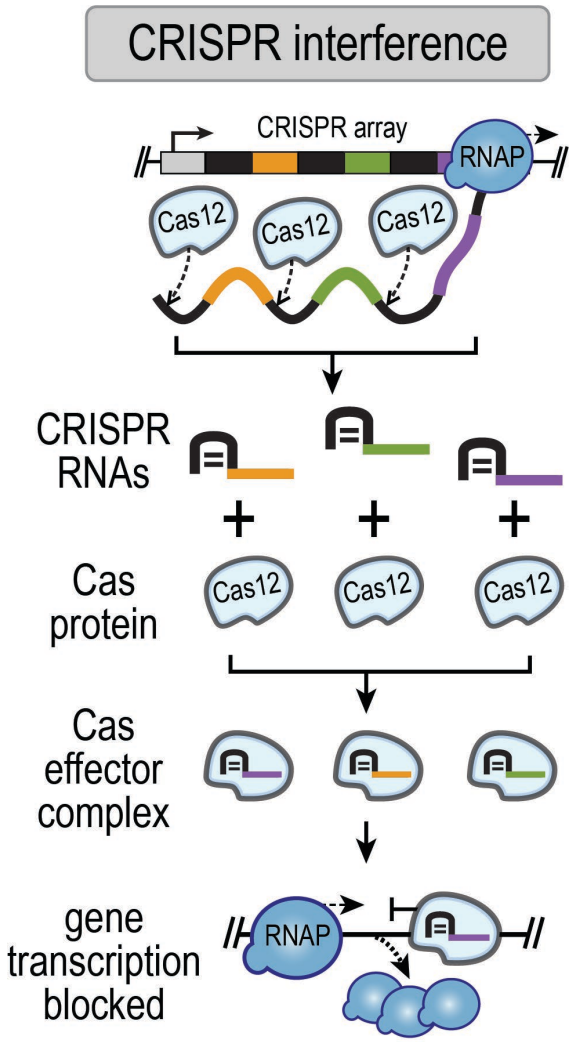

Figure 5. We use a deactivated Cas12 that processes a single CRISPR transcript into individual CRISPR RNAs that each enable Cas12 to bind to different DNA sequence. This allows the system to either target multiple genes, or as in this work, use several guides targeting a single gene to effectively 'turn off' the gene's expression.

Experiments revealed that repression of sfGFP by the regulated systems depended on the presence of CV in the medium, while the original system repressed sfGFP expression regardless of CV (Figure 6). Notably, when only Cas12 was regulated, we observed slight repression of sfGFP expression even without CV, suggesting low levels of Cas12 expression can still cause minor gene downregulation. Thus, to completely prevent off-target repression, both the Cas protein and guide RNA components should be regulated. Importantly, sfGFP repression was effective during both exponential growth and stationary phase, demonstrating the system's reliability across different bacterial growth stages — a critical factor for evaluating bioproduction genes like deacetylases.

To determine whether the genetic context (e.g., proximity to highly expressed neighboring genes) influences the efficiency of the regulated CRISPRi system, we performed analogous sfGFP repression experiments in the rhizobacterium *Pseudomonas fluorescens* SBW25. In *P. putida*, the Cas12 and guide RNA array were located at separate chromosomal loci, whereas in *P. fluorescens*, they were positioned adjacent to one another. Despite these differences in genetic context, the regulated CRISPRi system performed similarly well in both bacteria, indicating that it exhibits at least a partial insensitivity to chromosomal context.

Through these experiments, we successfully developed a broad-host CRISPRi system with inducible regulation using Jungle Express. This system demonstrates strong compatibility across diverse bacteria, tight control over gene regulation, and resilience to genetic context — making it a powerful tool for genetic studies that require precise regulation, such as targeting essential proteins or optimizing bioproduction processes across growth phases.

2.2 Development of growth phase-independent promoters

A significant goal for FY 2025 was to develop gene expression systems capable of maintaining consistent protein expression — such as for protein lysine deacetylases — across the major bacterial growth phases. While transcriptional promoter libraries have been developed to control transcriptional activity via reporter protein expression in various organisms, most of this work has relied on multicopy reporter plasmids. These plasmids do not accurately reflect transcription rates from chromosomally integrated genetic elements, which are more representative of real-world, single-copy gene expression. Furthermore, most transcriptional promoter libraries have only been evaluated during exponential growth, focusing primarily on promoters controlled by the organism's primary sigma factor (e.g., RpoD in proteobacteria). These classes of promoters typically exhibit high activity during exponential growth but experience reduced or even null expression during later growth phases, such as stationary phase. To our knowledge, large promoter libraries have not previously been characterized across multiple bacterial growth phases.

