



UNIVERSITÄT
DES
SAARLANDES



Leibniz-Institut
für Neue Materialien

Programming Probiotic Lactobacilli Towards Next- Generation Probiotics

Dissertation

zur Erlangung des Grades

des **Doktors der Naturwissenschaften**

der Naturwissenschaftlich-Technischen Fakultät

der Universität des Saarlandes

von

Marc Blanch Asensio

Saarbrücken

2025

Tag des Kolloquiums:	06.07.2026
Dekan:	Prof. Dr.-Ing. Dirk Bähre
Berichterstatter:	Prof. Dr. Aránzazu del Campo Bécares Prof. Dr. Bruce Morgan Prof. Dr. Matias D. Zurbriggen
Vorsitz:	Prof. Dr. Tsing-Young Dora Tang
Akad. Mitarbeiter:	Dr. Florian Hubrich

Summary

Lactiplantibacillus plantarum is a probiotic bacterium widely known for its health-promoting properties and fermentation capabilities through the production of natural metabolites. Despite its inherent advantages, its broader utility remains constrained by two limitations: i) a challenging genetic transformation and ii) a limited genetic toolbox for enhanced genetic programmability. This thesis addresses the first gap by identifying the barriers limiting genetic transformation and developing a time-saving, direct cloning method in *L. plantarum*. This method is used to address the second gap, which focuses on exploring sources of genetic parts, ranging from bacteriophages to unexplored endogenous parts, and evaluating their performance to expand the genetic toolbox of *L. plantarum*. In this thesis, valuable genetic parts are discovered and combined to achieve high levels of constitutive and inducible gene expression, improved protein secretion, and tunable plasmid retention. Ultimately, the potential of the engineered bacteria for responsive drug delivery is evaluated within the context of Engineered Living Materials (ELMs). Thus, this thesis describes the development of methods and tools leading to the first lactobacilli-based ELM capable of switchable protein release. Beyond living therapeutics, the outcomes of this thesis might contribute to expanding the programmability of *L. plantarum* towards advances in metabolism research, biotechnological, and imaging applications.

Kurzzusammenfassung

Lactiplantibacillus plantarum ist ein probiotisches Bakterium, das für seine positiven Eigenschaften und seine Fähigkeit zur Fermentation durch die Produktion natürlicher Metaboliten bekannt ist. Seine breitere Anwendung wird jedoch durch zwei Einschränkungen begrenzt: i) eine aufwendige genetische Transformation und ii) ein begrenztes genetisches Werkzeugset für verbesserte Modifikationen. Diese Arbeit identifiziert und überwindet Hindernisse für die genetische Transformation durch die Entwicklung einer direkten Klonierungsmethode. Diese Methode dient dazu, die zweite Beschränkung zu bewältigen, indem sie die Suche nach genetischen Bausteinen und deren Leistungsfähigkeit zur Erweiterung des genetischen Werkzeugsets von *L. plantarum* ermöglicht. In dieser Arbeit werden wertvolle Bausteine entdeckt und kombiniert, um eine verbesserte konstitutive und induzierbare Genexpression, Proteinsekretion und Plasmidretention zu erreichen. Das Potenzial der gentechnisch veränderten Bakterien für die gezielte Wirkstofffreisetzung wird im Kontext von Engineered Living Materials (ELMs) evaluiert. Diese Arbeit beschreibt die Entwicklung von Werkzeugen, die zu Laktobazillen-basierten ELMs mit schaltbarer Proteinfreisetzung führen. Über die Anwendung als lebende Therapeutika hinaus könnten die Ergebnisse dieser Arbeit dazu beitragen, das Engineering von *L. plantarum* im Hinblick auf Fortschritte in der Stoffwechselforschung und biotechnologischen Anwendungen auszuweiten.

Acknowledgements

As a unit of a set of twins, it was the first time I had to embark on a challenge completely on my own. Well, the truth is that I did not feel alone for long in this journey, and I met and collaborated with many people whom I would like to express my gratitude to.

Shirish. This thesis reflects what comes out when a PI and his/her student understand each other fully. Truly speaking, my PhD has not been as challenging as most PhD students claim. There have been no ups and downs. Everything has just gone gradually upwards. If there were any stressful moments, they were due to my stubbornness to do more, followed by your approval and belief in me. I will always be in debt to you for your unwavering support, your constant guidance whenever needed, and the freedom you have given me to purchase 200 gene fragments to keep IDT in business. I will always follow your research closely, and I wish you the best in the future.

The other members of BPM have also been pivotal in this accomplishment. Thanks to Priyanka, Florian, Anwesha, and Panos. I will always remember the good moments we spent together. My special appreciation also goes to Dr. Dey for the countless fruitful discussions in the lab and for other great moments. I wish you all the best on the other side of the Atlantic. Big thanks also to the real bosses of the lab, Christina and Sandi. Without you, the lab would not have been the same place. Your constant help has been invaluable.

Varun. It is hard to imagine Marc at INM without Varun and Varun at INM without Marc. We almost started this together. The first time I met you, I had to explain to you how to book the lab slots so you could work in the lab during the pandemic. Back then, we knew nothing about the 24 trips we would make together, exploring 14 countries, in the coming 4 years. I will not need to check our 4 publications together to remember you because you are the lifelong friend I made here. I am happy I will see your defense in person. I wish you a happy life next to Ketaki, to whom I am also thankful for all the moments we shared together these past 3 years at INM and all the amazing trips.

I would also like to extend my thanks to other colleagues at INM. First, to the people I interacted with on the first days, who became regular faces all these years. I am grateful to Sabine Müller, Bernd Rus, and Martina Bonnard for your support and help. I am also thankful to the members of the Dynamic Biomaterials Group, especially to Shardul, Sam, and Usama. Also, to María, Mokhammed, Zahra, Selim, and my man, Hanuman, who I consider friends, and to Sara for her support in the cell lab and for inviting me to her group activities. My deepest gratitude also goes to Prof. Dr. Aránzazu del Campo for the time she dedicated to my thesis and the wise advice she gave me, to which I have always listened carefully, and to Prof. Dr. Wilfried Weber for providing me with a very exciting opportunity ahead. I would also like to extend my gratitude to all the members of the Materials Synthetic Biology for their kind and warm reception.

In this journey, I also had the opportunity to collaborate with many people external to INM, and I am grateful to them. Thank you, Liang and Audrey, for the amazing time I had in Boston.

Thank you, Rahmi, for all the meetings and for being an inspiration to me for one of my projects. Thank you, Ilke, for coming not once but twice to Saarbrücken as a guest PhD student. The same goes for Mike. It was great having you here. I wish you all the best of luck, not only with our projects but with everything you do.

Família. Gràcies a tots, cosins, tietes i tiet, pel vostre suport durant aquests anys. Sempre us he tingut molt present. Manina, només vas poder seguir-me durant any i mig, però sé que allà on siguis sempre estaràs orgullosa dels teus ninos. Nenu, si estic escrivint això és en part gràcies a veure l'apassionat que et veia quan vas començar. Espero fer-ho tan bé com vas fer-ho tu. Continua sent una inspiració per mi, si us plau, i continua-ho fent al costat de la noia. Mama, papa, ara li toca al petit de casa, que segueix els passos del gran. Mami, sé que no és fàcil tenir els fills vivint per Europa, bé, ara un per Amèrica, però la veritat és que ens ho hem passat molt bé explorant el cor d'Europa, Turquia i els Estats Units junts aquests últims anys. Gràcies per tot el que has fet per nosaltres. Poques mares poden dir que tenen dos fills de doctors, però tu ho pots dir orgullosa ja que és mèrit teu. Papi, estic escrivint aquest text 12 anys després del dia que em va tocar dir-te adeu. Sé que no estaves massa convençut que decidís estudiar microbiologia fa 13 anys, però sembla que tot ha anat força bé. Continua cuidant-nos des d'allà on són afortunats de tenir-te. Aquesta tesi va per tu.

It is not easy to write back in English after writing in beautiful Catalan, but I am missing a person. A person who does not understand Catalan very well yet, but she will surely know how beautiful Catalan and Catalonia really are. Cansu, you were a great friend for 2 years. I will always remember our good chemistry and laughs. But little did I know that I would be writing this in my PhD thesis. Everything changed after you planned a trip to a country named Turkey. A trip that would mark my life forever, and a country I visit twice a year now. After that, everything became you. Not love at first sight, but true love. Because of you, I have adopted habits like having tea at night or adding yogurt to almost every meal. Now, I cannot live without them because I cannot imagine a life without you. The most meaningful thing that happened to me during my PhD at INM was crossing paths with you. I only wish for a happy life together. Seni çok seviyorum aşkim. T'estimo molt carinyo.

"Siguís un bon estudiant"

Antoni Blanch Verdú

13.06.2013

Table of contents

Motivation	10
State of the art.....	12
1. Lactobacilli bacteria.....	12
2. <i>Lactiplantibacillus plantarum</i>	15
3. Genetic transformation of <i>L. plantarum</i>	18
4. Genetic toolbox of <i>L. plantarum</i>	19
4.1 Genetic parts for plasmid maintenance	19
4.2 Genetic parts for gene regulation and protein secretion.....	23
5. Bacterial encapsulation within materials	33
Aims and Outline of the Thesis.....	36
Chapter 1.....	39
<i>Ease the genetic transformation of L. plantarum</i>	39
Publication and contributions.....	42
Chapter 2.....	57
<i>A strong promoter/repressor system for L. plantarum genetic engineering</i>	57
Publication and contributions.....	59
Chapter 3.....	73
<i>High levels of protein secretion from lactobacilli-based ELMs</i>	73
Publication and contributions.....	76
Chapter 4.....	91
<i>Switchable protein release from lactobacilli-based ELMs</i>	91
Publication and contributions.....	94
Chapter 5.....	110
<i>Genetic modules for antibiotic-free plasmid retention</i>	110
Publication and contributions.....	113
Chapter 6.....	127
<i>Development of L. plantarum for in vivo imaging and therapeutic applications</i>	127

Introduction.....	127
Materials and Methods.....	129
Results.....	133
Discussion and Conclusions	141
Conclusions and Outlook.....	142
References	146
Publications, scientific communications and patents	156
Peer-reviewed publications.....	156
Scientific talks.....	157
Patent applications	158
Abbreviations.....	159
Appendix I.....	160
<i>Expanding the genetic programmability of L. plantarum</i>	160
Publication and contributions.....	161
Appendix II.....	178
<i>Supporting Information of the publications</i>	178
II.1. Supporting Information Chapter 1	178
II.2. Supporting Information Chapter 2.....	183
II.3. Supporting Information Chapter 3.....	193
II.4. Supporting Information Chapter 4.....	205
II.5. Supporting Information Chapter 5.....	219

Motivation

Lactiplantibacillus plantarum are probiotic bacteria that play a significant role in human health, food fermentation, enhancing food safety, as well as in other biotechnology and biomedical applications. These properties and benefits are mainly attributed to a wide range of metabolites that these bacteria naturally produce. The natural properties of these bacteria can be complemented with novel functions and capabilities with the use of synthetic biology, leading to the development of next-generation probiotics. Nevertheless, even plasmid transformation in these bacteria, which is the first step to genetically transform them, remains tedious and time-consuming to date. Thus, this thesis first focuses on identifying the barriers limiting the genetic transformation in *L. plantarum* and using this knowledge to develop an alternative strategy to overcome them. That led to the development of a novel direct transformation method that facilitates and accelerates the genetic programmability of *L. plantarum*.

The next barrier to genetically programming *L. plantarum* is the lack of genetic tools for strong protein production, efficient secretion, and control over gene expression. To expand this toolbox, it is necessary to screen, test, and establish various genetic parts. A major question in this pursuit is which biological source is best adapted to offer high-performance genetic parts for *L. plantarum*. That can be addressed by investigating promising sources of genetic parts that are relevant to different molecular processes by adopting the widely used design-build-test framework in synthetic biology. These parts include promoters, repressors, and signal peptides, which can be screened from multiple sources, such as the genome of *L. plantarum*, other bacteria, and bacteriophages. Apart from expanding the toolbox, this strategy would also lead to a deeper understanding of the activity of genetic parts in *L. plantarum*, thereby enabling further optimization of genetic circuits for enhanced performance.

The discovery of novel, powerful genetic parts allows the programmability of bacteria with more complex capabilities, such as their use as living therapeutics through

therapeutically relevant protein release, as well as the assessment of these bacteria in different environments. Recombinant lactobacilli have barely been explored when encapsulated in a material. In the field of Engineered Living Materials (ELMs), living cells genetically programmed to perform specific functions are physically trapped within a material that securely contains them while providing a favorable environment for them to perform dynamic, adaptive, and responsive functions. However, little is known about the performance of the genetic parts when lactobacilli are mechanically restricted in materials as ELMs. Thus, this thesis explores the functioning of high-performance genetic parts in *L. plantarum* encapsulated within hydrogel materials, and the release of therapeutic proteins from lactobacilli-based ELMs.

State of the art

1. Lactobacilli bacteria

Lactobacilli are Gram-positive, acid-tolerant bacteria that form part of the lactic acid bacteria (LAB) group. This group of bacteria is extensively used in the food industry for fermentative processes and is associated with health benefits [1]. Lactobacilli are well-adapted to live symbiotically with higher-order organisms and are commonly isolated from a variety of plants, animals, and food products [2]. Due to the discovery of several new species, the *Lactobacillus* genus became very large (>260 species) and was reclassified into 25 separate genera in 2020 [3]. This new classification is based on the metabolic capabilities and ecological roles of each species instead of fermentation characteristics. Nevertheless, the generic term 'lactobacilli' remains valid for referring to all bacteria classified under the *Lactobacillaceae* family until 2020, and is used in this way throughout this thesis.

The fermentation capabilities of lactobacilli have made them valuable for the food industry [4]. These bacteria metabolize glucose through a fermentative process. The fermentation results in the final production of lactic acid and the subsequent acidification of their environment. This generates desirable alterations in the food (e.g., curdling of milk) and helps prevent food spoilage and improve shelf-life [5]. Lactobacilli are recognized as probiotics since they confer health benefits through various metabolites they produce, including the aforementioned lactic acid, phenyllactic acid, or short fatty acids [6]. The therapeutic effectiveness of these bacteria has been demonstrated by modulation of the immune system, antimicrobial activity against pathogens, or anticancer activity [7-9].

Apart from being probiotic, several lactobacilli species are human commensals. That means that their natural habitat is in the human body. Multiple lactobacilli species live in human saliva, respiratory tract, gut, vagina, or skin (**Figure 1**) [10, 11]. Among these commensal lactobacilli, *Lactiplantibacillus plantarum* is one of the most widely distributed

species in the human body, which is particularly relevant for healthcare or therapeutic applications.

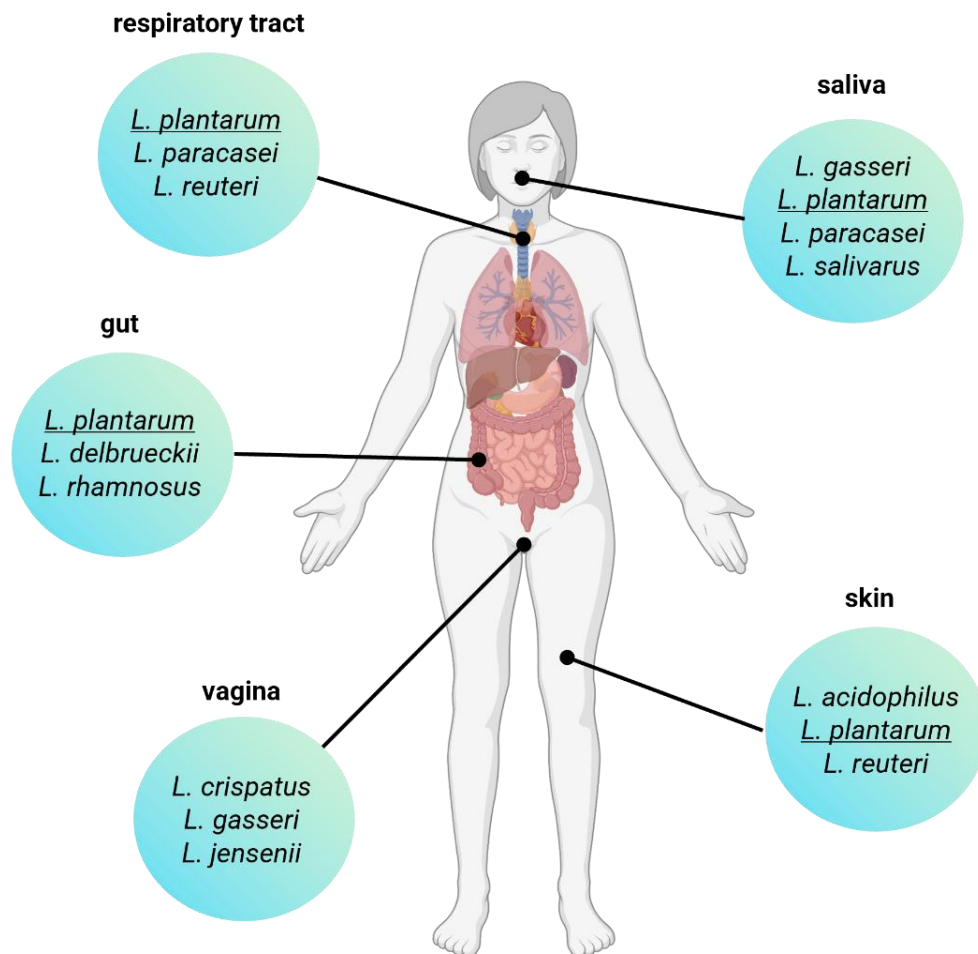


Figure 1. Illustration of a human body showing the natural habitats of multiple lactobacilli species. *L. plantarum* is the lactobacilli species that has colonized more different niches in the human body.

This trait has gained significant scientific interest in *L. plantarum*. **Figure 2** shows the number of publications based on the best-known lactobacilli species. Among these, *L. plantarum* is the species with the largest number of publications, which has been on the rise since 2010. That indicates growing interest in both *L. plantarum* fundamental and applied research.

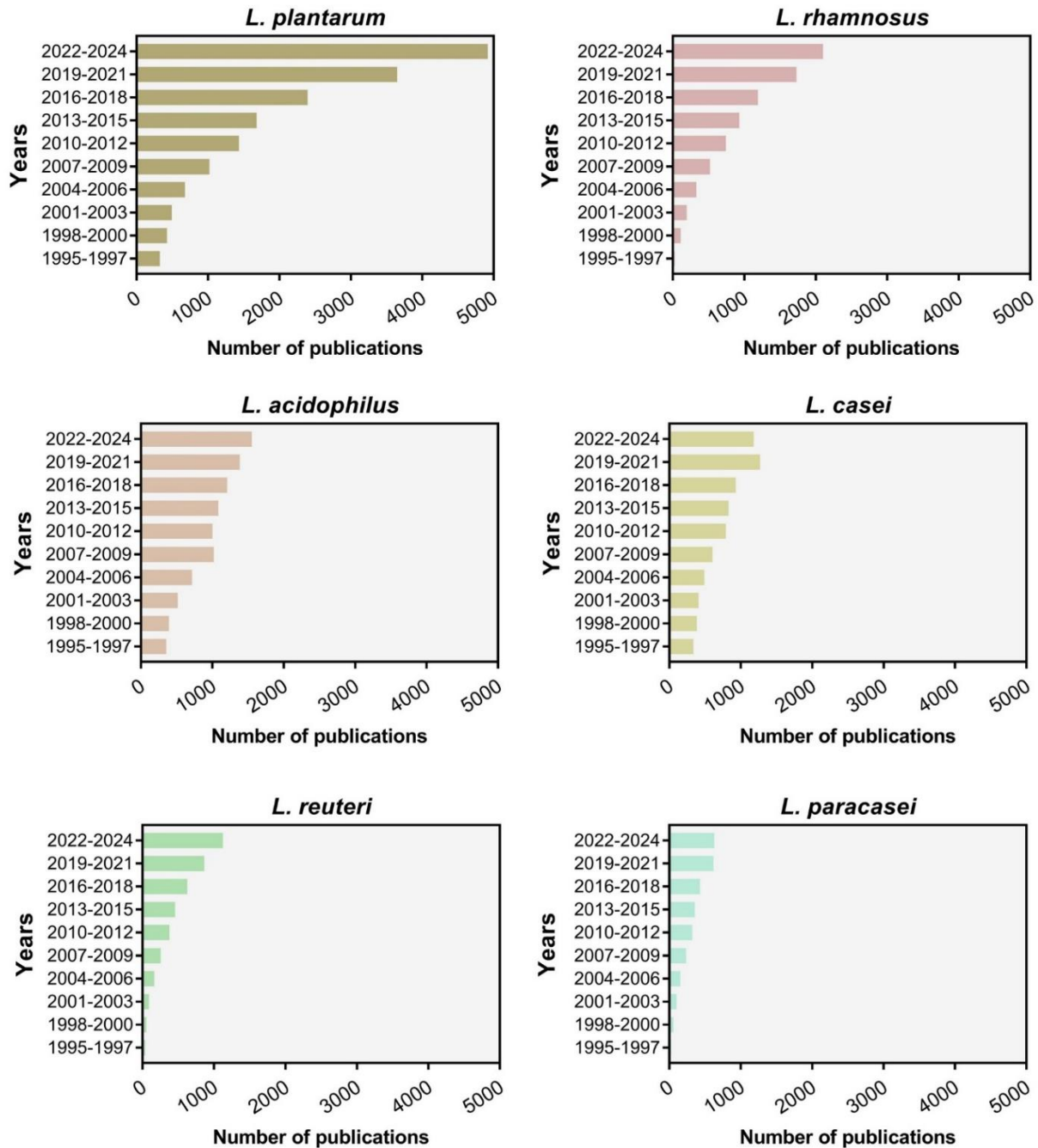


Figure 2. Plots showing the number of publications for *L. plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. reuteri*, and *L. paracasei* over the past 30 years. The data was collected from the Web of Science in May 2025. The species name before and after reclassification as search terms for the entire publication was used in the search.

2. *Lactiplantibacillus plantarum*

L. plantarum is an essential bacterium for the food industry (**Figure 3i**). This species has been isolated from a broad range of food products. For example, it has been naturally found in fermented olives, meat products, pickles, cabbage, kimchi, and tofu [12]. The metabolism of *L. plantarum* has been considerably investigated. It is well known that *L. plantarum* produces valuable metabolites as byproducts of its metabolism. Lactic acid is the best-known, and it has been commonly used as a natural food preservative given its ability to maintain acidic conditions and prevent food spoilage [13, 14]. Other metabolites produced by *L. plantarum* include short-chain fatty acids (SCFAs), mainly acetate, butyrate, and propionate [15]. These SCFAs provide health benefits and contribute to the probiotic properties of these bacteria (**Figure 3ii**). Multiple reports have shown that these acids exhibit both antioxidant activity and promising anti-tumor effects [15, 16].

In addition, *L. plantarum* is capable of producing extracellular polysaccharides (EPSs). These EPSs contain portions of mannose, fructose, galactose, and glucose, and have been used in the industry as gelling agents, emulsifiers, or stabilizers for food products [17]. The nature of these EPSs is also responsible for the probiotic properties of these LAB species, with studies showing that EPSs have antioxidant, antibiofilm, and antitumor activities [18, 19].

The production of antimicrobial peptides (e.g., plantaricin) is another factor that contributes to the natural probiotic properties of *L. plantarum* and lactobacilli bacteria [20]. For instance, *L. plantarum* has shown antimicrobial activity against food-borne pathogens such as *Listeria innocua* and *Listeria monocytogenes* [21, 22].

L. plantarum has also been subjected to genetic modifications to broaden its applications. These include the transformation of *L. plantarum* into probiotic cell factories for recombinant protein production (**Figure 3iii**). For example, *L. plantarum* has been programmed to produce industrially relevant enzymes such as lipases, cellulases, and xylanases [23, 24]. Yet, in more recent times, *L. plantarum* has been genetically modified to perform more complex functionalities such as delivery of therapeutically relevant

proteins in the body (**Figure 3iv**) or display of antigens on its cell walls to act as oral vaccines (**Figure 3v**) [25-29].

Concerning drug delivery, Ramírez et al. engineered *L. plantarum* to produce and secrete a phenylalanine hydroxylase, thus generating a bacterial probiotic system as a new therapeutic alternative for treating phenylketonuria [25]. More recently, Shen et al. demonstrated the use of *L. plantarum* engineered to produce anticancer prodrugs for inhibiting tumor growth in mice [26]. A year later, the same authors developed *L. plantarum* WCFS1 as an intranasal delivery vehicle to secrete hormones that regulate appetite, which significantly alleviated obesity-related symptoms in mice [27].

As for *L. plantarum*-based vaccines, Wang et al. surface-displayed the spike protein of the severe acute respiratory syndrome coronavirus 2, resulting in a potential food-grade oral candidate vaccine against this virus [28]. Rigaux et al. engineered *L. plantarum* to recombinantly produce Der p1, a cysteine proteinase derived from the house dust mite *Dermatophagoides pteronyssinus*, and used as a live vehicle against house dust mite atopic allergy [29].

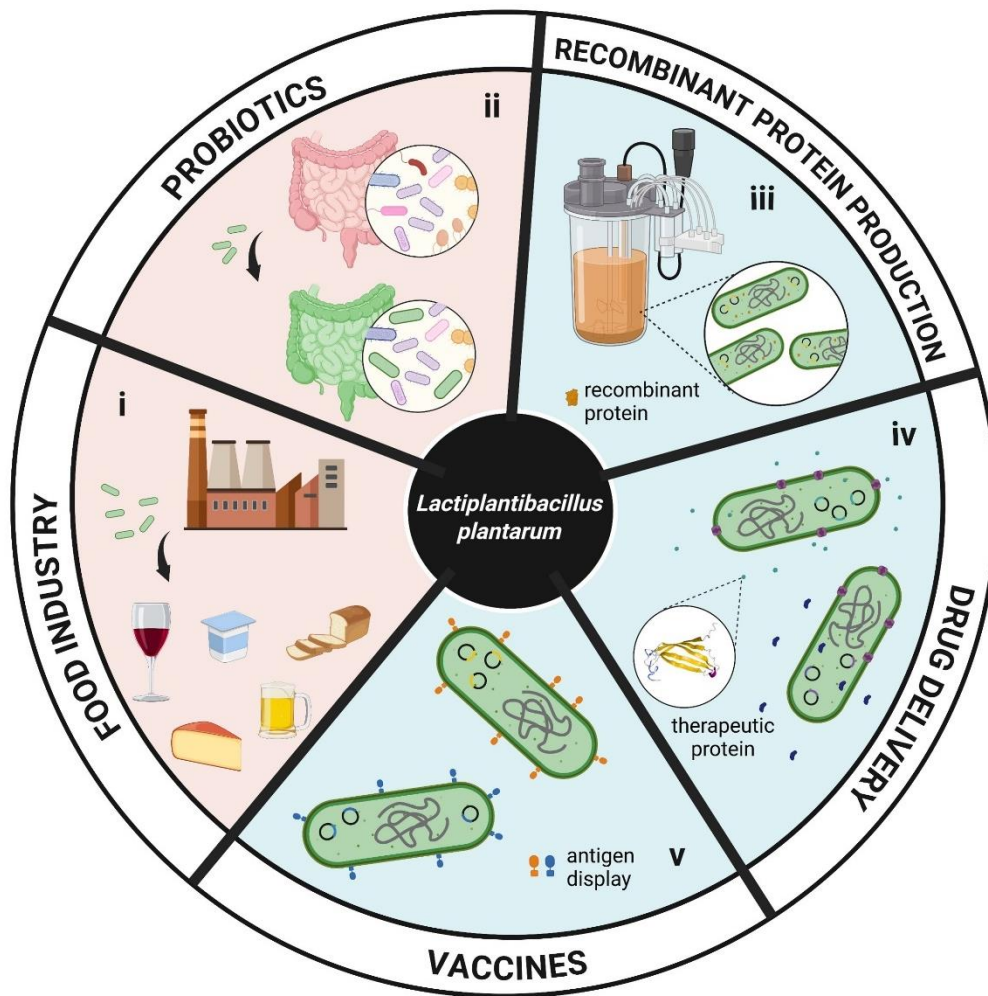


Figure 3. Illustration showing the five main applications of *L. plantarum*. Applications based on wild-type (non-modified) bacteria – depicted in a red background - include their use as probiotics and in the food industry. In parallel, *L. plantarum* has been genetically modified – depicted in a blue background – for recombinant protein production, as drug delivery vectors, and as vaccines as well.

All the previous studies illustrate the potential of leveraging a beneficial bacterial chassis and enhancing its capabilities through genetic modifications. More research groups have started investigating the genetic programming of *L. plantarum*. Nevertheless, the scope of these attempts is limited by two main challenges: the genetic engineering of *L. plantarum* and the genetic parts necessary for advanced programmability.

3. Genetic transformation of *L. plantarum*

The genetic programmability of any organism begins with the transfer of the desired genetic parts into the cell. For bacteria, there are three methods of deoxyribonucleic acid (DNA) transfer: transformation, transduction, and conjugation. Transduction and conjugation require additional viral or structural components apart from the vector DNA, which limits the applicability of these procedures to specific bacteria or strains [30]. Bacterial transformation is the most widely used method of DNA transfer. In this method, bacteria acquire exogenous extrachromosomal DNA, hereafter referred to as plasmids, from the surrounding medium and maintain it as self-replicating units independent of the genomic DNA, thus gaining novel genetic traits [31]. For a successful transformation, the most critical step is the uptake of the DNA by the bacteria. Naturally competent bacteria are capable of acquiring exogenous DNA themselves. However, most bacteria are not naturally competent, typically requiring a pre-treatment to weaken their membrane, followed by either a heat or electric shock (electroporation) to create pores in it, which enables the entry of DNA [32, 33].

Lactobacilli are commonly transformed via electroporation due to the thickness of their cell walls [34]. In this process, competent cells are incubated with plasmid DNA in a cuvette and subjected to a high-voltage electric pulse, causing a reversible disorganization in the membrane and making it more permeable to molecules and macromolecules [35]. Moreover, a higher concentration of exogenous plasmid DNA is required for successful DNA transformation in lactobacilli compared to *E. coli* [34]. For example, for *L. plantarum*, >500 nanograms (ng) of plasmid is generally used for transformation, in contrast with <100 ng required for *E. coli* [36, 37].

4. Genetic toolbox of *L. plantarum*

The genetic toolbox of one organism is a collection of parts (e.g., origins of replication, plasmid retention cassettes, promoters, ribosome-binding sites, transcriptional repressors and activators, signal peptides, etc.) that enable its precise and versatile genetic programming¹ [38, 39].

In this section, the genetic parts comprising the genetic toolbox of *L. plantarum* have been broadly divided into two sub-sections that represent their functionality: (i) plasmid maintenance and (ii) gene regulation and protein secretion.

4.1 Genetic parts for plasmid maintenance

These are the key genetic parts required for a plasmid to be retained in the bacterium after genetic transformation.

4.1.1 Origin of replication

The origin of replication (ORI) is essential for autonomous, self-controlled plasmid replication [40]. Moreover, ORIs determine the number of plasmid copies per cell, serving as a key part for fine-tuning the genetic programmability of bacteria [41].

ORIs have been widely studied in lactobacilli [42]. The p256 ORI is probably the most used in *L. plantarum* engineering. This ORI is derived from the p256 cryptic plasmid of *L. plantarum* NC7, and has shown plasmid stability in other species of *L. plantarum*, such as WCFS1 [43, 44].

¹ A collection of the state of the art regarding the genetic programmability of these bacteria, along with proposed strategies for further expanding their genetic programmability, has been compiled in a peer-reviewed review. This review is provided in **Appendix I** at the end of this dissertation.

4.1.2 Selectable marker: antibiotic resistance gene

Maintaining a plasmid is a metabolic burden for the host cell [45]. Hence, plasmids are maintained by bacteria only when they provide advantageous traits. Therefore, a mechanism is required to ensure that bacteria maintain the plasmid and retain the genetic parts to program them. For that, a selectable marker is introduced into the plasmid, which is typically a gene that confers resistance to an antibiotic. By adding an antibiotic to the growth media, only the bacteria that maintain the plasmid and therefore express the resistance can survive, exerting a selection pressure for retaining the plasmid.

There are two antibiotic resistances that are highly effective in *L. plantarum* and are commonly used for plasmid retention purposes. One is chloramphenicol acetyltransferase, which confers resistance to chloramphenicol. The other is erythromycin methyltransferase, which provides resistance to erythromycin. Both antibiotics have a similar mechanism of action, which focuses on the inhibition of protein synthesis by binding to the 50S ribosomal subunit and blocking the formation of additional peptide bonds [46, 47]. The chloramphenicol acetyltransferase covalently attaches an acetyl group to chloramphenicol, preventing its binding to the ribosome [48]. The enzyme erythromycin methyltransferase methylates an adenine residue of the 23S ribosomal ribonucleic acid (rRNA) subunit and significantly decreases the binding of the erythromycin to the ribosome [49, 50].

Despite providing efficient plasmid retention, antibiotic markers are not suitable for human applications since this approach raises concerns about horizontal gene transfer and requires the administration of antibiotics in the body. Additional genetic parts that can prove effective as an alternative to antibiotic resistance for plasmid retention are toxin/antitoxin systems.

4.1.3 Selectable marker: toxin/antitoxin systems

Toxin/antitoxin (TA) systems are genetic modules composed of two elements: a toxin-encoding gene and its cognate antitoxin-encoding gene [51]. There are eight different

types of TA systems based on molecular targets (either protein or non-coding RNA) and the mechanism of action. Yet, the most commonly studied is the type II TA system [52]. Here, both toxin and antitoxin are proteins. The function of the antitoxin is to sequester the toxin by a protein-protein interaction mechanism, which results in the neutralization of the toxicity associated with the toxin (**Figure 4A**) [53].

The systems were first discovered in plasmids, exerting a role in plasmid maintenance via a mechanism termed post-segregation killing [54]. Given that the stability of toxins tends to be higher than their cognate antitoxins, if plasmid loss occurs during cell division and the antitoxin cannot be further produced, non-neutralized toxins might remain in the cytoplasm of the bacteria, eventually leading to cell death (**Figure 4B**). Given this natural function, the possibility of using TA systems instead of antibiotic resistance genes for plasmid retention has been investigated. For instance, promising plasmid stability was accomplished both in vivo and in vitro in *E. coli* Nissle 1917 using the hok/sok and the axe/txe TA systems [55, 56].

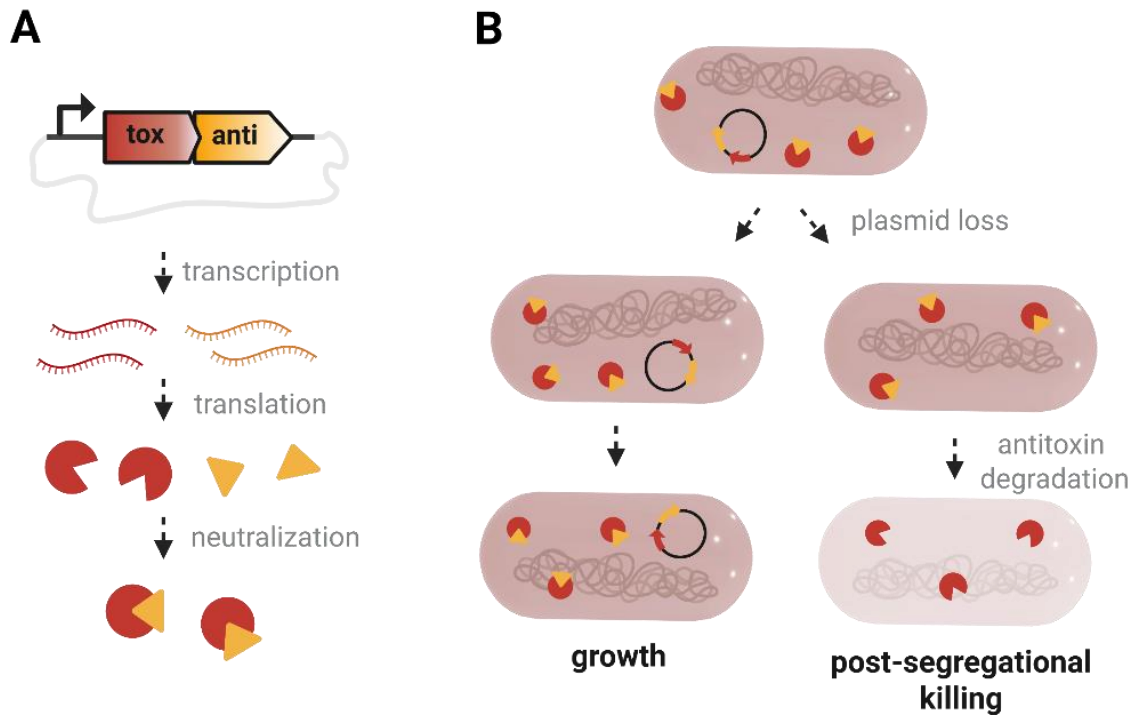


Figure 4. A) Schematic illustration of type-II TA systems encoded in a plasmid. First, both genes are transcribed into messenger RNAs (mRNAs), which are then translated into proteins. At the protein level, the antitoxin protein interacts with the toxin protein and neutralizes its toxic activity. **B)** Schematic illustration showing the post-segregation killing mechanism type-II TA systems. Without plasmid loss, both toxin and antitoxin are produced, and bacteria can grow through the neutralization of the toxin by the antitoxin. If plasmid loss occurs, production of toxins and antitoxins ceases. Due to the higher toxin stability, once the remaining antitoxin is degraded, the unbound toxin can exert its toxic activity and cause cell death.

In 2005, Sørvig et al. identified a TA system encoded in the endogenous p256 plasmid of *L. plantarum* NC7 and reported that this system is involved in the plasmid maintenance [43]. Besides, other TA systems have been identified in the genomes of other *L. plantarum* strains and other lactobacilli such as *L. rhamnosus*, *L. casei*, and *L. paracasei* [57-59].

These findings create an opportunity to further explore TA systems as genetic parts to mediate plasmid retention in lactobacilli.

All genetic engineering conducted in this thesis relies exclusively on plasmid engineering. The plasmids harbor the p256 ORI for plasmid replication and the erythromycin resistance gene. **Figure 5** shows the genetic architecture of a typical plasmid built for the genetic transformation of *L. plantarum* in this thesis. Beyond the origin of replication and the resistance, this plasmid harbors a gene of interest (GOI). This is one of the great benefits of plasmid engineering: the possibility to insert additional functional genetic parts into the plasmid, thereby equipping the engineered bacteria with advanced functionalities. Nevertheless, for these genetic parts to be adequately expressed, they need to be adapted, designed, and assembled for the host in which they are meant to function.

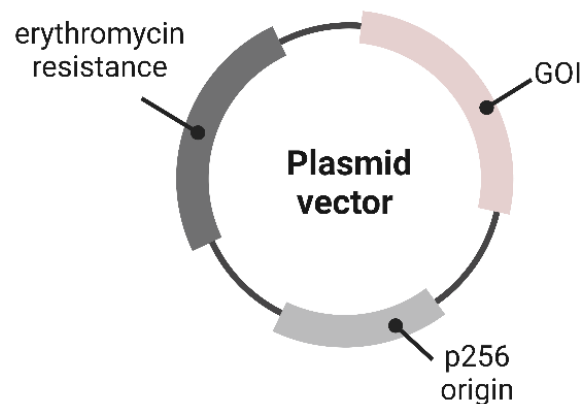


Figure 5. Genetic map of a plasmid vector adapted for *L. plantarum* engineering. The p256 ORI is responsible for the autonomous plasmid replication. The erythromycin resistance mediates plasmid retention when bacteria are grown in medium supplemented with erythromycin. In addition, a GOI can be cloned into the plasmid and expressed using the appropriate genetic tools.

4.2 Genetic parts for gene regulation and protein secretion

The genetic toolbox for regulating gene expression and protein secretion of model bacteria such as *Escherichia coli* is very extensive, with a wide range of well-characterized,

optimized genetic parts that encode various functions [38]. However, this is not the case for probiotic lactobacilli, which have a rather limited genetic toolbox.

Some of these genetic parts are DNA sequences that are not translated into proteins but are crucial for the initiation or regulation of transcription or translation. That includes promoters, ribosome-binding sites, and operators [38]. Other genetic parts are translated into proteins and are associated with specific functions, such as directing a protein to the extracellular environment. Transcriptional repressors, activators, and signal peptides are common examples of translated genetic parts within a genetic toolbox. Untranslated and translated genetic parts can be assembled to build genetic circuits that equip bacteria with advanced genetic programmability [39].

The genetic toolbox for *L. plantarum* engineering is probably the most expanded across all lactobacilli species, but remains limited compared to model organisms. Next, the state of the art of the most relevant genetic parts for *L. plantarum* genetic programming is discussed.

4.2.1 Promoters and Ribosome Binding Sites

Bacteria are commonly used as living factories for recombinant protein production because they can overproduce a protein of interest (POI) when instructed to do so [60, 61]. For such capabilities, the coding sequence of the POI needs to be placed downstream of a strong promoter. In prokaryotic promoters, there are two crucial regulatory elements, the -35 and -10 boxes, where the RNA polymerase binds to initiate the transcription process. To be precise, the sigma factor 70, a component of the RNA polymerase, is the regulatory protein with affinity towards the regulatory boxes. If the affinity is strong, the sigma factor 70 will recruit the RNA polymerase to that promoter. The stronger the affinity is, the higher the transcription rate. That results in higher levels of mRNA and, potentially, a larger amount of translated protein (**Figure 6**) [62, 63].

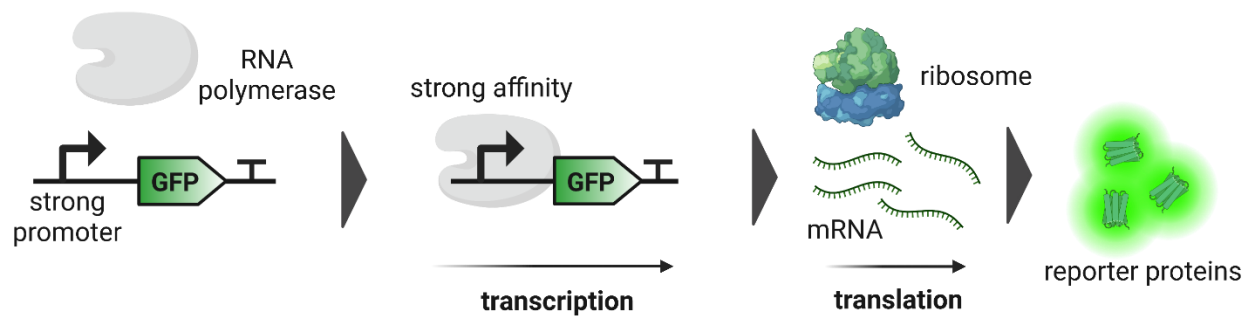


Figure 6. An example of engineering a gene encoding for a green fluorescent protein (GFP) under a strong promoter. The RNA polymerase exerts a strong affinity towards this promoter, leading to high levels of mRNA after transcription. This results in high-level GFP production after the ribosome-driven translation.

The amount of translated protein depends on another sequence named the ribosome-binding site (RBS). In bacteria, this sequence is frequently referred to as the Shine-Dalgarno sequence, named after its discoverers [64]. This sequence is placed downstream of the regulatory boxes of the promoter, as it operates at the RNA level, and its transcription is required. When in RNA form, the ribosome can bind to this RNA sequence and initiate protein translation [64, 65]. Translation efficiency greatly depends on the nucleotide composition and length of the RBS [66, 67]. As a result, these sequences are key regulatory elements for fine-tuning gene expression, acting in the translation process.

All things considered, strong promoters are valuable parts for the genetic programmability of any organism. For model bacteria such as *E. coli*, *Corynebacterium glutamicum*, *Bacillus subtilis*, or *Streptomyces coelicolor*, extensive work has focused on generating and testing large libraries of natural or synthetic promoters to achieve high levels of protein production [68-75].

Similar but less exhaustive work has been done on *L. plantarum* [76]. For example, in 2006, Rud et al. Created a synthetic promoter library, thus generating a range of promoters exerting different levels of activity [77]. The strongest promoter of this library, P_{48} , has been used in this thesis for protein expression. A recent study by Meng et al. reported another constitutive promoter used in this thesis, P_{23} , and made variants by introducing

random mutations adjacent to the RBS [78]. More recently, in 2025, Yu et al. followed up this work by creating 247 mutants of P_{23} via error-prone polymerase chain reaction (PCR), some of which performed better than wild-type P_{23} [68].

Spangler et al. and Tauer et al. reported more constitutive promoters in *L. plantarum* and additionally investigated the importance of the spacer sequence between the RBS and the start codon [79, 80]. This genetic trait has been considered when designing genes adapted for *L. plantarum* for this thesis, despite its moderate strength. In terms of overall levels of protein production, *L. plantarum*'s levels remain low compared to those achieved with model bacteria. That is mainly because strong promoters were unknown in *L. plantarum* before the start of this thesis, which provided opportunities for further work in this direction by experimentally testing unexplored promoters from different biological sources.

Apart from the -35 and -10 boxes and the RBS, promoters can additionally include regulatory sequences such as operators. These operators are relevant for the functionality of other genetic parts commonly used in synthetic biology for programming organisms: the transcriptional repressors.

4.2.2 Transcriptional repressors

Transcriptional repressors are proteins that bind to a specific DNA sequence termed an operator, which is commonly placed within the promoter region, close to the regulatory boxes. This binding repression of gene expression [81]. In bacteria, typical repressors are composed of two protein domains: the DNA-binding domain, normally based on helix-turn-helix motifs, is responsible for binding tightly to the operator, and a dimerization domain, which allows monomers to form a dimer and favor a tight interaction with the DNA [82]. Tight binding of the repressor to the operator sequence obstructs the RNA polymerase from interacting with the regulatory sequences of the promoter, resulting in reduced mRNA synthesis from the gene under that promoter (**Figure 7**).

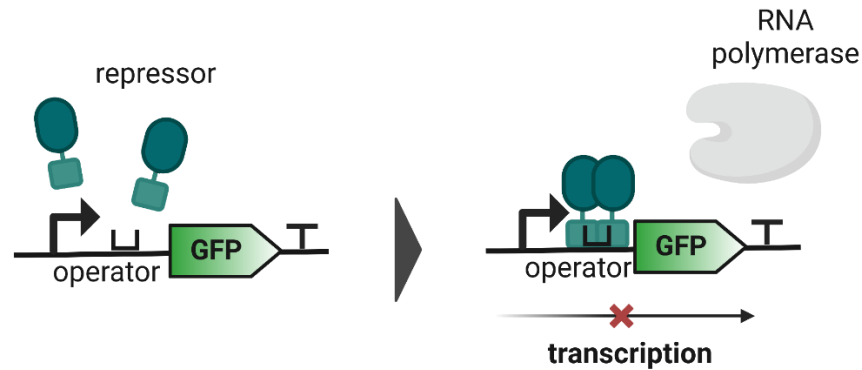


Figure 7. Repression of GFP expression (transcription) by placing an operator sequence within a promoter sequence and expressing the cognate repressor in parallel.

Repressors are common parts of genetic circuits since they allow temporal regulation of gene expression [83, 84]. For instance, Riglar et al. built a genetic circuit in *E. coli* to detect tetrathionate, produced during inflammation, and developed an inflammation-living diagnostic [85]. Two transcriptional repressors, *cro* and *cl*, were part of that genetic circuit. These repressors are naturally encoded in the *E. coli lambda* phage genome [86, 87].

In nature, numerous transcriptional repressors can modulate their activity in response to external stimuli and are therefore referred to as switchable repressors [88]. The *lacI* repressor was one of the first repressors to be thoroughly characterized. When the repressor is exposed to the effector molecule or inducer, in this case, allolactose, it undergoes a conformational change that decreases its binding affinity with the operator sequence (**Figure 8**) [89].

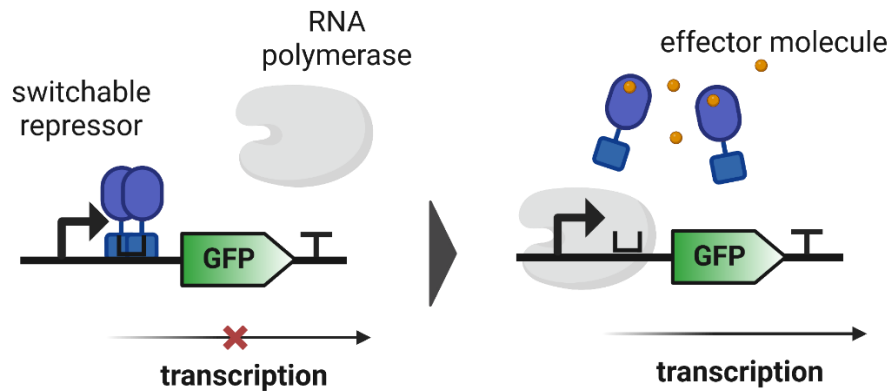


Figure 8. Switchable repression of GFP expression (transcription) by the addition of the effector molecule, which triggers a conformational change to the repressor and impairs gene repression.

In the past two decades, advances in structural and computational biology have permitted the development of repressor engineering strategies and the modification of non-responsive repressors into switchable repressors that respond to sugars or physical stimuli such as heat or light [90-92].

Switchable repressors have been extensively used in genetic circuits to regulate gene expression on demand [93]. That is especially relevant for recombinant protein production, where high levels of expression might impose a metabolic burden that can be overcome by making such production inducible [94, 95]. These switchable repressors are also integral parts of living biosensors or used for therapeutic applications [96, 97].

All the previously mentioned examples are based on *E. coli*. However, limited knowledge exists concerning repressors in *L. plantarum*. Attempts to adapt well-functioning genetic switches from other bacteria have resulted in poorly performing switches in lactobacilli.

In 2016, Heiss et al. tested genetic switches responsive to Isopropyl β -D-1-thiogalactopyranoside and xylose in *L. plantarum*. Despite being well-established genetic switches in other bacteria, they exhibited leaky expression and poor induction (below 10-fold change) [98]. In another study, Zhang et al. built genetic switches based on endogenous inducible promoters from *L. plantarum* WCFS1 responsive to lactose and

galactose [99]. The switches achieved fold changes of 10-12, but the performance was low compared to model bacteria. For instance, the pBAD system in *E. coli* achieves fold-changes greater than 1000 upon induction with L-arabinose [100, 101]. These differences are due to the existence of strong promoters and repressors in the *E. coli* genetic toolbox. Nevertheless, these studies shed light on promoter/repressor designs in *L. plantarum*. They also indicate that additional effort is required to build or identify reliable promoter/repressor systems that can be engineered into genetic switches in *L. plantarum*.

In addition to the transcriptional repression, gene expression can be regulated through genetic parts that exert the opposite effect: transcriptional activation. These are called activators and are introduced in the next section.

4.2.3 Transcriptional activators

Transcriptional activators are proteins that positively regulate gene expression. Activators can operate via two mechanisms: by direct protein–protein interaction with the RNA polymerase, or by introducing a conformational change in the promoter region, making it more accessible to the RNA polymerase [102, 103].

Common activators in bacteria are the response regulators from two-component signal transduction systems [104]. These systems are composed of a histidine kinase receptor and a response regulator. In canonical systems, the histidine kinase acts as a sensor that perceives the input signal and autophosphorylates on a conserved histidine residue. Next, the phosphate group is transferred to the response regulator, which subsequently becomes activated. Once activated, the response regulators assist in enhancing transcription from a cognate inducible promoter [105, 106].

The most reliable and widely used genetic switch in lactobacilli is based on a two-component regulatory system originally from a *L. sakei* operon. This switch, termed the pSIP system, consists of the genes *sppK* and *sppR*, encoding for a histidine kinase receptor and its cognate response regulator, respectively. Induction takes place upon the

presence of an autoinducer peptide, which results in the phosphorylated activation of the response regulator and initiation of gene expression from the inducible *sppIP* promoter (Figure 9) [107].

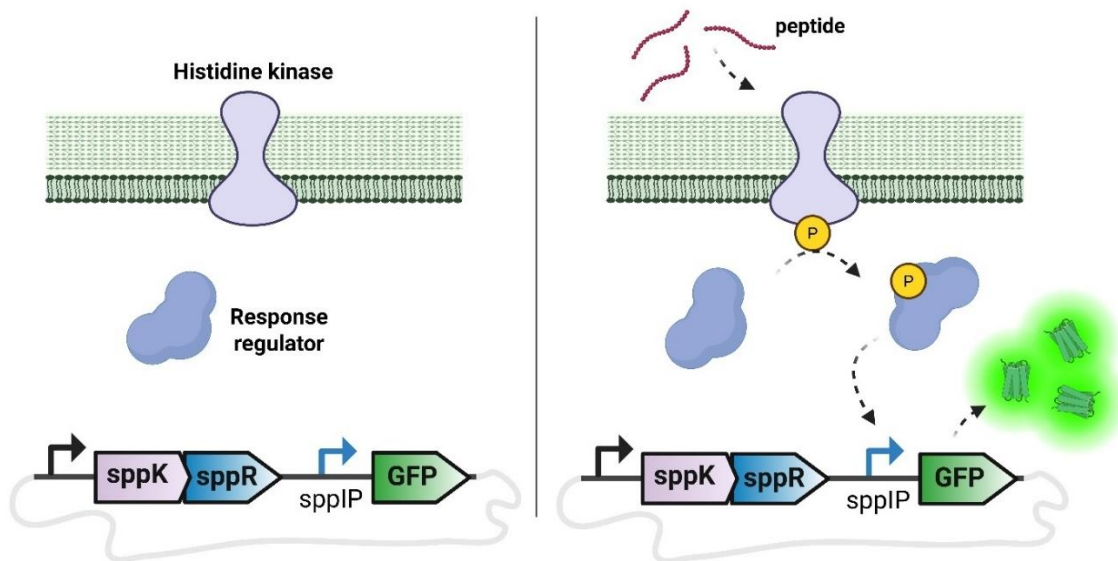


Figure 9. *pSIP* system in *lactobacilli*. Only in the presence of the autoinducer peptide does the histidine kinase become phosphorylated and initiate a cascade that results in the activation of the response regulator *sppR*. The activated *sppR* response regulator positively regulates the transcription of the GFP-encoding gene from the inducible promoter *sppIP*.

The pSIP switch has proven functional at activating gene expression in multiple lactobacilli species, including *L. sakei*, *L. plantarum*, *L. curvatus*, *L. brevis*, *L. rhamnosus*, *L. gasseri*, *L. acidophilus*, *L. casei*, and *L. reuteri* [108-110].

In the past decades, this switch has been employed to regulate gene expression in *L. plantarum*. For instance, the pSIP system has enabled the production of enzymes like β -galactosidases or nucleases and antigens like the oncofetal antigen or an antigen from the porcine transmissible gastroenteritis virus [108, 111-115].

Nonetheless, the pSIP system i) is composed of two components adapted to the transcription machinery in lactobacilli, which restricts its portability and adaptability, ii) relies on a peptide as an inducer, making it relatively cost-intensive as an inducible

system, and iii) only supports a moderate level of protein production after induction. Altogether, there is still a critical need for a genetic switch offering better functionality.

The pSIP-based genetic switch shown in **Figure 9** regulates the intracellular production of GFP. However, intracellular protein production is not always the desired output. In some cases, recombinant proteins are meant to perform their activity in the extracellular space. For example, when probiotic bacteria are engineered to continuously secrete therapeutic proteins that interact with host-cell receptors to modulate specific pathways. To mediate the transport of the translated protein into the extracellular space, the GOI must be equipped with another genetic part: a signal peptide (SP).

4.2.4 Signal peptides for protein secretion

Gram-positive bacteria such as *B. subtilis*, *Bacillus licheniformis*, or *C. glutamicum* are attractive hosts for industrial processes due to their naturally high protein secretion capacity [116-119]. The mechanism underlying protein secretion in Gram-positive bacteria has been thoroughly investigated and is well-understood [120]. There is a major route that most proteins take [121].

During translation, the polypeptide chain emerging from the ribosome initially remains in an unfolded state, a condition that must be preserved for proteins destined for secretion via the Sec pathway. Recognition of these proteins by the host secretory machinery is mediated through a cleavable N-terminal SP. A chaperone identifies the SP and assists in maintaining the protein in an unfolded state, which is then transferred to SecA, the motor domain of the Sec translocase. Following that, this protein is translocated across the membrane through the SecYEG channel. SecDF subsequently pulls the protein through the channel to the periplasmic space, where a signal peptidase cleaves the SP. Finally, secreted proteins interact with the bacterial cell wall and pass through it into the external environment (**Figure 10**) [122-124].

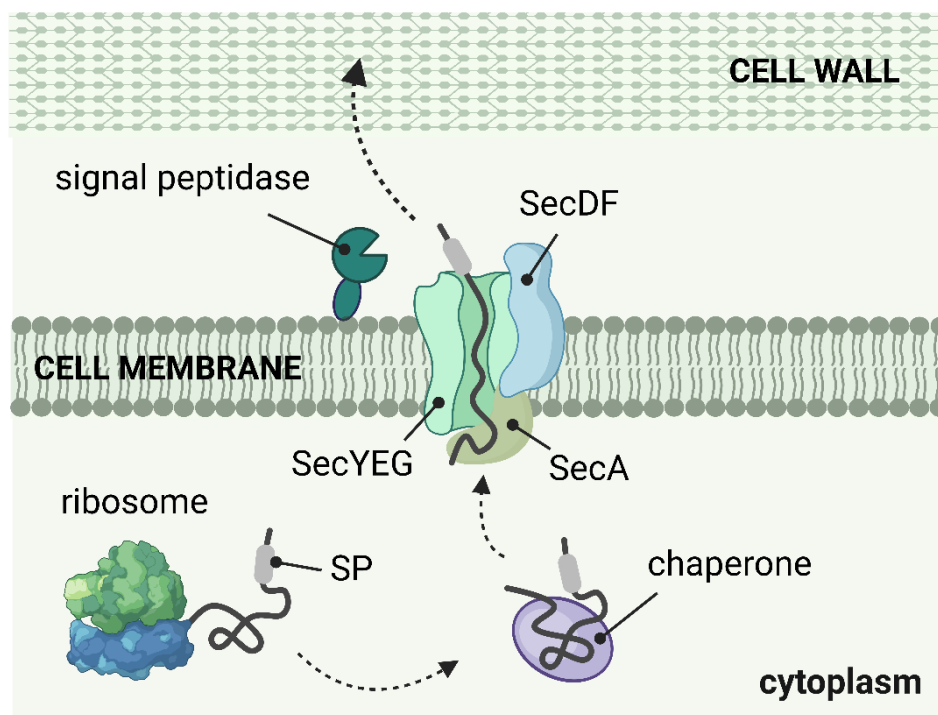


Figure 10. The Sec pathway in Gram-positive bacteria. Unfolded SP-tagged proteins are recognized by a chaperone and translocated through SecYEG with the assistance of SecA and SecDF. In the periplasmic space, a signal peptidase cleaves the SP, and the protein is free to be folded and pass through the cell wall into the extracellular space.

Secretion efficiency is strongly influenced by the SP sequence [125]. Beyond elucidating the core components of the Sec pathway machinery, researchers have focused on identifying SPs capable of driving high levels of protein secretion. These SPs are incorporated into the recombinant proteins of interest intended to be secreted. For example, in the Gram-positive model bacterium *B. subtilis*, large libraries based on natural and engineered SPs have been generated. Fu et al. tested up to 173 SPs in a single study and identified 15 SPs that led to promising levels of protein secretion [126]. From LAB, the best secreter species is *Lactococcus lactis*, which is considered to be an efficient cell factory for protein secretion [127]. In *L. lactis*, there is a gold-standard signal for protein secretion, the Usp45sp [128, 129]. Ng et al. generated a mutant library of the Usp45sp, resulting in enhanced secretion levels [130].

Related work has been carried out in *L. plantarum*. In 2008, Mathiesen et al. tested seven endogenous SP in *L. plantarum* WCFS1. They assessed the secretion of two proteins. The first one was an α -amylase, an enzyme with industrial relevance for starch hydrolysis. The second one was a nuclease A (NucA) from *Staphylococcus aureus*, which has been commonly used as a secretion reporter in LAB [131]. From these SPs, Lp_0373 and Lp_0600 showed promising results for directing protein secretion in *L. plantarum* WCFS1 [132]. One year later, the same authors developed a library composed of 76 endogenous SPs mined from the genome of *L. plantarum* WCFS1, as well. These SPs were computationally predicted to be cleaved by the signal peptidase I and follow the Sec secretion pathway. From this library, Lp_3050 was the most efficient SP [133]. This SP is regarded as the current gold-standard SP for protein secretion in *L. plantarum*, and is commonly used in *L. plantarum*-based secretion studies as a reference [134].

The overall protein secretion levels reported for *L. plantarum* and lactobacilli are low compared to the best-secreting Gram-positive bacteria, such as *B. subtilis* or *C. glutamicum*. Even if *L. plantarum*'s endogenous secretory machinery does not naturally secrete as much protein as the best secreters, there are still opportunities for improvement.

So far, the most relevant genetic parts for the genetic programmability of bacteria, including *L. plantarum*, have been listed and discussed. All these parts are newly presented in more detail in the following chapters with the intention of exploring, discovering, and optimizing novel parts to expand the genetic programmability of *L. plantarum*.

5. Bacterial encapsulation within materials

The intersection of synthetic biology involving living cells and materials sciences is commonly referred to as engineered living materials (ELMs). In ELMs, a living component (e.g., bacteria, fungi, mammalian cells) is embedded within a material component, thereby mechanically restraining growth and, therefore, their escape, while maintaining

their genetically programmed functionality [135, 136]. This field has gained considerable attention in the past decade, targeting a wide range of applications, including biotherapeutics and other biomedical applications such as biosensors, environmental remediation, eco-friendly architecture, or sustainable energy generation [137-139]. An example based on a living biosensor is illustrated in **Figure 11**. In this setup, living bacterial cells are physically constrained within material matrices and can sense an extracellular molecule and produce a red fluorescent intracellular reporter in response.

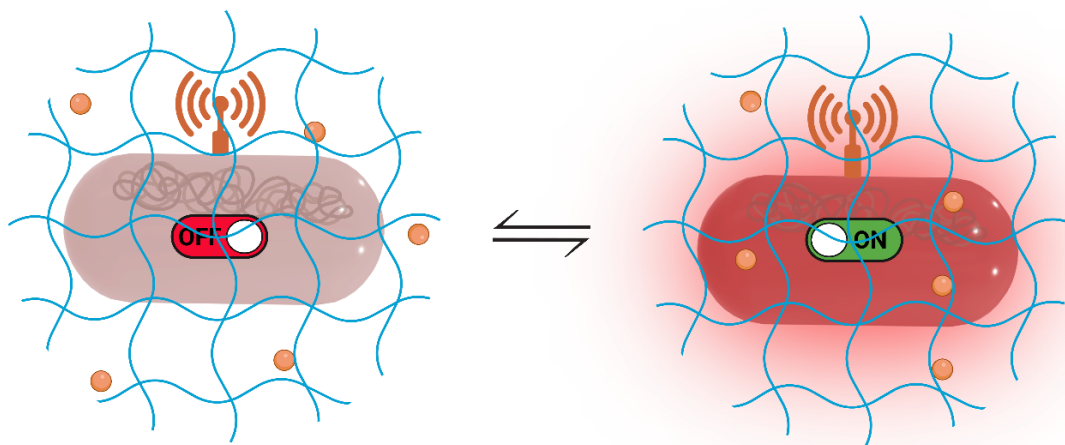


Figure 11. *Living biosensor. Encapsulated bacteria sense the molecule and respond accordingly by expressing a fluorescent reporter gene, serving as a biosensor for that molecule.*

Bacterial encapsulation within hydrogels is a widely employed ELM format, as their high water content enables great tunability [140]. These materials can be fabricated with various functionalities to generate chemical gradients while offering a mechanical confinement for the encapsulated living cells. That has broadened the applicability of hydrogels in fields ranging from biosensors to delivery of therapeutics [141, 142]. The works by Liu et al. are representative examples of hydrogel-based ELMs. These authors created 3D-printed living tattoos and wearable devices such as gloves for chemical detection on human skin [143, 144].

Hydrogels can be synthesized by crosslinking various polymer precursors, including poly(N-isopropyl acrylamide), poly(N, N-dimethyl acrylamide), alginate, hyaluronic acid, gelatin, agarose, among others [145]. Among these, alginate is low-cost, easy to handle,

and biocompatible [146, 147]. One example of an alginate-based ELM is reported by Li et al. These authors engineered bacteria to sense an autoinducer molecule secreted by the opportunistic pathogen *P. aeruginosa* and encapsulated them in alginate microgels. The presence of the autoinducer in the environment could activate the encapsulated bacteria, showcasing the promise of this ELM format for bacterial detection [148].

Most hydrogel-based ELM studies to date rely on the encapsulation of *E. coli*, the most frequently employed bacterial chassis for living materials. This prevalence is due to the wide array of capabilities these bacteria can be genetically programmed to perform.

From LAB, *L. lactis*-based ELMs are the most reported in the literature. For instance, in 2020, Yang et al. engineered *L. lactis* to secrete interferon- γ in response to light exposure and encapsulated these bacteria within alginate beads. This ELM slowed down tumor progression in mice [149]. Two years later, Pan et al. developed light-responsive *L. lactis*-containing alginate microspheres that secreted granulocyte-colony-stimulating factors produced by the bacteria. This system relieved Parkinson's disease symptoms in mice [150]. Both studies showcase the potential of combining genetically enhanced probiotic bacteria with a biomaterial-based containment to create smart, responsive materials.

Wild-type lactobacilli, including *L. plantarum*, have also been encapsulated within various materials in recent decades. However, given that the activity of these bacteria is required in the gastrointestinal tract, the primary objective of such encapsulation has been to preserve the cell viability during the harsh conditions encountered before reaching the gastrointestinal tract. For example, alginate and other polymers have shown potential to promote bacterial survival when exposing living materials to acidic conditions and bile salts [151, 152].

More advanced functionalities within ELMs can be realized by expanding the *L. plantarum* genetic toolbox, along with a reliable biocompatible containment to create ELMs with non-model probiotic bacteria. However, to achieve this, it is first necessary to discover high-performance genetic parts, followed by implementing an encapsulation strategy that preserves both functional integrity and performance of the genetic parts.

Aims and Outline of the Thesis

The following four aims have been pursued in this thesis to i) achieve simpler, more reliable genetic transformation and ii) enhance the genetic programmability of *L. plantarum* for therapeutic and biotechnology applications.

- 1. Identify the barriers limiting genetic transformation in *L. plantarum* and develop strategies to overcome them.**
- 2. Identify promising sources of high-performance genetic parts for strong protein expression and secretion, and evaluate their performance by applying a design-build-test framework.**
- 3. Demonstrate the applicability of novel genetic parts by using them to efficiently produce and secrete therapeutically relevant proteins, and to mediate antibiotic-free plasmid retention.**
- 4. Assess the performance of these genetic parts when *L. plantarum* bacteria are encapsulated within alginate hydrogel beads as an ELM.**

The aims have been addressed in the following six chapters of this thesis.

Chapter 1 is about making the genetic transformation of *L. plantarum* more straightforward, less time-consuming, and suitable for toxic genes to intermediate hosts. Toward that end, the barriers limiting the drawbacks of the conventional method for transforming these bacteria were identified, and strategies to overcome them were proposed. The result was a novel, flexible, direct cloning method, supposing a time-saver and intermediate-free alternative to the conventional cloning method for *L. plantarum* WCFS1.

Chapter 2 is based on identifying promoter/repressor systems as components of a protein production module. Here, the source of genetic parts is first discussed, and the

rationale behind the selection is justified. Next, genetic parts, namely transcriptional repressors, from lactobacilli-infecting bacteriophages were investigated to shut down the gene expression in *L. plantarum*. A design-build-test approach is employed to generate genetic variants and improve their performance, resulting in the discovery of an outstanding promoter/repressor system.

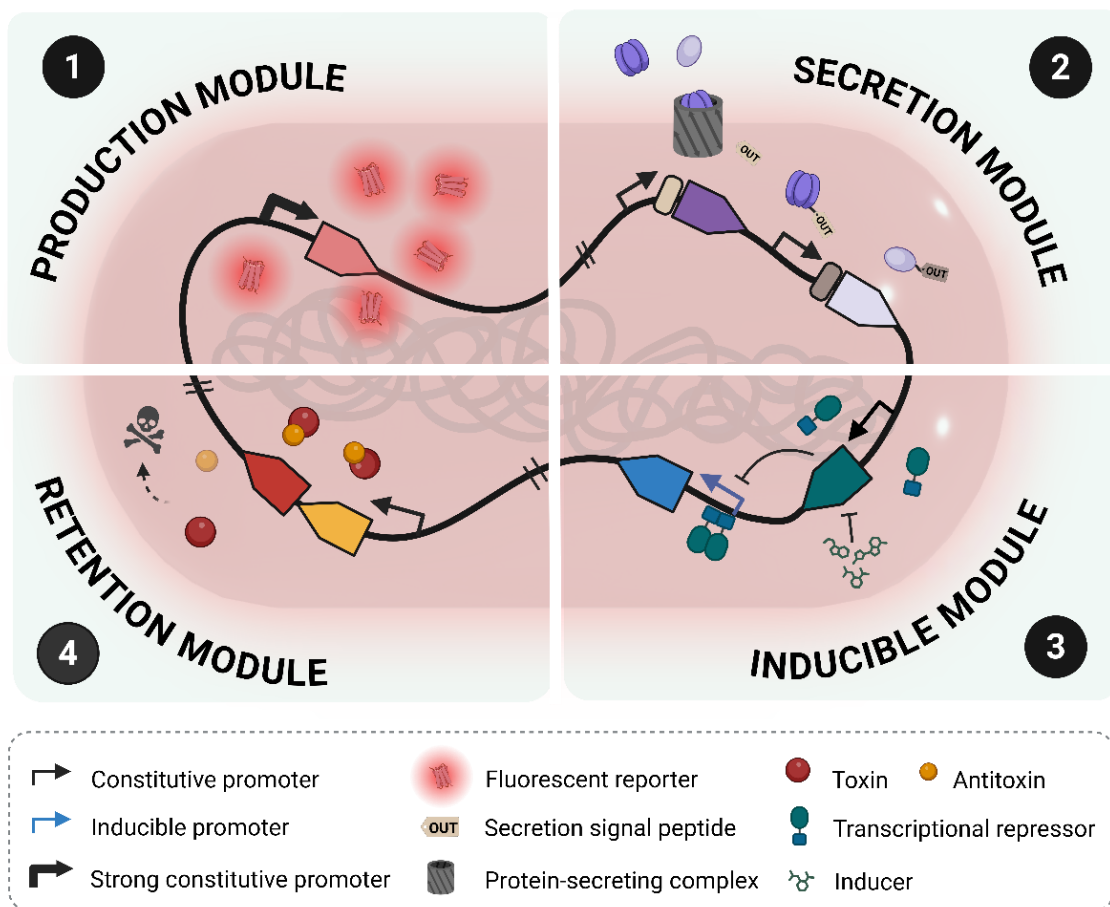
Chapter 3 embarks on protein secretion by using the newly discovered promoter for driving the expression of genetic secretion modules. This chapter investigates whether the novel promoter can be used as a key component of a secretion module to achieve higher levels of protein secretion. The other components of the secretion modules (SPs) are mined from the endogenous gene machinery of *L. plantarum*. The performance of secretion modules is assessed from both bacterial cultures and, for the first time, in *L. plantarum*-based ELMs. Engineered *L. plantarum* bacteria are effectively and functionally encapsulated within alginate beads, giving rise to the PEARL format: “Protein Eluting Alginate with Recombinant Lactobacilli”, and secretion of therapeutically relevant proteins is accomplished.

Chapter 4 focuses on engineering the novel strong constitutive promoter into an inducible one, thus generating an inducible module adapted for *L. plantarum*. This chapter further investigates the question related to the performance of the genetic parts in material-encapsulated bacteria. Switchable protein production and secretion are studied for the first time in *L. plantarum*-containing ELMs, and an enhancement effect driven by the material encapsulation is described.

Chapter 5 is about the development of a plasmid retention module that does not require antibiotics. In this chapter, a strong promoter discovered by us is used to evaluate the performance of other genetic tools mediating plasmid retention. Here, multiple TA systems never tested in *L. plantarum* were cloned, and their effect on mediating antibiotic-free plasmid retention was evaluated.

Chapter 6 further examines the applicability of the novel genetic parts for the production and secretion of valuable proteins. In this final chapter, relevant recombinant *L. plantarum* strains are generated. First, the strong constitutive promoter is engineered to drive

expression of not one but three reporters simultaneously by employing a polycistronic approach. One of the reporters is a luciferase capable of generating high levels of bioluminescent light, the performance of which is being evaluated through *in vivo* studies in mice. Second, a strain that continuously secretes a nerve growth factor into the environment is described.



Plasmid map showing the genetic modules built and explored in the upcoming chapters of this thesis. The genetic parts relevant for each module are shown in the legend. All these parts play central roles in the chapters that follow. In brief, the **production module** (explored in Chapters 2 and 5) features a strong constitutive promoter able to very strongly drive the expression of a fluorescent protein. The **secretion module** (Chapter 3) builds on this and showcases the secretion of proteins by efficient SPs. Downstream is the **inducible module** (Chapter 4), which adds a switchable repressor to achieve inducible gene expression. Finally, the **retention module** (explored in Chapter 5) incorporates a TA system to mediate retention of the whole plasmid in an antibiotic-free manner.

Chapter 1.

*Ease the genetic transformation of *L. plantarum**

This chapter identifies the limitations of the genetic transformation of *L. plantarum* and explores a strategy to overcome them. The major barrier to successful genetic transformation of *L. plantarum* is the large amount of DNA required. For plasmid DNA, this is around 500-1000 ng as opposed to <100 ng in the case of *E. coli*. Common methods of assembling DNA into a plasmid, including Gibson Assembly, Golden Gate Assembly, or Restriction Digestion and Ligation, typically yield <50 ng of correctly assembled plasmid. To obtain sufficient amounts of such plasmid DNA for successful transformation of *L. plantarum*, the standard method relies on employing intermediate hosts such as *E. coli* that can replicate the plasmid while dividing [36]. However, involving an intermediate host has certain disadvantages. First, additional genetic parts needed for plasmid replication and maintenance in the intermediate host must be integrated into the plasmid. That makes the plasmid size bigger, more tedious to engineer, and more challenging to transform into the final host. Also, these additional parts are present but have no functionality in the final host, which increases the risk of horizontal gene transfer. Second, the genetic parts meant to function in *L. plantarum* are generally adapted for it. For example, the DNA sequences are codon-optimized based on the codon usage of lactobacilli, which varies from that of *E. coli*. In addition, due to the phylogenetic distance between *E. coli* and *L. plantarum*, other genetic traits such as guanine-cytosine content and distinct methylation patterns can also represent genetic incompatibilities [153]. Finally, certain DNA sequences, including SPs for protein secretion or constitutive promoters for intracellular recombinant protein production, can function distinctively (e.g., stronger expression in *E. coli*) in each species, which might lead to unwanted

mutations in the *L. plantarum*-adapted genetic parts. Therefore, using *E. coli* as an intermediate host for plasmid DNA amplification can result in unusable plasmid DNA and limit the genetic sequences that can be transformed into *L. plantarum*. These hurdles have been reported by others and faced by me at the start of this doctoral thesis work.

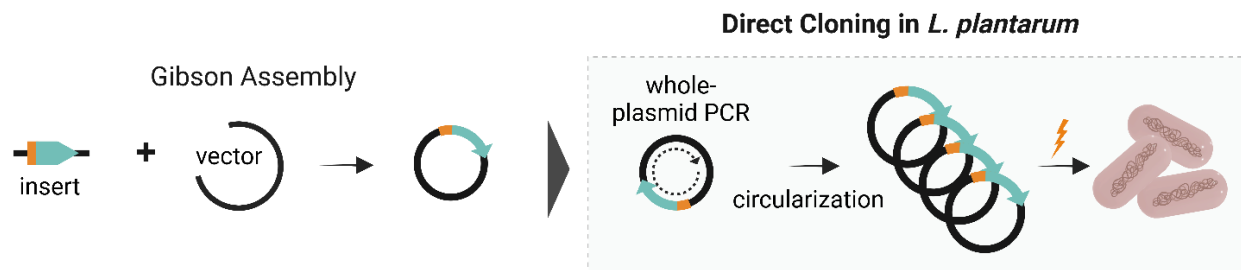
One simple alternative to avoid all these incompatibilities is to use a phylogenetically closer, easy-to-transform intermediate host. The widely used probiotic bacteria *L. lactis* are sometimes selected for this approach. However, this alternative is more time-consuming, as it additionally requires the preparation of *L. lactis* competent cells and electroporation of such [154]. As another option, in 2012, Spath et al. reported a cloning approach for direct transformation in *L. plantarum* CD033. This direct cloning relied on restriction enzymes, where high concentrations of PCR-amplified DNA fragments were digested with restriction enzymes before being ligated to obtain sufficient circular DNA [153]. Despite working well, this method requires the presence of multiple restriction sites across DNA fragments, limiting the versatility of the DNA assembly process and potentially requiring placement in key sequences of the gene (e.g., promoter region), which can interfere with the gene's overall expression performance. Hence, no current method allows both DNA assembly versatility and direct transformation of the lactobacilli-adapted genetic parts into *L. plantarum* WCFS1.

To ease the genetic programmability of *L. plantarum* WCFS1, a novel direct cloning method was developed. This method allows both assembly versatility and direct transformation. For versatility, the method first relies on Gibson assembly to join the DNA fragments into circular DNA. Next, a PCR-based *in vitro* DNA amplification of the assembled product is performed, followed by subsequent phosphorylation and ligation reactions leading to sufficient plasmid DNA amounts for successful *L. plantarum* transformation. The transformation efficiency of this direct cloning drops by roughly 61% compared to *E. coli*-derived plasmid DNA transformation, but transformants were obtained even with 300 ng of the ligated plasmid. This method saves 2 days compared to the conventional method involving an intermediate host, as it allows the transformation of high concentrations of the final plasmid DNA after 2 (vs. 4) days. This method proved

effective at delivering a mutation-free plasmid DNA into *L. plantarum* that could not be cloned when *E. coli* was used as an intermediate host. The plasmid encoded a human elastase inhibitor, elafin, which was toxic for *E. coli*, resulting in DNA mutations in crucial parts of the gene because of selective pressure. That served as a key example of when the direct cloning approach provided a major advantage.

Overall, the direct cloning method may be an attractive alternative for the transformation of not only *L. plantarum* but also any other challenging-to-transform bacteria, as it allows scale-up of the assembled circular final product by increasing the volumes of PCR, phosphorylation, and ligation reactions. The only limiting factor is the host's acceptance of unmethylated circular DNA, which is not an issue in the case of *L. plantarum*. This direct cloning has been used for the transformation of multiple genetic parts described in the following chapters.

Graphical Abstract



Gibson assembly is used to join together the insert and vector DNA fragments with compatible ends. Following that, a PCR is performed to amplify the whole assembled plasmid in vitro. Circularization of the amplified linear DNA is performed following phosphorylation and ligation reactions, which ultimately leads to sufficient circular DNA amounts for successful transformation of L. plantarum WCFS1.

Publication and contributions

Article Type: Research article.

Title: *In vitro* assembly of plasmid DNA for direct cloning in *Lactiplantibacillus plantarum* WCSF1².

Year Published: 2023.

Research Journal: PLOS One.

Authors: Marc Blanch-Asensio, Sourik Dey, and Shrikrishnan Sankaran.

First authors: Marc Blanch-Asensio and Sourik Dey (joint co-authorship).

Author contributions: Marc Blanch-Asensio performed all the genetic engineering work. Marc Blanch-Asensio conducted most of the experimental procedures. Marc Blanch-Asensio and Sourik Dey wrote the draft of the manuscript. Marc Blanch-Asensio made the illustrations. Sourik Dey analyzed and plotted the data. All authors took part in the conceptualization. Shrikrishnan Sankaran led the supervision, reviewed, and edited the manuscript.

² The supporting information of this publication is provided in Appendix II.1 at the end of this dissertation.

LAB PROTOCOL

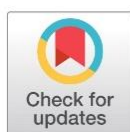
In vitro assembly of plasmid DNA for direct cloning in *Lactiplantibacillus plantarum* WCSF1

Marc Blanch-Asensio¹, Sourik Dey¹, Shrikrishnan Sankaran^{1*}

Bioprogrammable Materials, INM—Leibniz Institute for New Materials Campus D2 2, Saarbrücken, Germany

¹ These authors contributed equally to this work.

* Shrikrishnan.sankaran@leibniz-inm.de



Abstract

Lactobacilli are gram-positive bacteria that are growing in importance for the healthcare industry and genetically engineering them as living therapeutics is highly sought after. However, progress in this field is hindered since most strains are difficult to genetically manipulate, partly due to their complex and thick cell walls limiting our capability to transform them with exogenous DNA. To overcome this, large amounts of DNA (>1 µg) are normally required to successfully transform these bacteria. An intermediate host, like *E. coli*, is often used to amplify recombinant DNA to such amounts although this approach poses unwanted drawbacks such as an increase in plasmid size, different methylation patterns and the limitation of introducing only genes compatible with the intermediate host. In this work, we have developed a direct cloning method based on in-vitro assembly and PCR amplification to yield recombinant DNA in significant quantities for successful transformation in *L. plantarum* WCSF1. The advantage of this method is demonstrated in terms of shorter experimental duration and the possibility to introduce a gene incompatible with *E. coli* into *L. plantarum* WCSF1.

OPEN ACCESS

Citation: Blanch-Asensio M, Dey S, Sankaran S (2023) *In vitro* assembly of plasmid DNA for direct cloning in *Lactiplantibacillus plantarum* WCSF1. PLoS ONE 18(2): e0281625. <https://doi.org/10.1371/journal.pone.0281625>

Editor: Hari S. Misra, Bhabha Atomic Research Centre, INDIA

Received: September 28, 2022

Accepted: January 27, 2023

Published: February 16, 2023

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pone.0281625>

Copyright: © 2023 Blanch-Asensio et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All raw data including images and sequencing files related to results described in this paper have been added to the OSF data repository and can be accessed at this DOI - DOI: [10.17605/OSF.IO/C6X3D](https://doi.org/10.17605/OSF.IO/C6X3D).

Introduction

Lactobacilli are a group of Gram-positive bacteria of great importance to the food and healthcare industries with numerous strains identified as being beneficial for humans, and used as probiotics [1–3]. Furthermore, since they naturally colonize almost every site of the human body that hosts a healthy microbiome, e.g. the gastrointestinal tract [4,5], urogenital tracts [6], oral cavity [7] and nasal cavity [8], lactobacilli are an excellent foundational candidate for the development of live biotherapeutic products (LBPs) [9]. Beyond their natural health benefits, there is considerable interest in engineering them with heterologous genes for therapeutic applications like drug delivery [10,11] and mucosal vaccinations [12,13]. However, one of the crucial factors slowing down progress in lactobacilli engineering is difficulties in transforming them with exogenous DNA [14]. This is largely due to their thick and complex cell wall structures, which prevent successful bacterial transformation if the concentration of plasmid DNA is less than >1 µg [15]. To obtain such high plasmid DNA quantities, shuttle vectors are often used that can be amplified in intermediate hosts, predominantly *E. coli* [16]. To facilitate the construction of recombinant plasmids, several shuttle vectors have been identified, which can

Funding: This work was supported by a the Deutsche Forschungsgemeinschaft (DFG) Research grant [Project # 455063657 - <https://gepris.dfg.de/gepris/projekt/455063657>] for M.B. A., the DFG Collaborative Research Centre, SFB 1027 [Project # 200049484 - <https://gepris.dfg.de/gepris/projekt/466932240>] for S.S. and the Leibniz-Gemeinschaft's Leibniz Science Campus on Living Therapeutic Materials [LifeMat - <https://www.lscifemat.de/>] for S.D. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

undergo stable replication in both the cloning host, *E. coli* and the relevant Lactobacilli strains [17–19]. Nevertheless, since *E. coli* is a Gram-negative bacterium that is phylogenetically distant from Lactobacillus genera, this strategy can lead to genetic sequence incompatibilities due to GC-content differences [20], DNA methylation [21], repetitive sequence insertions [22] and toxic protein buildup in the *E. coli* cloning host [23]. Alternatively, the Gram-positive lactic acid bacterium, *Lactococcus lactis*, can also be used as an intermediate host for recombinant plasmid construction [24]. However, the availability of functional replication origins in *L. lactis* is limited [25] and inclusion of additional broad-range replicons significantly increases the size of the plasmid. The excessive increase in the size of the plasmid might lead to segregational instability [26] and thereby limit the size of the heterologous genes that can be included in it. Hence, it is desirable to be able to directly transform circular plasmid dsDNA into the lactobacilli strains without relying on intermediate bacterial hosts like *E. coli* and *L. lactis*. To avoid the need for an intermediate host, Spath et al. developed a direct cloning approach based on the assembly of PCR-amplified DNA fragments by restriction digestion and ligation to obtain optimal quantities of circular dsDNA for transformation in *Lactiplantibacillus plantarum* CD033 [27]. They further show that the unmethylated plasmid DNA allowed for transformation in a strain (*L. plantarum* DSM20174) that could not be transformed using methylated DNA, possibly due to native restriction-modification systems. However, the method still requires the presence of restriction sites within the DNA sequences, which can limit the versatility of combining heterologous genes in the plasmid and needs to be accounted for when dealing with strains that may harbor unknown restriction-modification systems.