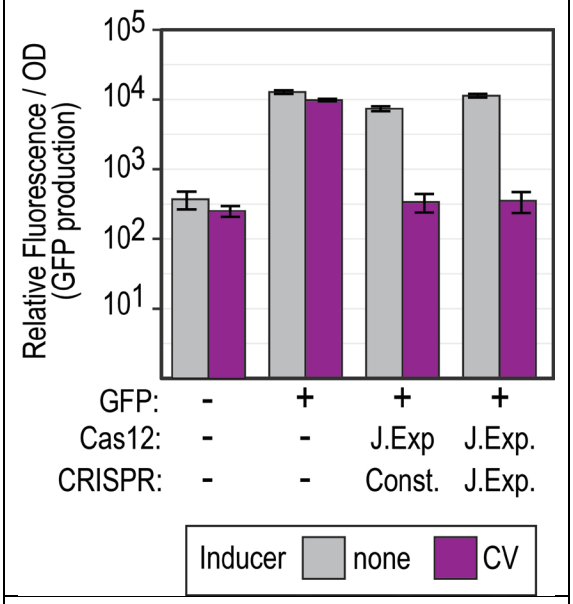

Figure 6. This plot compares mNeonGreen production with and without a CRISPRi system. *Const.* represents a constitutive expression and *J. Exp* represents regulated expression of the indicated CRISPRi component. The production of mNeonGreen is measured using a multi-well fluorescent plate reader. Error bars represent the standard error in three biological replicates.

Challenges of Protein-Based Reporter Systems

Characterizing promoter activity using protein-based reporters poses inherent challenges. Reporter proteins (e.g., sfGFP) are often highly stable, with half-lives spanning hours to days[10]. This stability can obscure the distinction between active transcription in the current growth phase and residual reporter protein produced during earlier phases. Addressing this limitation generally requires costly and specialized techniques, such as RNA-seq, PTM proteomics[11], BONCAT[12] (Bioorthogonal Non-Canonical Amino Acid Tagging), or host-specific solutions (e.g., use of organism-specific proteolysis tags).

In contrast, mRNA molecules are far less stable, with half-lives typically ranging from seconds to minutes [13]. Measuring mRNA abundance provides high temporal resolution, allowing direct assessment of transcriptional activity during specific growth phases. Thus, for this study, we employed a promoter characterization method that measures transcript abundance rather than relying on protein-based reporter measurements.

High-Throughput Transcriptional Measurement for Promoter Characterization

To characterize a library of chromosomally integrated promoters, we utilized an approach that combines SAGE with a modified high-throughput sequencing-based transcriptional assay (Figure 7)[7]. This allowed efficient construction and reliable evaluation of promoter variant libraries.