In this work, we report a direct cloning method that leverages the Gibson assembly strategy and takes advantage of recent advances in cost-effective oligonucleotide synthesis. By doing so, we avoid the need for restriction sites and improve the feasibility of combining diverse DNA sequences to construct versatile recombinant plasmids. We demonstrate this direct cloning method in *Lactiplantibacillus plantarum* WCFS1, one of the commonly engineered probiotic *Lactobacillus* strains for which improved engineering methods are highly sought [28,29]. Furthermore, this direct cloning method is considerably quicker in comparison to indirect cloning methods requiring an intermediate host. We have characterized the efficiency and accuracy of this approach and have demonstrated the successful cloning of a gene expressing the medically relevant protein, Elafin which showed a high failure rate when being cloned through the intermediate host, *E. coli*. Thus, this direct cloning method will be instrumental in enabling the cloning of Lactobacilli with a wider variety of heterologous genes and with greater versatility than previously possible.

Materials and methods

Bacterial strains and growth conditions

L. plantarum WCFS1 was used as the parent strain in this study. The strain was grown in the De Man, Rogosa and Sharpe (MRS) media (Carl Roth GmbH, Germany, Art. No. X924.1). Recombinant *L. plantarum* WCFS1 strains were grown in MRS media supplemented with 10 µg/mL of erythromycin (Carl Roth GmbH, Art. No. 4166.2) at 37°C and 250 rpm shaking for 16 h. For the indirect cloning experiments, NEB 5-alpha Competent *E. coli* cells were used (New England Biolabs GmbH, Germany, Art. No. C2987). This strain was grown in Luria-Bertani (LB) medium (Carl Roth GmbH, Art. No. X968.1). Recombinant *E. coli* DH5α strains were grown in LB media supplemented with 200 µg/mL of erythromycin at 37°C, 250 rpm shaking conditions for 16 h.

Molecular biology

Q5 High Fidelity 2X Master Mix (New England Biolabs GmbH [NEB], Germany, No. M0492S) was used to perform DNA amplification. Amplified DNA products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega GmbH, Germany, Art. No. A9282). Foragarose gels, 1 kb Plus DNA Ladder (Catalog Number 10787018) and Generuler 100 bp Plus DNA Ladder (Catalog Number (SM0321) was procured from ThermoFisher Scientific™, Germany and used for reference. Primers were synthesized by Integrated DNA Technologies (IDT) (Louvain, Belgium) and the elafin gene fragment was ordered as eBlock from IDT (Coralville, USA). All primers used in this work are shown in S1 Table in [S2 File](#). The mCherry gene fragment was amplified by PCR from a plasmid previously generated in our lab. The genetic sequences of mCherry and elafin genes are shown in S2 Table in [S2 File](#). The plasmid *pLp3050sNuc* (Addgene plasmid # 122030) [30] was used as the vector backbone in this study. The schematic for the recombinant plasmids, pLp_mCherry and pLp_elafin constructed in this study have been highlighted in S1 Fig in [S2 File](#). The Codon Optimization tool from IDT (Choice Host Organism—*L. acidophilus*) was used to optimize the codon bias for mCherry coding segment. The Java Codon Adaptation Tool (JCat) [31] was used to codon-optimize the gene encoding for the human peptidase inhibitor 3, elafin (GenBank ID: AAX36874.1) using the codon optimization database for *L. plantarum* WCFS1. DNA assembly was performed using the HiFi Assembly Master Mix (NEB GmbH, Germany, Art. No. E5520S). For plasmid circularization, the Quick Blunting Kit (NEB GmbH, Germany, Art. No. E1201S) and the T4 DNA Ligase enzyme (NEB GmbH, Germany, Art. No. M0318S) were used.

L. plantarum WCFS1 electrocompetent cell preparation

Wild-type *L. plantarum* WCFS1 was cultured overnight in 5 mL of MRS media and at 37°C with shaking (250 rpm). After 16h, 1 mL of the culture ($OD_{600} = 2$) was added to 20 mL of MRS media and 5 mL of 1% (w/v) glycine. This secondary culture was incubated at 37°C and 250 rpm until the OD_{600} reached 0.8. The cells were then harvested by centrifugation at 4000 rpm (3363 X g) for 12 min at 4°C. After discarding the supernatant, the pellet was washed twice with 5 mL of ice-cold 10 mM $MgCl_2$. The pellet was then washed twice with ice-cold Suc/Gly solution (1 M sucrose and 10% (v/v) glycerol mixed in a 1:1 (v/v) ratio), first with 5 mL and second with 1 mL. Next, the supernatant was discarded, and the bacterial pellet was resuspended in 450 μ L of ice-cold Suc/Gly solution. Finally, 60 μ L aliquots were prepared and immediately used for DNA electroporation or stored at -80°C for future use.

Electroporation based transformation in *L. plantarum* WCFS1

For electroporation transformation, plasmids were first mixed with 60 μ L of electrocompetent cells at quantities (300–1200 ng) specified in the Results section. After a short incubation on ice, the mixture was transferred to ice-cold electroporation cuvettes with a 2 mm gap (Bio-Rad Laboratories GmbH, Germany, #1652086). Electroporation was performed using the Micro-Pulser Electroporator (Bio-Rad Laboratories GmbH, Germany), with a single pulse (5 ms) at 1.8 kV. Immediately after the pulse, 1 mL of MRS media was added to the bacterial mixture and then transferred to a 1.5 mL Eppendorf tube for further incubation at 37°C, 250 rpm for 3 h. Following this incubation, cells were pelleted down at 4000 rpm (3363 X g) for 5 min. 800 μ L of the supernatant was discarded, and the remaining 200 μ L was used for cell resuspension by slow pipetting. Finally, the resuspended pellet was plated on MRS Agar plates supplemented with 10 μ g/mL of Erythromycin, and plates were incubated at 37°C for 48 h for colonies to grow.

Direct cloning method in *L. plantarum* WCFS1

This study created and optimized a novel direct cloning method based on amplifying and circularizing in vitro-assembled gene fragments to be directly transformed in *L. plantarum* WCFS1 (Fig 1). For the Gibson HiFi Assembly reaction, complementary overhangs were included by PCR using a set of primers that contained the corresponding overhangs at the 5' ends. In the Gibson HiFi assembly reaction mixture, 50 ng of the PCR-amplified linear vector with overlapping DNA fragments and 10 ng of the corresponding eBlock were mixed along with 10 μ l of the HiFi DNA Assembly Master Mix (Mili-Q water was added up to 20 μ l). The reaction was incubated at 50°C for 30 minutes. After that, 5 μ l of the assembled product was used as template for an additional round of PCR, using a set of primers that annealed specifically to the insert region. The final volume of this PCR was 120 μ l, and the amplification cycle threshold was set at 22. 5 μ l of this reaction was run on an agarose gel to confirm amplification (S2 Fig in S2 File). After purifying the linear PCR product, 3500 ng of DNA were phosphorylated using the Quick Blunting Kit. This reaction was performed as suggested in the standard reaction protocol. 2.5 μ l of the 10X Quick Blunting buffer and 1 μ l of the Enzyme Mix were mixed with the purified DNA (3500 ng). Milli-Q water was added up to 25 μ l. The reaction was incubated for 30 minutes at 25°C to allow the blunting reaction and then kept at 70°C for

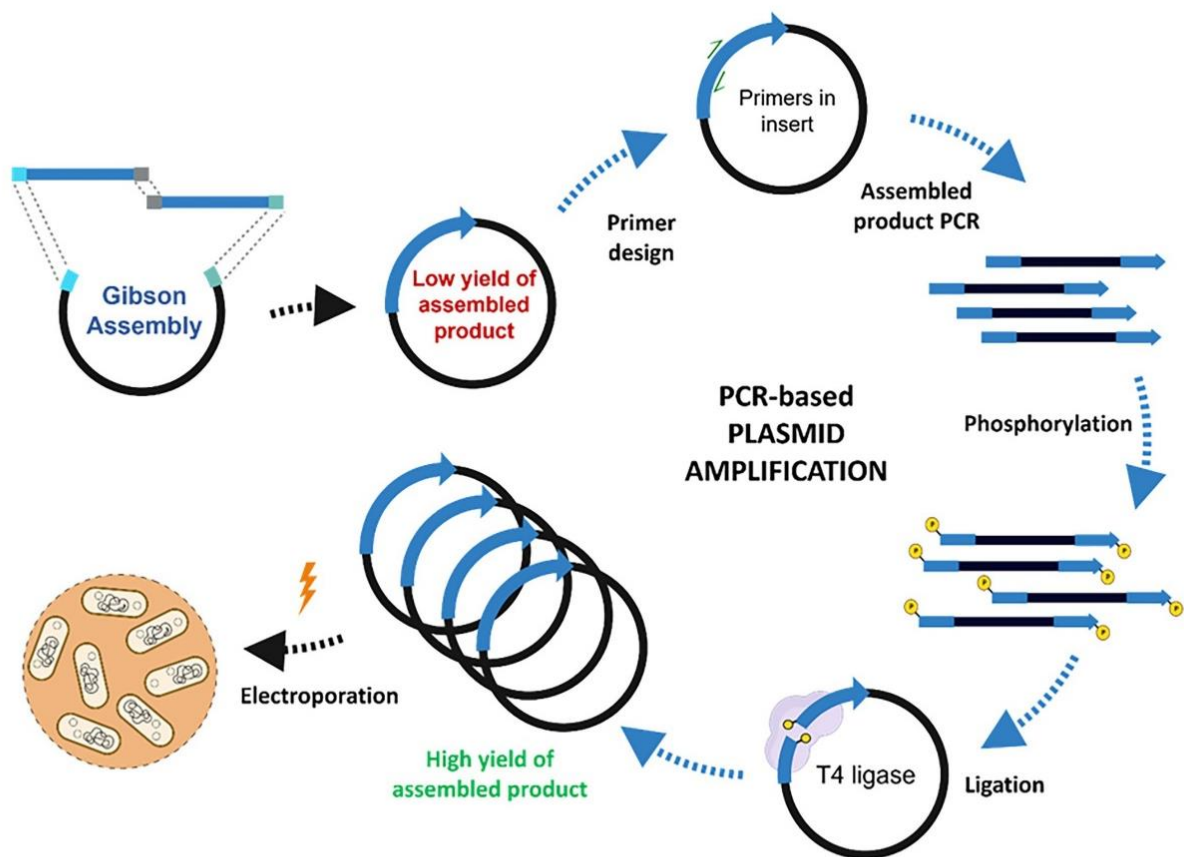


Fig 1. Scheme of the PCR-based plasmid amplification in the direct cloning method. The scheme was generated using BioRender.

<https://doi.org/10.1371/journal.pone.0281625.g001>

10 minutes to inactivate the enzyme. In the next steps, phosphorylated DNA was ligated using the T4 ligase enzyme. This reaction was slightly modified from the standard protocol because a higher amount of DNA was added to the reaction. Per ligation, 500 ng of phosphorylated DNA (3.6 μ l of the Quick Blunting reaction) were mixed with 1.5 μ l of T4 Ligase enzyme and 2.5 μ l of 10X T4 Ligase Buffer. Milli-Q water was added up to 25 μ l. The number of ligation reactions depended on the amount of DNA intended to be circularized. The ligation reactions were incubated for 2.5 hours at 25°C and then 10 minutes at 70°C to inactivate the enzyme. Following the incubation, the ligation mixtures were combined, and the ligated dsDNA was purified using the Promega kit. In this purification, DNA was eluted 3 times with 9 μ l of Milli-Q water each time to obtain the highest DNA concentration. The DNA concentration of the ligated mixture was measured (absorbance at 260 nm) using a NanoDrop Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific GmbH, Germany). The purified ligated products were then transformed into *L. plantarum* WCFS1 electrocompetent cells.

The protocol described in this peer-reviewed article is published on protocols.io, <https://doi.org/10.17504/protocols.io.ewov1o82olr2/v1> and is included for printing as S1 File with this article.

Sequence verification was performed by PCR amplification of the target gene conducted directly from the bacterial pellet. To do so, the selected bacteria were inoculated in MRS media supplemented with 10 μ g/mL of erythromycin and incubated overnight at 37°C and 250 rpm. The following day, 1 mL of the bacterial culture was collected in a 1.5 mL Eppendorf tube and centrifuged at 4°C for 3 min at 8400 X g. The supernatant was discarded, the residual pellet fraction was scratched off with a sterile pipette tip and used as a template for the PCR (100 μ l as final reaction volume, 28 cycles). Alternatively, a colony grown on the MRS plate can also be used to for PCR amplification of the target gene segment. The PCR settings involved an additional initial denaturation step for 10 minutes at 98°C to ensure maximum bacterial lysis. After the PCR, 5 μ l of the PCR products were assessed by agarose gel electrophoresis to confirm amplification at the expected size. Next, the PCR product was purified, and the DNA concentration was measured using the Nanodrop. Finally, 2000 ng of the purified PCR product was sent for sequencing to Eurofins Genomics GmbH (Ebersberg, Germany). An additional DNA purification step before Sanger sequencing was employed for obtaining efficient results (Additional Service: PCR Purification).

Indirect cloning via the intermediate host strain *E. coli*

For the indirect cloning, an additional Gibson HiFi reaction was performed (identical to the Gibson HiFi reaction set for the direct protocol). However, an additional step was done before setting this reaction, which involved the restriction enzyme digestion with DpnI (NEB GmbH, Germany, Art. No. R0176S). In this reaction, 500 ng of the purified PCR product were mixed with 1 μ l of DpnI enzyme and 1 μ l of rCutSmart buffer (Milli-Q water was added up to 10 μ l). Incubation was performed for 30 minutes at 37°C followed by 10 minutes at 70°C. The digested product was used for the Gibson HiFi Assembly reaction. Once the reaction was done, it was transformed into NEB 5-alpha Competent cells (50 μ l). In this transformation, NEB 5-alpha Competent cells were first thawed on ice for 10 minutes. After that, 8 μ l of the reaction were properly mixed with the competent cells by pipetting and incubated on ice for 20 minutes. Following the incubation, a 60-second heat shock was performed by placing the cells at a 42°C water bath. Next, cells were again incubated on ice for 5 minutes. After that, 950 μ l of SOC media was added to the cell mixture and kept for incubation for 1 hour at 37°C. Finally, 150 μ l of the culture was plated on an LB agar plate supplemented with 200 μ g/mL of erythromycin and incubated at 37°C overnight.

For the pLp_mCherry cloning, the screening of positive clones was done using the Gel Documentation System Fluorchem Q (Alpha Innotech Biozym GmbH, Germany). Bacterial colonies expressing mCherry were imaged in the Cy3 channel (Exλ/Emλ = 554 nm/568 nm) and the corresponding brightfield image was taken using the ethidium bromide channel (Exλ/Emλ = 300 nm/600 nm). One red colony was inoculated in LB media supplemented with 200 µg/mL of erythromycin and incubated at 37°C overnight. The following day, plasmid extraction was performed using the Plasmid extraction miniprep kit (Qiagen GmbH Germany, Art. No. 27104). The plasmid DNA concentration was measured using the 260 nm absorbance setting on the NanoDrop Microvolume UV-Vis Spectrophotometer. The recombinant plasmid was then transformed into *L. plantarum* WCFS1 electrocompetent cells.

For the pLp_elafin cloning, 20 colonies were streaked on a fresh LB agar plate supplemented with 200 µg/mL of erythromycin and incubated at 37°C overnight. The following day, positive clones were screened by PCR using a forward primer that annealed to the vector and a reverse primer that annealed to the elafin gene (100 µl as final reaction volume, 28 cycles). In the PCR, 10 minutes at 98°C were set for the initial denaturation of the samples. Three positive clones confirmed by colony PCR were inoculated in LB media supplemented with 200 µg/mL of erythromycin and incubated at 37°C overnight. The following day, the respective plasmids were extracted and sent for sequencing to Eurofins Genomics GmbH (Ebersberg, Germany).

Results and discussion

The Gibson assembly approach to combining DNA fragments requires the fragments to contain terminal overhangs that complementarily overlap by ~20 bases. The method was originally developed to stitch together the first artificial genome due to the high level of flexibility it provided compared to restriction digestion-based methods [32]. It is a single reaction assembly method that can be performed without thermal cycling and within an hour. In this work, we employ Gibson reaction to conduct in-vitro assembly of circular dsDNA constructs for direct cloning in *L. plantarum* WCFS1. As shown in Fig 1, our method involves PCR amplification of a vector and an insert with overlapping arms, followed by their Gibson reaction-based assembly that yields a low quantity (50–80 ng) of the assembled dsDNA. To obtain optimal concentration of dsDNA for transformation in Lactobacilli, a second amplification and recircularization step was performed, yielding >1 µg of the desired construct. This method was characterized and optimized in terms of transformation efficiency, accuracy, and capability for cloning challenging genes in *Lactiplantibacillus plantarum* WCFS1. Different plasmid constructs were assembled and compared with indirect cloning using *E. coli* as an intermediate host.

Transformation efficiency and accuracy of the direct cloning method

Transformation efficiency (TE) indicates the extent to which cells can take up DNA from the extracellular space and express the genes encoded by it [33]. While it is possible to transform *L. plantarum* WCFS1 with extracellular DNA, TE is typically poor [27]. To demonstrate this, a simple plasmid construct, pLp_mCherry, with gene sequences ideal for indirect cloning through *E. coli* was used. This plasmid consisted of a p256 replicon, an erythromycin resistance cassette and the mCherry gene driven by a strong constitutive promoter P_{ilpA} , [34] all of which are compatible in both *E. coli* and *L. plantarum* WCFS1. The plasmid was constructed through Gibson reaction-based assembly of the pLp-3050sNuc plasmid backbone and P_{ilpA} -mCherry insert and transformed in *E. coli*. One correctly sequenced plasmid, extracted from an *E. coli* DH5α clone, was transformed in *L. plantarum* WCFS1 at different concentrations (300, 600, 900 and 1200 ng), yielding low TE values that increased with higher DNA concentrations (20–

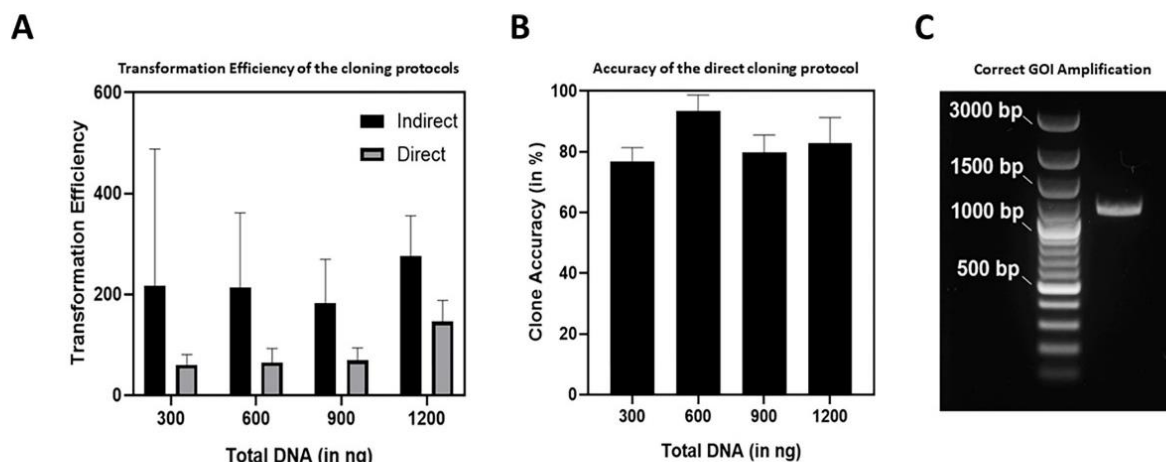


Fig 2. (A) Transformation Efficiency comparison against the total concentration of DNA transformed for the indirect and direct cloning protocol in *L. plantarum* WCFS1 (The standard deviations correspond to three independent biological replicates) (B) Percentage accuracy of correct recombinant clones against the total concentration of DNA transformed in *L. plantarum* WCFS1 using the direct cloning protocol (The whiskers correspond to values from three independent biological replicates). (C) Agarose gel showing the colony PCR product (1140 bp) corresponding to the mCherry gene of interest (GOI). A red *L. plantarum* colony obtained after direct cloning was used as the template DNA for the PCR reaction. Generuler 100 bp Plus DNA Ladder (ThermoFisher Scientific™) was used for the reference.

<https://doi.org/10.1371/journal.pone.0281625.g002>

500 cfu/μg) (Fig 2A). In the direct cloning method, the Gibson reaction based assembled dsDNA was PCR amplified using complementary primers within the insert region and the resulting linear fragments were circularized by phosphorylation and ligation to yield high amounts of plasmid DNA suitable for transformation in *L. plantarum* WCFS1 (> 3μg). Following the transformation of the circularized plasmid mix in *L. plantarum* WCFS1 at different DNA concentrations as mentioned above, TE values were found to be lower than that of the indirect method. However, despite being lower the overall TE values were within the same order of magnitude and increased drastically when the net DNA concentration used for transformation was above 1 μg (Fig 2A). The lower TE values could be due to incomplete circularization of the PCR-amplified plasmid fragments, due to which the final quantity of the circularized constructs might have been lower than the total DNA that was quantified [35]. The accuracy of clones generated from the direct cloning method, determined by their ability to express mCherry, was estimated after checking the fluorescence of 418 colonies that grew across all the DNA quantities tested. Colonies were streaked on fresh plates and the following day they were examined for the presence of fluorescent protein. Overall, 347 out of 418 colonies were red, giving an accuracy of 83% (Fig 2B). The accuracy in the indirect cloning method with the correctly sequenced recombinant plasmid isolated from *E. coli* DH5α was found to be above 99%, as expected.

Furthermore, we wanted to prove that a DpnI digestion prior to the Gibson HiFi Assembly reaction would not have an impact on the number of positive clones obtained through the direct cloning method since we assumed that the backbone vectors used as template for the PCR would be extremely diluted during the multiple purification steps involved in the protocol. Therefore, we repeated the pLp_mCherry direct cloning with and without a DpnI digestion of the insert and vector PCR products prior to the Gibson HiFi Assembly reaction. We screened 10 red colonies for both experimental conditions by PCR amplification of a partial gene segment within the mCherry reporter. The proportion of red colonies obtained was

similar in both conditions and colony PCR amplification of a part of the mCherry gene (10 red colonies from each) yielded PCR products of the same expected size from all clones. This confirmed that a DpnI digestion prior to the HiFi Assembly reaction is not necessary (S3 Fig in [S2 File](#)).

Notably, since *L. plantarum* WCFS1 contains 3 endogenous plasmids [36], sequencing-based verification of desired regions in the recombinant plasmid was done by PCR amplification of the entire mCherry gene of 1140 base pairs (bp) (Fig 2C). The gene segments were directly amplified from bacterial cell pellets, and the amplicon sequencing was outsourced to an external service provider, Eurofins Genomics GmbH, where their additional DNA purification option was employed. An initial purification of the PCR amplified product by us seemed to improve the quality of the sequencing chromatograms but was not absolutely necessary to get the correct results (S5A and S5B Fig in [S2 File](#)). As expected, all clones expressing mCherry yielded the correct sequences without any mutations or deletions. The whole mCherry gene was also amplified from 10 non-red colonies using the same set of primers and PCR conditions as in Fig 2C. We obtained amplification for 7 out of 10, nevertheless, the PCR product was either bigger or smaller than expected (S4 Fig in [S2 File](#)). These results suggest that mutations might have occurred during the PCR amplification steps or a minor proportion of wrongly assembled products were formed during Gibson assembly which can result in recombinant clones with the mutated gene of interest (GOI).

Time requirement for the direct and indirect cloning methods

The direct cloning method is considerably quicker and less labor-intensive than the indirect cloning method. All steps in the direct cloning method can be completed in 4 days after which PCR-amplified sequences can be sent for sequencing. In contrast, the indirect cloning method requires 5 to 6 days, depending on the time allocated for growth of bacteria on the master plate (Fig 3). Note that a master plate is needed to be made from transformed *E. coli* colonies in our case due to the use of erythromycin as the antibiotic resistance marker. *E. coli* has natural resistance to this antibiotic at low dosages which can be surpassed by supplementing the growth media with higher concentration of erythromycin. On one hand, this will prevent the growth of non-transformed cells but on the other hand it will also lead the transformed colonies to grow slowly making the colonies too small for reliable use in colony PCR analysis. In the case of *L. plantarum* WCFS1, a master plate was not required since colonies grown for 48 hours were large enough to handle both colony PCR analysis and inoculation in liquid cultures.

Direct cloning of a gene incompatible with *E. coli*

The main advantage of the direct cloning method is demonstrated in the ability to transform genes in lactobacilli that are challenging using the indirect method. Genes encoding proteins that are toxic to *E. coli*, for example, often result in mutations or complete deletions of the GOI in the plasmid when transformed into *E. coli* [37]. We therefore tested the cloning of a plasmid containing the human peptidase inhibitor 3 gene, known as elafin, encoded downstream of a strong constitutive promoter (P_{ilpA}). This protease has been reported to exert anti-microbial activity with *E. coli*, so its constitutive expression is expected to be toxic [38,39]. Transformation of the assembled plasmid containing the constitutively expressed elafin in *E. coli* yielded very few colonies. The screening of positive clones were done by PCR amplification using a primer set, where one specifically annealed to the vector and the other one was complementary to the insert region. Only 3 clones showed amplification, but the amplified product was shorter than expected (524 bp) (Fig 4A). Sequencing of plasmids extracted from these clones revealed several mutations and deletions. The whole P_{ilpA} was deleted from all three plasmids, and two

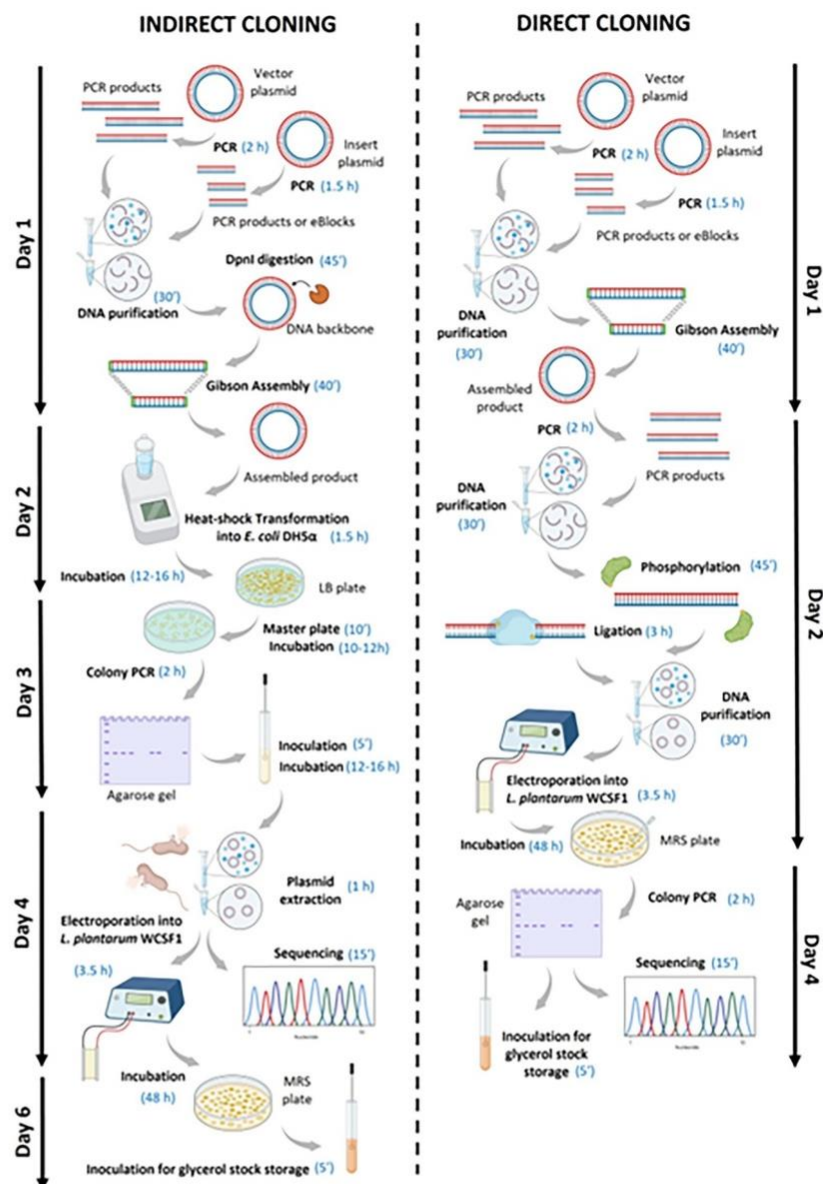


Fig 3. Schematic representation of the steps and temporal requirements for the direct and indirect cloning methods used for *L. plantarum* WCFS1. The scheme was generated using BioRender.

<https://doi.org/10.1371/journal.pone.0281625.g003>

clones had the elafin coding sequence truncated (Fig 4B, S6 Fig in S2 File). On the other hand, the direct cloning method yielded over 124 colonies after transformation with 1000 ng of phosphorylated and ligated dsDNA. 10 colonies were screened by PCR, and all of them showed amplification at the expected size of 524 bp (Fig 4A). Sequencing of the gene amplified from randomly selected 3 clones revealed no mutations or deletions (Fig 4B).

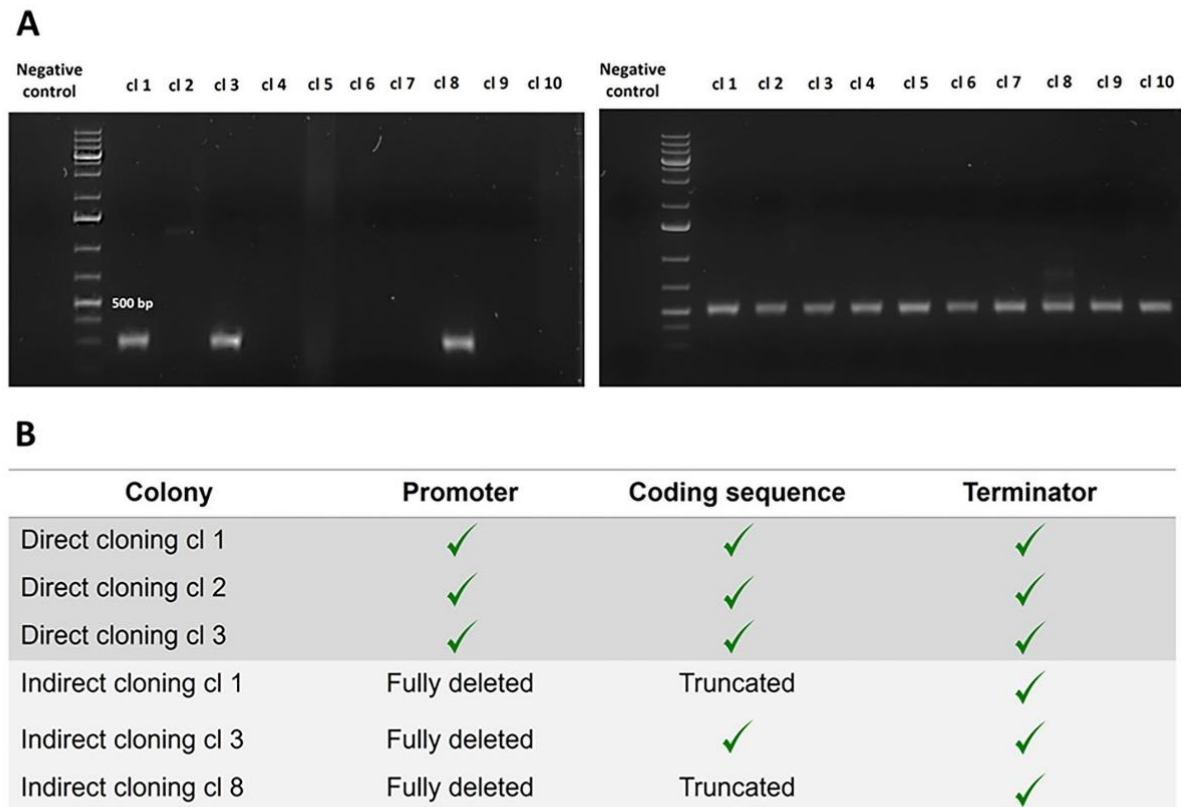


Fig 4. (A) The agarose gel (left) shows the colony PCR result of 10 randomly selected *E. coli* pLp_elafin clones. The agarose gel (right) corresponds to the colony PCR of 10 randomly selected *L. plantarum* WCFS1 pLp_elafin clones. All the colony PCR reactions were performed under the same conditions. The expected amplicon size for the pLp_elafin clones were 524 bp. The reference ladder used was the 1 kb Plus DNA ladder (ThermoFisher™) (B) Table listing the results obtained after Sanger Sequencing of the isolated pLp_elafin plasmids. The (✓) suggests a complete match to the expected sequence. Three plasmids per cloning method (direct and indirect) were sent for analysis.

<https://doi.org/10.1371/journal.pone.0281625.g004>

Conclusions

The direct cloning method developed in this paper has proved effective in transforming circular dsDNA (plasmid) DNA into *L. plantarum* WCFS1 without any intermediate host requirement for plasmid amplification. We demonstrate that this method provides two major benefits for lactobacillus engineering—(i) it saves time of at least 2 days compared to commonly used indirect cloning methods involving intermediate hosts and (ii) enables the cloning of genetic constructs that might be toxic or incompatible with the intermediate host. Since this method relies on PCR-amplification based *in vitro* assembly of DNA fragments, it must be noted that the accuracy can be affected by mutations that occur during PCR amplification and the possible formation of unspecific assembly fragments. To minimize the risk of mutations, a high-fidelity polymerase (Q5 DNA polymerase) was used in this study [40]. To accelerate the identification of positive colonies, a visible reporter like mCherry can be included or colony PCR can be performed. Using these methods, we confirmed that the accuracy of the transformed clones was above 80%. While we have tested this direct cloning method only in *L. plantarum* WCFS1, we believe this strategy can also be expanded to other hard-to-transform lactobacilli,

in which similar plasmids have been previously transformed using the indirect method [41]. When testing the direct cloning method on different strains, it is important to note that success will depend on whether they accept unmethylated DNA. Furthermore, if transformation is hindered by restriction-modification systems in these strains, DNA design strategies can be employed to overcome this challenge [42]. Finally, while we have used modest-sized plasmid (<4 kb) with a low copy number replicon (P256 replicon, copy number 3–5). Based on previous studies [43], it is expected that bigger plasmids with higher copy number replicons can be transformed using the direct cloning method although further investigations are definitely needed to test the effect of plasmid size on transformation efficiency and accuracy of the transformed clones.

Supporting information

S1 File. Supporting information file containing the step-by-step protocol generated in this study.

(PDF)

S2 File. Supporting information file containing all supporting information tables and figures.

(DOCX)

Acknowledgments

The plasmid *pLp3050sNuc* was a kind gift from Prof. Geir Mathiesen.

Author Contributions

Conceptualization: Marc Blanch-Asensio, Sourik Dey, Shrikrishnan Sankaran.

Data curation: Marc Blanch-Asensio, Sourik Dey.

Formal analysis: Marc Blanch-Asensio, Sourik Dey.

Funding acquisition: Shrikrishnan Sankaran.

Investigation: Sourik Dey.

Methodology: Marc Blanch-Asensio, Sourik Dey.

Project administration: Shrikrishnan Sankaran.

Resources: Shrikrishnan Sankaran.

Supervision: Shrikrishnan Sankaran.

Visualization: Marc Blanch-Asensio, Sourik Dey, Shrikrishnan Sankaran.

Writing – original draft: Marc Blanch-Asensio, Sourik Dey, Shrikrishnan Sankaran.

Writing – review & editing: Shrikrishnan Sankaran.

References

1. Ganji-Arjenaki M, Rafieian-Kopaei M. Probiotics are a good choice in remission of inflammatory bowel diseases: A meta analysis and systematic review. *Journal of cellular physiology*. 2018 Mar; 233(3):2091–103. <https://doi.org/10.1002/jcp.25911> PMID: 28294322
2. Abad CL, Safdar N. The role of lactobacillus probiotics in the treatment or prevention of urogenital infections—a systematic review. *Journal of Chemotherapy*. 2009 Jun 1; 21(3):243–52. <https://doi.org/10.1179/joc.2009.21.3.243> PMID: 19567343

3. Hakansson A, Molin G. Gut microbiota and inflammation. *Nutrients*. 2011 Jun 3; 3(6):637–82. <https://doi.org/10.3390/nu3060637> PMID: 22254115
4. Hayashi H., Takahashi R., Nishi T., Sakamoto M. and Benno Y., 2005. Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. *Journal of medical microbiology*, 54(11), pp.1093–1101. <https://doi.org/10.1099/jmm.0.45935-0> PMID: 16192442
5. Valeur N., Engel P., Carbajal N., Connolly E. and Ladefoged K., 2004. Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. *Applied and Environmental Microbiology*, 70(2), pp.1176–1181. <https://doi.org/10.1128/AEM.70.2.1176-1181.2004> PMID: 14766603
6. Aragon IM, Herrera-Imbroda B, Queipo-Ortuño MI, Castillo E, Del Moral JS, Gomez-Millan J, et al. The urinary tract microbiome in health and disease. *European urology focus*. 2018 Jan 1; 4(1):128–38. <https://doi.org/10.1016/j.euf.2016.11.001> PMID: 28753805
7. Sedghi L, DiMassa V, Harrington A, Lynch SV, Kapila YL. The oral microbiome: Role of key organisms and complex networks in oral health and disease. *Periodontology* 2000. 2021 Oct; 87(1):107–31. <https://doi.org/10.1111/prd.12393> PMID: 34463991
8. Dimitri-Pinheiro S, Soares R, Barata P. The microbiome of the nose—Friend or foe?. *Allergy & rhinology*. 2020 Mar; 11:2152656720911605. <https://doi.org/10.1177/2152656720911605> PMID: 32206384
9. Vargason AM, Anselmo AC. Clinical translation of microbe-based therapies: Current clinical landscape and preclinical outlook. *Bioengineering & translational medicine*. 2018 May; 3(2):124–37. <https://doi.org/10.1002/btm2.10093> PMID: 30065967
10. Motta JP, Bermúdez-Humarán LG, Deraison C, Martin L, Rolland C, Rousset P, et al. Food-grade bacteria expressing elafin protect against inflammation and restore colon homeostasis. *Science translational medicine*. 2012 Oct 31; 4(158):158ra144–. <https://doi.org/10.1126/scitranslmed.3004212> PMID: 23115353
11. Pusch O, Kalyanaraman R, Tucker LD, Wells JM, Ramratnam B, Boden D. An anti-HIV microbicide engineered in commensal bacteria: secretion of HIV-1 fusion inhibitors by lactobacilli. *Aids*. 2006 Oct 3; 20(15):1917–22. <https://doi.org/10.1097/01.aids.0000247112.36091.f8> PMID: 16988512
12. Cortes-Perez NG, Azevedo V, Alcocer-González JM, Rodríguez-Padilla C, Tamez-Guerra RS, Corthier G, et al. Cell-surface display of E7 antigen from human papillomavirus type-16 in *Lactococcus lactis* and in *Lactobacillus plantarum* using a new cell-wall anchor from lactobacilli. *Journal of drug targeting*. 2005 Feb 1; 13(2):89–98. <https://doi.org/10.1080/10611860400024219> PMID: 15823960
13. Kuczkowska K, Kleiveland CR, Minic R, Moen LF, Øverland L, Tjåland R, et al. Immunogenic properties of *Lactobacillus plantarum* producing surface-displayed *Mycobacterium tuberculosis* antigens. *Applied and environmental microbiology*. 2017 Jan 1; 83(2):e02782–16. <https://doi.org/10.1128/AEM.02782-16> PMID: 27815271
14. Samperio S, Guzmán-Herrador DL, May-Cuz R, Martín MC, Álvarez MA, Llosa M. Conjugative DNA Transfer From *E. coli* to Transformation-Resistant *Lactobacilli*. *Frontiers in microbiology*. 2021 Feb 11; 12:606629. <https://doi.org/10.3389/fmicb.2021.606629> PMID: 33643236
15. Trevors J.T., Chassy B.M., Dower W.J. and Blaschek H.P. (1992) Electroporation of bacteria by plasmid DNA. In *Guide to Electroporation and Electrofusion* ed. Chang D.C., Chassy B.M., Saunders J. A. and Sowers A.E. pp. 265–290. San Diego, CA: Academic Press Inc
16. Karlskås IL, Maudal K, Axelsson L, Rud I, Eijsink VG, Mathiesen G. Heterologous protein secretion in lactobacilli with modified pSIP vectors. *PLOS one*. 2014 Mar 10; 9(3):e91125. <https://doi.org/10.1371/journal.pone.0091125> PMID: 24614815
17. Spath K, Heint S, Egger E, Grabherr R. *Lactobacillus plantarum* and *Lactobacillus buchneri* as expression systems: evaluation of different origins of replication for the design of suitable shuttle vectors. *Molecular biotechnology*. 2012 Sep; 52(1):40–8. <https://doi.org/10.1007/s12033-011-9471-x> PMID: 22081307
18. Pérez-Arellano I, Zúñiga M, Pérez-Martínez G. Construction of compatible wide-host-range shuttle vectors for lactic acid bacteria and *Escherichia coli*. *Plasmid*. 2001 Sep 1; 46(2):106–16. <https://doi.org/10.1006/plas.2001.1531> PMID: 11591136
19. Shimizu-Kadota MA, Shibahara-Sone HA, Ishiwa HI. Shuttle plasmid vectors for *Lactobacillus casei* and *Escherichia coli* with a minus origin. *Applied and environmental microbiology*. 1991 Nov; 57(11):3292–300. <https://doi.org/10.1128/aem.57.11.3292-3300.1991> PMID: 1781687
20. Lee J, Kaletunc G. Evaluation of the heat inactivation of *Escherichia coli* and *Lactobacillus plantarum* by differential scanning calorimetry. *Applied and Environmental Microbiology*. 2002 Nov; 68(11):5379–86. <https://doi.org/10.1128/AEM.68.11.5379-5386.2002> PMID: 12406728

21. Teresa Alegre M, Carmen Rodríguez M, Mesas JM. Transformation of *Lactobacillus plantarum* by electroporation with in vitro modified plasmid DNA. *FEMS microbiology letters*. 2004 Dec 1; 241(1):73–7. <https://doi.org/10.1016/j.femsle.2004.10.006> PMID: 15556712
22. Godiska R, Mead D, Dhodda V, Wu C, Hochstein R, Karsi A, et al. Linear plasmid vector for cloning of repetitive or unstable sequences in *Escherichia coli*. *Nucleic acids research*. 2010 Apr 1; 38(6):e88–. <https://doi.org/10.1093/nar/gkp1181> PMID: 20040575
23. Zhang K, Ni Y. Tyrosine decarboxylase from *Lactobacillus brevis*: soluble expression and characterization. *Protein expression and purification*. 2014 Feb 1; 94:33–9. <https://doi.org/10.1016/j.pep.2013.10.018> PMID: 24211777
24. O'Sullivan DJ, Klaenhammer TR. High-and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene*. 1993 Dec 31; 137(2):227–31. [https://doi.org/10.1016/0378-1119\(93\)90011-q](https://doi.org/10.1016/0378-1119(93)90011-q) PMID: 8299952
25. De Vos WM. Gene cloning and expression in lactic streptococci. *FEMS microbiology reviews*. 1987 Sep 1; 3(3):281–95.
26. Kiewiet R, Bron S, de Jonge K, Venema G, Seegers JF. Theta replication of the lactococcal plasmid pWVO2. *Molecular microbiology*. 1993 Oct; 10(2):319–27.
27. Spath K, Heintz S, Grabherr R. Direct cloning in *Lactobacillus plantarum*: electroporation with non-methylated plasmid DNA enhances transformation efficiency and makes shuttle vectors obsolete. *Microbial cell factories*. 2012 Dec; 11(1):1–8. <https://doi.org/10.1186/1475-2859-11-141> PMID: 23098256
28. Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, et al. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Sciences*. 2003 Feb 18; 100(4):1990–5. <https://doi.org/10.1073/pnas.0337704100> PMID: 12566566
29. Nguyen TT, Nguyen HM, Geiger B, Mathiesen G, Eijssink VG, Peterbauer CK, et al. Heterologous expression of a recombinant lactobacillal β -galactosidase in *Lactobacillus plantarum*: effect of different parameters on the sakacin P-based expression system. *Microbial Cell Factories*. 2015 Dec; 14(1):1–1.
30. Mathiesen G, Sveen A, Brurberg MB, Fredriksen L, Axelsson L, Eijssink VG. Genome-wide analysis of signal peptide functionality in *Lactobacillus plantarum* WCFS1. *BMC genomics*. 2009 Dec; 10(1):1–3. <https://doi.org/10.1186/1471-2164-10-425> PMID: 19744343
31. Grote A., Hiller K., Scheer M., Münch R., Nörtemann B., Hempel D.C. et al., 2005. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic acids research*, 33 (suppl_2), pp.W526–W531. <https://doi.org/10.1093/nar/gki376> PMID: 15980527
32. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature methods*. 2009 May; 6(5):343–5. <https://doi.org/10.1038/nmeth.1318> PMID: 19363495
33. Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. *Journal of molecular biology*. 1983 Jun 5; 166(4):557–80. [https://doi.org/10.1016/s0022-2836\(83\)80284-8](https://doi.org/10.1016/s0022-2836(83)80284-8) PMID: 6345791
34. Dey S, Asensio MB, Kuttar SB, Sankaran S. Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic lactobacilli. *bioRxiv*. 2022 Jan 1.
35. Guo YY, Shi ZY, Fu XZ, Chen JC, Wu Q, Chen GQ. A strategy for enhanced circular DNA construction efficiency based on DNA cyclization after microbial transformation. *Microbial cell factories*. 2015 Dec; 14(1):1–3. <https://doi.org/10.1186/s12934-015-0204-x> PMID: 25896825
36. Van Kranenburg R, Golc N, Bongers R, Leer RJ, De Vos WM, Siezen RJ, et al. Functional analysis of three plasmids from *Lactobacillus plantarum*. *Applied and environmental microbiology*. 2005 Mar; 71(3):1223–30. <https://doi.org/10.1128/AEM.71.3.1223-1230.2005> PMID: 15746322
37. Renda BA, Hammerling MJ, Barrick JE. Engineering reduced evolutionary potential for synthetic biology. *Molecular BioSystems*. 2014; 10(7):1668–78. <https://doi.org/10.1039/c3mb70606k> PMID: 24556867
38. King AE, Critchley HO, Sallenave JM, Kelly RW. Elafin in human endometrium: an antiprotease and antimicrobial molecule expressed during menstruation. *The Journal of Clinical Endocrinology & Metabolism*. 2003 Sep 1; 88(9):4426–31. <https://doi.org/10.1210/jc.2003-030239> PMID: 12970320
39. Sallenave JM. Antimicrobial activity of antiproteases. *Biochemical Society Transactions*. 2002 Apr; 30(2):111–5. PMID: 12023836
40. Potapov V. and Ong J.L., Examining sources of error in PCR by single-molecule sequencing. *PloS one*. 2017, 12(1), p.e0169774. <https://doi.org/10.1371/journal.pone.0169774> PMID: 28060945
41. Karliskás I.L., Maudal K., Axelsson L., Rud I., Eijssink V.G. and Mathiesen G. Heterologous protein secretion in lactobacilli with modified pSIP vectors. *PLOS one*. 2014 9(3), p.e91125. <https://doi.org/10.1371/journal.pone.0091125> PMID: 24614815

42. Johnston C.D., Cotton S.L., Rittling S.R., Starr J.R., Borisy G.G., Dewhirst F.E. et al. Systematic evasion of the restriction-modification barrier in bacteria. *Proceedings of the National Academy of Sciences*, 2019 116(23), pp.11454–11459. <https://doi.org/10.1073/pnas.1820256116> PMID: 31097593
43. Mathiesen G., Øverland L., Kuczkowska K. and Eijsink V.G. Anchoring of heterologous proteins in multiple *Lactobacillus* species using anchors derived from *Lactobacillus plantarum*. *Scientific Reports*. 2020 10(1), pp.1–10.

Chapter 2.

*A strong promoter/repressor system for *L. plantarum* genetic engineering*

Strong promoter/repressor systems are essential for fine-tuning the gene expression of any organism. Nonetheless, strong promoters and repressors remain unknown in *L. plantarum*. That supposes a bottleneck for achieving high levels of recombinant protein production or shutting down the gene expression of any GOI fully.

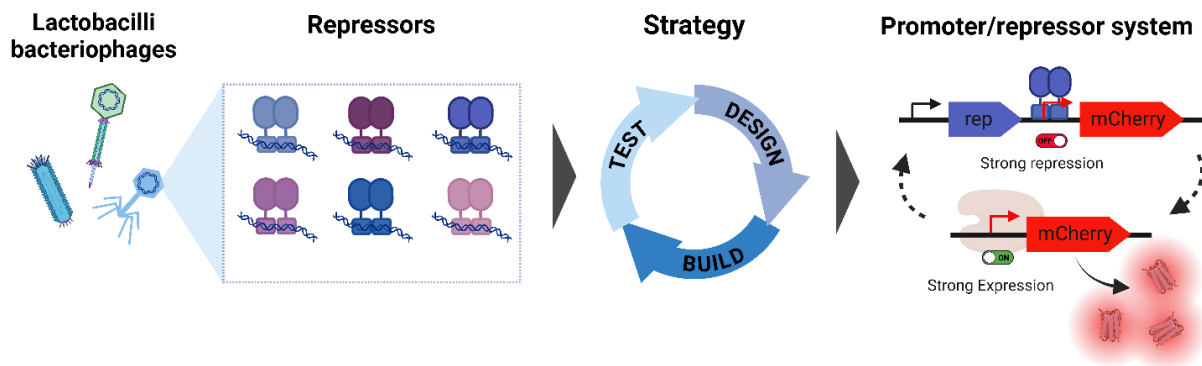
As a mining source of genetic parts, phages have proven to be an invaluable source of useful genetic parts for the genetic programmability of other bacteria. Their genetic parts are naturally adapted to their bacterial hosts as a result of an evolutionary arms race between bacteria and phages [155]. The best example is the T7 RNA polymerase from the *lambda* phage, which drives extremely high levels of gene expression from the T7 promoter in *E. coli* [156]. Other examples include transcriptional repressors that are part of genetic switches that control the lysogenic and lytic cycles of phages [157]. Nonetheless, phage-derived parts remain largely unexplored in *L. plantarum*, offering an attractive opportunity for discovering genetic parts of interest. This chapter explores the question of whether bacteriophages specific to LAB are a promising source of genetic parts for regulating gene expression in *L. plantarum*. Specifically, promoter/repressor systems from multiple bacteriophages were investigated to expand the genetic toolbox of *L. plantarum*.

In this work, six transcriptional repressors encoded in the genome of lactobacilli prophages (bacteriophages integrated into the genome of bacteria) were tested in *L. plantarum* WCFS1. To evaluate the performance of the repressors, a standardized genetic architecture was designed for all, with the corresponding cognate operator sequences

placed downstream of the strong promoter recently discovered by our group, P_{tlpA} . Hence, strong repressor binding would result in decreased levels of P_{tlpA} -driven reporter gene expression. Following rounds of screening and optimization, one repressor, Rep from the mv4 prophage in *L. delbrueckii*, exhibited outstanding levels of P_{tlpA} repression, postulating itself as a useful part for *L. plantarum* engineering. Given these results, the endogenous promoter from the mv4 prophage naturally regulated by Rep, namely P_{tec} , was also tested in *L. plantarum* WCFS1. Not surprisingly, the rep repressor can fully repress P_{tec} 's driven expression. However, a highly interesting finding was observed upon the release of P_{tec} from Rep-mediated repression: P_{tec} exhibited extraordinarily high levels of fluorescent protein production.

All in all, a strong promoter/repressor system has been discovered, which can greatly enhance *L. plantarum* engineering, provided that P_{tec} proves effective at expressing genes other than fluorescent reporters.

Graphical Abstract



Transcriptional repressors were mined from the genomes of lactobacilli prophages. The performance of six repressors (at repressing a fluorescent reporter gene) was assessed by designing, building, and testing genetic circuits. The rep repressor showed strong repression levels. On the other hand, the natural cognate promoter of rep showed outstanding gene expression levels when released from rep-mediated repression.

Publication and contributions

Article Type: Research article.

Title: Discovery of a high-performance phage-derived promoter/repressor system for probiotic lactobacillus engineering ³.

Year Published: 2024.

Research Journal: Microbial Cell Factories.

Authors: Marc Blanch-Asensio, Varun Sai Tadimarri, Alina Wilk, and Shrikrishnan Sankaran.

First author: Marc Blanch-Asensio.

Author contributions: Marc Blanch-Asensio performed all the genetic engineering work. Alina Wilk assisted in the genetic engineering experiments. Marc Blanch-Asensio conducted most of the experimental procedures. Varun Sai Tadimarri performed the experiments involving *E. coli* and wrote the corresponding sections in the manuscript. Marc Blanch-Asensio made the illustrations and analyzed and plotted the data. Marc Blanch-Asensio wrote most of the draft of the manuscript. Marc Blanch-Asensio and Shrikrishnan Sankaran took part in the conceptualization. Shrikrishnan Sankaran led the supervision, reviewed, and edited the manuscript.

³ The supporting information of this publication is provided in Appendix II.2 at the end of this dissertation.

RESEARCH

Open Access



Discovery of a high-performance phage-derived promoter/repressor system for probiotic lactobacillus engineering

Marc Blanch-Asensio^{1,2}, Varun Sai Tadimarri^{1,2}, Alina Wilk^{1,2} and Shrikrishnan Sankaran^{1*}

Abstract

Background The *Lactobacillaceae* family comprises many species of great importance for the food and healthcare industries, with numerous strains identified as beneficial for humans and used as probiotics. Hence, there is a growing interest in engineering these probiotic bacteria as live biotherapeutics for animals and humans. However, the genetic parts needed to regulate gene expression in these bacteria remain limited compared to model bacteria like *E. coli* or *B. subtilis*. To address this deficit, in this study, we selected and tested several bacteriophage-derived genetic parts with the potential to regulate transcription in lactobacilli.

Results We screened genetic parts from 6 different lactobacilli-infecting phages and identified one promoter/repressor system with unprecedented functionality in *Lactiplantibacillus plantarum* WCFS1. The phage-derived promoter was found to achieve expression levels nearly 9-fold higher than the previously reported strongest promoter in this strain and the repressor was able to almost completely repress this expression by reducing it nearly 500-fold.

Conclusions The new parts and insights gained from their engineering will enhance the genetic programmability of lactobacilli for healthcare and industrial applications.

Keywords Lactobacilli, *L. Plantarum*, Probiotic bacteria, Bacteriophage, Promoter, Repressor.

Background

Lactobacilli are ubiquitous in and around humans, providing numerous health benefits. They are commensals in multiple organs (gut, skin, urinary tract, vagina, lungs, etc.), and are one of the largest families of probiotics [1]. Several probiotic strains are being clinically tested as Live Biotherapeutic Products to treat diseases like ulcerative colitis [2], mastitis [3], atopic dermatitis [4], bacterial

vaginosis [5], and periodontitis [6]. Apart from this, lactobacilli are also vital in the food industry for producing fermented products like yogurt [7], cheese [8], beer [9], wine [10]. etc. Due to this close association with our lives, there is considerable interest to genetically enhance and expand their capabilities for both medical [11] and industrial applications [12]. In the medical sphere, lactobacilli are being engineered as drug delivery vehicles to treat diseases like ulcerative colitis [13], HIV infection [14], respiratory infections [15], etc. or as oral vaccine candidates that display antigens on their cell surface [16]. In the industrial sphere, lactobacilli are being considered as alternative recombinant expression hosts to *E. coli* since (i) they don't produce endotoxins and many strains have

*Correspondence:

Shrikrishnan Sankaran

Shrikrishnan.sankaran@leibniz-inm.de

¹Bioprogrammable Materials, INM - Leibniz Institute for New Materials, Campus D2 2, 66123 Saarbrücken, Germany

²Saarland University, 66123 Saarbrücken, Germany



© The Author(s) 2024. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

Generally Recognized as Safe (GRAS) status, minimizing the toxicity risks for pharmaceutical protein production [17], (ii) infrastructure for their culture is well established in the food industry, and (iii) they are good at secreting proteins that can be purified from culture supernatants [18].

To realize such applications, considerable progress has been made in identifying a handful of genetic parts including constitutive promoters [19], peptide-inducible promoters [20], ribosome binding sites (RBS) [21], signal peptides for protein secretion [18], origins of replication [22], and food-grade plasmid retention systems [23, 24]. However, the programmability of lactobacilli is severely limited by the scarcity of genetic parts that allow control over gene expression, especially strong promoters and effective repressors that are needed to realize important functions like inducible expression.

Synthetic biology tools enable us to genetically program microorganisms with functions analogous to electronic circuits [25, 26]. The most basic element for such programming is a switch that can regulate the expression of genes between ON and OFF states depending on the presence and absence of a trigger. These switches are typically inducible gene expression systems, with which the production of recombinant proteins can be activated by an inducer molecule (e.g., IPTG) [27] or stimulus (e.g., light) [28]. These switches most often involve repressors, whose function is to block gene expression driven by a promoter [29]. While such screening and identification of repressors has been extensively done in model organisms like *Escherichia coli* [30, 31], lactobacilli severely lack reliable repressors.

Heiss and colleagues performed a study in *L. plantarum* in which they tested several promoter/repressor systems either derived from *Bacillus megaterium*, *E. coli* or other lactic acid bacteria (LAB) but were found to be considerably leaky and the promoters associated with them were weak [32]. For example, an endogenous promoter/repressor ($P_{lacA}/lacR$) system showed a ~8-fold induction capability in response to 2% (w/v) lactose in *L. plantarum* 3NSH. The system was completely repressed in the presence of monomeric sugars (glucose and galactose). In the same study, the orthogonal xylose inducible promoter/repressor system ($P_{xylA}/xylR$), derived from *B. megaterium*, was responsive to xylose supplementation but showed significant leaky expression in the absence of the inducer. This study also tested the promoter/repressor system $P_{lacA}/lacI$ from *E. coli* in combination with T7 RNA Polymerase system. Post induction, the dynamic range of the system accounted for a ~6-fold higher reporter expression. However, the reporter expression levels were low compared to a moderately strong constitutive promoter. Overall, these results highlight the need for identifying promoter/repression systems that work

reliably in *L. plantarum* (e.g., a strong repressor able to fully repress a strong promoter). In this study, we looked for repressors in another promising source for genetic parts – bacteriophages that infect LABs. By screening through multiple phage-derived repressors that control their lytic and lysogenic cycles in LABs, for the first time, we identified one candidate that efficiently and reliably represses gene expression in *L. plantarum* WCFS1. Interestingly, the native promoter associated with the repressor was found to drive the highest reported levels of gene expression in *L. plantarum* WCFS1. The discovery of this promoter and repressor combination lays the foundation for creating inducible gene expression systems and achieving advanced programming capabilities in lactobacilli.

Materials and methods

Strain, media and plasmids

L. plantarum WCFS1 was used as the parent strain in this study. The strain was maintained in the De Man, Rogosa and Sharpe (MRS) media (Carl Roth GmbH, Germany, Art. No. X924.1). Genetically engineered *L. plantarum* WCFS1 strains were grown in MRS media supplemented with 10 µg/mL of erythromycin (Carl Roth GmbH, Art. No. 4166.2) at 37 °C and 250 revolutions per minute (rpm) for approximately 16 h. NEB 5-alpha Competent *E. coli* cells were used (New England Biolabs GmbH, Germany, Art. No. C2987) for the cloning of certain plasmids. This strain was maintained in Luria-Bertani (LB) media (Carl Roth GmbH, Art. No. X968.1). Genetically engineered *E. coli* DH5α strains were grown in LB media supplemented with 200 µg/mL of erythromycin at 37 °C, 250 rpm shaking conditions for approximately 16 h. The pLp_3050sNuc plasmid, which was used as the backbone vector in this study, was a kind gift from Prof. Geir Mathiesen (Addgene plasmid # 122,030). *E. coli* Nissle was a kind gift from Prof. Rolf Müller. *L. plantarum* φg1e was a kind gift from Dr. Makiko Kakikawa.

Molecular biology

Polymerase Chain Reaction (PCR) was performed using Q5 High Fidelity 2X Master Mix (NEB) with primers synthesized by Integrated DNA Technologies (IDT) (Leuven, Belgium) or Eurofins Genomics GmbH (Köln, Germany). Primers are listed in Table S2. Synthetic genes were purchased as eBlocks from IDT (Coralville, USA). The eBlocks were codon optimized using the IDT Codon Optimization Tool (Coralville, USA). NEBuilder® HiFi DNA Assembly Cloning Kit, Quick Blunting Kit and the T4 DNA Ligase enzyme were purchased from New England BioLabs (NEB, Germany). The plasmid extraction kit was purchased from Qiagen GmbH (Hilden, Germany). The DNA purification kit was purchased from Promega GmbH (Walldorf, Germany). Generuler 1 Kb

DNA Ladder (Thermo Fisher Scientific) was used as a reference for the agarose gels.

***L. plantarum* WCFS1 competent cell preparation and DNA transformation**

L. plantarum WCFS1 was inoculated in 5 mL of MRS media without any antibiotic and grown overnight at 37 °C with shaking (250 rpm). The next day, 1 mL of the bacterial culture was transferred into a secondary culture based on 20 mL of MRS and 5 mL of 1% (w/v) glycine. The secondary culture was incubated at 37 °C, 250 rpm until the optical density of the sample measured at a wavelength of 600 nm (OD_{600}) reached approximately 1. The cells were pelleted down by centrifuging at 4000 rpm for 12 min at 4 °C. Next, the bacterial pellet was washed several times, in each, bacteria were centrifugated for 8 min at 4000 rpm. The first two washes were done with 5 mL of ice-cold 10 mM $MgCl_2$. The next two washes were performed with 5 mL of ice-cold Sac/Gly solution [10% (v/v) glycerol and 1 M sucrose mixed in a 1:1 (v/v) ratio]. Lastly, after discarding the supernatant, the pellet was resuspended in 500 μ L of Sac/Gly solution, and the competent cells were distributed in 60 μ L aliquots for DNA transformation. For transformation, 1 μ g of dsDNA was added to the competent cells and incubated on ice for 10 min. The mixture was transferred to an ice-cold 2 mm gap electroporation cuvette (Bio-Rad Laboratories GmbH, Germany), and cells were electroporated with a single pulse at 1.8 kV, after which 1 mL of MRS medium was immediately added. The mixture was then incubated at 37 °C, 250 rpm for a recovery period of 3 h. After the recovery, the cells were centrifuged at 4000 rpm for 5 min, and 800 μ L of the supernatant was discarded. The remaining 200 μ L were used to resuspend the pellet, and the entire 200 μ L were plated on MRS Agar supplemented with 10 μ g/mL of erythromycin. The plates were incubated at 37 °C for 1–3 days to allow the growth of bacterial colonies.

Direct cloning in *L. plantarum* WCFS1

Plasmid engineering of *L. plantarum* WCFS1 was done using the direct cloning method previously developed by us [33], which involved PCR-based amplification and circularization of recombinant plasmids, which were then transformed in the bacteria by electroporation. In brief, complementary overhangs for HiFi Assembly were either synthesized as custom-designed eBlocks or generated by PCR. The HiFi DNA Assembly reaction was performed following the manufacturer's protocol. Then, 5 μ L of the assembled HiFi product was used as a DNA template in the PCR reaction (100 μ L final volume). After purifying the PCR product, 1000 to 2000 ng of linear DNA was phosphorylated using the Quick Blunting Kit and following the manufacturer's protocol. Next, phosphorylated

products were ligated using the T4 ligase enzyme. Two ligation reactions were set per cloning, each based on 500 ng of phosphorylated DNA, 2.5 μ L of 10X T4 Ligase Buffer and 1.5 μ L of T4 Ligase enzyme (autoclaved Milli-Q water was added to make the reaction volume to 25 μ L). The ligations were incubated at 25 °C for 3 to 5 h and then at 70 °C for 30 min for enzyme inactivation. After the incubation, the ligations were mixed and purified, performing three rounds of elution to concentrate the DNA (each with 10 μ L of autoclaved Milli-Q water). The entire eluted mix (approximately 1000 ng) was transformed into *L. plantarum* WCFS1 electrocompetent cells.

The same strategy was used to achieve site-directed mutagenesis, where specific DNA sequences were removed from the plasmid by PCR using the bacterial pellet as a template for the PCR and primers covering the whole region but the targeted sequence. The linear PCR product was circularized as described before using the Quick Blunting Kit and the T4 Ligase. For sequence verification, DNA sequences of interest were amplified (100 μ L final volume) using a bacterial pellet as a template. The PCR product was purified and sent for Sanger sequencing to Eurofins Genomics GmbH (Köln, Germany) by selecting the additional DNA purification step prior to sequencing.

***E. coli* Nissle 1917 competent cell preparation**

Wild-type *E. coli* Nissle 1917 bacteria was grown overnight in LB media at 37 °C, 250 rpm. The next day, bacteria were subcultured in 100 mL of fresh LB media and incubated at 37 °C and 250 rpm until the OD_{600} reached 0.4. Bacteria were pelleted down by centrifugation at 4000 rpm for 5 min. After discarding the supernatant, the pellet was washed twice with 10 mL of ice-cold $CaCl_2$ (200 mM) and once with a 10 mL of 1:1 combination of $CaCl_2$ (200 mM) and glycerol (10% w/v). Following the final wash, the pellet was resuspended in 1 mL of $CaCl_2$ +glycerol mixture and 100 μ L aliquots were prepared and stored at -80 °C unless used immediately.

***E. coli* DH5 α and *E. coli* Nissle 1917 DNA transformation**

E. coli DH5 α DNA transformation was performed following the manufacturer's protocol for the NEBuilder® HiFi DNA Assembly Cloning Kit.

For *E. coli* Nissle 1917 DNA transformation, 200 ng of plasmid DNA were mixed well with the competent cells by pipetting gently and incubated on ice for 30 min. Following the incubation, a 45-second heat shock was performed by placing the cells at a 42 °C water bath. Next, cells were again incubated on ice for 5 min. After that, 900 μ L of SOC media was added to the cell mixture and kept for incubation for 1 h at 37 °C. Next, the mixture was pelleted down by centrifugation at 4000 rpm for 5 min. 600 μ L were immediately discarded, and the

remaining 300 μ L were used to resuspend the mixture. Finally, 150 μ L were plated on an LB agar plate supplemented with 200 μ g/mL of erythromycin and incubated at 37 °C overnight.

Flow cytometry analysis

Engineered strains were grown in 5 mL of MRS media supplemented with 10 μ g/mL erythromycin at 37 °C with shaking, 250 rpm. The next day, bacteria were subcultured to an OD₆₀₀ of 0.01 in 5 mL of MRS media (supplemented with 10 μ g/mL erythromycin) and grown at 37 °C with shaking (250 rpm) for 16 h. The following day, 1 mL of the bacterial suspensions were harvested by centrifugation at 10,000 rpm. After discarding the supernatant carefully, the pellet was resuspended in 1 mL of sterile Dulbecco's 1X PBS. The mixtures were then serially diluted by a 10⁴ Dilution Factor, and 5,000 bacteria events were recorded for analysis using Guava easyCyte BG flow-cytometer (Luminex, USA). A predesigned gate based on forward side scatter and side scatter thresholding was employed to get rid of debris and doublets during the collection of events. The fluorescence intensity of mCherry was measured using excitation by a green laser at 532 nm (100 mW) and the Orange-G detection channel 620/52 nm filter was used for signal analysis. The gain settings used for the data recording were as follows: Forward Scatter (FSC) – 11.8, Side Scatter (SSC) – 4, and Orange-G Fluorescence – 1.68. The compensation control for fluorescence recording was set at 0.01, with an acquisition rate of 5 decades. The Luminex GuavaSoft 4.0 software for EasyCyte was used for the analysis and representation of data.

Microplate reader setup for reporter gene expression quantification

L. plantarum WCSF1 engineered strains were grown in the same manner as described for the Flow Cytometry Analysis. 200 μ L of the 1000- μ L resuspended mixture (PBS containing engineered bacteria) was added to a UV STAR Flat Bottom 96 well microtiter plate (Greiner BioOne GmbH, Germany). Next, the samples were analyzed in the Microplate Reader Infinite 200 Pro (Tecan Deutschland GmbH, Germany) and both the absorbance (600 nm wavelength), and mCherry fluorescence intensity (Ex_{λ} / Em_{λ} = 587 nm/625 nm) were measured. The Z-position and gain settings were set to 19,000 μ m and 100, respectively. The readings were taken using the top read setting. The fluorescence values were normalized with the optical density of the bacterial cells to calculate the Relative Fluorescence Units (RFU) (formula $RFU = \text{Fluorescence} / OD_{600}$). The same procedure was followed for *E. coli* Nissle engineered strains, but those were grown in LB media supplemented with 200 μ g/mL of erythromycin at 37 °C with shaking (250 rpm).

Experiments were performed in triplicates on three different days.

Growth rate measurements and biomass calculation

To measure the growth curves of the engineered strains, they were cultivated overnight in antibiotic supplemented MRS media at 37 °C with shaking (250 rpm). The following day, the bacterial cultures were subcultured in 3-mL secondary cultures at an initial OD₆₀₀=0.01 and incubated at 30 °C with shaking (250 rpm) until reaching an OD₆₀₀ of 0.4–0.5. Then, 200 μ L of the cultures were distributed in a UV STAR Flat Bottom 96 well microtiter plate. The plate was placed in the Microplate Reader with constant shaking conditions at an incubation temperature of 37 °C. The kinetic assay was set to record the absorbance (600 nm) of the bacterial cultures with an interval of 15 min for 16 h. The experiment was conducted twice on two independent days, keeping two technical duplicates per experiment.

To estimate the bacterial media-free wet biomass of the engineered strains, they were cultivated overnight in antibiotic supplemented MRS media at 37 °C with shaking (250 rpm). The following day, the bacterial cultures were subcultured in 5-mL secondary cultures at an initial OD₆₀₀=0.01 and incubated at 37 °C with shaking (250 rpm) for 16 h. The following day, all 5-mL cultures were pelleted down in several rounds of centrifugation (10,000 rpm), and the biomass of the bacterial pellets was measured using an analytical balance (Denver Instrument). Experiments were performed in triplicates on three different days.

Fluorescence microscopy analysis

Engineered strains were grown in the same manner as described for the Flow Cytometry Analysis. 10 μ L of the 1000- μ L resuspended mixture (PBS containing engineered bacteria) was placed on glass slides of 1.5 mm thickness (Paul Marienfeld GmbH, Germany) and 1.5 H glass coverslips (Carl Roth GmbH, Germany) were placed on top of it. The samples were then observed under the Plan Apochromat 100 $\times$ oil immersion lens (BZ-PA100, NA 1.45, WD 0.13 mm) of the Fluorescence Microscope BZ-X800 (Keyence Corporation, Illinois, USA). The mCherry signal was captured in the BZ-X TRITC filter (model OP-87,764) at an excitation wavelength of 545/25 nm and an emission wavelength of 605/70 nm with a dichroic mirror wavelength of 565 nm. The images were adjusted for identical brightness and contrast settings. ImageJ2 software was used to process the images.

Statistical and bioinformatics analysis

Statistical analysis was performed using GraphPad Prism 7.0 software. Student's T-tests were used to determine if there were significant differences between the means of

the groups. InterPro was used to identify the DBD of rep. AlphaFold was used to predict the 3D structures of the rep repressor.

Results

Strategy to identify reliable promoter/repressor systems

A strategy to find and optimize reliable promoter/repressor systems in *L. plantarum* WCFS1 was developed as shown in Fig. 1. The search for potential transcriptional repressors was limited to repressors encoded in the genetic switches that regulate lytic and lysogenic cycles in bacteriophages. The strategy involved (1) identifying bacteriophages that infect lactobacilli with characterized genetic switches, (2) selecting 6 repressors with known

operator sequences, (3) designing all the genetic parts required for (4) building a genetic platform, (5) testing the repression mediated by each repressor, and finally (6) improving such repression by introducing certain modifications to the operator/promoter regions.

Screening of six bacteriophage repressors

The six different phage-derived repressors that we tested in this study are: *cng* and *cpg* repressors from the ϕ g1e phage infecting *L. plantarum* [34, 35], *tec* and *rep* repressors from the mv4 phage infecting *Lactobacillus delbrueckii* [36], and *cI* and *cro* repressors from the A2 phage infecting *Lactocaseibacillus casei* (formerly *Lactobacillus casei*) [37, 38] (Table S2). A standard, simple

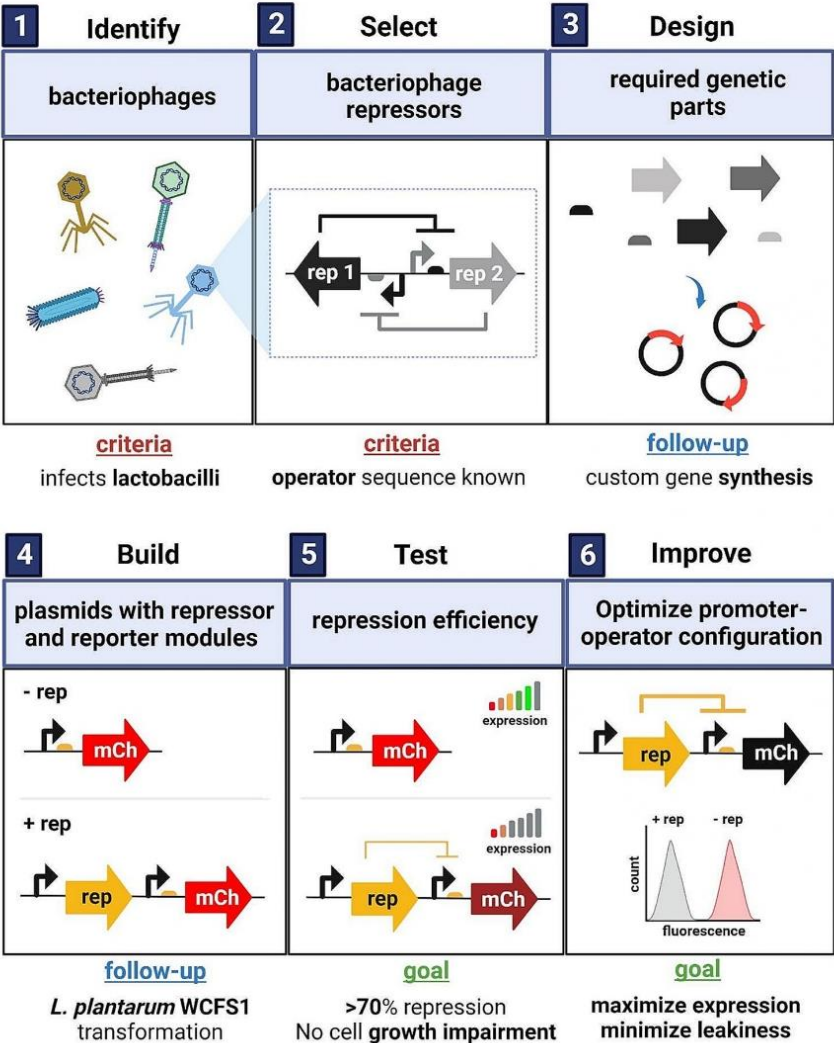


Fig. 1 Panels showing the strategy and all the steps that were defined and followed to find novel bacteriophage-derived promoter/repressor systems in *L. plantarum* WCFS1.

genetic module was designed and built to test the repression mediated by each repressor. The module was based on our previously reported strongest constitutive promoter in *L. plantarum* WCFS1 (P_{tlpA}) [39], which enabled reliable characterization of repressor activity. Notably, previous studies attempting to identify efficient repressors in lactobacilli were limited by promoters driving moderate levels of expression [32]. Our repressor-testing module included (i) P_{tlpA} driving the expression of the reporter gene mCherry, (ii) repressor-specific operators

inserted between the -10 box and the RBS (Figure S1) and (iii) repressors constitutively expressed by a moderately strong promoter (P_{48}). The module was constructed through two rounds of cloning to encode both the operator and repressor in the plasmid. In the first round, the operator was inserted within the promoter, and in the second round, each repressor was cloned in the plasmid containing the corresponding operator (Fig. 2A). Repression of mCherry production was first assessed by quantifying the drop in fluorescence intensity using

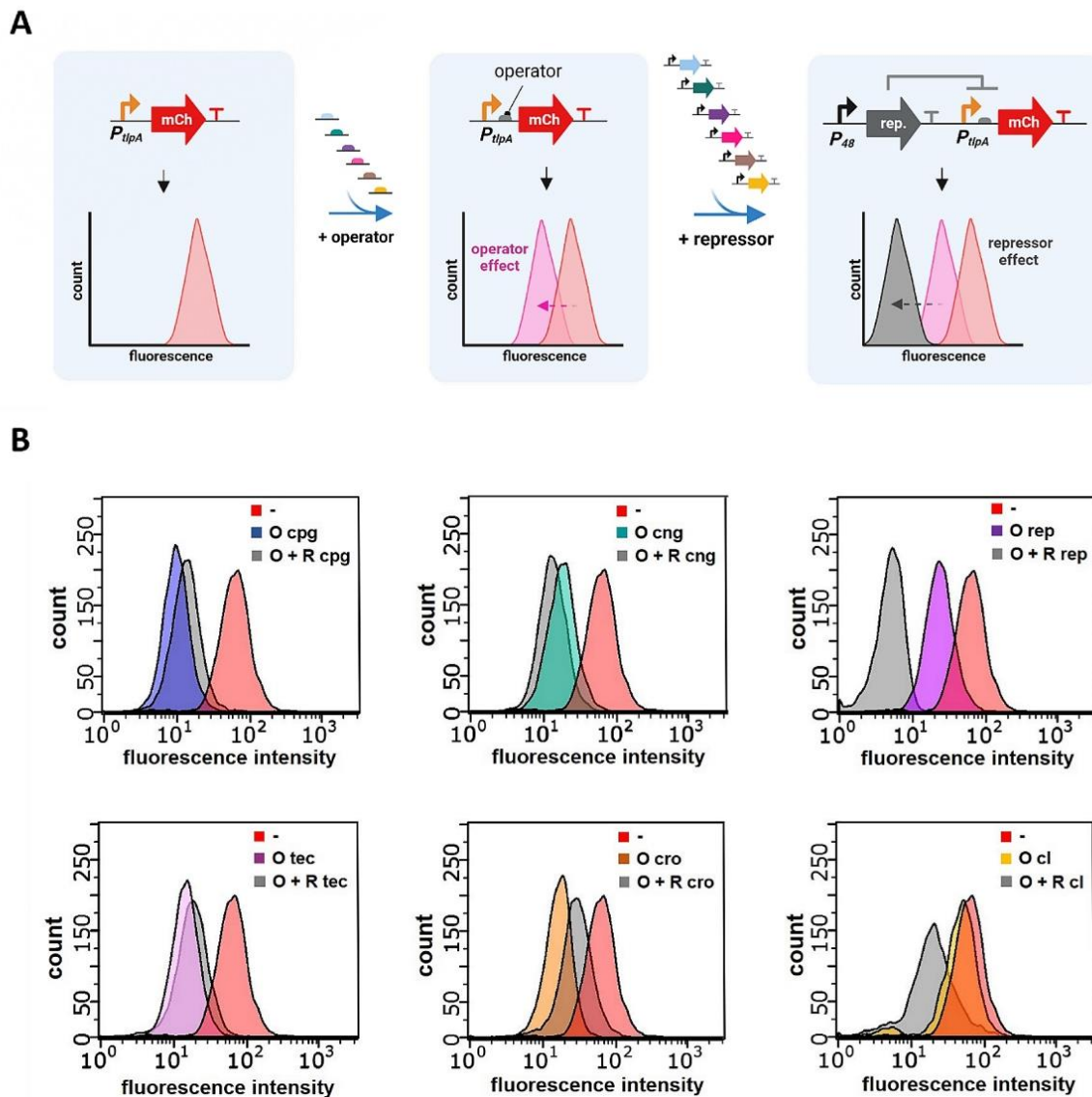


Fig. 2 (A) Cloning workflow design. In the first round of cloning, the operators were inserted within the promoter P_{tlpA} . In the second round, the repressors were cloned in the corresponding plasmid. All genetic fragments were based on IDT synthetic eBlocks. (B) Flow cytometry data showing the effect on mCherry expression of cloning each operator (O) and each operator plus repressor (O + R) in the plasmid

flow cytometry (Fig. 2C). This analysis first revealed that insertion of the operator sequences weakened the strength of P_{tlpA} in all cases between ~1.3 and 4.3-fold (Figure S2), although fluorescence intensities remained high enough to assess repressor activity. When the repressors were encoded in the plasmids containing the operator sequences, drop in fluorescence was observed only with the repressors cng, rep and cI (Fig. 2C). Notably, rep was found to be the strongest repressor among the three with fluorescence intensity values comparable to the wild-type strain that was not modified to produce mCherry (Figure S3).

Quantification of repression levels

Next, we proceeded to quantify the repression levels and the effect on cell growth imposed by cng, rep and cI.

We set a couple of requirements that potential repressors should meet in order to proceed with the next step of optimizing repression. Repressors (i) should be able to repress at least up to 70% mCherry expression and (ii) do not considerably impair bacterial growth. Concerning repression, only rep showed levels of repression above 70%, precisely, rep-driven repression was found to be up to 92% (Fig. 3A and B). As for the effect on cell growth, we measured the growth curves of bacteria encoded with all three repressors when bacteria were in the log phase. We used a microplate reader and compared the curves to wild-type bacteria. (Fig. 3C). Only the growth curve of the cI repressor was noticeably lower than the others. Nevertheless, differences in the OD₆₀₀ after 16 h of growth in the micro plate reader were found to be non-significant (Figure S4). It is also worth noting that the lag

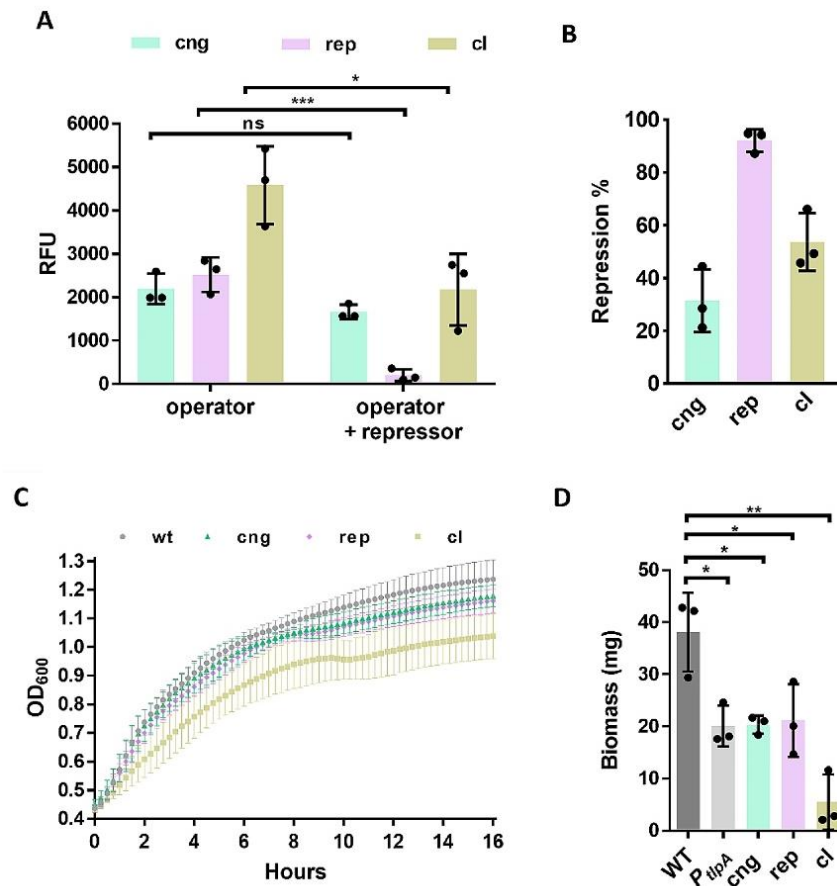


Fig. 3 (A) Expression levels of mCherry in relative fluorescence units (RFU) for all three operators (O cng, O rep and O cI) and all three operators plus repressors (cng, rep and cI). (B) Percentage of repression mediated by each repressor. (C) Growth curves of all three repressors and wild-type bacteria over 16 h. (D) Bacterial biomass of wild-type, P_{tlpA} -mCherry, and bacteria carrying each repressor after overnight growth in the incubator. Each sample is based on a 5-mL culture. Experiments for Figures A, B and D were performed as experimental triplicates ($N=3$). Experiments for Figure C were performed as experimental duplicates, each with two technical replicates ($N=2$, $n=2$). Column heights and error bars represent the means and standard deviations (SD). ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

phase of bacteria encoding the cI repressor was longer than that of the other bacteria.