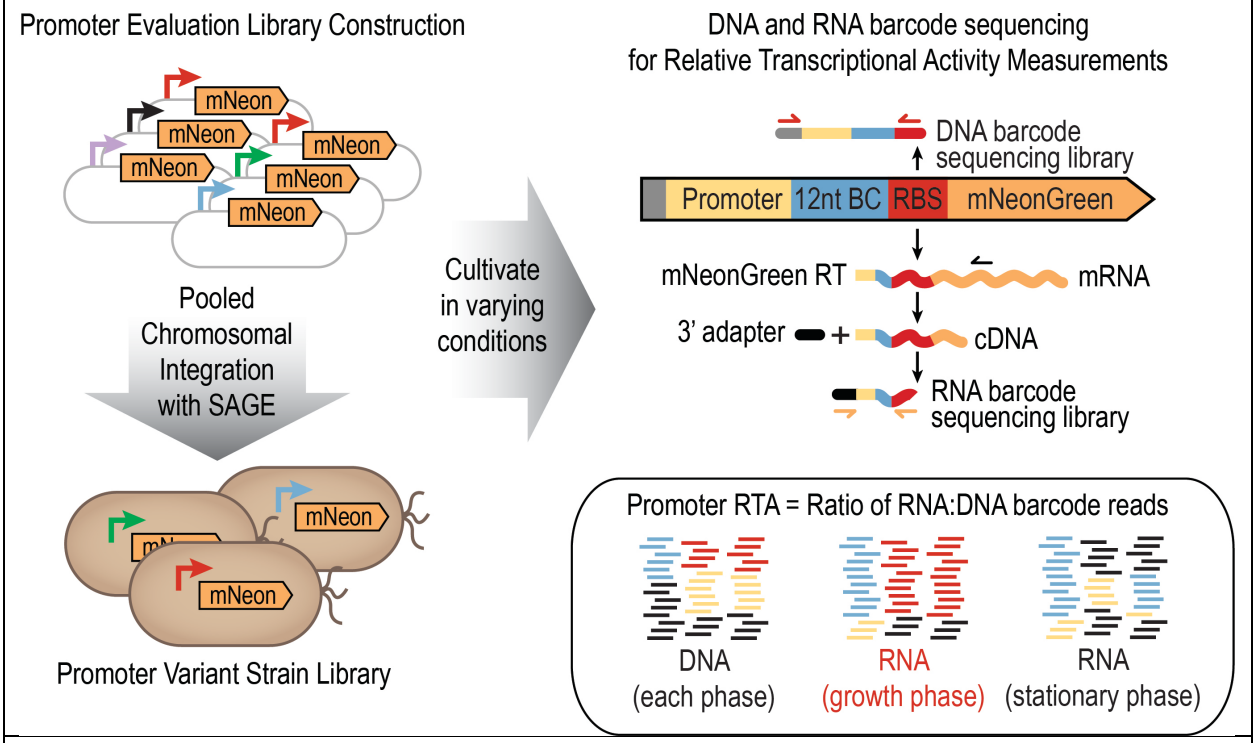

Figure 7. By utilizing a pooled collection of strains that each contain a distinct barcoded transcription promoter variant we can evaluate hundreds to thousands of individual promoters in a single experiment. Using high-throughput barcode sequencing on both the DNA and RNA fractions of the same sample we can determine the relative transcriptional activity (RTA) of each promoter in the pooled strain library. Comparing the changes in the RTA for each promoter across conditions or stages of growth allows us to determine how consistent or condition-specific the promoters are.

Using this method, we characterized a collection of 445 promoters that included native *Pseudomonas putida* promoters, engineered variants thereof, and widely used synthetic promoters. Importantly, instead of focusing solely on promoters controlled by RpoD — the sigma factor that regulates housekeeping genes during exponential growth — we targeted a diverse set of promoters controlled by other sigma factors, such as RpoE, RpoS, RpoN, and RpoH. These sigma factors regulate genes involved in stress responses, stationary-phase functions, and other physiological processes beyond routine exponential-phase housekeeping.

The promoter library was integrated into a *P. putida* strain engineered for itaconic acid bioproduction. Transcript abundance measurements were taken across three distinct growth phases: exponential growth, early stationary phase, and late stationary phase. Additionally, we evaluated transcriptional activity under conditions with and without crystal violet (CV) — used as an inducer in prior experiments.

Our measurements revealed that the majority of tested promoters exhibited variability in expression across growth phases or between replicates under identical conditions. However, a small subset of promoters demonstrated consistent expression across all experimental conditions and growth phases, while others showed intriguing expression patterns, such as transient spikes during early stationary phase (Figure 8).

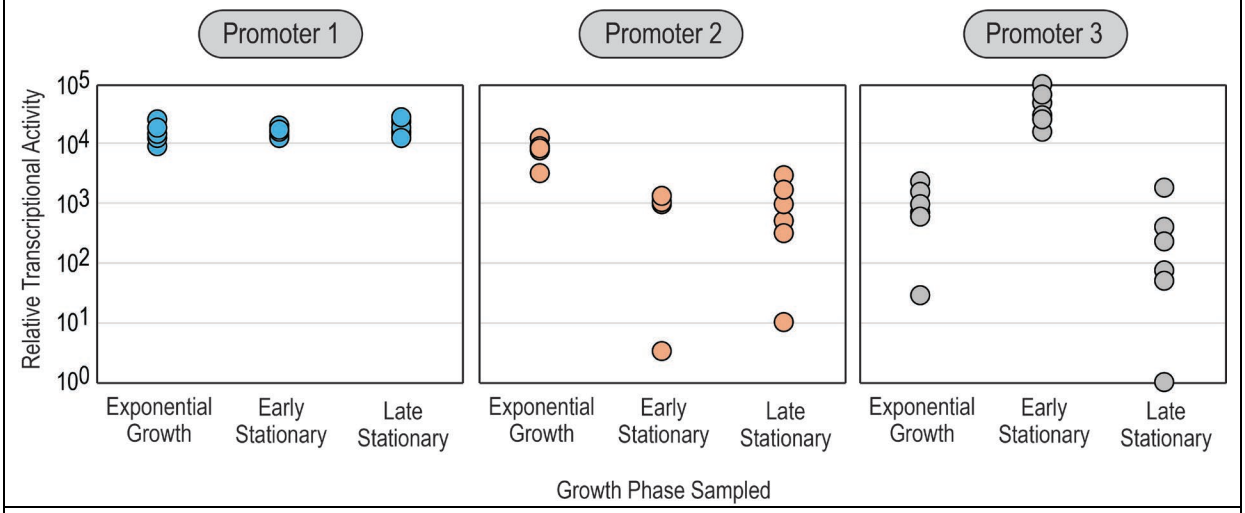

Figure 8. Example RTA data for promoters exhibiting three distinct expression profiles. Promoter 1 displayed consistent expression across three replicates in two conditions, promoter 2 expression decreased and increasingly noisy expression across growth phases, and promoter 3 expression was largely limited to a spike in early stationary phase.

These results yield dual benefits. First, the identified stable promoters will serve as invaluable tools for bacterial engineering, enabling researchers to maintain consistent gene expression during bioprocesses spanning multiple growth phases. Second, this work provides insights into how different classes of promoters are regulated across growth phases, offering a deeper understanding of sigma factor-mediated transcriptional control and promoter dynamics.

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