The operator is essential for repression

We proceeded to confirm that rep repression was dependent on the presence of the operator sequence. This sequence is generally required for the DNA-binding domain (DBD) of the repressors to bind and favor repression. For that, we removed the operator from the plasmid by site-directed mutagenesis PCR using the bacterial pellet as a template for the amplification (Fig. 4A). The PCR product was then cloned through direct cloning into *L. plantarum* WCFS1 (Figure S5). After removing the operator, mCherry expression increased drastically owing to both the inability of rep to bind to the operator and thus repress, and the excision of the operator from P_{tlpA} (Fig. 4B), which was known to have a negative impact on the expression (Figure S2). We also employed Interpro to confirm the presence of a DBD based on a helix-turn-helix domain at the N-terminal of the protein. In addition, AlphaFold predicted the 3D structure of rep, which showed to have the typical structure of a repressor, a DBD at the N-terminal and a dimerization domain at the C-terminal (Fig. 4C). AlphaFold also predicted the protein-protein interaction of rep monomers forming a dimer with the dimerization domains and DBDs from one monomer associated with those from the other monomer, which is typically needed for repression (Fig. 4D).

Optimization of rep-based repression

Next, we attempted to increase the levels of repression by introducing certain modifications to the promoter region. The first approach involved P_{tlpA} engineering by introducing modifications in the placement of the operator. Thus, three new variants were cloned: (i) removing 14-bp between the operator and the RBS (O1), (ii) placing the operator within P_{tlpA} , between the -35 and -10 boxes (O2), and (iii) adding an additional operator upstream of P_{tlpA} (O3) (Fig. 5A). Removing part of the spacer between the operator and the RBS and placing the operator within P_{tlpA} considerably decreased the expression of mCherry, even in the absence of the rep (Fig. 5B). On the other hand, adding an extra operator sequence upstream of P_{tlpA} only mildly affected mCherry expression but significantly improved repression, from 92% (initial clone, Fig. 3B) to above 99% (Fig. 5C). We tested this double-operator approach for the cng repressor by adding an extra operator (O cng) by PCR upstream the P_{tlpA} promoter (Figure S6A). However, surprisingly no repression could be detected after the addition of an extra operator in this repression module (Figure S6B). This suggests that even though this double-operator approach is a valid strategy to improve repressor performance, it is not generally applicable to all repressors.

The second approach attempted to improve repression by replacing P_{tlpA} with the native promoter (P_{tec}) associated with rep (Fig. 5D). This promoter is encoded in the lysogeny region of the *L. delbrueckii* bacteriophage mv4, and it drives the expression of the tec repressor. The rep operator is placed between the -10 and -35 regions of

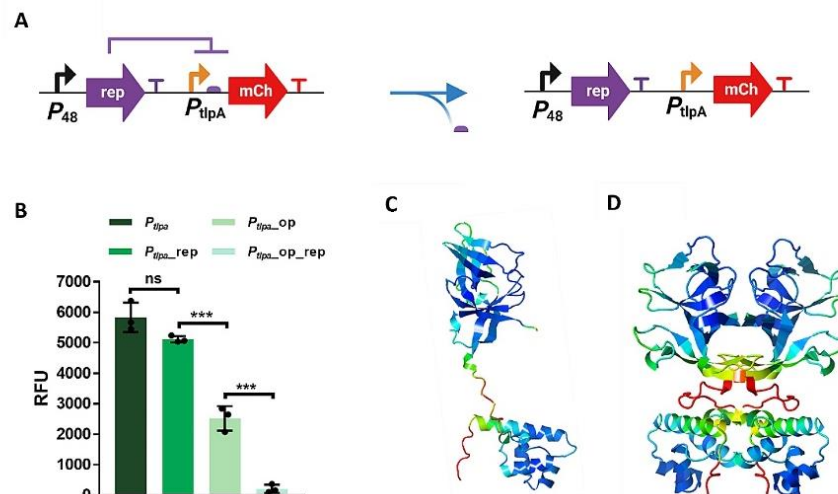


Fig. 4 (A) Scheme of the site-directed mutagenesis done to remove the operator from the plasmid. (B) Expression levels of mCherry in terms of RFU for P_{tlpA} (unmodified), P_{tlpA} -rep (only repressor), P_{tlpA} -op (only rep operator) and P_{tlpA} -op_rep (operator plus rep). All the experiments were performed as experimental triplicates ($N=3$). Column heights and error bars represent the means and SD. ns = not significant, *** $p < 0.001$. (C) AlphaFold 3D structure prediction of the rep as a monomer. (D) AlphaFold 3D structure prediction of the rep as a dimer

the promoter [36]. In this cloning, we first replaced the rep-operated P_{tlpA} with P_{tec} in the plasmid encoding for rep and repression was almost complete. Surprisingly, when the repressor was removed from the plasmid by site-directed mutagenesis (Figure S7), the level of mCherry expression was extremely high, due to which the repression efficiency was determined as $>99.7\%$ (Fig. 5F and E).

We also evaluated the effect of these new clones (P_{tlpA} -O3_rep and P_{tec} _rep) on cell growth. It was observed that growth was only slightly decreased

compared to wild-type bacteria (Figure S8), similar to that of rep with a single operator and the cng repressor, and better than that of the cI repressor (Fig. 3C).

These results proved that repression can be enhanced by either engineering the operator placement within P_{tlpA} or by introducing the endogenous P_{tec} promoter. Whereas the original clone showed a fold-change of ~ 15 (P_{tlpA} -O_rep), the optimized clones showed a fold-change of ~ 117 (P_{tlpA} -O3_rep_mCherry) and ~ 475 (P_{tec} _rep) (Fig. 5G).

Characterization of a strong constitutive promoter

Next, owing to the surprisingly high expression levels of P_{tec} , we further characterized it and compared it to P_{tlpA} . After overnight growth at 37°C , we could observe a change in the MRS media color due to high levels of mCherry production. This color change was more pronounced when growing these bacteria in LB media supplemented with glucose to favor bacterial growth in this non-optimal media. Such visible color change of the medium due to the expression of a fluorescent protein is regularly observed when expressing these proteins in *E. coli* but has not been reported in lactobacilli. Also, when spinning these bacteria down, the pellet was bright red, similar to what is observed in *E. coli* (Fig. 6A). As expected, such a strong constitutive promoter had an effect on cell growth compared to wild-type bacteria. This effect was evident when bacteria were grown overnight in the incubator with shaking and not in the plate reader (Figure S9). However, biomass was higher than bacteria carrying P_{tlpA} -mCherry, cng repressor and rep repressor.

In comparison with P_{tlpA} , P_{tec} drove a considerably higher level of gene expression, which was visible by eye (Fig. 6B) and microscopy, (Fig. 6C) and was confirmed by flow cytometry (Fig. 6D). Fluorescence spectroscopy using a plate reader revealed that P_{tec} was ~ 9 times stronger than P_{tlpA} (Fig. 6E), confirming it to be the strongest constitutive promoter discovered for heterologous gene expression in *L. plantarum* WCFS1. Since the visible color changes of the liquid culture and bacterial pellets were comparable to expression in *E. coli*, we compared P_{tec} -driven mCherry expression levels in *L. plantarum* WCFS1 with the expression in probiotic *E. coli* Nissle 1917 driven by the strong P_{tlpA} promoter. After quantifying the expression levels of mCherry with the microplate reader, we observed that while P_{tlpA} in *E. coli* 1917 was 17-fold stronger than in *L. plantarum*, P_{tlpA} in *E. coli* was only 2-fold stronger than P_{tec} in *L. plantarum* (Fig. 6E).

In light of this finding, we attempted to test the strength of the promoter, P_{cpg} , associated with the 2nd best repressor (cng) identified in this study. Yet, this promoter was only of moderate strength comparable to constitutive

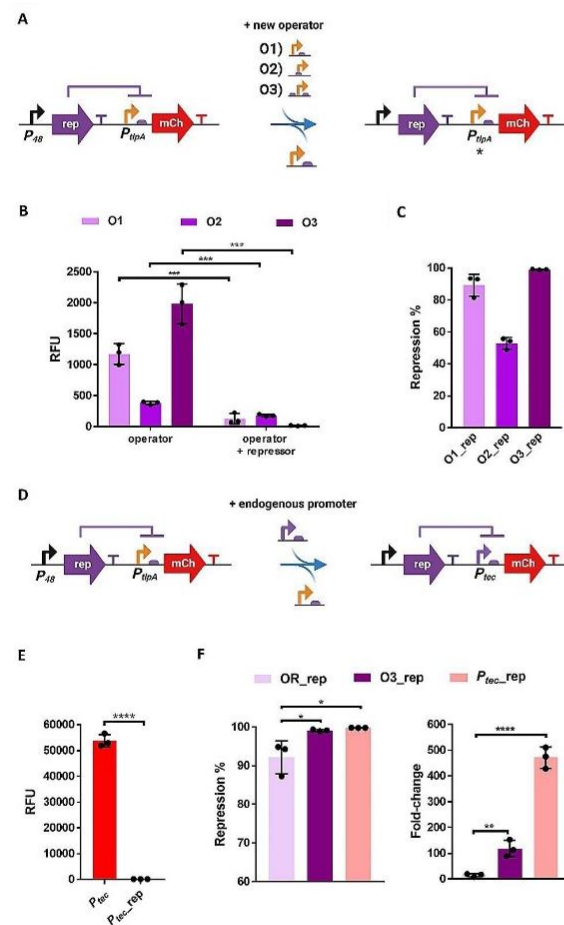


Fig. 5 (A) Scheme of the genetic modifications that introduced the operator of rep at different locations in and around the promoter P_{tlpA} . (B) Expression levels of mCherry for all three operator variants (with and without the rep repressor in the plasmid). (C) Percentage of repression mediated by each operator variant (O1, O2 and O3). (D) Scheme of the substitution of P_{tlpA} by P_{tec} . (E) Expression levels of mCherry for P_{tec} -mCherry and P_{tec} -rep_mCherry bacteria. (F) Percentage of repression and fold-changes for P_{tlpA} -O_rep (OR_rep), P_{tlpA} -O3_rep (O3_rep) and P_{tec} _rep. All the experiments were performed as experimental triplicates ($N=3$). Column heights and error bars represent the means and SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

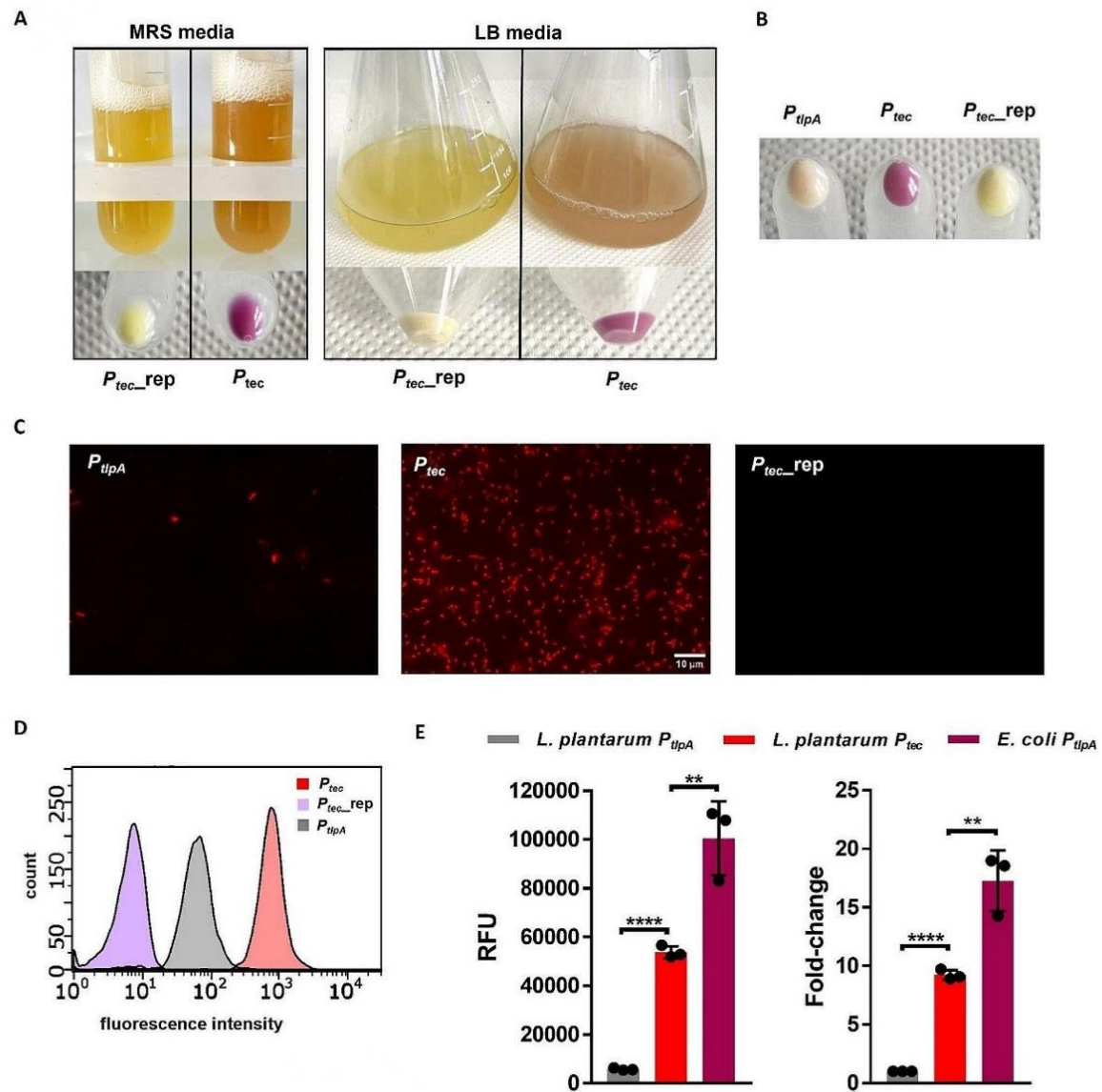


Fig. 6 (A) Left: 5-ml MRS cultures based on P_{tec_rep} and P_{tec} bacteria. Cells were pelleted down, and pellets are shown at the bottom. Right: 95-ml LB (supplemented with 5 ml 1 M glucose) cultures based on P_{tec_rep} and P_{tec} bacteria. Cells were pelleted down, and the corresponding pellets are shown at the bottom. (B) Pellets of bacteria encoded to express mCherry driven by P_{tec_rep} , P_{tec} and P_{tlpA} after overnight growth. (C) Fluorescence microscopy images of bacteria encoded to express mCherry driven by P_{tec_rep} , P_{tec} and P_{tlpA} . (D) FACS data showing the comparison between bacteria encoded to express mCherry driven by P_{tec_rep} , P_{tec} and P_{tlpA} . (E) Expression levels of mCherry driven by P_{tec} in $L. plantarum$ WCSF1, P_{tlpA} in $L. plantarum$ WCSF1 and P_{tlpA} in $E. coli$ Nissle 1917. Fold changes of mCherry expression driven by P_{tec} in $L. plantarum$ WCSF1 and P_{tlpA} in $E. coli$ Nissle 1917 normalized to the expression in P_{tlpA} $L. plantarum$ WCSF1. All the experiments were performed as experimental triplicates ($N=3$). Column heights and error bars represent the means and SD. ** $p < 0.01$, *** $p < 0.001$

promoters like P_{48} in this strain as it was ~6 fold weaker than P_{tlpA} and ~55 fold weaker than P_{tec} (Figure S10).

Discussion

Bacteriophages have proven to be an extensive and diverse source of genetic parts to expand the synthetic biology toolbox of bacteria [40], including transcriptional systems [41], integrases [42], anti-CRISPR proteins [43], endolysins [44], and repressors [45]. Such parts are naturally adapted to their bacterial hosts due to the coevolution and the arms race between bacteriophages and bacteria for millions of years [46]. However, bacteriophage parts remain largely unexplored for building genetic circuits in lactobacilli. In this study, we have established a genetic platform for testing transcriptional repressors from lactobacilli-infecting bacteriophages. Relying on operating the strong constitutive P_{tlpA} promoter proved to be a prudent strategy since the strength and compatibility of the natural phage promoters are unpredictable. For example, P_{cpg} , the natural promoter associated with the partially effective cng repressor, only showed a moderate expression in *L. plantarum* WCFS1, which might not have been enough to assess repression. Out of six different repressors encoded in genetic switches in lactobacilli prophages, only the rep repressor showed promising results in terms of efficient and reliable repression of the reporter gene without impacting bacterial growth. These results highlight that lactobacilli prophages can be a promising yet challenging source of genetic parts to expand the genetic toolbox. Furthermore, the endogenous promoter associated with rep exhibited unprecedented levels of gene expression, significantly narrowing the gap in terms of expression levels between model (*E. coli* Nissle 1917) and non-model probiotic bacteria engineered for therapeutic applications. P_{tec} could also be operated and used as a genetic platform to identify more repressors in this strain since even low repression could be more readily assessed than with P_{tlpA} . The highly efficient P_{tec} /rep promoter/repressor system could now be applied in combination with other genetic parts for building genetic circuits. One limitation of the system is that it is currently not inducible. However, this provides the opportunity for future work to employ repressor engineering strategies and modify rep into a switchable repressor that responds to sugars [47] or physical stimuli such as light [48] or heat [49]. Such switchable repressors will enable inducible gene expression, which would be desirable to circumvent the notable metabolic burden and stress that P_{tec} might be causing to the cells due to its transcriptional expression strength. Also, the unmodified repressor can be combined with other inducible gene expression systems to invert the induction system as a NOT logic gate [50, 51]. In combination with the P_{tec} promoter, such induction or

inversion functions can be achieved at a remarkably high level of performance.

Conclusions

We identified a novel, strong and efficient promoter/repressor system in the probiotic bacterium *L. plantarum* WCFS1 by screening for such genetic parts in lactobacilli-infecting bacteriophages. After improving the system, we achieved repression levels of >99% and fold-changes of >100. Moreover, we discovered a super strong constitutive promoter, P_{tec} , which can drive levels of expression never achieved before in this strain, precisely ~9 times higher than the previously reported strongest promoter, P_{tlpA} . These novel genetic parts will be instrumental in expanding the capabilities to engineer gene expression regulation in *L. plantarum*.

Abbreviations

LAB	Lactic Acid Bacteria
MRS	De Man, Rogosa and Sharpe
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
RFU	Relative Fluorescent Units
RBS	Ribosome Binding Sites
OD ₆₀₀	Optical density at 600 nm
SD	Standard deviations

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12934-024-02302-7>.

Supplementary Material 1

Acknowledgements

The Lactiplantibacillus plantarum WCFS1 strain was a kind gift from Prof. Gregor Fuhrmann (Helmholtz Institute for Pharmaceutical Research, Saarland). The plasmid pLp_3050sNuc was a kind gift from Prof. Geir Mathiesen (Addgene plasmid # 122030). *E. coli* Nissle was a kind gift from Prof. Rolf Müller. *L. plantarum* ϕ g1e was a kind gift from Dr. Makiko Kakikawa. Biorender was used for generating the schemes for the figures of this publication.

Author contributions

MBA and SS developed the initial concept. MBA conducted most of the experiments and wrote the manuscript, partially assisted by AW. VST conducted the experiments related to *E. coli* Nissle 1917 engineering and analysis. SS supervised the study and revised the manuscript. All authors have read and approved the final manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL. This work was supported by a Deutsche Forschungsgemeinschaft (DFG) Research grant [Project # 455063657 - <https://gepris.dfg.de/gepris/projekt/455063657>] and the Leibniz Science Campus Living Therapeutic Materials. Open Access funding enabled and organized by Projekt DEAL.

Data availability

The protein and genetic sequences used in this study have been provided in the supplementary information files. The processed results have been comprehensively provided within the manuscript or supplementary information files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

A patent application has been filed based on the results of this work (Application no. is DE 102022 119024.2).

Received: 5 December 2023 / Accepted: 10 January 2024

Published online: 07 February 2024

References

1. Probiotics, Sept. NIH Office of Dietary Supplements 2023. Probiotics - Health Professional Fact Sheet (nih.gov). Accessed 2023.
2. Zocco MA, Dal Verme LZ, Cremonini F, Piscaglia AC, Nista EC, Candelli M, Novi M, Rigante D, Cazzato IA, Ojetti V, Armuzzi A. Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis. *Aliment Pharmacol Ther.* 2006;23(11):1567–74.
3. Jiménez E, Fernández L, Maldonado A, Martín R, Olivares M, Xaus J, Rodríguez JM. Oral Administration of Lactobacillus Strains Isolated from breast milk as an alternative for the treatment of infectious mastitis during Lactation. *Appl Environ Microbiol.* 2008;74(15):4650.
4. Rosenfeldt V, Benfeldt E, Nielsen SD, Michaelsen KF, Jeppesen DL, Valerius NH, Pærregaard A. Effect of probiotic Lactobacillus strains in children with atopic dermatitis. *J Allergy Clin Immunol.* 2003;111(2):389–95.
5. Mastromarino P, Macchia S, Meggiorini L, Trinchieri V, Mosca L, Perluigi M, Midulla C. Effectiveness of Lactobacillus-containing vaginal tablets in the treatment of symptomatic bacterial vaginosis. *Clin Microbiol Infect.* 2009;15(1):67–74.
6. Teughels W, Durukan A, Ozelik O, Pauwels M, Quirynen M, Haytac MC. Clinical and microbiological effects of Lactobacillus reuteri probiotics in the treatment of chronic periodontitis: a randomized placebo-controlled study. *J Clin Periodontol.* 2013;40(11):1025–35.
7. Ashraf R, Shah NP. Selective and differential enumerations of Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium spp. in yoghurt—A review. *Int J Food Microbiol.* 2011;149(3):194–208.
8. Kasimoğlu A, Göncüoğlu MU, Akgün S. Probiotic white cheese with Lactobacillus acidophilus. *Int Dairy J.* 2004;14(12):1067–73.
9. Chan MZ, Chua JY, Toh M, Liu SQ. Survival of probiotic strain Lactobacillus paracasei L26 during co-fermentation with *S. Cerevisiae* for the development of a novel beer beverage. *Food Microbiol.* 2019;82:541–50.
10. du Toit M, Engelbrecht L, Lerm E, Krieger-Weber S. Lactobacillus: the next generation of malolactic fermentation starter cultures—an overview. *Food Bioprocess Technol.* 2011;4:876–906.
11. Pedrololi DB, Ribeiro NV, Squizzato PN, de Jesus VN, Cozetto DA, Tuma RB, Gracindo A, Cesar MB, Freire PJ, da Costa AF, Lins MR. Engineering microbial living therapeutics: the synthetic biology toolbox. *Trends Biotechnol.* 2019;37(1):100–15.
12. de Vos WM. Systems solutions by lactic acid bacteria: from paradigms to practice. *Microb Cell Fact.* 2011;10(1):1–3.
13. Watterlot L, Rochat T, Sokol H, Cherbuy C, Bouloufa I, Lefèvre F, Gratadoux JJ, Honvo-Hueto E, Chilmoneczyk S, Blugeon S, Corthier G. Intragastric administration of a superoxide dismutase-producing recombinant Lactobacillus casei BL23 strain attenuates DSS colitis in mice. *Int J Food Microbiol.* 2010;144(1):35–41.
14. Janahi EM, Haque S, Akhter N, Wahid M, Jawed A, Mandal RK, Lohani M, Areeshi MY, Almalki S, Das S, Dar SA. Bioengineered intravaginal isolate of Lactobacillus plantarum expresses algal lectin scytovirin demonstrating anti-HIV-1 activity. *Microb Pathog.* 2018;122:1–6.
15. Wang M, Fu T, Hao J, Li L, Tian M, Jin N, Ren L, Li C. A recombinant Lactobacillus plantarum strain expressing the spike protein of SARS-CoV-2. *Int J Biol Macromol.* 2020;160:736–40.
16. Zhang Y, Yang L, Zhang J, Huang K, Sun X, Yang Y, Wang T, Zhang Q, Zou Z, Jin M. Oral or intranasal immunization with recombinant Lactobacillus plantarum displaying head domain of Swine Influenza A virus hemagglutinin protects mice from H1N1 virus. *Microb Cell Fact.* 2022;21(1):185.
17. Plavec TV, Berlec A. 2020. Safety aspects of genetically modified lactic acid bacteria. *Microorganisms*, 8(2), p.297.
18. Mathiesen G, Sveen A, Brurberg MB, Fredriksen L, Axelsson L, Eijsink VG. Genome-wide analysis of signal peptide functionality in Lactobacillus plantarum WCFS1. *BMC Genomics.* 2009;10:1–3.
19. Rud I, Jensen PR, Naterstad K, Axelsson L. A synthetic promoter library for constitutive gene expression in Lactobacillus plantarum. *Microbiology.* 2006;152(4):1011–9.
20. Halbmayer E, Mathiesen G, Nguyen TH, Maischberger T, Peterbauer CK, Eijsink VG, Haltrich D. High-level expression of recombinant β -galactosidases in Lactobacillus plantarum and Lactobacillus sakei using a sakacin P-based expression system. *J Agric Food Chem.* 2008;56(12):4710–9.
21. Tauer C, Heintz S, Egger E, Heiss S, Grabherr R. Tuning constitutive recombinant gene expression in Lactobacillus plantarum. *Microb Cell Fact.* 2014;13(1):1–1.
22. Spangler JR, Caruana JC, Phillips DA, Walper SA. Broad range shuttle vector construction and promoter evaluation for the use of Lactobacillus plantarum WCFS1 as a microbial engineering platform. *Synth Biol.* 2019;4(1):ysz012.
23. Takala T, Saris P. A food-grade cloning vector for lactic acid bacteria based on the nisin immunity gene nisl. *Appl Microbiol Biotechnol.* 2002;59:467–71.
24. Nguyen TT, Mathiesen G, Fredriksen L, Kittl R, Nguyen TH, Eijsink VG, Haltrich D, Peterbauer CK. A food-grade system for inducible gene expression in Lactobacillus plantarum using an alanine racemase-encoding selection marker. *J Agric Food Chem.* 2011;59(10):5617–24.
25. Khalil AS, Collins JJ. Synthetic biology: applications come of age. *Nat Rev Genet.* 2010;11(5):367–79.
26. Xia PF, Ling H, Foo JL, Chang MW. Synthetic genetic circuits for programmable biological functionalities. *Biotechnol Adv.* 2019;37(6):107393.
27. Dubendorf JW, Studier FW. Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. *J Mol Biol.* 1991;219(1):45–59.
28. Baumschlager A, Khammash M. Synthetic biological approaches for optogenetics and tools for transcriptional light-control in bacteria. *Adv Biology.* 2021;5(5):2000256.
29. Brophy JA, Voigt CA. Principles of genetic circuit design. *Nat Methods.* 2014;11(5):508–20.
30. Lee JY, Cha S, Lee JH, Lim HG, Noh MH, Kang CW, Jung GY. Plug-in repressor library for precise regulation of metabolic flux in Escherichia coli. *Metab Eng.* 2021;67:365–72.
31. Chang HJ, Mayonove P, Zavala A, De Visch A, Minard P, Cohen-Gonsaud M, Bonnet J. A modular receptor platform to expand the sensing repertoire of bacteria. *ACS Synth Biol.* 2018;7(1):166–75.
32. Heiss S, Hörmann A, Tauer C, Sonnleitner M, Egger E, Grabherr R, Heintz S. Evaluation of novel inducible promoter/repressor systems for recombinant protein expression in Lactobacillus plantarum. *Microb Cell Fact.* 2016;15:1–7.
33. Blanch-Asensio M, Dey S, Sankaran S. In vitro assembly of plasmid DNA for direct cloning in Lactobacillus plantarum WCFS1. *PLoS ONE.* 2023;18(2):e0281625.
34. Kakikawa M, Watanabe N, Funawatashi T, Oki M, Yasukawa H, Taketo A, Kodaira KI. Promoter/repressor system of Lactobacillus plantarum phage ϕ g1e: characterization of the promoters pR49–pR–pL and overproduction of the cro-like protein cng in Escherichia coli. *Gene.* 1998;215(2):371–9.
35. Kakikawa M, Ohkubo S, Syama M, Taketo A, Kodaira KI. The genetic switch for the regulatory pathway of Lactobacillus plantarum phage ϕ g1e: characterization of the promoter PL, the repressor gene cpg, and the cpg-encoded protein cpg in Escherichia coli. *Gene.* 2000;242(1–2):155–66.
36. Coddeville M, Auvray F, Mikkonen M, Ritzenthaler P. Single independent operator sites are involved in the genetic switch of the Lactobacillus delbrueckii bacteriophage mv4. *Virology.* 2007;364(2):256–68.
37. García P, Ladero V, Alonso JC, Suárez JE. Cooperative interaction of CI protein regulates lysogeny of Lactobacillus casei by bacteriophage A2. *J Virol.* 1999;73(5):3920–9.
38. Ladero V, García P, Alonso JC, Suárez JE. A2 cro, the lysogenic cycle repressor, specifically binds to the genetic switch region of Lactobacillus casei bacteriophage A2. *Virology.* 1999;262(1):220–9.
39. Dey S, Blanch-Asensio M, Balaji Kutta S, Sankaran S. Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic lactobacilli. *Microb Biotechnol.* 2023 Feb 1.
40. Lemire S, Yehl KM, Lu TK. Phage-based applications in synthetic biology. *Annual Rev Virol.* 2018;5:453–76.

41. Wang W, Li Y, Wang Y, Shi C, Li C, Li Q, Linhardt RJ. Bacteriophage T7 transcription system: an enabling tool in synthetic biology. *Biotechnol Adv.* 2018;36(8):2129–37.
42. Fogg PC, Colloms S, Rosser S, Stark M, Smith MC. New applications for phage integrases. *J Mol Biol.* 2014;426(15):2703–16.
43. Marino ND, Pinilla-Redondo R, Csörgő B, Bondy-Denomy J. Anti-CRISPR protein applications: natural brakes for CRISPR-Cas technologies. *Nat Methods.* 2020;17(5):471–9.
44. Abdelrahman F, Easwaran M, Daramola OI, Ragab S, Lynch S, Oduselu TJ, Khan FM, Ayobami A, Adnan F, Torrents E, Sanmukh S. Phage-encoded endolysins. *Antibiotics.* 2021;10(2):124.
45. Hochschild A, Lewis M. The bacteriophage λ CI protein finds an asymmetric solution. *Curr Opin Struct Biol.* 2009;19(1):79–86.
46. Hampton HG, Watson BN, Fineran PC. The arms race between bacteria and their phage foes. *Nature.* 2020;577(7790):327–36.
47. Dimas RP, Jiang XL, Alberto de la Paz J, Morcos F, Chan CT. Engineering repressors with coevolutionary cues facilitates toggle switches with a master reset. *Nucleic Acids Res.* 2019;47(10):5449–63.
48. Li X, Zhang C, Xu X, Miao J, Yao J, Liu R, Zhao Y, Chen X, Yang Y. A single-component light sensor system allows highly tunable and direct activation of gene expression in bacterial cells. *Nucleic Acids Res.* 2020;48(6):e33.
49. Xiong LL, Garrett MA, Buss MT, Kornfield JA, Shapiro MG. Tunable temperature-sensitive transcriptional activation based on lambda repressor. *ACS Synth Biol.* 2022;11(7):2518–22.
50. Ohlendorf R, Vidavski RR, Eldar A, Moffat K, Möglich A. From dusk till dawn: one-plasmid systems for light-regulated gene expression. *J Mol Biol.* 2012;416(4):534–42.
51. Taton A, Ma AT, Ota M, Golden SS, Golden JW. NOT gate genetic circuits to control gene expression in cyanobacteria. *ACS Synth Biol.* 2017;6(12):2175–82.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Chapter 3.

High levels of protein secretion from lactobacilli-based ELMs

The previous publication reported an engineered *L. plantarum* strain that was highly fluorescent due to remarkable levels of mCherry intracellular protein production, which accumulates in the cytoplasm of the bacteria. However, in certain cases, the produced proteins are intended to be secreted into the external environment, and for that, SPs come into play.

In *L. plantarum*, the Sec pathway is the most well-characterized route for protein secretion. Proteins can be secreted through this pathway when carrying one SP in the N-terminal domain. A SP of great value should meet two criteria: i) drive significant levels of protein secretion, and ii) function effectively for potentially any protein, regardless of the origin, structure, and, ideally, size. Nevertheless, SPs that meet both criteria in *L. plantarum* remain unknown to date, supposing an important bottleneck for the use of *L. plantarum* in biotechnological and biomedical applications.

This chapter investigates the questions of whether the newly discovered promoter can be applied to efficiently enhance protein secretion, and also to assess the performance of this genetic part when bacteria are encapsulated within alginate beads. In the first part of the following work, protein secretion is addressed by testing five novel SPs in *L. plantarum*. For that, the commonly used secreted reporter protein NucA from *Staphylococcus aureus* is used [133]. As a result, an efficient SP is discovered, capable of driving more NucA levels than the most widely used SP in *L. plantarum*. Next, an attempt to further increase protein secretion by involving the newly discovered strong constitutive promoter is performed. Interestingly, the use of the P_{tec} gene expression led to

significantly higher secretion levels. These results highlighted another important application of this promoter: to allow enhanced protein secretion.

However, the secretion of reporter proteins is not meaningful from an application perspective. Therefore, in the second part of the work, the compatibility of this novel secretion platform to secrete valuable proteins other than a reporter protein was evaluated. For that, *L. plantarum* was engineered to secrete two therapeutically relevant proteins independently, elafin and α -melanocyte-stimulating hormone (α -MSH). Secretion of both proteins was detected, thus confirming the development of two potentially relevant engineered *L. plantarum* strains for biomedical applications.

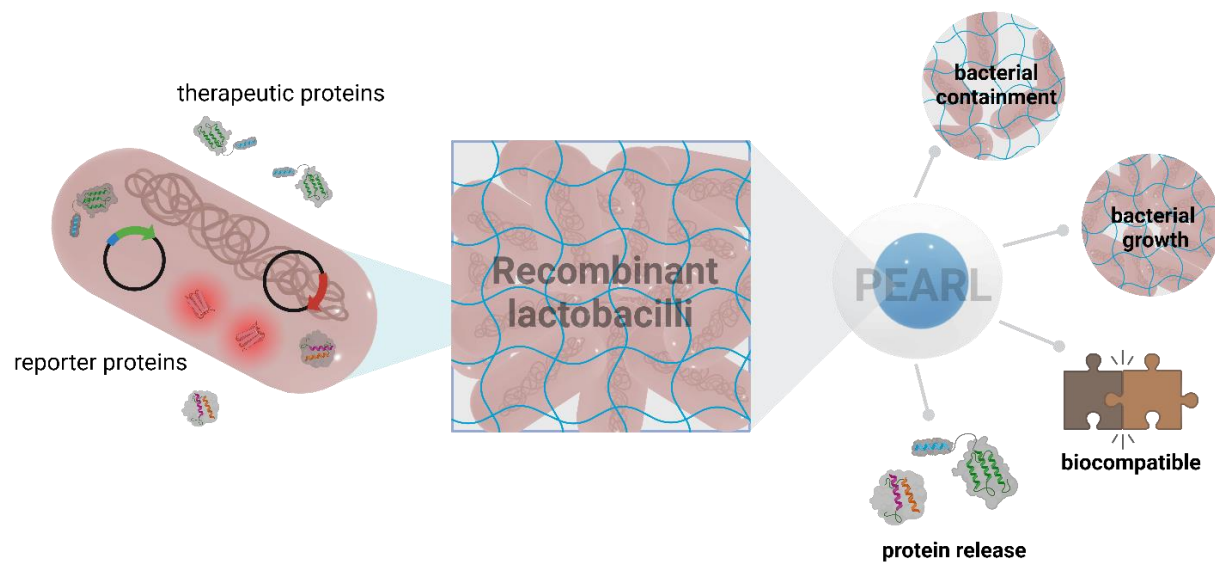
Despite the potential of these strains, their direct use in human-based applications is not realistic as they are non-contained genetically modified organisms (GMOs). To bring these strains closer to more clinically feasible scenarios, the encapsulation of *L. plantarum* within biocompatible materials as an ELM format was explored in the third part of the publication. Engineered *L. plantarum* bacteria were successfully encapsulated within manually fabricated alginate core-shell beads. Encapsulation kept *L. plantarum* contained while allowing bacterial growth and activity. For example, the production of the fluorescent protein mScarlet3 was observed by the eye in the core of the alginate bead after 24 hours of growth in growth media. Additionally, protein release from the beads was confirmed for all engineered *L. plantarum* strains and detected even after 14 days of incubation. This promising combination of alginate beads and engineered *L. plantarum* gave rise to the PEARL format, named after Protein Eluting Alginate with Recombinant Lactobacilli.

Finally, in the last part of the publication, the biocompatibility and toxicity of PEARLs were assessed in co-culturing experiments with human fibroblasts and zebrafish embryos, respectively. The results showed that encapsulation effectively reduced the toxicity induced by the metabolites produced by the bacteria, further highlighting the benefits and importance of using materials for bacterial containment.

Overall, the identification of a novel SP and the implementation of the P_{tec} promoter to drive gene expression resulted in increased protein secretion in *L. plantarum*. These parts

can potentially be used to attempt the secretion of any POI. Furthermore, high, sustained protein release was detected over time when engineered *L. plantarum* was successfully encapsulated in an ELM format for the first time. Based on these findings, PEARL marks the beginning of the next generation of lactobacilli-based ELMs.

Graphical Abstract



*PEARL is a biocompatible ELM format based on recombinant *L. plantarum* bacteria that allows bacterial growth in a structurally contained manner. Furthermore, PEARL enables protein release when *L. plantarum* bacteria are genetically programmed to continuously secrete reporter or therapeutic proteins.*

Publication and contributions

Article Type: Research article.

Title: PEARL: Protein Eluting Alginate with Recombinant Lactobacilli ⁴.

Year Published: 2025.

Research Journal: Small.

Authors: Marc Blanch-Asensio, Varun Sai Tadimarri, Ketaki Deshpande, Sara Trujillo, Carole Baumann, Jonas Baumann, Rolf Müller, and Shrikrishnan Sankaran.

First authors: Marc Blanch-Asensio and Varun Sai Tadimarri (joint co-authorship).

Author contributions: Marc Blanch-Asensio and Varun Sai Tadimarri performed all the genetic engineering work. Marc Blanch-Asensio and Varun Sai Tadimarri conducted all the experimental procedures involving bacteria and PEARLs. Varun Sai Tadimarri performed the screening of SPs and fabricated all the alginate beads. Marc Blanch-Asensio and Varun Sai Tadimarri performed the data analysis. Marc Blanch-Asensio made the illustrations and plotted the data. Marc Blanch-Asensio and Varun Sai Tadimarri wrote most of the draft of the manuscript. Ketaki Deshpande performed most of the mammalian cell experiments and wrote corresponding sections in the manuscript. Sara Trujillo performed a few cell experiments and reviewed and edited the corresponding sections in the manuscript. Carole and Jonas Baumann performed all the zebrafish experiments and wrote the corresponding sections in the manuscript. Rolf Müller reviewed the manuscript. Marc Blanch-Asensio and Varun Sai Tadimarri led the conceptualization of the project with support from Shrikrishnan Sankaran. Shrikrishnan Sankaran led the supervision, reviewed, and edited the manuscript.

⁴ The supporting information of this publication is provided in Appendix II.3 at the end of this dissertation.

PEARL: Protein Eluting Alginate with Recombinant Lactobacilli

Varun Sai Tadimarri, Marc Blanch-Asensio, Ketaki Deshpande, Jonas Baumann, Carole Baumann, Rolf Müller, Sara Trujillo, and Shrikrishnan Sankaran*

Engineered living materials (ELMs) made of bacteria in hydrogels have shown considerable promise for therapeutic applications through controlled and sustained release of complex biopharmaceuticals at low costs and with reduced wastage. While most therapeutic ELMs use *E. coli* due to its large genetic toolbox, most live biotherapeutic bacteria in development are lactic acid bacteria due to native health benefits they offer. Among these, lactobacilli form the largest family of probiotics with therapeutic potential in almost all sites of the body with a microbiome. A major factor limiting the use of lactobacilli in ELMs is their limited genetic toolbox. This study expands on recent work to expand the genetic programmability of probiotic *Lactiplantibacillus plantarum* WCFS1 for protein secretion and encapsulate it in a simple, cost-effective, and biocompatible core-shell alginate bead to develop an ELM. The controlled release of recombinant proteins is demonstrated, even up to 14 days from this ELM, thereby terming it PEARL – Protein Eluting Alginate with Recombinant Lactobacilli. Notably, lactobacillus encapsulation offered benefits like bacterial containment, protein release profile stabilization, and metabolite-induced cytotoxicity prevention. These findings demonstrate the mutual benefits of combining recombinant lactobacilli with alginate for the controlled and sustained release of proteins.

1. Introduction

Engineered living materials (ELMs) are composites made of living entities integrated with materials.^[1,2] The living entities in ELMs enable them to be designed with life-like abilities of adaptation, regeneration, biosynthesis, growth, etc. Additionally, the possibility to genetically reprogram these abilities to optimally suit a desired application has propelled the rapid development of ELMs in various domains ranging from architecture to medicine. ELMs have advanced in the medical field, especially due to the benefits they offer to deliver therapeutics.^[3–5] Since the human body naturally supports microbial survival, ELM activities can potentially be sustained in the long term. The microbes can be engineered to produce high-value biopharmaceutical drugs (proteins, peptides, biomacromolecules) for delivery at the disease site. In most studies conducted to date, *E. coli* has been the predominant organism encapsulated within ELMs. For example, an ELM, termed PATCH, encapsulated lysostaphin-secreting *E. coli* to

inhibit the growth of *Staphylococcus aureus*, including a methicillin-resistant *S. aureus* (MRSA) strain.^[6] In another study, an *E. coli* strain engineered to light-responsively produce an anti-bacterial compound, deoxyviolacein. After encapsulation, the strain demonstrated drug release over 40 days.^[7] This approach avoids the need to externally produce, purify, encapsulate, and store these often fragile biopharmaceuticals, thereby reducing the costs and waste associated with these processes.

To achieve these benefits, therapeutic ELMs primarily use bacteria as their living entity, since they grow fast, have simple nutrient requirements, can be encapsulated at high densities, and are often naturally beneficial to the host in the form of probiotics. In fact, bacteria have been developed for over two decades as engineered live biotherapeutics that can be administered directly within the body to produce and deliver drugs on-site.^[8] While clinical trials have shown that the engineered bacteria are safe for use in humans, proving their efficacy has been a major hurdle blocking their approval as therapeutic products.^[9] A fundamental challenge in achieving therapeutic efficacy is ensuring that the right density of the engineered bacteria is viable and active when they reach the target site. Furthermore, this must

V. S. Tadimarri, M. Blanch-Asensio, K. Deshpande, S. Trujillo, S. Sankaran
INM – Leibniz Institute for New Materials
Saarland University
Campus D2 2, 66123 Saarbrücken, Germany
E-mail: Shrikrishnan.sankaran@leibniz-inm.de

V. S. Tadimarri, M. Blanch-Asensio, K. Deshpande, R. Müller
Saarland University
66123 Saarbrücken, Germany
J. Baumann, C. Baumann, R. Müller
Helmholtz Institute for Pharmaceutical Research Saarland (HIPS)
Helmholtz Center for Infection Research (HZI)
Saarland University
Campus E8 1, 66123 Saarbrücken, Germany
R. Müller
German Centre for Infection Research (DZIF)
Partner Site Hannover, Braunschweig, Germany

 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/smll.202408316>

© 2025 The Author(s). Small published by Wiley-VCH GmbH. This is an open access article under the terms of the [Creative Commons Attribution License](#), which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

DOI: 10.1002/smll.202408316

be balanced with the need for biocontainment of the bacteria, preventing them from invading unwanted sites in the body or environmental niches where they can potentially disturb the local ecology. ELMs satisfy these needs since they can provide a conducive and protected environment for the bacteria to grow to large population sizes while physically confining them within the material.^[10–13] Most therapeutic ELMs are based on probiotic *E. coli* and only a few studies cover other probiotic or GRAS (generally recognized as safe) strains like *Lactococcus lactis*,^[14] *Bacillus subtilis*^[15] and *Corynebacterium glutamicum*.^[16] Interestingly, most engineered live biotherapeutic candidates currently in clinical trials are lactic acid bacteria. This is because lactic acid bacteria are the largest group of commensals and probiotics, beneficial not only for the gut but also for other organs hosting a microbiome. In particular, the *Lactobacillus* family has the largest number of probiotic strains, many of which are being explored for therapeutic applications.^[17] Among these, *Lactiplantibacillus plantarum* has been well investigated and is the most studied strain to date.^[18] Several *L. plantarum* strains have GRAS status and none are reported as pathogenic to date, whereas only *E. coli* Nissle 1917 is probiotic with GRAS status and almost all other strains are pathogenic or endotoxic. Because of this, beneficial effects of *L. plantarum* strains have been demonstrated in multiple sites of the body beyond the gut, like the oral cavity, female reproductive organs, bladder, skin, etc., whereas the applicability of *E. coli* is largely limited to the gastrointestinal tract. Even there, *L. plantarum*'s thick cell wall and metabolic robustness enable it to survive harsh conditions like low pH, lytic enzymes, and bile salts, better than *E. coli*. Overall *L. plantarum* for ELMs offers significant advantages in terms of safety, health benefits, biocompatibility, and robustness. However, ELMs containing lactobacilli have not yet been reported despite these benefits, possibly due to their limited genetic programmability.

Recently, a few research groups, including ours, have made major strides in expanding the genetic programmability of *L. plantarum* WCFS1.^[18] We developed a direct cloning methodology to speed up the plasmid-based engineering of this strain by circumventing the need for an intermediate host.^[19] Using this strategy, we have identified the strongest constitutive promoter and repressor reported to date and characterized several toxin-antitoxin systems for antibiotic-free plasmid retention.^[20,21] Other groups have reported using sakacin-inducible, xylose-inducible, and lactose-inducible systems to express heterologous proteins in *L. plantarum* WCFS1.^[22] To facilitate the secretion of these proteins, various endogenous and exogenous signal peptides have been explored.^[23] In addition to secretion, the anchoring of these secreted proteins has garnered significant interest in recent years, particularly in the development of bacterial vaccines. In this context, antigens have been secreted and anchored to various components of the bacterial cell surface using endogenous LysM and LPXTG moieties, enhancing their potential as vaccine candidates.^[24] Nevertheless, the performance and containment of these engineered bacteria within biocompatible materials under physiologically relevant conditions is yet to be shown. Encapsulation of *L. plantarum* in hydrogels and food-based materials has been done for their delivery as probiotics.^[6,25] These materials were not designed to contain the bacteria but rather to deliver them in the gut.

In this study, we have investigated how *L. plantarum* WCFS1 can be securely contained within a hydrogel. To do this, core-shell alginate beads were fabricated using a simple manual strategy that can be easily adapted by other labs even without experience in hydrogel fabrication. Interestingly, we observed that alginate in this format is particularly well suited to contain *L. plantarum* while maintaining its viability for at least 14 days, while *E. coli* was found to leak out within a day. We further expanded the genetic programmability of this bacterium by optimizing the genetic parts needed to drive high-level secretion of a nuclease protein. This optimization involved identifying and testing multiple signal peptides to determine which ones most compatibly function with our recently discovered strong constitutive promoters. Encapsulation of the engineered lactobacilli in alginate beads helped to stabilize the release profiles of the secreted protein and sustained this activity over at least 14 days. Due to this prolonged protein elution capability, we hereafter term this ELM as PEARL – Protein Eluting Alginate with Recombinant Lactobacilli. With these PEARLS, we also demonstrate the secretion of two therapeutically relevant proteins, α -Melanocyte-stimulating hormone (α -MSH) and elafin, a neutrophil elastase inhibitor. Finally, encapsulation additionally aids in preventing cytotoxicity that can potentially be caused by metabolites of *L. plantarum* as confirmed by biocompatibility tests performed using mammalian cells and highly sensitive Zebrafish embryos. These results set the stage for the development of PEARLS toward therapeutic applications.

2. Results and Discussion

2.1. PEARL Fabrication and Characterization of Encapsulated *L. plantarum*

To study the behavior, secretory performance, and biocompatibility of encapsulated probiotic lactobacilli, we developed the PEARL format, which is an easy-to-fabricate alginate core-shell macrobead ELM as shown in Figure 1a. *L. plantarum* WCFS1 was encapsulated in an alginate bead by manually dropping a viscous alginate-bacteria mix into a calcium chloride bath to form the core gel as previously described.^[13] A polysaccharide-staining dye, Alcian Blue, was added to this mix to improve the visibility of the core gel for the next step. These beads were then submerged in a viscous alginate solution. The core bead surrounded by the alginate solution was then dropped into the calcium chloride bath to form a thick shell layer (Figure 1a).

To study the growth and containment of probiotic lactobacilli within PEARLS, *L. plantarum* WCFS1 was engineered to constitutively express mScarlet3, a highly bright and stable fluorescent protein.^[26] For that, we exchanged mCherry for the mScarlet3 gene (Figure S3a, Supporting Information), and we could visually confirm an increase in the pellet brightness intensity by eye (Figure S3b, Supporting Information) and flow cytometry (Figure S3c, Supporting Information).

After the fabrication of PEARLS with roughly 10^6 cells (equivalent to OD₆₀₀ of 0.01) per bead, they were incubated in MRS, the optimal media, or LB media at 37 °C. We assess the performance of the PEARLS in LB since it is a non-optimal and minimal nutrient media for these bacteria. Ideally, this ELM format is meant to be performed in environments where nutrients might

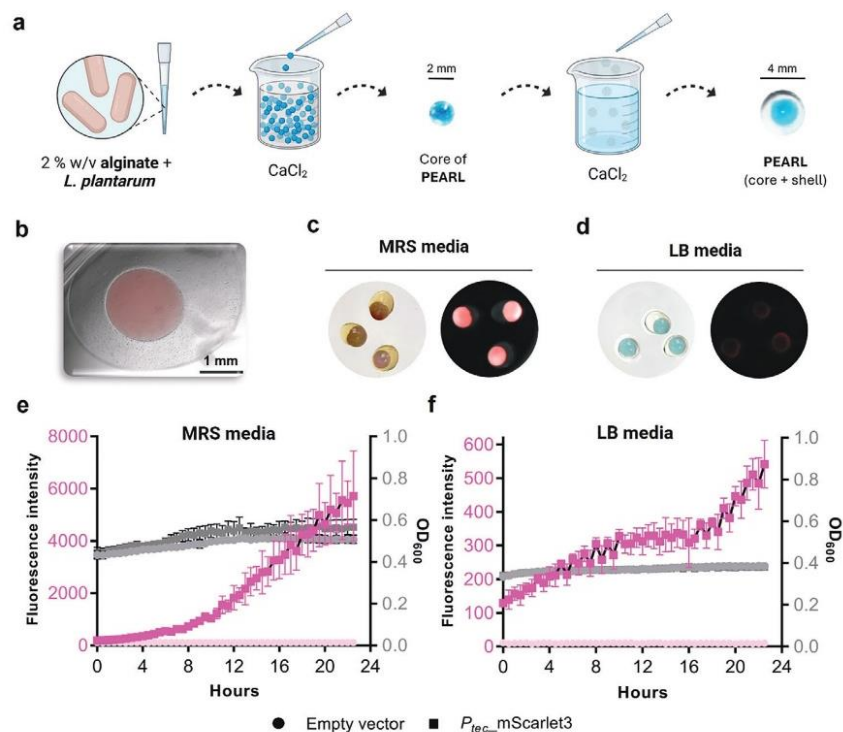


Figure 1. Lactobacilli-based alginate macro beads design and fabrication: a) Schematic representation of manual fabrication of alginate-based PEARL. It depicts the step-by-step making of PEARL. b) 4x overlay microscopic image of the PEARL with a core comprising mScarlet3 expressing *L. plantarum* and a shell surrounding the core. c) Macroscopic images of PEARLs after 24 h of incubation in MRS media; To the left is the brightfield image depicting the bacterial core and shell, and to the right is the CH3 fluorescent image showing the strong fluorescence of mScarlet3 in the core. The circular field of view is ≈ 15 mm. d) Macroscopic images of PEARLs after 24 h of incubation in LB media; To the left is the brightfield image depicting the bacterial core and shell, and to the right is the CH3 fluorescent image showing the fluorescence of mScarlet3 in the core. The circular field of view is ≈ 15 mm. e) 24-h kinetic measurements of fluorescence intensity and OD_{600} values of encapsulated *L. plantarum* expressing mScarlet3 in PEARLs grown in MRS media ($n = 4$, mean $\pm$ SD). f) 24-h kinetic measurements of fluorescence intensity and OD_{600} values of encapsulated *L. plantarum* expressing mScarlet3 in PEARLs grown in LB media ($n = 4$, mean $\pm$ SD).

be scarce. Therefore, we wanted to confirm the activity of bacteria within PEARLs incubated in LB media. By fluorescence microscopy, we observed that mScarlet3 fluorescence was only detected in the core of the PEARL (Figure 1b) indicating the secure containment of bacteria within the core. Furthermore, we could also see differences when PEARLs were incubated in either MRS or LB media. Only in MRS the core was visually colorful due to prominent levels of mScarlet3 production (Figure 1c). However, when the PEARLs were incubated in LB media, mScarlet3 fluorescence could be confirmed by the BioRad Gel Documentation System (Figure 1d).

To quantify bacterial growth and mScarlet3 expression in PEARLs incubated in both MRS and LB media, optical density at 600 nm and fluorescence intensity were measured. A slight increase in optical density at 600 nm (OD_{600}) and a significant increase in fluorescence intensity (Ex/Em – 569/600 nm) were measured over 24 h in a microplate reader (Figure 1e,f), indicating that bacteria are active but stay contained within the core as outgrowth into the media was not observed.

In contrast, this ELM format was unable to contain *E. coli* Nissle 1917, neither in LB agar plates (Figure S4a, Supporting Information) nor in culture (Figure S4b, Supporting Information) beyond 8 h, as observed by bacterial growth around the beads and the rapid increase in OD_{600} values. This indicates that alginate is particularly suited to contain *L. plantarum* when fabricated in a core-shell format.

Apart from ensuring containment, supporting biomass growth, and enabling protein expression, the PEARLs also help prevent the pH of the media from dropping below 6, which occurs when the bacteria are cultured without encapsulation (Figure S4c, Supporting Information). While the specific causes for this surprising outcome are unknown, possible reasons could include restricted diffusion of acidic byproducts through the calcium alginate matrix,^[27] or potential alterations in bacterial metabolism due to the encapsulated environment.^[10]

These promising results showed that *L. plantarum* could grow and produce a heterologous protein in a contained manner within the PEARLs. For application as a therapeutic ELM, it is desirable that the bacteria secrete proteins, and these proteins

diffuse out of the material. In the next sections, we engineer robust protein secretion from *L. plantarum* using strong promoters recently discovered by our group and demonstrate their functionality in PEARLs.^[20]

2.2. Enhancing Protein Secretion in *L. plantarum*

2.2.1. Identification and Selection of Endogenous Signal Peptides (SPs)

Protein secretion in bacteria is enabled by SPs at the N-terminal of peptides that are recognized by the cellular secretion machinery, which transports the proteins across the cell membrane. While several SPs have been identified in *L. plantarum*, it was necessary to identify optimal candidates compatible with our previously discovered promoters and drive higher secretion of protein of interest compared to the reported signal peptides. For this, we screened the protein sequences encoded by more than 200 endogenous genes via InterProScan until we identified 40 SPs. As a reporter protein, we chose a nuclease (NucA) from *Staphylococcus aureus*, which can be easily detected in the media using a simple DNA degradation assay and has been extensively used to characterize secretion in gram-positive bacteria.^[23,28,29] SignalP6.0 was used to predict NucA secretion mediated by all SPs.^[30] The predictions of 5 SPs with varying secretion levels, henceforth termed SP1-5 (SP1: lp_2175, SP2: lp_2959, SP3: lp_1132, SP4: lp_0819, SP5: lp_3151) were experimentally verified. We additionally included one of the most efficient and used SPs from the literature, Lp_3050 in the testing for comparison.^[24] NucA secretion was assessed using DNase agar and media assays (Figure 2a).

2.2.2. Characterization of Novel Endogenous SPs Using NucA Assay

The promoters for *L. plantarum* recently discovered in our lab are P_{ilpA} and P_{tec} (5x and 40x stronger than previously reported).^[21,20] To avoid the risk of saturating the secretion machinery in *L. plantarum*, we initially tested the ability of the weaker promoter out of the two, P_{ilpA} , to drive the production and secretion of NucA fused to each of the SP (SP1-5 and Lp_3050) (Figure 2b). Secretion was semi-quantitatively characterized using a DNase agar plate assay in which active extracellular NucA creates a dark halo around secreting colonies by degrading fluorescently labeled DNA in the agar. Only three SPs: Lp_3050, SP3, and SP5 were found to secrete detectable amounts of active NucA after 24 h of growth on DNase agar plates. Two parameters were measured to compare the performance of each SP in the DNase agar plates – the area of the halo and the decrease in fluorescence intensity within this halo. Based on this analysis, SP3 was found to generate the biggest halo ($\approx 4 \text{ cm}^2$) and the largest drop in fluorescence (Figure S5a, Supporting Information), followed by Lp_3050. Next, we tested the possibility of increasing SP3 and Lp_3050-driven secretion using the P_{tec} promoter (Figure 2c). We observed that both SP3 and Lp_3050_NucA-driven secretion could be increased when P_{tec} was the promoter driving the gene expression since bigger and darker halos (Figure S5b, Supporting Information) were detected for P_{tec} compared to P_{ilpA} . These results proved that NucA secretion can be increased by replacing the Lp_3050 signal peptide for SP3 under P_{ilpA} or swapping P_{ilpA} by P_{tec} for both

Lp_3050 and SP3. Yet, the best promoter-SP combination was found to be P_{tec} -Lp_3050.

To quantify the amount of NucA secreted by these promoter-SP combinations, we used liquid DNase media instead of the agar, in which the bacteria were allowed to grow for 24 h at 37 °C with shaking (Figure 3d). This enabled us to accurately measure the drop in fluorescence intensity in the media and interpolate the values in a NucA standard curve (Figure S6a, Supporting Information). Once again, NucA secretion was observed only with SP3, SP5, and Lp_3050, validating the results obtained with the agar plates (Figure 2b). SP3 mediated better secretion than Lp_3050 when driven by P_{ilpA} (≈ 20 and $\approx 15 \text{ nM}$, respectively). However, both SP3 and Lp_3050-driven secretion levels could significantly increase when P_{ilpA} was swapped by P_{tec} ($\approx 24 \text{ nM}$ and $\approx 55 \text{ nM}$, respectively) (Figure 3e). Interestingly, with P_{tec} , Lp_3050 performed better than SP3, indicating that SPs may perform differently when the expression is driven by different promoters.

Based on the growth curve of *L. plantarum* WCFS1 in LB media (Figure 1g), it is evident that the bacteria grow exponentially for about 6 h beyond which they reach their saturation phase due to limitation of nutrients. This change in growth phase can influence protein production and secretion, so we quantified NucA secretion at mid (3 h) and late log phase (6 h) in addition to the late saturation phase (24 h). We observed that for all the promoter/SP combinations, most of the protein (>60% approximately) is secreted within first 6 h, suggesting that the promoters are most active in the log phase of growth (Figure 2a). Furthermore, most of the secretion from the P_{ilpA} constructs occurs over the first 6 h, whereas from the P_{tec} constructs, most secretion occurs in the first 3 h (Figure S6b, Supporting Information). We examined both the promoter's activity using another reporter, mCherry, in the different growth phases, and we observed that P_{tec} is much more active in the early and mid-log phase, which supports our results obtained with the NucA where most of the protein is produced (Figure S6c, Supporting Information). Such high levels of production and secretion in the hour's timescale are well-suited for short-term protein delivery within the body.

2.3. Assessing Protein Release from PEARLs

NucA release from PEARLs was assessed by two different methods: 1) by placing the PEARLs in the center of a DNase agar plate and imaging the agar plate at regular time intervals, and 2) by placing the PEARLs in a well containing DNase media followed by fluorescence measurements (Figure 3a). In DNase agar plates, we observed halo formation at 24 h after placing the bead in the center of the plate for all the NucA-secreting clones based on the P_{ilpA}/P_{tec} promoters and SP3/Lp_3050 SPs (Figure 3b). The halos kept increasing over the next 14 days, suggesting continuous NucA secretion from the bead. We observed the same secretion trend for the different promoter/SP combinations as observed in culture (Figure 3b), with the P_{tec} -3050 strain producing the biggest and darkest halos. We also noticed that the bacteria remained contained in the bead and secretion was sustained for at least 14 days (Figure 3c). However, it is to be noted that since the PEARLs in this study are manually fabricated, if the core is misaligned in the shell leading to a thin shell on one edge, outgrowth

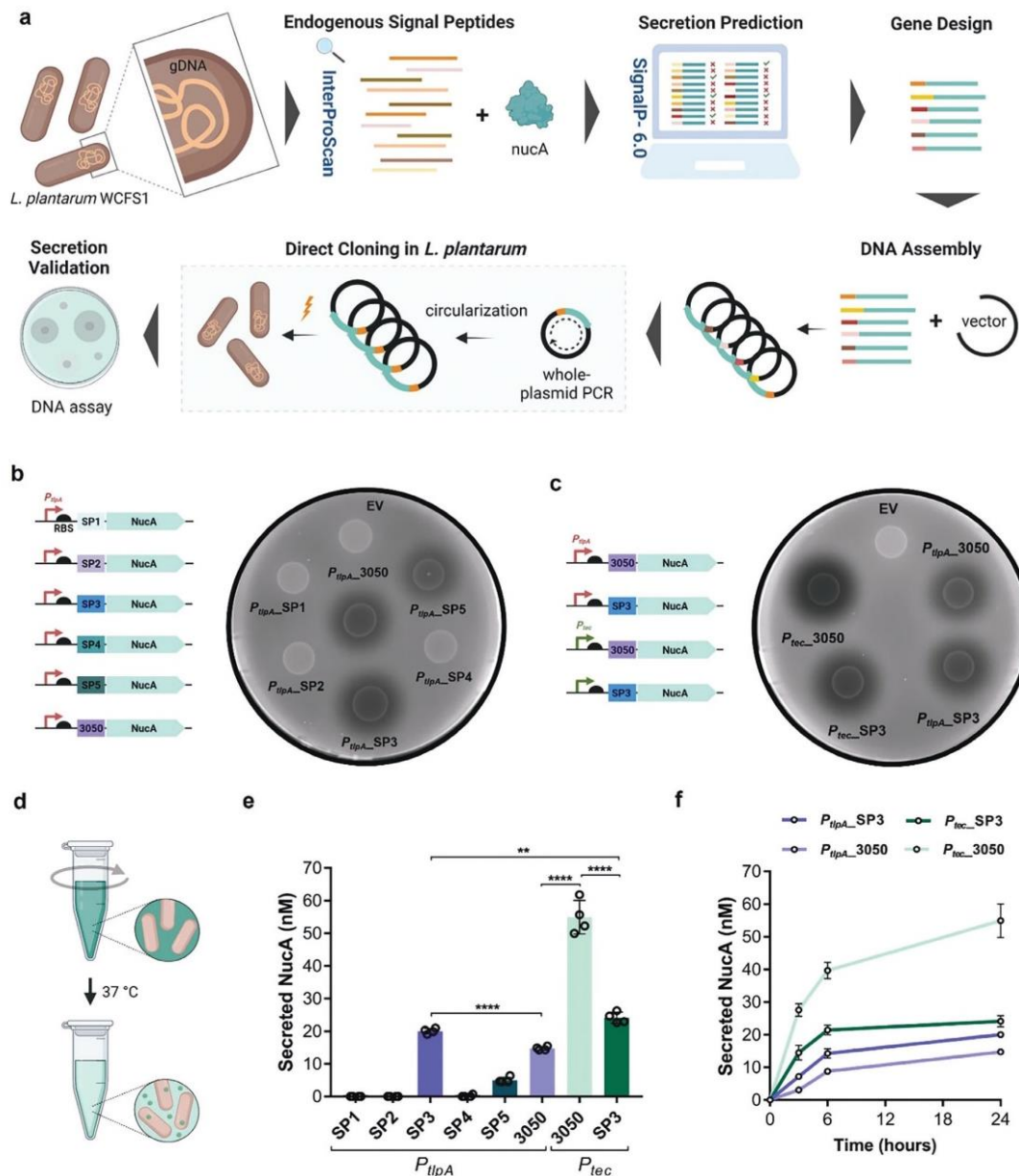


Figure 2. Workflow depicting quantification of recombinant protein secretion in DNase agar and DNase media: a) Flow scheme representing the screening of endogenous signal peptides followed by gene design and molecular cloning in *L. plantarum* WCFS1 to validate the secretion of staphylococcal NucA (17 kDa), a reporter protein commonly used for secretion studies. b) Schematic of genetic constructs of P_{tlpA} promoter driving the expression of NucA, each tagged with one of six different signal peptides and EV bacteria (as negative control); to the right is the image of DNase agar plate illustrating the secretion of NucA from 10 μ L of bacterial spots. c) Schematic of genetic constructs of P_{tlpA} and P_{tec} promoters driving the expression of NucA, each tagged with two best SPs (P_{3050} and SP3) from this study; to the right is the image of DNase agar plate illustrating the secretion of NucA from 10 μ L bacterial spots of these genetic constructs. d) Graphical representation of liquid culture-based assay to quantify the NucA secretion from engineered bacterial strains: NucA secreted from bacteria depolymerizes the DNA, thereby breaking down the methyl green-DNA complex in media resulting in loss of green coloration. e) Quantification of secreted NucA in DNase media after 24 h of incubation for all the secretion-based genetic constructs screened in this study ($n = 3$, mean $\pm$ SD). f) Timepoint-based quantification ($n = 3$, mean $\pm$ SD) of secreted NucA (in nanomolar, nM) in DNase media for two best constructs of each promoter (P_{tec_3050} , P_{tec_SP3} , P_{tlpA_3050} , P_{tlpA_SP3}).

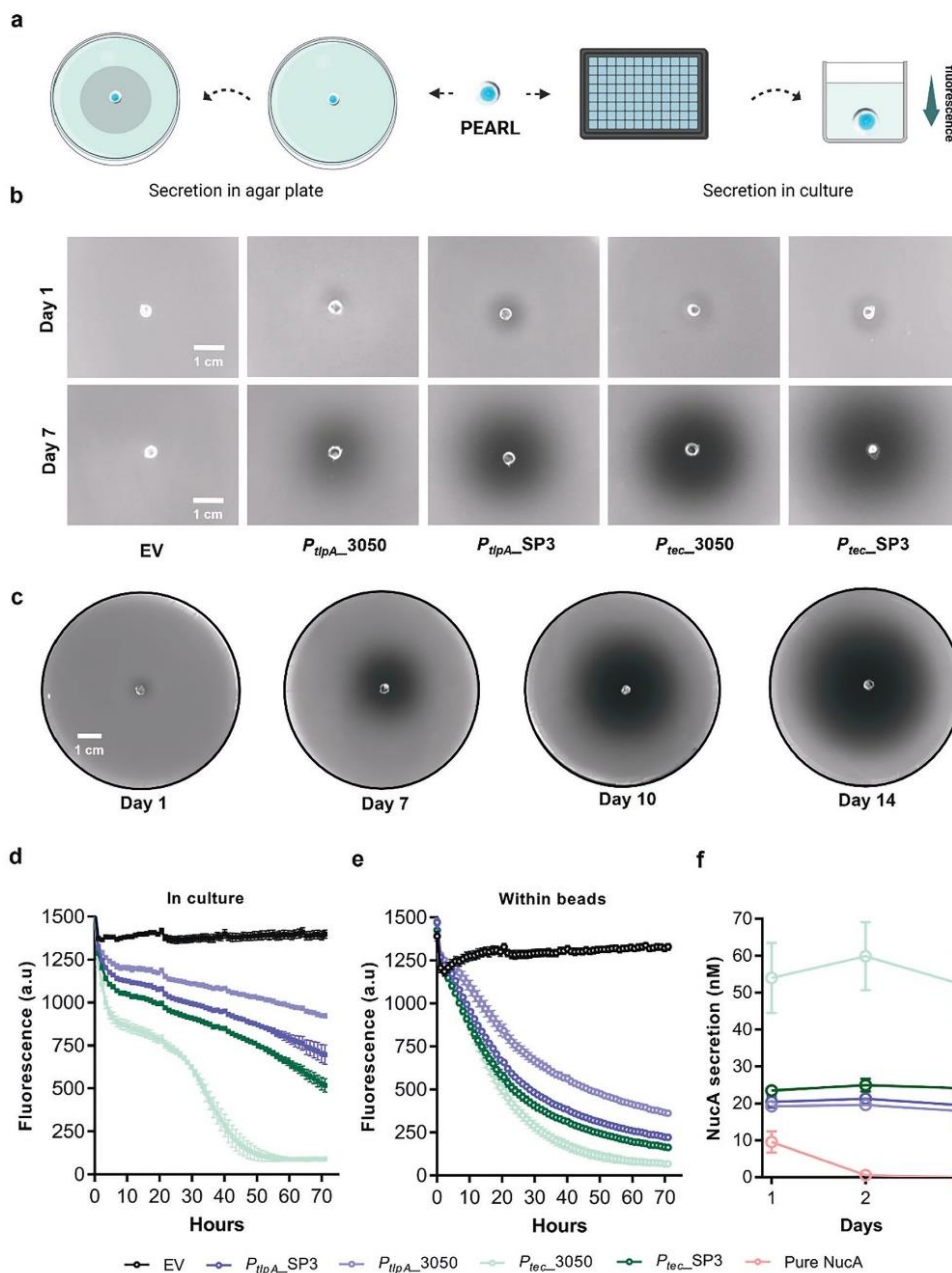


Figure 3. Characterization of PEARL's release profile in DNase agar and DNase media: a) Graphical representation of assays done to validate and quantify the secreted NucA from alginate beads using DNase agar plate and by fluorescence kinetics in culture. b) DNase agar plate-based qualitative assay to track the NucA secretion and bacterial containment within PEARLs on Day 1 and Day 7. c) NucA secretion from PEARL embedded in DNase agar plate captured on Day 1, 7, 10, 14 indicating the bacterial viability up to 14 days d) Quantification of NucA secretion with reference to the drop in fluorescence in wells with culture for 72 h (3 days) ($n = 5$, mean $\pm$ SD). e) Quantification of NucA secretion with reference to the drop in fluorescence in wells with alginate beads for 72 h (3 days) ($n = 5$, mean $\pm$ SD). f) Plot showing the comparison of secreted NucA in nanomolar ($n = 5$, mean $\pm$ SD) on Day 1, 2 & 3 from PEARLs for P_{tec_3050} , P_{tec_SP3} , P_{tlpA_3050} and P_{tlpA_SP3} .

of the bacteria, indicated by colonies outside the bead, is observed within 1–2 days. This occurred in $\approx 10\%$ of the PEARLs that were fabricated, and these constructs were not included in any of the analyses.

To quantify NucA secretion from the PEARLs, DNA degradation in liquid DNase media was measured over 3 days under static incubation conditions. Non-encapsulated bacterial cultures with the same number of cells as in the alginate core of the PEARLs (10^6) were included in the experiment for comparison. Interestingly, NucA secretion from non-encapsulated bacteria was distinctly non-monotonic, with a rapid increase within the first 8 h and then a much slower increase over the next 72 h (Figure 3d). In the case of P_{tec} _3050, another increase in secretion rate was observed around 24 h, and the final plateauing around 45 h is attributed to the complete degradation of the probe DNA in the DNase media. It is suspected that these different secretion profiles are related to the different growth phases that these bacteria go through during this period associated with metabolic changes and possible biofilm formation (Figure S6c, Supporting Information).^[31] In contrast, NucA release profiles from the PEARLs were found to be much more stable with a largely monotonic rate observed till at least 24 h followed by a gradual plateauing most likely caused by the decrease in DNA substrate availability (Figures 3e and 7a–d.). Even the differences in protein secretion rates observed between the different promoter-signal peptide combinations seem to have considerably reduced during the first 24 h.

Furthermore, we wanted to study whether bacteria actively sustained NucA secretion for days when encapsulated in PEARLs or if most of the secretion takes place within the first day after encapsulation and the secreted NucA remains active for several days. For that, we incubated the PEARLs in DNase media, collected the media after 24 h, measured the levels of NucA, performed several washes with PBS, added fresh DNase media, and recorded the data up to 3 days. We observed that the PEARLs secrete very similar levels of NucA day after day (Figure 3f). On the other hand, pure NucA encapsulated in core-shell alginate beads did not exert NucA activity outside the bead beyond the first day. This confirmed that pure NucA diffused quickly from the beads and is then washed away, whereas PEARLs keep secreting NucA (Figure 3f).

Considering our previous results indicating that the P_{tec} and P_{tlpA} are most active in the early- to mid-log phases (Figure 2f), the results with the PEARLs suggest that the bacteria remain in a state equivalent to these growth phases within the alginate. This is possible since bacterial growth is restricted by mechanical forces imposed by the hydrogel matrix rather than nutrient unavailability.^[10,11] Thus, while the population size is not necessarily increasing, the bacteria probably have access to nutrient levels similar to what they would have in liquid culture at the early- or mid-log phase. Thus, in the PEARL format, the most productive phase of the engineered bacteria is prolonged.

2.4. Assessing the Compatibility of PEARLs for Therapeutically Relevant Proteins

To test the compatibility of the PEARL platform to secrete therapeutically relevant bioactive proteins, we engineered *L. plantarum*

to secrete elafin (also known as Trappin-2) and α -melanocyte stimulating hormone (α -MSH) as two independent secretory strains. Elafin, an 11 kDa protein, inhibits serine proteases such as neutrophil elastase and proteinase-3. By inhibiting these proteases, elafin protects tissues from protease-mediated damage in inflammatory conditions and plays a protective role in different parts of the human body, including the skin, lungs, and gastrointestinal tract. Previous studies have demonstrated the secretion of elafin from engineered *Lactococcus lactis* and *Lactobacillus casei* to explore its potential in treating inflammatory bowel disease.^[32]

To identify the genetic construct capable of secreting a complex protein like elafin (with 4 disulfide bonds) in *L. plantarum* WCFS1, we tested the same circuit combinations as previously tested for NucA. Using the P_{tec} promoter, we could not obtain a mutation-free construct of elafin, likely due to the high strength of the promoter causing metabolic stress in bacteria to produce such complex protein. However, with the P_{tlpA} promoter, the cloning was possible without mutations and we detected the secretion of elafin in combination with the SP3 but not with Lp_3050 (Western blot, Figure S9b, Supporting Information). Therefore, we used the *L. plantarum* strain harboring P_{tlpA} _SP3_elafin construct for further experiments to demonstrate the functionality of the PEARL system.

Western blot analysis of denatured culture supernatants demonstrated successful secretion of elafin in DMEM media, which is more relevant for mammalian cells and tissues (Figure S9b, Supporting Information). To quantify the amount of secreted protein in DMEM, we optimized a split NanoLuc Luciferase assay based on the interaction of two complementary subunits, LgBiT (large bit) and SmBiT (small bit).^[33] The 11-amino-acid SmBiT tag was fused at the C-terminal of elafin, which can bind to the LgBiT fragment added in the culture supernatant to generate a luminescent signal in the presence of furimazine (Figure 4b). Based on the luminescence values obtained in this analysis (Figure 4c), we correlated the values to a standard curve (Figure S8a, Supporting Information) and quantified that ≈ 30 nM of the elafin_SmBiT fusion protein was detected from culture and PEARL supernatants (Figure S8b, Supporting Information, Figure 4e). To assess the bioactivity of the secreted elafin in both DMEM culture and PEARLs incubated in DMEM, we performed an in-house optimized neutrophil elastase inhibitory assay (Figure 4f, described in materials & methods). The results confirmed that elafin secreted from PEARLs was functional and effectively inhibited neutrophil elastase (NE) (Figure 4g).

To demonstrate the functionality of the P_{tec} promoter in secreting a therapeutic protein, we tested two combinations of α -MSH (tagged with Lp_3050 and SP3). α -MSH is an endogenous peptide hormone expressed by keratinocytes in the human body, that stimulates melanogenesis in melanocytes providing photoprotective effects against ultraviolet (UV) radiation among other health benefits.^[34] We could not obtain a mutation-free construct for P_{tec} _3050_ α -MSH_SmBiT but could do so for P_{tec} _SP3_ α -MSH_SmBiT. Based on quantification from the split Nano Luciferase assay, 10 nM of α -MSH was secreted by P_{tec} _SP3_ α -MSH_SmBiT construct in DMEM media after 24 h, and 2.5 nM of α -MSH was detected in the PEARL supernatant.

Depending on the genetic compatibility resulting from the promoter's strength, the signal peptide's efficiency, and the protein's nature, some genetic combinations work efficiently,

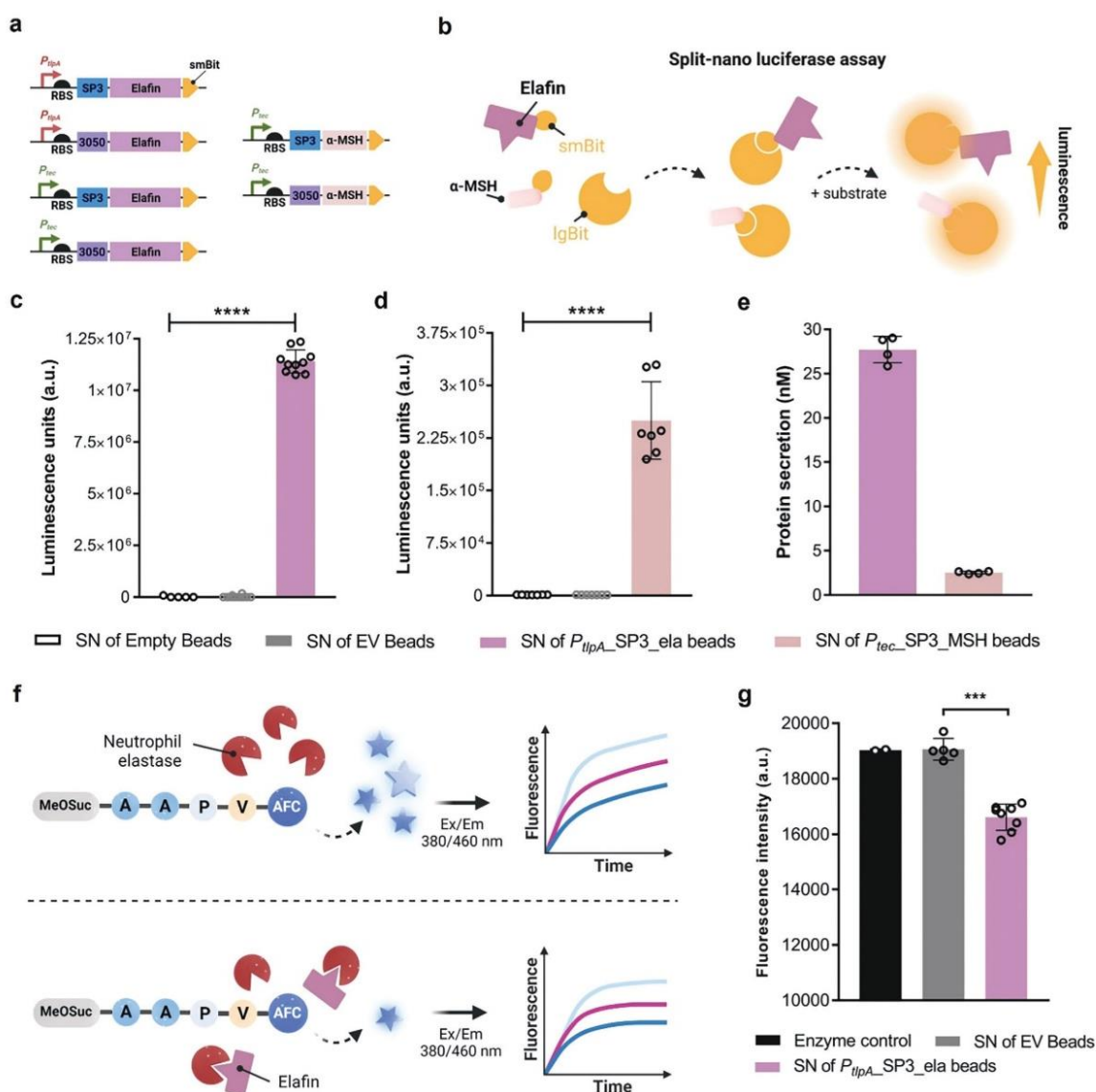


Figure 4. Genetic constructs were designed, and assays were performed in DMEM media to assess the secretion of therapeutically relevant recombinant proteins. a) Schematic of genetic constructs of *P_{tipA}* and *P_{tec}* promoters driving the expression of elafin and α-MSH, each tagged with either 3050 or SP3 signal peptide and SmBiT fragment. b) Graphical illustration of Nano Luciferase assay wherein when LgBiT (large bit) comes in close proximity to elafin-SmBiT or α-MSH-SmBiT, reassembles into a functional luciferase enzyme that produces a luminescent signal upon the addition of a substrate. c) A plot representing luminescence values was obtained from a Nano Luciferase assay performed with supernatants of elafin-secreting PEARLs incubated in DMEM media for 24 h. (*n* = 10, mean ± SD) d) Plot representing luminescence values obtained from Nano Luciferase assay performed with supernatants of α-MSH-secreting PEARLs incubated in DMEM media for 24 h. (*n* = 7, mean ± SD) e) Quantification of elafin and α-MSH secreted into PEARL supernatants after 24 h. f) Graphical representation of Neutrophil elastase inhibition assay optimized to detect the bioactivity of elafin; Presence of elafin results in decreased fluorescence. g) Plot comparing the fluorescence values at 10 min-time point showing the decreased fluorescence in elafin-PEARL supernatant indicating the NE inhibition by elafin. (*n* = 5, mean ± SD).

leading to the secretion of significant amounts of recombinant proteins, while others do not. This highlights the importance of having and testing multiple options of promoters and signal peptides for the production and secretion of different proteins. In all cases where successful protein secretion could be encoded in *L. plantarum*, the PEARL format supports this functionality and release of these differently sized proteins into the surrounding media (α -MSH_SmBiT: 5 kDa, elafin_SmBiT: 11 kDa, NucA: 17 kDa).

2.5. Polycistronic Gene Expression from PEARLs

Since our strongest promoter, P_{lec} was capable of driving high levels of NucA secretion and fluorescent protein expression, we tested its ability to do both simultaneously. Being able to encode strong production of two proteins at once would improve the versatility of programming *L. plantarum*. This would be better than encoding separate copies of the promoter for each gene since i) it reduces the overall size of the genetic module and the resulting plasmid, which is an important parameter affecting transformation efficiency in *L. plantarum*, plasmid retention, and metabolic burden during replication, ii) eases further genetic manipulation within the plasmid by avoiding the inclusion of repetitive sequences, and iii) simplifies the regulatory control of both genes at once since only a single promoter needs to be modified to alter their expression levels. For this, we inserted the mScarlet3 gene downstream of the NucA gene, separated by a ribosome binding site (Figure 5a). With non-encapsulated bacteria, we observed that the expression of mScarlet3 dropped to roughly half when compared to the strain encoding only mScarlet3 production. In contrast, NucA secretion did not significantly decrease compared to the strain only encoding NucA secretion (Figure 5b). Both reporters could also be detected well in a DNase agar plate when grown in static (Figure 5c). In the PEARL format, mScarlet3 could be detected inside the bead and continuous secretion over 7 days was also detected using DNase agar plates (Figure 5d).

Furthermore, we assessed the detection of both proteins from the PEARLs by tracking the fluorescence with the microplate reader. We could detect a drop in fluorescence due to NucA secretion (Figure 5e) or an increase in mScarlet3-driven fluorescence (Figure 5f) only when bacteria encode for NucA or mScarlet3 as a single gene or polycistronically. In PEARLs, the differences in protein production after 20 h of growth were only significant for the NucA (Figure 5g). Overall, these experiments confirmed that the P_{lec} promoter can be used to drive strong expression of two independent genes in parallel, secretion is minimally compromised by doing this, and this functionality is maintained in the PEARLs. Given the lack of incredibly strong constitutive promoters in *L. plantarum* WCFS1, this polycistronic approach supposes an attractive alternative to express two genes of interest highly.

2.6. Cytocompatibility Investigation of Bacterial Alginate Gels Secreting NucA

L. plantarum is known to produce lactic acid and other metabolites that can be cytotoxic to eukaryotic cells.^[35] Several reports suggest that such cytotoxicity can be selective for cancer cells but

we found it important to test if encapsulation of these bacteria in the PEARLs would alter this cytotoxicity. To evaluate this, we co-cultured the PEARLs along with human fibroblasts in a media optimized to support the growth of *L. plantarum* and human cells. Thus, during the 24-h period of this experiment, the bacteria proliferate within the PEARLs and secrete NucA. These co-cultures were used to assess the impact of PEARLs on the viability and metabolic activity of human fibroblasts (Figure 6a). We compared conditions containing the P_{lec} -3050 (a high NucA producer, >25 nM, Figure 3f) and P_{tlpA} -3050 (a low NucA producer, <5 nM, Figure 3f) strains. We used empty alginate macro beads, wild-type lactobacilli entrapped in the alginate macro beads, NucA-laden alginate macro beads (50 nM), and media containing 0.5, 5, and 50 nM of NucA as controls. First, we quantified the percentage of cell death by measuring the number of cells with damaged cell membranes using the lactate dehydrogenase assay (Figure 6b). A lysis condition where all cells were damaged with Triton X-100 was used as a positive control. All conditions investigated presented similar values of cell death around 10%, which were comparable to the negative control, untreated cells. Cell viability was also quantified as the number of metabolically active cells using the alamar Blue assay (Figure 6c). Results show similar values of viability to the negative control. The metabolic activity of fibroblasts decreased slightly for P_{tlpA} -3050, but this was not statistically significant compared to the negative control. Some conditions presented slightly higher metabolic activity than the negative control, which was observed for conditions containing bacteria and without bacteria (i.e., Nuc control 5 nM). This points to a different proliferation rate unrelated to the presence of bacteria in the co-cultures. All measurements were within a similar range.

LDH assay and alamar blue assay were performed with PEARL supernatants as well and the results show similar values as negative control (Figure S10a,b Supporting Information). We also stained the actin cytoskeleton of the fibroblasts with supernatants of PEARLs (Figure 6d) to assess cell morphology (Figure 6e). From the staining, we observed similar morphologies across conditions, with spread cells and mature actin fibers developed. Nuclei staining was quantified to obtain values of cell densities for the conditions evaluated. Cell densities were similar across conditions (1600 cells mm⁻²) indicating comparable cell confluency after treatment to the negative control (Figure S11, Supporting Information).

2.7. Zebrafish Embryo Toxicity Test

Zebrafish (ZF) embryos <5 days post fertilization (dpf) were used as a highly sensitive in vivo acute toxicity invertebrate test model. Following OECD guideline 236,^[36] such tests are usually performed in 24-well plates with one ZF embryo per well in a volume of 2 mL media. In order to achieve a more robust exposure scenario, the test protocols were adapted in the following ways to be better suited for PEARLs:

Usually, the zebrafish embryos are exposed to test compounds while they remain inside the egg from which they hatch later during the test. However, the chorion can prevent substances from accessing the embryo.^[37] To ensure that the embryos came into direct contact with any bacterial products released in the media,

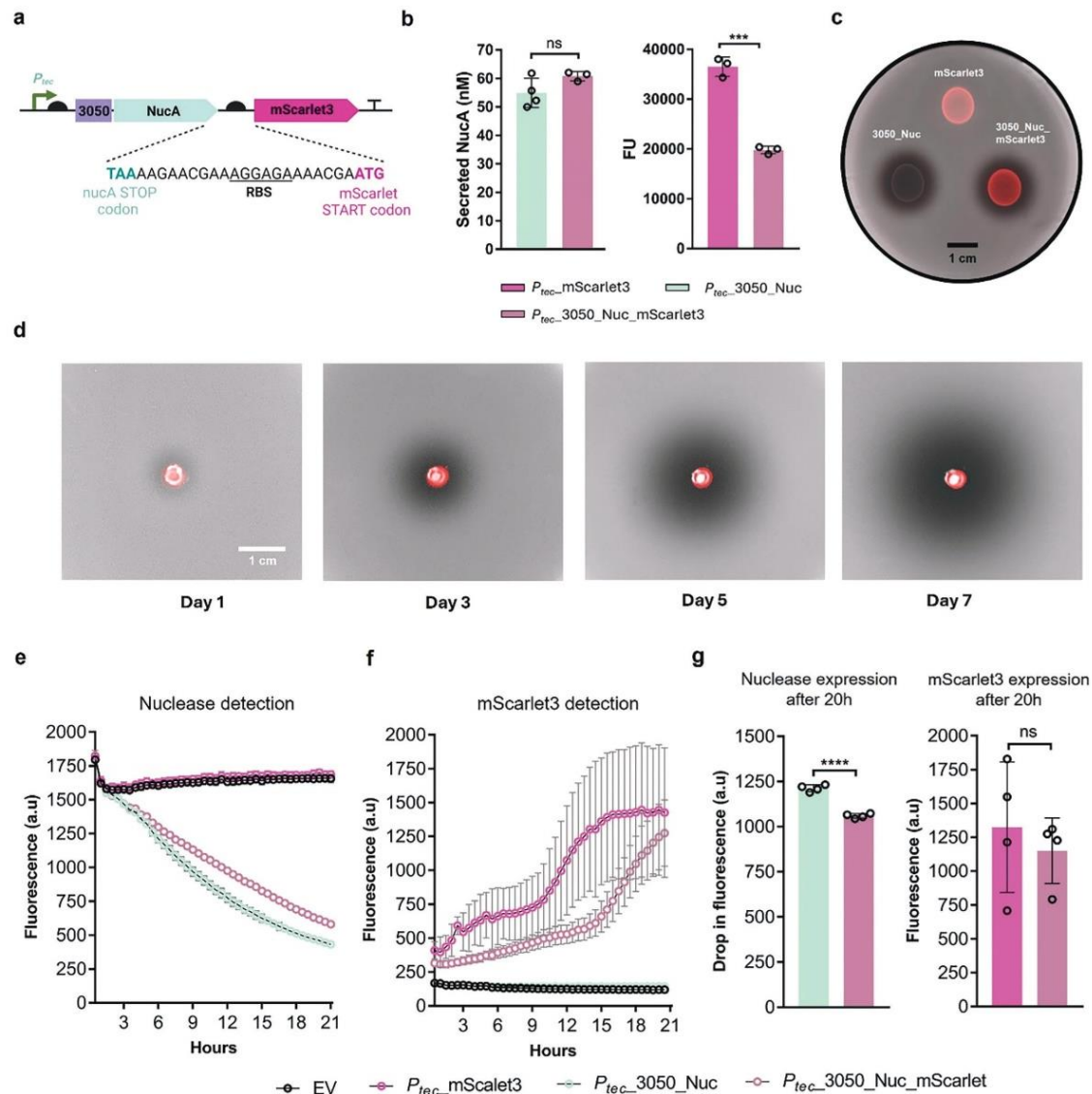


Figure 5. Polycistronic gene expression from PEARLs. a) Scheme of the polycistronic gene based on NucA and mScarlet3 genes. The DNA sequence between the stop codon of NucA and the start codon of mScarlet3 is shown. b) The left plot shows the levels of secreted NucA ($n = 3/4$, mean $\pm$ SD) for the polycistronic-engineered bacteria and single NucA secreting bacteria (P_{tec} -3050) when bacteria were grown in DNase media. The right plot shows the decrease in fluorescence expression (mean $\pm$ SD) by placing the mScarlet3 gene downstream NucA gene (in comparison to mScarlet3-producing *L. plantarum* WCFS1) when bacteria were grown in MRS media. c) DNA agar plate showing bacteria expressing only mScarlet3, secreting only NucA, and expressing and secreting mScarlet3 and NucA, respectively, after 24 h of growth. d) PEARL containing the polycistronic engineer strain. Expression of both genes could be detected from day 1 and kept increasing over the days. e) Detection of NucA secretion as drop in fluorescence from PEARLs ((mean $\pm$ SD) incubated in DNase media. f) Detection of mScarlet3-driven fluorescence from PEARLs (mean $\pm$ SD) incubated in LB media. g) Single gene versus polycistronic gene expression levels comparison after 20 h of growth in PEARLs. Significant differences were only obtained for NucA (left plot).

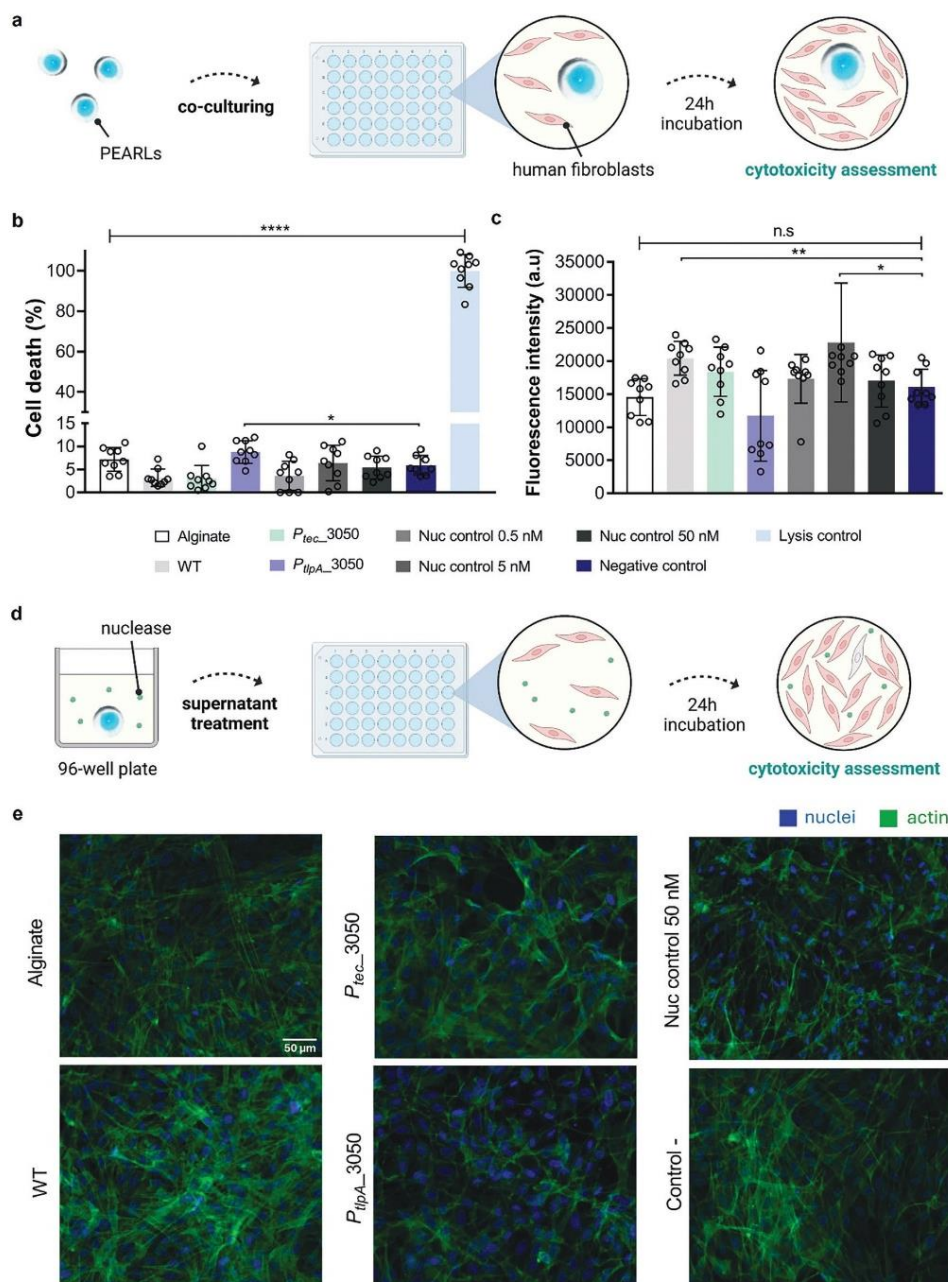


Figure 6. Cytocompatibility evaluation of NucA-secreting PEARLs on human fibroblasts. a) Schematic showing PEARLs were co-cultured along with human fibroblasts in growth media (for fibroblasts) for 24 h to evaluate the compatibility potential on human fibroblasts. The collected supernatants were used for viability assessment. b) Percentage of cell death quantified after co-culture treatment via LDH assay (mean $\pm$ SD). c) Fluorescence intensity measured during alamarBlue assay after co-culture treatment (mean $\pm$ SD, arbitrary units (a.u.)). d) Schematic showing PEARLs incubation in growth media (for fibroblasts) overnight for evaluation by immunostaining. The collected supernatants were used to treat fibroblasts in culture for 24 h, after which immunostaining was performed. e) Representative fluorescence images of human fibroblasts after treatment with PEARLs supernatants (scale bar: 50 μ m, blue: nuclei, green: actin).

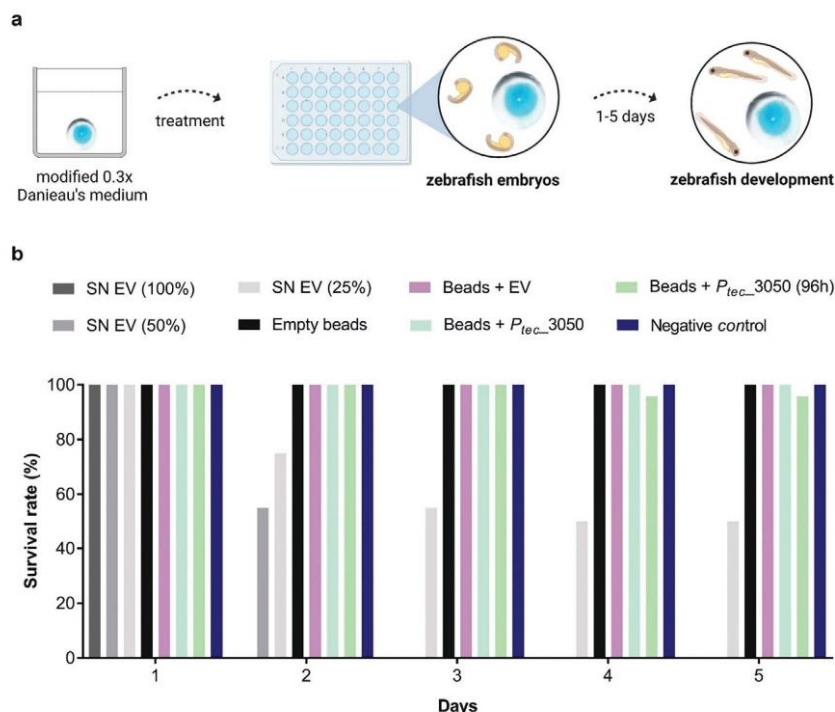


Figure 7. Cytotoxicity evaluation of NucA-secreting alginate beads upon Zebrafish embryos. a) Graphical representation of alginate beads incubated in 0.3x Danieau's media followed by incubation of zebrafish embryos with beads in a 48-well flat bottom plate up to 5 days. b) Survival rates of zebrafish embryos exposed to three different concentrations of the cell-free supernatant (SN) containing the complete bacterial secretome (SN EV (100%), SN EV (50%), SN EV (25%) as a positive control ($n = 20$ /treatment group), four different kinds of alginate beads: empty beads, beads + EV (containing wild-type *L. plantarum*), beads + *P_{tec}-3050* (secreting the highest level of NucA), beads + *P_{tec}-3050* (96 h) (pre-incubated for 96 h before adding ZF embryos) and the negative control ($n = 24$ /treatment group).

they were released from the chorion before the start of the test. The de-chorionation took place at 1 dpf since this is the earliest time at which this treatment can be carried out reliably without harming the embryos, and this is also a common practice in drug screening.^[38]

The ZF media was adapted to support *L. plantarum* growth and metabolism by adding saccharose. Different sugars (Glucose, Galactose, Maltose, Arabinose, Fructose, Sorbitol, Xylose, Mannose, and Saccharose) were added to the *L. plantarum* culture at 1% v/v concentration and after 24 h, OD₆₀₀ and pH of the cultures were measured. Saccharose supplementation was the only condition in which significant bacterial growth (up to OD₆₀₀ of 2) was seen along with the least drop in pH to 6. In all the remaining conditions with other sugars, either bacterial growth was compromised, or the pH decreased below 6, even down to 3.

The potential toxicity of non-encapsulated *L. plantarum* on ZF embryos was investigated using a cell-free supernatant containing the complete bacterial secretome. To decrease the production volumes needed, the test was performed in a 96-well plate, thereby decreasing the test volume from 2 to 0.3 mL per replicate. We observed that the pure supernatant instantly killed all ZF embryos within the first 24 h (Figure 7b). On dilution to 50% of the culture supernatant, 45% of the ZF embryos died within the first

24 h. After 48 h all embryos were dead. On dilution to 25% of the culture supernatant, 75% survived after 24 h, 55% after 48 h, and 50% after 72 and 96 h. The results indicate that metabolic byproducts of *L. plantarum* are toxic to ZF embryos, similar to their reported effect on cancer cells.^[35] Notably, the supernatant decreased the pH from 8 to values around 6, possibly due to lactic acid production by the bacteria. This low pH was measured in the 50% diluted samples but in 25% dilution, the pH was closer to neutral (Table S2, Supporting Information). However, ZF embryos have a relatively high pH tolerance and should tolerate the observed pH changes.^[39] Therefore, the observed mortality induced by the supernatant is considered to be induced rather by substances produced by *L. plantarum* than by decreased pH.

The effect of the PEARLs on ZF embryos was tested in 48-well plates to reduce the volume and increase the concentration of potentially released bacterial metabolic byproducts. The size of a well was small enough to establish direct contact between embryos and beads, but still big enough to prevent mechanical interferences such as squeezing of the embryos. 8 PEARLs were tested for each condition with 3 embryos in the same well as one PEARL. The scheme of the experiment is shown in Figure 7a.

Survival of the ZF embryos in the presence of the PEARLs was tested over 96 h and compared to a negative control group that did

not contain any PEARLs or alginate. In the first test group, empty alginate beads without bacteria were tested. In the s group, core-shell alginate beads contained wild-type *L. plantarum*. The next group included PEARLs containing the *P_{tec}*-3050 strain, which secretes the highest level of NucA. In all these groups, all embryos survived the 96-h incubation period (Figure 7b). In an additional treatment group, the PEARLs containing the *P_{tec}*-3050 strain were incubated for 96 h without ZF before the start of the test to increase the concentration of secretion products in the surrounding media. In this treatment, one embryo died between test days 3 and 4 resulting in a survival rate of 95.8% (Figure 7b). Such effects with a value lower than 10% are usually considered natural variation and are not related to toxicity.^[40] Even though a toxic effect cannot be completely ruled out, the death of a single embryo is attributed to natural variance rather than a toxic effect, supported by the absence of any other adverse effects. Thus, no significant reduction of survival due to the presence of PEARLs was observed during the 96 h test duration. This was quite a surprising result considering the toxicity of the non-encapsulated bacteria. It is to be noted that the PEARLs did not considerably reduce the pH of the media as it remained ≈ 7.5 in all cases (Table S2, Supporting Information). This indicated that encapsulation of the bacteria lowered its lactic acid production rate. While it is possible that alginate encapsulation slowed down metabolite diffusion into the media, the results from Figure 3d indicate that diffusion of a secreted protein occurs within a matter of hours, so the 4–8 day periods of the last two test groups should have been sufficient for metabolite accumulation in the media. Furthermore, we were able to clearly detect NucA secretion into the ZF media after 24 h (Figure S12a, Supporting Information).

In parallel, the PEARLs incubated in ZF media for 14 days were dissolved using EDTA to recover the viable bacteria and plated on an MRS-agar plate. The number of colony-forming units (CFU) was found to be around 10^5 CFU/mL (Figure S12c, Supporting Information). Though this is lower than the initial number of cells that were encapsulated in the PEARLs ($\approx 10^6$), it is to be noted that EDTA treatment could have killed some of the bacteria.^[41] We confirmed this by dissolving PEARLs 1 h after their fabrication and determining the bacterial counts using the same method, which was in the same order of magnitude ($< 4 \times 10^5$ CFU/mL). However, this was still higher than the 14-day-old PEARLs, so it is possible that bacterial viability or robustness dropped over this time period. Additionally, six random colonies from the MRS agar plate were spotted on the DNase-agar plate, and secretion was observed from all colonies after 24 h (Figure S12d, Supporting Information). These results indicated that PEARLs could maintain the viability and activity of a bacterial population over 14 days and that encapsulation of *L. plantarum* in alginate possibly reduces or prevents the production of metabolites that can be cytotoxic to the zebrafish. Extensive analysis of the secretome from non-encapsulated bacteria and the PEARLs using metabolomic and proteomic methods could shed light on this mechanism.

3. Conclusion

To our knowledge, this is the first study that comprises an ELM based on probiotic lactobacilli. We have developed a rapid, cost-effective, and safe encapsulation method that keeps probiotic bac-

teria viable and active while containing them. On top of that, the manual approach of fabricating PEARLs is cost-effective and easy to implement for small-batch production at the lab scale. Characterization of these PEARLs has revealed interesting insights concerning protein secretion and cytotoxicity. For example, the exact amount of secreted protein from both non-encapsulated and encapsulated bacteria was measured in the range of 5–50 nM and, a majority of this was secreted within the first 6 h. Encapsulation as PEARLs offered multiple benefits like supporting long-term protein release (at least 14 days), stabilizing the release profiles, and improving release rates. Additionally, we discovered and characterized new genetic parts that will enhance the programmability of *L. plantarum* for protein secretion. Two constitutive promoters, *P_{tec}* and *P_{ilpA}* secreted significant amounts of three different proteins (NucA, elafin, and α -MSH) from PEARLs. Finally, the cytotoxicity results using human cell lines and ZF suggest considerable suppression of toxic metabolite release when lactobacilli are encapsulated in alginate. Altogether, these results indicate that PEARLs are a promising platform for further development toward therapeutic applications.

4. Experimental Section

Strain, Media, and Plasmids: *L. plantarum* WCFS1 strain was used in this study. De Man, Rogosa, and Sharpe (MRS) media (Carl Roth GmbH, Germany, Art. No. X924.1) was used to grow the strain. Engineered *L. plantarum* WCFS1 strains were grown in MRS media with $10 \mu\text{g mL}^{-1}$ of erythromycin (Carl Roth GmbH, Art. No. 4166.2) at 37°C and 250 revolutions per minute (rpm) for 16 to 20 h. *E. coli* Nissle 1917 was maintained in Luria-Bertani (LB) media (Carl Roth GmbH, Art. No. X968.1). Engineered *E. coli* Nissle 1917 was grown in LB media supplemented with $200 \mu\text{g mL}^{-1}$ of erythromycin at 37°C , 250 rpm for 16 to 20 h. The pLp_3050sNuc plasmid was the backbone vector used in this study (Addgene plasmid # 122 030).

Molecular Cloning Reagents: The following enzymes and kits were purchased from NEB: Q5 High Fidelity 2X Master Mix for PCRs, NEBuilder HiFi DNA Assembly Cloning Kit for Gibson Assembly, Quick Blunting Kit for DNA phosphorylation, and the T4 DNA Ligase enzyme for DNA ligation. DNA oligos were purchased from Eurofins Genomics GmbH (Köln, Germany) (Table S1, Supporting Information). Synthetic genes were synthesized as eBlocks from Integrated DNA Technologies (IDT) (Coralville, USA) and were codon optimized using the IDT Codon Optimization Tool. Plasmids were extracted using the plasmid extraction kit from Qiagen GmbH (Hilden, Germany). The DNA purification kit was purchased from Promega GmbH (Walldorf, Germany). The ladder used for agarose gels was the Generuler 1 Kb DNA Ladder from Thermo Fisher Scientific.

Plasmid Construction: The direct cloning method for *L. plantarum* WCFS1, was performed to assemble and obtain enough plasmid amount required for a successful transformation.^[19] In brief, Gibson Assembly was used to assemble the synthetic genes into the plasmid backbone. Instead of transforming the assembled product into an *E. coli* cloning strain, a PCR-based plasmid amplification, followed by phosphorylation and ligations of the PCR products was performed for circularization. Finally, a final purification was performed to concentrate the circular DNA before *L. plantarum* WCFS1 transformation. For sequence verification, the gene of interest was amplified by PCR (Q5 high-fidelity polymerase), and the product was sent for sequencing at Eurofins Genomics GmbH (Köln, Germany).

***L. plantarum* WCFS1 Transformation:** *L. plantarum* WCFS1 electrocompetent cells were prepared as described in.^[19] *L. plantarum* WCFS1 transformation was done following the protocol described.^[19]

Media Selection: When inoculated from glycerol stock, *L. plantarum* was always grown in MRS media. To assess the containment of bacteria within PEARLs, both MRS and LB media were used for subculturing and incubation. For all NucA detection studies, bacteria were then subcultured

in DNase media. For elafin and α -MSH experiments, DMEM media was used for subculturing of bacteria and incubation of PEARLs. For fibroblast experiments, fibroblast growth media was used for subculturing of bacteria and incubation of PEARLs. For zebrafish experiments, 0.3x Danieau's media was used for subculturing of bacteria and incubation of PEARLs.

NucA Assay (in DNase media with Methyl green) – Qualitative Assay: To analyze the secretion of NucA by genetically modified bacteria, the bacteria were first grown overnight in MRS media supplemented with $10 \mu\text{g mL}^{-1}$ erythromycin at 37°C , 250 rpm, and the OD_{600} was measured the following day. The samples were subcultured in 4 mL of antibiotic-supplemented fresh MRS media at an initial OD_{600} of 0.1 and incubated at 37°C until an OD of 1. Then, $10 \mu\text{L}$ of the undiluted bacterial cultures were spotted on DNase agar (Altmann Analytik GmbH, Germany) supplemented with $10 \mu\text{g mL}^{-1}$ of erythromycin and incubated at 37°C for 24 h. The discoloration zone surrounding the spotted area, indicative of NucA activity was captured and documented using the BioRad Gel Documentation System.

NucA Assay (in DNase media with Methyl green) – Quantitative Assay: To measure the concentration of the secreted NucA, overnight bacterial cultures (in MRS broth) were subcultured to an OD_{600} of 0.1 in MRS broth. Once the OD reached 1, bacteria equivalent to OD 0.1 were transferred to an autoclaved DNase media supplemented with erythromycin of 1 mL volume and incubated at 37°C and 250 rpm shaking conditions for 24 h. The DNase media was obtained by agar precipitation of the commercial DNase Agar and the final formulation comprised Tryptose (20 g/L), Sodium Chloride (5 g L^{-1}), Calf Thymus DNA (2 g L^{-1}), Methyl Green (0.05 g L^{-1}). After 24 h, the bacterial cultures were centrifuged at $13\,000 \text{ rpm}$ ($15\,700 \times g$) and $200 \mu\text{L}$ of the respective supernatants were added to the clear bottom 96-well microtiter plate (Corning 96 well clear bottom black plate, USA). The samples were analyzed in the Microplate Reader Infinite 200 Pro for methyl green-DNA complex-based fluorescent intensity ($\text{Ex}/\text{Em} = 633/668 \text{ nm}$). The Z-position and gain settings for recording the fluorescence were set to $19\,000 \mu\text{m}$ and 100, respectively. The raw data obtained was correlated and calculated based on the standard curve explained below.

NucA Assay (in DNase media with Methyl green) – Standard curve: Overnight grown culture of WCF51 – Empty vector (EV, bacteria carrying a plasmid based on the p256 origin of replication and the erythromycin resistance) was diluted to the OD 0.1 in DNase agar + methyl green media. Using the 0.1 OD DNase media prepared in the previous step as the solvent, serial dilutions of NucA protein (Sigma-Aldrich) were made. In the experiment, the standard curve was plotted in the range of $200\text{--}1.56 \text{ nM}$ ($200, 100, 50, 25, 12.5, 6.25, 3.12, \text{ and } 1.56 \text{ nM}$). The final volume of each concentration was 1 mL and was prepared in 1.5 mL Eppendorf tube. Once the protein dilutions were transferred to the Eppendorf tube, they were covered with aluminum foil to protect the bleaching of methyl green from exposure to light. The tubes were stacked in a 37°C incubator with 250 rpm shaking for 24 h.

After 24 h, $200 \mu\text{L}$ from each Eppendorf/concentration were pipetted in duplicates into a black 96-well plate with transparent bottom. The fluorescence of samples was measured at Ex/Em of $633/668 \text{ nm}$ at Z position of $19\,000 \mu\text{m}$ and Gain of 100 in the Microplate Reader Infinite 200 Pro (Tecan Deutschland GmbH, Germany).

Flow Cytometry: The fluorescence intensity of the reporter proteins mCherry and mScarlet3 in bacterial cultures was measured using the Guava easyCyte BG flow-cytometer (Luminex, USA). Samples were prepared as described in.^[20] The Luminex GuavaSoft 4.0 software for Easy-Cyte was employed for the analysis of the data.

PEARL Fabrication – Encapsulation in Alginate Core-Shell Hydrogels – Fabrication of Alginate Cores: About 3 wt% alginate solution was prepared by dissolving sodium alginate (Sigma-Aldrich 71 238) in sterile MilliQ water followed by sterilization by autoclaving it at 120°C for 15 min. A bacterial culture ($\approx 10^8$ cells in LB media) was mixed with 3 wt% alginate solution in a 1:2 volume ratio to obtain a bacterial-alginate core solution of 2 wt%. The bacterial culture was supplemented with 1% v/v Alcian blue to enhance the visualization of alginate-bacteria core beads during manual fabrication. The bacteria-alginate core mixture was vortexed thoroughly to ensure uniformity. Droplets, each with a volume of $10 \mu\text{L}$, were pipetted and dropped into a 5 wt% CaCl_2 solution (Ionic crosslinker). The droplets

were left to polymerize and solidify in CaCl_2 solution for 15 min. Solidified core beads (roughly 2 mm in diameter) were collected and washed thrice with sterile Milli-Q water to remove the remnant CaCl_2 solution and bacteria on the surface of the beads.

PEARL Fabrication – Encapsulation in Alginate Core-Shell Hydrogels – Shell Coating: About 1.5 wt% alginate solution for shell coating was prepared by mixing 3 wt% alginate stock solution and Milli-Q water in a 1:1 volume ratio followed by vortexing. Alginate cores from the last step of the previous section were dipped into the shell solution. Alginate bead surrounded by shell solution (roughly $25 \mu\text{L}$) was collected with a pipette tip, whose tip was cut with a sterile blade to increase the size of the opening, followed by dropping into the cross-linker solution. The core-shell alginate beads were left to solidify for 15 min. The beads were collected, washed with sterile Milli-Q water, and transferred to a well plate with a nutrient media.

Tracking Changes in PEARLs Using Microplate Reader: PEARLs (alginate beads) after fabrication were immediately transferred to a 96-well plate containing wells filled with $200 \mu\text{L}$ of respective media (LB media for OD_{600} measurements and DNase media for NucA quantification). Growth kinetics along with tracking of fluorescence expression was done for 72 h using Tecan microplate reader with settings for Excitation/Emission wavelengths [Ex/Em for mCherry ($587/625 \text{ nm}$)/mScarlet3 ($569/600 \text{ nm}$)/NucA ($633/668 \text{ nm}$)], Manual gain 80–100 (depending on the magnitude of signal, a particular gain value has been selected for individual assays, between 80 and 100), Z-position $19\,000 \mu\text{m}$ and integration time of $20 \mu\text{s}$. Data was normalized and analyzed using GraphPad prism.

Western Blot: Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) of denatured protein samples (Culture supernatants in the experiments) were performed. PVDF membrane (Immobilon-P, Merck Millipore) was cut to the same size as polyacrylamide gel immersed in 99% methanol for 10 s followed by soaking the membrane in sterile MQ water for 10 s and finally dispersed in 50 mL of cold 1x Transfer buffer (25 mM Tris Base-pH 8.3, 192 mM Glycine, 200 mL methanol and rest of MQ water upto 1L) for a few minutes. Parallely, components needed for cassette assembly (foam pad, filter paper, polyacrylamide gel) were equilibrated in cold 1x Transfer buffer for 10 min. The cassette was assembled in the order of Support grid/Cathode (–) $\rightarrow$ foam pad $\rightarrow$ filter paper $\rightarrow$ polyacrylamide gel $\rightarrow$ PVDF membrane $\rightarrow$ filter paper $\rightarrow$ foam pad $\rightarrow$ Support grid/anode (+). The cassette was placed in a Bio-Rad buffer tank set on a magnetic stirrer, filled with 1x Transfer buffer, a magnetic stirring bar was placed underneath the cassette and the whole setup was connected to a power unit with the voltage set to 100 V for a duration of 90 min and the transfer was initiated by switching on the power supply. After 90 min, the PVDF membrane was peeled out (make sure that protein ladder got transferred from PAGE gel onto the PVDF membrane, indicating the successful transfer of proteins) and placed in a 50 mL amber-colored falcon by rolling the membrane inward such that the side of the membrane with transferred proteins was in contact with the blocking buffer (15 mL of 3% Bovine Serum Albumin in 1x Tris-buffered saline (TBS)) inside. The falcon was placed in a rotary shaker and incubated at room temperature for 1 h. The spent blocking buffer was discarded and 10 mL of fluorophore-tagged antibody (elafin antibody H-2 conjugated to Alexa Fluor 647 from Santa Cruz Biotechnology, in the ratio of 1:500) in 3% Bovine Serum Albumin made with 1x TBS was added to the falcon. The falcon was set in a rotary shaker and incubated overnight at 4°C . Next morning, the membrane was taken out of the falcon and washed thrice with 1x TBST (TBS supplemented with 0.05% Tween-20) followed by capturing the blot in optimized RGB settings in a Bio-Rad gel doc device.

Neutrophil Elastase Inhibition Assay: Confirmation of elafin's bioactivity was performed by measuring the inhibition of Neutrophil elastase (NE) enzyme. An in-house assay was optimized using human neutrophil elastase (324681-Sigma-Aldrich), neutrophil elastase specific fluorogenic substrate (MeOSuc-AAPV-AFC, from Abcam), and commercial recombinant human elafin (R&D systems). In the absence of NE inhibitors, NE recognizes & binds to a specific tetrapeptide sequence (AAPV) and cleaves the bond between valine and AFC (7-amino-4-trifluoromethylcoumarin), a fluorescent moiety at the C-terminus of substrate. Upon cleavage, AFC is released and exhibits strong fluorescence which can be measured using a

Chapter 4.

Switchable protein release from lactobacilli-based ELMs

The promoters are the genetic parts that determine gene expression levels by influencing the affinity with the RNA polymerase. All the promoters mentioned in the previous chapters were constitutive. These promoters are defined as “unregulated promoters that allow for continual transcription of their associated genes”. In other words, they remain active under normal conditions and, in principle, do not respond to inducers. In bacteria, using promoters that are active full-time can lead to bacterial growth impairment, undesired mutations, and other disadvantages. In the first part of this thesis, this was commonly observed in *E. coli*, which led to the idea of developing the direct cloning method described in Chapter 2. However, it also started to be the case in *L. plantarum* after the discovery of P_{tec} . For instance, the sequence-verified recombinant strain encoding the elafin gene under P_{tec} has always failed, possibly due to high levels of elafin resulting in toxicity for *L. plantarum*, as shown previously for *E. coli* [158].

Inducible genetic switches are typically used to circumvent such issues [95, 98]. These switches are based on inducible promoters that generally become active upon induction, thus turning on gene expression on demand. There is one widely used inducible switch in *L. plantarum*, the pSIP system, which was developed in 2005 [107]. However, this switch has certain limitations, including i) being a two-component system, ii) requiring a cost-intensive peptide as an inducer, and iii) not achieving high levels of protein production after induction. Therefore, there is a need for novel inducible genetic switches and a plethora of opportunities to explore.

This chapter further investigates the question of whether the newly discovered genetic parts are able to perform well when bacteria are encapsulated within alginate beads. However, the primary objective is not protein secretion but rather the regulation of inducible gene expression. To achieve this, in the following work, effort is invested in turning the novel constitutive P_{tec} promoter into an inducible one that responds to food-grade small molecules. To that end, three genetic switches, so far unexplored in *L. plantarum*, were adjusted for optimal expression in these bacteria and designed to repress P_{tec} -driven expression of the fluorescent protein mCherry. Interestingly, all three genetic switches showed responsiveness towards the cognate inducer molecule, but only one, the cumate switch, was further investigated due to its promising results.

The temporal profile of the cumate switch showed growth phase dependency and an inverse correlation between a drop in the pH of the media, mediated by the metabolic activity of *L. plantarum*, and an increase in the leakiness of the switch. Yet, we reported two strategies to reduce leakiness significantly: i) thermally aiding the switch by growing the uninduced bacteria at 30°C reduced significantly leakiness (from 2-3x to >200x fold-changes for the uninduced and induced states of the switch after 10 hours of incubation), and ii) grow the bacteria in nutrient-limited conditions where their metabolism is restricted, and pH is more stable.

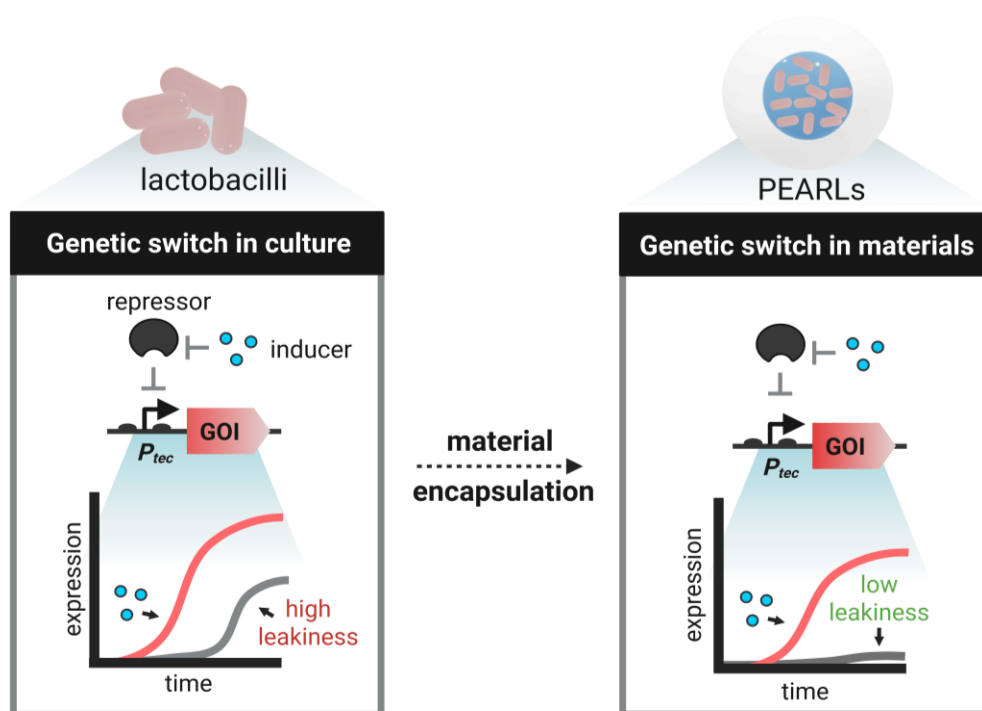
More interesting results were observed after encapsulation of the cumate-responsive bacteria within PEARLs. Encapsulation within materials almost completely reduced the leakiness of the cumate switch while allowing proper induction upon the addition of the inducer and further diffusion into the cores of the PEARLs. Switchable protein secretion was reported for the first time in lactobacilli-based ELMs, even after 9 days of incubation. Furthermore, that was not only proved for the cumate switch but also for another switch responsive to vanillate, which showed even higher levels of leakiness in the uninduced state in culture. The co-encapsulation of diverse inducible populations enabled the development of dual-mode multi-switching and multi-functional ELMs.

In the last part of the publication, the PEARL format is redesigned and fabricated using a more automated, scaled-up, and microencapsulation approach more suitable for

biomedical applications. The new format, termed micro-PEARLs, showed prominent protein release and cumate-specific responsiveness, establishing this new format as an attractive alternative for next-generation lactobacilli-based ELMs.

Altogether, this study reports a novel genetic switch for highly inducible gene expression in *L. plantarum*. It is envisaged that this responsive ELM format is promising for developing *L. plantarum* towards advanced applications in biotechnology, pharmaceuticals, and living therapeutics.

Graphical Abstract



*The strong constitutive promoter P_{tec} was engineered to be responsive to the food-grade small molecule cumate, developing a novel genetic switch in *L. plantarum*. This switch exerted a moderate degree of P_{tec} -driven inducibility but strong leakiness in the uninduced state after a given bacterial growth. However, encapsulation within PEARLs suppressed leaky expression while allowing induction upon the addition of cumate, pointing towards an encapsulation-enhanced induction.*

Publication and contributions

Article Type: Research article.

Title: Encapsulation-enhanced switchable protein release from engineered probiotic lactobacilli ⁵.

Year Published: 2025.

Research Journal: Journal of Controlled Release.

Authors: Marc Blanch-Asensio, Varun Sai Tadimarri, Roberto Martinez, Gurvinder Singh Dahiya, Cao Nguyen Duong, Rahmi Lale, and Shrikrishnan Sankaran.

First author: Marc Blanch-Asensio.

Author contributions: Marc Blanch-Asensio performed all the genetic engineering work and most of the experimental procedures. Varun Sai Tadimarri fabricated all the PEARLs and micro-PEARLs. Roberto Martinez assisted in micro-PEARLs fabrication. Rahmi Lale, Gurvinder Singh Dahiya, Marc Blanch-Asensio, and Shrikrishnan Sankaran took part in the conceptualization. Rahmi Lale and Gurvinder Singh Dahiya reviewed the manuscript. Cao Nguyen Duong performed the confocal microscopy imaging and analysis. Marc Blanch-Asensio made the illustrations, analyzed, and plotted the data. Marc Blanch-Asensio wrote most of the draft of the manuscript. Shrikrishnan Sankaran led the supervision, reviewed, and edited the manuscript.

⁵ The supporting information of this publication is provided in Appendix II.4 at the end of this dissertation.



Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Encapsulation-enhanced switchable protein release from engineered probiotic lactobacilli[☆]

Marc Blanch-Asensio^{a,b}, Varun Sai Tadimarri^{a,b}, Roberto Martinez^a, Gurvinder Singh Dahiya^c, Cao Nguyen Duong^a, Rahmi Lale^{c,d}, Shrikrishnan Sankaran^{a,*}

^a INM - Leibniz Institute for New Materials, Saarland University, Campus D2 2, 66123 Saarbrücken, Germany

^b Saarland University, 66123 Saarbrücken, Germany

^c Syngens AS, 7089, Norway

^d Department of Biotechnology and Food Science, Faculty of Natural Sciences, Norwegian University of Science and Technology, Trondheim N 7491, Norway

ARTICLE INFO

Keywords:

Engineered living materials
Probiotic lactobacilli
Genetic switch
Alginate
Protein secretion

ABSTRACT

Living microbial therapeutics promise precise, programmable interventions at disease sites, yet most demonstrations of on demand drug release still rely on *Escherichia coli*, whose rich genetic toolkit is unmatched among probiotics. In particular, genetic parts to regulate *in situ* protein production are severely lacking in non-model probiotic bacteria like lactobacilli. Here, we equip the probiotic *Lactiplantibacillus plantarum* with high-performance genetic switches and show how material encapsulation can further enhance their behavior. By integrating cumate or vanillate-responsive operators and repressors with the strongest constitutive promoter in *L. plantarum* (*P_{tec}*), we generated two switches that support micromolar range induction. In rapidly growing culture conditions, acidification-associated leakiness of the switch was observed, which could compromise their applicability for precise on-demand delivery of drugs. Furthermore, such leakiness also limits the duration for which these engineered probiotics can be reliably used. By restricting growth through mild temperature or nutrient limitation, acidification and leakiness were suppressed. Strikingly, immobilizing the engineered cells in core-shell alginate beads (Protein Eluting Alginate with Recombinant Lactobacilli, PEARLS) almost eliminated leakiness, enabling day-scale, reversible control of intracellular reporters and secreted enzymes. This leakiness suppression persisted when two strains carrying orthogonal switches were co-encapsulated and even after miniaturization to submillimeter beads. These results expand the genetic toolbox of probiotic *L. plantarum*, demonstrate the synergy between genetic circuit design and material encapsulation, and advance lactobacilli toward stimuli-responsive therapeutic platforms.

1. Introduction

Engineered living therapeutics are microorganisms that are genetically programmed to deliver biopharmaceuticals within the body [1]. They are emerging as a powerful complement to small molecules and biologic medicines due to their ability to self-replicate, navigate complex host environments, and continuously produce macromolecular cargos or bioactive metabolites to treat chronic pathologies [2–4]. Furthermore, they can be genetically programmed to sense external triggers or biomarkers, process these inputs, and control the release profiles of their therapeutic payloads *in situ* [5]. While several engineered bacteria have made it to clinical trials and show promise for the

future of this technology, *in situ* control over release has mainly been demonstrated with *Escherichia coli* due to its well-equipped genetic toolbox. Extending such capabilities beyond *E. coli* to probiotic species that naturally inhabit human mucosa would broaden the applicability of this technology and improve safety and colonization profiles.

A central barrier to this broader vision is the limited genetic programmability of non-*E. coli* probiotics [6]. While *E. coli* benefits from decades of synthetic biology tool development with numerous well-characterized promoters, repressors, and transcription factors, most other probiotic genera only have access to a handful of basic genetic parts. Furthermore, in several taxa like *Lactobacillus*, efforts to port heterologous parts often falter because of divergent intracellular and

[☆] This article is part of a Special issue entitled: 'Microbial Therapeutics' published in Journal of Controlled Release.

* Corresponding author.

E-mail address: Shrikrishnan.sankaran@leibniz-inm.de (S. Sankaran).

<https://doi.org/10.1016/j.jconrel.2025.114264>

Received 2 July 2025; Received in revised form 17 September 2025; Accepted 23 September 2025

Available online 24 September 2025

0168-3659/© 2025 Elsevier B.V. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

metabolic environments [7]. Consequently, sophisticated genetic circuits that enable control over cargo production in response to sensing and processing external triggers have remained out of reach for most clinically relevant probiotics.

A second challenge is achieving reliable spatiotemporal control over the activity of microbial therapeutics once they are released into the body [8]. Freely administered bacteria experience several impediments to their function, like immune pressure, microbial competition, phage infections, and environmental stresses like changes in pH, osmotic pressure, and nutrient availability [9,10]. To maintain probiotic viability and functionality, material-assisted strategies like encapsulation in hydrogels, protective coatings, or layer-by-layer approaches have been employed [11–13]. This strategy aids in physically preventing attacks from immune cells, other microbes, or phages, and buffers environmental stresses, while permitting nutrient diffusion to the cells and release of therapeutic factors [14,15]. The effective application of this strategy for therapeutically engineered microbes is being extensively explored in the field of Engineered Living Materials (ELMs) [16,17].

Within this landscape of engineered probiotics for therapeutic applications, *Lactiplantibacillus plantarum* stands out as a versatile chassis candidate [7]. Several strains within this species are GRAS-designated, intrinsically acid-tolerant, and exert beneficial effects in the oral, gastrointestinal, urinary, skin, and reproductive niches, like immunomodulation and barrier-strengthening [18–20]. Their extensive use in fermented foods and probiotic supplements further speaks to their safety profile in humans [21,22]. Despite these advantages, the translation of *L. plantarum* into a living therapeutic platform has been hampered by a shallow genetic toolbox, which our laboratory has recently expanded [7,23]. For therapeutic applicability, we identified the strongest constitutive promoter characterized to date in *L. plantarum*, P_{tec} , and paired it with signal peptides to drive high-level secretion of heterologous proteins [24,25]. Second, we demonstrated that encapsulating these engineered lactobacilli inside core-shell alginate beads as “Protein-Eluting Alginate with Recombinant Lactobacilli (PEARLs)” stabilizes their metabolism, sustains protein release for weeks, prevents bacterial escape, and improves their biocompatibility [25]. Encapsulation, therefore, provides both a containment layer and a physicochemical micro-environment that may be exploited to adjust gene expression.

Yet, one key piece of the puzzle remains missing – high-performance genetic switches that can be controlled by clinically compatible triggers/inducers while exhibiting minimal leaky expression. Genetic switches are vital for the genetic programmability of an organism since they allow temporal control over gene expression, which can enhance productivity and reduce metabolic burden [26,27]. The well-established peptide-inducible pSIP system affords tight repression but only moderate output and requires costly synthetic peptides for induction [28,29]. Carbohydrate-responsive promoters like P_{xyI} (xylose-inducible), P_{lac} (lactose-inducible), and P_{gal} (galactose-inducible) are inexpensive yet display modest dynamic ranges and can be difficult to control *in vivo* because of host sugar metabolism [30,31]. Bile-induced promoters have also been explored in lactobacilli, but their induction relies on intestinal conditions, which restrict their applicability [32]. Our own efforts to adapt thermo-responsive genetic switches that function incredibly well in *E. coli* completely failed in *L. plantarum* due to unknown causes. Consequently, despite considerable efforts to create genetic switches in *L. plantarum*, reliable control over strong protein production has remained elusive, and the underlying causes behind the failures are poorly understood.

In the present study, we address this unmet need by modifying the strong P_{tec} promoter into genetic switches responsive to the food-grade small molecules, cumate and vanillate. The genetic switches supported strong protein production but were found to be leaky under conditions that supported rapid bacterial growth, leading to a drop in the pH. This leakiness could be greatly reduced using temperature or nutrient conditions that limited growth and pH change, but still facilitated strong protein production on induction. More interestingly, we show that

alginate encapsulation in the PEARL format drastically suppresses leakiness of these switches, yielding fold-changes exceeding ninety-fold for intracellular reporters and enabling day-scale, reversible control of secreted enzymes. Furthermore, such encapsulation enabled the co-encapsulation of strains with orthogonal switches to develop dual-mode PEARLs capable of independently regulating fluorescent protein production and enzyme secretion. Finally, we show that the leakiness-suppressing effect is sustained even when the PEARLs are miniaturized by an order of magnitude. Together, these advances expand the genetic toolbox of *L. plantarum* with parts offering temporal control over protein production and underscore the synergy between genetic circuit design and material encapsulation for the next generation of living drug carriers.

2. Experimental section

2.1. Strains, media, and plasmid vector

L. plantarum WCFS1 was used in this study. The strain was grown in De Man, Rogosa, and Sharpe (MRS) medium (Carl Roth GmbH, Germany, art. no. X924.1). The strain was incubated at either 30 °C or 37 °C (indicated in each case), and 250 rpm. The medium of the engineered strains was supplemented with 10 µg/mL of erythromycin (Carl Roth GmbH, Germany, art. No. 4166.2). For the cloning of all the plasmids, NEB DH5-α competent *E. coli* cells were used (New England Biolabs GmbH, Germany, art. No. C2987). Engineered *E. coli* was grown in Luria-Bertani (LB) medium (Carl Roth GmbH, art. No. X968.1) supplemented with 200 µg/mL of erythromycin at 37 °C and 250 rpm. The pTec_mCherry plasmid (Addgene plasmid #229145) was the backbone vector for all the plasmids used in this work. The backbone of this plasmid is derived from the pLp_3050sNuc plasmid (Addgene plasmid #122030).

2.2. Molecular cloning reagents

Primers were synthesized by Eurofins Genomics GmbH (Köln, Germany). All the primers used in this study are listed in Table S1. Synthetic gene fragments were ordered as eBlocks from Integrated DNA Technologies (IDT) (Coralville, USA). The codon optimization tool of IDT was used to codon-optimize the eBlocks (selecting the option for *Lactobacillus acidophilus*). All the eBlocks used in this study are listed in Fig. S0. For the polymerase chain reaction (PCR), Q5 High Fidelity 2× Master Mix (NEB GmbH, Germany, No. M0492S) was used. 1 kb Plus DNA Ladder (Catalog Number 10787018) was purchased from ThermoFisher Scientific (Germany) and used as a reference in gel electrophoresis. The Wizard® SV Gel and PCR Clean-Up System (Promega GmbH, Germany, Art. No. A9282) was used to purify the PCR products. DNA assembly was performed using the HiFi Assembly Master Mix (NEB GmbH, Germany, Art. No. E5520S). The Qiagen GmbH (Hilden, Germany) plasmid extraction kit was used to lyse and extract the plasmids from *E. coli* DH5-α.

2.3. Plasmid construction

The eBlocks were designed with compatible ends (20 base-pair overhangs) to the backbone vector. Each eBlock was resuspended with 20 µL of previously autoclaved Milli-Q water and used directly in the HiFi assembly reaction following the manufacturer's protocol. Right after, the assembled products were transformed into NEB DH5-α *E. coli* competent cells following the manufacturer's heat shock protocol except for the NucA cloning, where bacteria were grown at 28 °C instead of 37 °C. Plasmids were extracted from *E. coli* DH5-α cells and used for transforming *L. plantarum* WCFS1 after sequence verification by Sanger sequencing (Eurofins Genomics GmbH (Köln, Germany)). All the plasmids and the engineered strains generated in this study are listed in Table S2.

2.4. *L. plantarum* WCSF1 competent cell preparation and DNA transformation

The preparation of *L. plantarum* WCSF1 electrocompetent cells and the plasmid DNA transformation were done as described in [33]. In brief, *L. plantarum* WCSF1 was inoculated in 5 mL of MRS medium and cultured overnight at 37 °C and 250 rpm. After overnight growth, 1 mL of the culture was mixed with 20 mL of MRS medium and 5 mL of 1 % (w/v) glycine and incubated at 37 °C and 250 rpm for approximately 4 h until the OD_{600nm} reached roughly 1. Next, the cells were pelleted down by centrifuging at 4000 rpm for 10 min at 4 °C, followed by two washes with 5 mL of ice-cold 10 mM MgCl₂ and two additional washes with 5 mL of ice-cold Sac/Gly solution [1 M sucrose mixed with 10 % (v/v) glycerol in a 1:1 (v/v) ratio]. After that, cells were resuspended in 500 µL of Sac/Gly solution, and 60 µL aliquots of competent cells were prepared for DNA transformation. For the electroporation, 1 µg of plasmid DNA was added to a competent cell aliquot prior to transferring the mixture into an ice-cold 2 mm gap electroporation cuvette (Bio-Rad Laboratories GmbH, Germany). After 10 min of incubation on ice, a single pulse at 1.8 kV was applied, followed by the addition of 1 mL of MRS medium. The mixture was incubated at 37 °C and 250 rpm for 3 h. Finally, all the cells were plated on MRS Agar supplemented with 10 µg/mL erythromycin and incubated at 37 °C for 40–48 h.

2.5. Gene expression switches induction

Vanillic acid (vanillate) and 4-isopropylbenzoic acid (cumate) were purchased at Sigma Aldrich (Catalog number H36001 and 268402, respectively). Anhydrotetracycline (aTc) was purchased at Cayman Chemical (Item no. 10009542). All inducers were resuspended in pure ethanol. The concentrations used for inductions were: 50, 100, or 200 µM of vanillate for the vanillate switch, 0.2, 0.5, 1, or 4 µM of aTc for the aTc switch, and 0.1 to 100 µM of cumate for the cumate switch (indicated in each experiment). The induction time is indicated in each experiment. The pSIP system induction was done using the IP673 peptide (GeneCust, Boyes, France), to a final concentration of 100 ng/mL.

2.6. Flow cytometry analysis

Engineered *L. plantarum* WCSF1 was grown in 4 mL of MRS medium supplemented with 10 µg/mL erythromycin at 37 °C and 250 rpm. The next day, bacteria were sub-cultured to an OD_{600nm} of 0.01 in 2 mL of MRS medium supplemented with 10 µg/mL erythromycin and grown at 37 °C and 250 rpm for 8 h. For the induced conditions, inducers were added along with the erythromycin. After that, 1 mL of the bacterial cultures was pelleted down by centrifugation at 6000 rpm for 2 min. The supernatant was discarded carefully, and the pellets were resuspended in 1 mL of sterile Dulbecco's 1 × PBS (DPBS). Finally, the mixtures were serially diluted by a 10⁴-dilution factor, and 5000 bacterial events were recorded for analysis using the Guava easyCyte BG flow-cytometer (Luminex, USA). The fluorescence intensity of mCherry was measured using excitation by a green laser at 532 nm (100 mW) and the Orange-G detection channel 620/52 nm filter was used for signal analysis. The Luminex GuavaSoft 4.0 software for EasyCyte was used for the analysis and representation of data.

2.7. Microplate reader setup for induction levels assessment

Engineered *L. plantarum* WCSF1 was grown in 4 mL of MRS medium supplemented with 10 µg/mL erythromycin at 37 °C and 250 rpm. After overnight growth, bacteria were sub-cultured to low OD_{600nm} (indicated in the results section) in MRS medium supplemented with 10 µg/mL erythromycin. For the induced conditions, the inducers were added along with the erythromycin, and bacteria were incubated for a period of 5 to 8 h (the exact time and cumate concentration are indicated in the results section). After that, bacterial pellets were resuspended in 1 mL of

DPBS, and 200 µL of the mixture was transferred to a UV STAR Flat Bottom 96-well microtiter plate (Greiner BioOne GmbH, Germany). The microplate reader Infinite 200 Pro (Tecan Deutschland GmbH, Germany) was used to quantify the levels of fluorescence. The mCherry fluorescence intensity was measured using the excitation/emission values: Ex_λ / Em_λ = 587 nm/625 nm. The fluorescence values were normalized with the OD_{600nm} of the mixtures (quantified by measuring the absorbance at 600 nm) to calculate the Relative Fluorescence Units (RFU) (Fluorescence/OD_{600nm}). The RFU values for *L. plantarum* WCSF1 carrying an empty vector (a plasmid based on the origin and the antibiotic resistance) were used as blank. The settings for the microplate reader were as follows: the Z-position was set at 19000 µm, the gain settings at 80–100 (indicated in the results section), and the readings were taken from the top.

2.8. Microplate reader kinetics setup for induction assessment

Engineered *L. plantarum* WCSF1 was grown in 4 mL of MRS medium supplemented with 10 µg/mL erythromycin at 37 °C and 250 rpm. After overnight growth, bacteria were sub-cultured to low OD_{600nm} and incubated at 37 °C and 250 rpm.

- **In MRS medium:** after 8 h of growth, bacteria were again diluted to an OD_{600nm} of 0.01 with MRS supplemented with 10 µg/mL erythromycin. For the induced conditions, 5 µM of cumate, 50 µM of vanillate, or 100 ng/mL of IP673 peptide was added for induction. Next, bacteria were transferred to a 96-well plate, and a kinetic cycle was set at 37 °C or 30 °C for up to 16 h (indicated in the results section). Fluorescence intensity and OD_{600nm} values were taken every 30 min. RFU values were calculated as described in the previous section.
- **In LB or LBG media:** when bacteria reached an OD_{600nm} of 0.1 or 0.3, bacteria were transferred into either LB medium or LBG medium (LB supplemented with 0.1 M of glucose) supplemented with 10 µg/mL erythromycin. For the induced conditions, 5 µM of cumate was added. Next, bacteria were transferred to a 96-well plate, and a kinetic cycle was set at 37 °C for up to 21 h. Fluorescence intensity and OD_{600nm} values were taken every 30 min. RFU values were calculated as described in the previous section.

2.9. pH measurements

For non-encapsulated bacteria experiments, the pH of the media was measured after 0, 10, 15, and 20 h (indicated in each experiment). For encapsulated bacteria experiments, PEARLs were kept for one to three days in the incubator. The pH of the LB medium was measured every 24 h. In all cases, the supernatants were collected and centrifuged for 3 min at 10000 rpm. The supernatants were transferred into new tubes, and the pH was measured using a calibrated Elite pH Spear Pocket Tester (Thermo Scientific, Germany).

2.10. NucA Assay (in DNase medium with Methyl green)

2.10.1. Qualitative assay

Engineered *L. plantarum* WCSF1 was grown overnight in MRS medium supplemented with 10 µg/mL erythromycin at 37 °C and 250 rpm. The next day, the OD_{600nm} was measured, and bacteria were subcultured in 4 mL of MRS medium supplemented with 10 µg/mL erythromycin at an OD_{600nm} of 0.05 and incubated for approximately 5 h at 30 °C or 37 °C until the OD_{600nm} was close to 1. For the induced conditions, 5 µM of cumate was added during the subculturing. 5 h later, 10 µL of the cultures were spotted on DNase agar with Methyl green (per liter: 20 g of tryptose, 5 g of sodium chloride, 2 g of deoxyribonucleic acid, 0.05 g of methyl green, and 13 g of agar; Altmann Analytik GmbH, Germany) supplemented with 10 µg/mL of erythromycin and incubated at 37 °C for 40 h. After this incubation, the BioRad Gel Documentation System

was used to capture images of the DNase agar plates, and the discoloration of the halo generated around the spotted area was measured using ImageJ (version 1.53 k).

2.10.2. Quantitative assay

Engineered *L. plantarum* WCSF1 was grown as described in the qualitative assay section. When bacteria reached an OD_{600nm} of approximately 1, bacteria equivalent to OD_{600nm} of 0.1 were transferred to 1 mL of DNase medium supplemented with 10 µg/mL erythromycin and incubated at 30 °C or 37 °C and 250 rpm for 24 h. For the induced conditions, 5 µM of cumate was added during the subculturing. The DNase medium was obtained as described in [25]. After 24 h, the cultures were centrifuged at 7000 rpm, and 200 µL of the supernatants were added to the clear-bottom 96-well plate. The samples were analyzed in the microplate reader Infinite 200 Pro to measure the fluorescence intensity of the supernatants (Ex/Em = 633/668 nm). The Z-position was set at 19000 µm, and the gain settings at 100. The NucA concentration was correlated and calculated using the standard curve at 24 h described in [25].

2.10.3. Kinetics in the microplate reader

Engineered *L. plantarum* WCSF1 was grown in 4 mL of MRS medium supplemented with 10 µg/mL erythromycin at 37 °C and 250 rpm. After overnight growth, bacteria were sub-cultured to an OD_{600nm} of 0.01 in MRS medium supplemented with 10 µg/mL erythromycin. When bacteria reached an OD_{600nm} of approximately 0.1 and 0.3, bacteria were transferred to DNase medium with and without cumate and supplemented with 10 µg/mL erythromycin. 200 µL of the mixture was transferred to a 96-well plate, and a kinetic study was set for 20 h. NucA secretion was measured by taking measurements of the DNase medium at Ex/Em = 633/668 nm and absorbance at 600 nm. Measurements were taken every 30 min. The manual gain was set at 100, Z-position at 19000 µm, the temperature was 37 °C, and the integration time was 20 µs.

2.11. PEARLs fabrication and cumate-responsiveness

2.11.1. Fabrication

PEARLs were fabricated as described in [25]. In brief, a 3 wt% alginate solution was prepared by dissolving alginate in sterile Milli-Q water, followed by autoclaving the solution for 15 min at 121 °C. Equal amounts of 3 wt% alginate and *L. plantarum* WCSF1 were resuspended in LB medium supplemented with 10 µg/mL erythromycin (approximately 10⁸ cells per mL) and vortexed to obtain the core solution (1.5 wt%). Equal amounts of 3 wt% alginate and sterile Milli-Q water were mixed to obtain the shell solution (1.5 wt%). 10 µL droplets of core solution were pipetted and dropped into a 5 wt% CaCl₂ solution (ionic crosslinker). The droplets were left to polymerize in the ionic crosslinker for 15 min. After that, the core beads were collected and washed three times with sterile Milli-Q water. Alginate core beads were dipped into a 2-mL eppendorf with 1.5 wt% shell alginate solution, and the core bead with approximately 25 µL of shell solution was pipetted and dropped into a 5 wt% cross-linker solution. Core-shell PEARLs were left to polymerize in the cross-linker solution for 15 min before the induction experiments.

2.11.2. Cumate induction and quantification

PEARLs were transferred into a 96-well plate, and 200 µL of LB medium, or Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Germany) supplemented with 10 µg/mL erythromycin was added. For the induced conditions, 100 µM of cumate or 50 µM of vanillate was added. Next, the microplate reader was used to track bacterial growth (bacterial growth as an increase in absorbance at 600 nm) and mCherry production (Ex/Em = 587/625 nm) for 24 to 70 h, depending on the experiments. Measurements were taken every 30 min. The manual gain was set at 100, the Z-position at 19000 µm, the

temperature was 37 °C, and the integration time was 20 µs. The BioRad Gel Documentation System was also used to capture macroscopic images of the PEARLs after induction. Images were processed with ImageJ.

For the NucA experiments, 200 µL of DNase medium (supplemented with 10 µg/mL erythromycin) without or with cumate (100 µM) was added. NucA secretion was measured by taking measurements of the DNase medium surrounding the PEARLs at Ex/Em = 633/668 nm and absorbance at 600 nm after 24 h at 37 °C. The manual gain was set at 100, the Z-position at 19000 µm, and the integration time was 20 µs. After that, the wells were washed three times with DPBS. Next, 200 µL of fresh DNase medium (supplemented with 10 µg/mL erythromycin) medium without or with cumate (100 µM) was added, and measurements were taken 24 h after (day 2). The same procedure was repeated until day 7 or day 9, depending on the experiment.

2.12. Live/Dead staining and imaging of PEARLs

A sterile scalpel was used to carefully detach the shells from the cores of the PEARLs and to split the cores in half. Next, the half cores were stained with a mixture of SYTO9 and propidium iodide (PI) (Thermo Fisher Scientific) at final concentrations of 5 µM and 30 µM in DPBS, respectively. The mixture was incubated for 30 min at room temperature in static conditions. After that, the stained cores were washed with DPBS before imaging.

Imaging was performed using a Zeiss Cell Discoverer 7 microscope equipped with Zen 3.11 software (Zeiss, Oberkochen, Germany). A Zeiss Plan-Apochromat 20×/0.95 objective with an optovar 0.5× Tubelens was used for image acquisition. Excitation/emission wavelengths were set to 488 nm/410–546 nm and 561 nm/605–700 nm for detecting live and dead bacterial populations, respectively. A tile scan of the entire PEARL sample was acquired at a Z-stack interval of 5.52 µm, taken 50 µm above the plate surface. The 3D volumes of live and dead bacterial colonies were quantified using the Blob Finder tool (ZEISS Arivis Pro, Zeiss, Oberkochen, Germany). In addition to colonies stained exclusively with SYTO9 (live) or PI (dead), some colonies showed partial staining with both markers. For these SYTO9/PI-positive colonies, the volume was evenly divided between live and dead categories. The percentage of live cells (Live %) was calculated as the volume of live cells divided by the total volume of live and dead cells. Representative images were generated using orthographic maximum intensity projections (MIP) of the Z-stacks (Zen 3.11, Zeiss, Oberkochen, Germany).

2.13. Micro-PEARLs fabrication, secretion assessment, and cumate responsiveness

2.13.1. Fabrication

The core and shell solutions were prepared in the same compositions as prepared for PEARLs. 5 mL of core solution was filled in a 5 mL syringe, and 20 mL of shell solution was filled in a 20 mL syringe. Micro-PEARLs were fabricated using the microencapsulator B-390 (Buchi, Switzerland). The autoclaved reaction vessel was arranged with a 150 µm nozzle (for the core) and a 300 µm nozzle (for the shell). Syringes with core and shell alginate solutions were secured to the respective entry points of the aseptic reaction vessel. 200 mL of crosslinker solution (5 wt% CaCl₂ solution) was introduced into the reaction vessel through the hose with a filter (Sartorius, Germany). The microencapsulator device was switched on and voltage (1600 V), frequency (910 Hz), syringe pump for core (20 mL/s), syringe pump for shell (7 mL/s), and a stirrer (40 %) were enabled. The micro-PEARLs with core and shell were dropped into the reaction vessel until the core solution ran out. The micro-PEARLs were left in the reaction vessel for up to 15 min for polymerization. The micro-PEARLs were collected in a sterile schott flask connected to the device through an efflux port at the bottom of the reaction vessel. The micro-PEARLs were finally washed thrice with sterile Milli-Q water. The reaction vessel and the nozzles were cleaned by pumping sterile water, and the device was autoclaved at 121 °C.

2.13.2. *NucA* secretion assessment

After the washes, the micro-PEARLs were collected with a sterile spatula and placed in the center of DNase agar (Altmann Analytik GmbH, Germany) plates supplemented with 10 µg/mL of erythromycin and incubated at 37 °C for five days. The next day, the discoloration zone was captured using the BioRad Gel Documentation System. The images were processed using ImageJ.

2.13.3. *Cumate* induction

After the washes, the micro-PEARLs were collected with a sterile spatula and placed in either 2-mL tubes or a 12-well plate. Equal weights of micro-PEARLs were distributed for all the conditions. For the induction in the tubes, 1 mL of LB medium supplemented with 10 µg/mL of erythromycin and 100 µM of cumate was added. Induction was performed for approximately 24 h. After that, induction was assessed with the BioRad Gel Documentation System or the Keyence microscope (BZ-X800E). For induction in the plates, 500 µL of LB supplemented with 10 µg/mL of erythromycin and 100 µM of cumate was added per well. The plate was placed in the microplate reader, and an increase in mCherry production (Ex/Em = 587/625 nm) was tracked for approximately 19 h. The absorbance at 600 nm was also measured for normalization of the values. Measurements were taken every 30 min. The manual gain was set at 100, Z-position at 23000 µm, the temperature was 37 °C, and the integration time was 20 µs.

2.13.4. *Fluorescence microscopy*

The Keyence microscope (BZ-X800E) was used to perform microscopy of micro-PEARLs. After the induction, micro-PEARLs were transferred to a 12-well plate with a glass bottom for better imaging. Multichannel images of micro-PEARLs were captured in 4× and 10× (S plan fluor objective) magnification using the CH3 (red fluorescence) and CH4 (brightfield) channels. The images were processed and analyzed using ImageJ.

2.14. *Statistical analysis*

Statistical analysis was done using GraphPad Prism 7.0 software. Student's *t*-tests were used to determine statistically significant differences among the means of the groups. The differences among groups are indicated as: no statistically significant difference (ns), *p*-values <0.05 (*), *p*-values <0.01 (**), *p*-values <0.005 (***), *p*-values <0.001 (****).

3. Results and discussion

3.1. *Developing a genetic switch in L. plantarum*

To create a genetic switch in *L. plantarum*, we leveraged our previously discovered phage-derived strong constitutive promoter, *P_{tec}* [24]. We modified the promoter to make it responsive to three switchable repressors from small-molecule inducible expression systems with the prospect of being used in a therapeutic context. The first one was the TetR repressor from the anhydrotetracycline (aTc) inducible system, proven to work in multiple bacteria such as *E. coli*, *Vibrio cholerae*, or *Streptococcus suis*, and commonly used in inducible expression studies in mammalian cells [34–36]. Besides, the aTc inducer has been demonstrated in mouse experiments to activate administered bacteria in vivo [37]. The second one was the CymR repressor from the cumate inducible system, which responds to cumate, a food-grade molecule, and has been adapted for bacteria such as *E. coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, as well as for mammalian cells [38–41]. The last one was the VanR repressor from the vanillate inducible system, which showed promising results in *E. coli*, *Sphingomonas*, and *Caulobacter crescentus*, and its inducer, vanillate, is also a food-grade molecule [40,42–44]. Accordingly, the *P_{tec}* promoter was flanked by the corresponding operator sequences, one placed upstream and the other downstream of the promoter sequence (Fig. S1A and S1B).

All three switches were designed with the same plasmid architecture, which harbored a *P₂₅₆* origin of replication and erythromycin resistance gene. The *mCherry* gene was positioned downstream of the operator-flanked *P_{tec}* promoter, while the repressor for each genetic switch was placed under the control of a moderate-strength constitutive promoter, *P₂₃* [45] (Fig. S1A). This was done to ensure that the repressor gene was continuously expressed, while mCherry expression could be switched ON and OFF upon the addition of the respective inducers (aTc, cumate, and vanillate) (Fig. 1A). Using flow cytometry, we assessed the responsiveness of each genetic switch to the given inducer after 6 h of induction, seeking a candidate that strongly represses *P_{tec}* in the absence of the inducer and triggers strong protein production upon induction (Fig. 1B). The aTc switch (*P₂₃_tetR_mCherry* variant) showed low leaky mCherry expression in the absence of the inducer but also very weak levels of expression upon induction with 0.2–4 µM of aTc (~3× fold-change vs uninduced, Fig. 1B, C and S1C). The cumate switch (*P₂₃_cymR_mCherry* variant) was more promising, with low levels of leakiness when uninduced (similar to wild-type *L. plantarum* WCFS1) and significantly higher levels of mCherry expression upon induction with 100 µM of cumate (~27× fold-change vs uninduced, Fig. 1B and C). Finally, the vanillate switch (*P₂₃_vanR_mCherry* variant) showed elevated levels of leakiness when uninduced and only slightly higher levels of expression with 50–200 µM vanillate (~2× fold-change vs uninduced, Fig. 1B, C and S1D). Overall, all three switches were responsive to their inducers, but since the cumate switch offered the best performance, we proceeded to further characterize and improve this switch.

For that, we first tested whether the genetic switch responds to cumate only due to inhibition of the CymR repressor. Hence, in the plasmid encoding this genetic switch, we removed the *cymR* gene and observed that bacteria constitutively produced as much mCherry as the variants with the repressor that were induced by cumate (100 µM). This not only proved that induction was mediated by the repressor but also suggested that cumate fully inactivated it (Fig. 1D). By varying the inducer concentration, we further determined that 5 µM cumate was sufficient for maximal induction, below which expression levels dropped in a seemingly linear manner (Fig. 1E). This suggested the possibility of tuning expression levels by varying the concentration of cumate in the culture.

Nevertheless, we observed considerable leakiness in the switch, indicating that repression of the inducible gene is incomplete in the absence of cumate. Notably, leakiness seemed to increase with longer culture times, as indicated by the different fold-changes at 5 h (Fig. 1C) and 8 h (Fig. 1D) after induction. We speculated that this difference may be associated with bacterial density in the cultures. So, we studied the performance of the cumate switch over time with cultures having different starting bacterial densities - OD_{600nm} of 0.3, 0.1, and 0.01, and correlated the data with kinetic measurements of bacterial growth. Interestingly, regardless of the initial bacterial density, leaky expression was observed in the uninduced cultures once bacteria crossed a density of 0.4 OD_{600nm} (measured in a microplate reader) (Fig. S2A and S2C). This was in the late log-phase of growth, indicating that the operated *P_{tec}* promoter is repressed during the early and mid-log phases of growth (Fig. S2B).

We speculated that the leakiness could be associated with insufficient expression of the CymR repressor driven by the *P₂₃* promoter. Hence, we developed two additional variants by replacing *P₂₃* with *P_{thpA}*, a stronger constitutive promoter (*P_{thpA}_cymR_mCherry* variant), and with the natural (non-operated) *P_{tec}* promoter (*P_{tec}_cymR_mCherry* variant) (Fig. S3A). We observed similar trends as with the *P₂₃_cymR_mCherry* variant (Fig. 1F), with leakiness occurring in the uninduced state after 6–8 h of growth (Fig. S3B and S3C). Prominently, all variants supported similar levels and kinetics of protein production on induction. This suggested that failure of the repressor was not due to its quantity in the cell, but to some other factor associated with growth.

Since building a genetic switch based on two independent genes (the

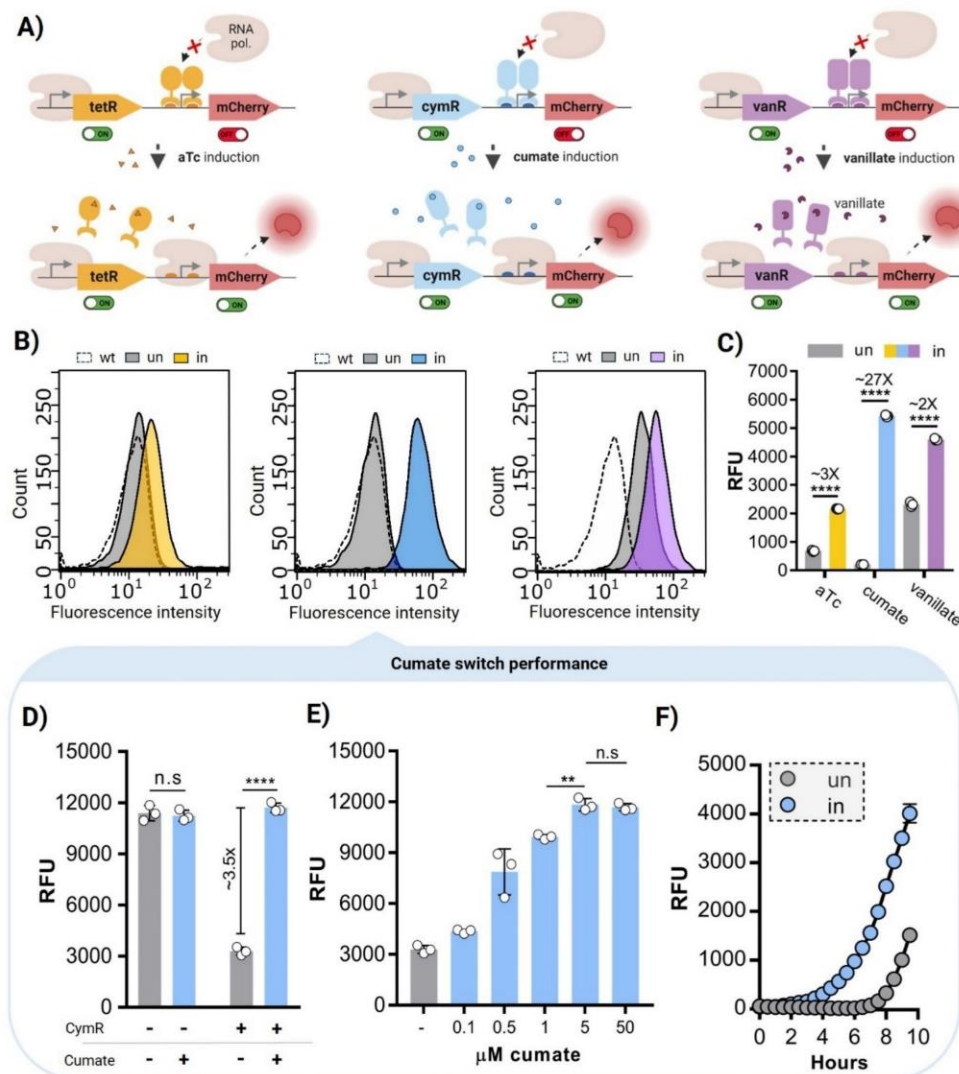


Fig. 1. A) Schemes showing the mode of action of the three inducible switches, and the expected outcome after the addition of the inducers. B) Histograms obtained by flow cytometry showing the red fluorescence intensity of the uninduced and induced states of each switch. For all, the fluorescence intensity of non-engineered *L. plantarum* is shown as a reference for leakiness. Bacteria were subcultured to an OD_{600nm} of 0.05 and induced for 6 h with 0.2 μM of aTc, 100 μM of cumate, and 50 μM of vanillate, respectively. C) RFU values for the aTc, cumate, and vanillate switches after 5 h of induction (starting OD_{600nm} = 0.05, n = 3, mean ± SD). “un” = uninduced condition, “in” = induced condition (0.2 μM of aTc, 100 μM of cumate, and 50 μM of vanillate). D) RFU values for the *P₂₃-cymR-mCherry* variant after 8-h induction with 100 μM of cumate. The presence of the repressor cymR in the plasmid and the addition of cumate is shown as “+”. The absence of the repressor cymR and cumate are shown as “-” (starting OD_{600nm} = 0.05, n = 3, mean ± SD). E) RFU values for the *P₂₃-cymR-mCherry* after inducing with different cumate concentrations for 8 h (starting OD_{600nm} = 0.05, n = 3, mean ± SD). F) RFU values for the cumate switch after inducing with 5 μM of cumate for 10 h (starting OD_{600nm} = 0.01, n = 3, mean ± SD). “un” = uninduced condition, “in” = induced condition (5 μM of cumate). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

repressor gene and the inducible gene) did not address the leakiness problem, we redesigned the switch employing a feedback circuit where both the repressor (*cymR*) and gene of interest (*mCherry*) are under the same operated *P_{lec}* promoter, hypothetically achieving gene expression only in the presence of cumate (Fig. S3D). This feedback mechanism has been used in genetic switches in *E. coli* to improve the stability of the switch, but it has never been attempted in *L. plantarum* [46,47]. To ensure the production of both proteins, we included the *P₂₃* ribosome-binding site between the STOP codon of the *mCherry* gene and the

START codon of the *cymR* gene (Fig. S3D). We termed this variant *P_{lec}-cymR-mCherry* (polycistronic). This variant showed similar results concerning the leakiness of the switch. However, interestingly, the overall levels of production were higher (Fig. S3E). The early leakiness could be associated with a compromise in the production levels of the downstream gene (*cymR*) compared to the upstream gene (*mCherry*), as already reported by us in *L. plantarum* with polycistronic gene expression [25].

When comparing all these constructs in terms of induced fold-change

over uninduced leaky expression, the $P_{23_cymR_mCherry}$ switch emerged as the superior system with a maximal fold-change above $60\times$ at 6 h (Fig. S3F). However, it is to be noted that fold-changes for all switches vary over time, being low at the start before induced expression is detectable, increasing to a maximum while leaky expression remains poor, and reducing again as leaky expression increases.

Next, we compared the performance of $P_{23_cymR_mCherry}$ (best fold-change switch) and $P_{tec_cymR_P_mCherry}$ (highest production switch) with the most widely used genetic switch in *L. plantarum*, the pSIP system. This is a two-component system in which an inducer peptide (SppIP) binds a transmembrane receptor kinase, which in turn phosphorylates a response regulator that acts as an activator to initiate

transcription from the P_{spp} promoter [28]. As expected, we observed clear differences between the performance of the cumate and the pSIP switches. Firstly, the pSIP switch showed negligible leakiness at all the assessed bacterial densities (0.3, 0.1, and 0.01). However, the overall levels of expression were significantly lower compared to the cumate switches (Fig. S4A). For instance, after 12 h of induction, mCherry production by the pSIP_409 system was 8 to 17 times lower than that of the cumate switches (Fig. S4B). Thus, the cumate switches based on the strong P_{tec} promoter outperformed the pSIP switch in terms of protein production but were also leakier. Nevertheless, these results showed that it is possible to repress the operated P_{tec} promoter during the early and mid-log phases of growth. Finding a way to decrease the leakiness of the

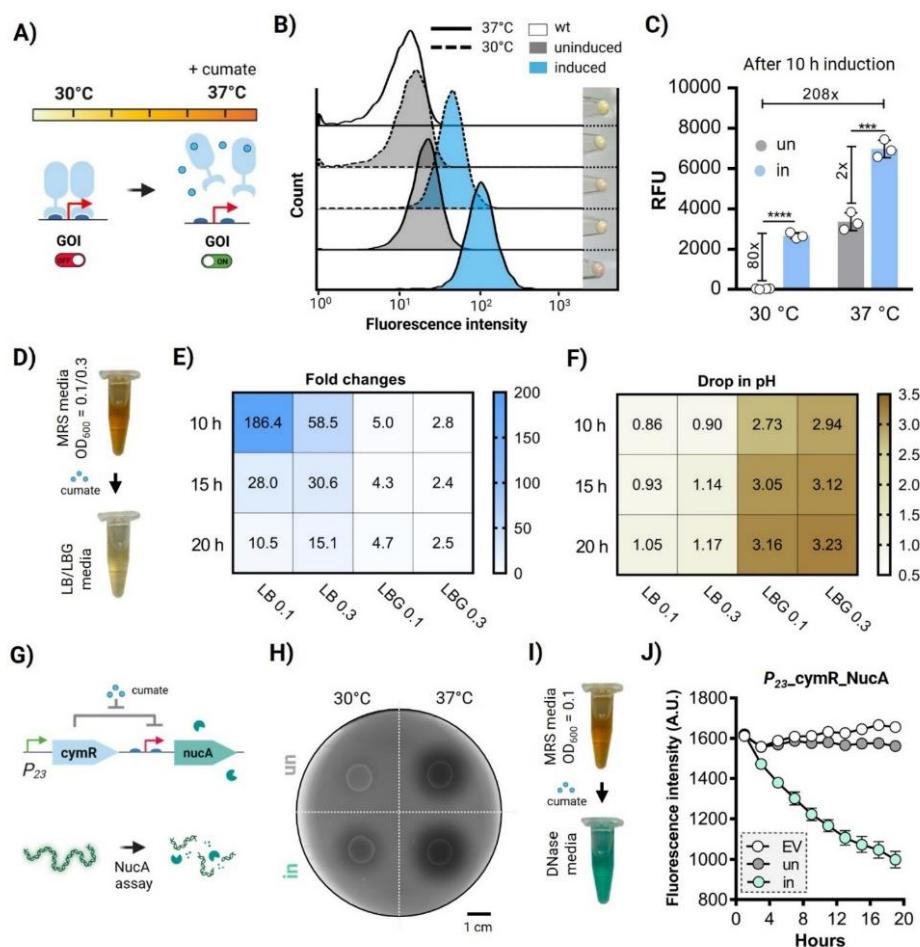


Fig. 2. A) Scheme depicting the thermally-aided switching strategy involving both temperature and cumate. Proper turning on of the gene is achieved via both the incubation at 37 °C and the addition of cumate. B) Histograms showing the fluorescence intensity (after 8 h) of the $P_{23_cymR_mCherry}$ variant at 30 °C and 37 °C and with and without the addition of cumate. Bacterial pellets are shown on the right of each condition. C) RFU values corresponding to hour 10 after induction for the $P_{23_cymR_mCherry}$ variant when induced at 37 °C and 30 °C ($n = 3$, mean $\pm$ SD). “un” = uninduced condition, “in” = induced condition (5 μ M of cumate). D) Illustration showing the procedure of bacterial growth and subculturing. Bacteria were subcultured in MRS medium and grown until an OD_{600nm} of either 0.1 or 0.3, and then transferred to LB or LBG media with and without cumate. E) Heatmap plot depicting the fold-changes between the induced and uninduced conditions in LB and LBG media with an initial OD_{600nm} of 0.1 and 0.3 after 10, 15, and 20 h of incubation at 37 °C. Bacteria were induced with 5 μ M of cumate ($n = 4$, mean $\pm$ SD). F) Heatmap depicting the drop in pH after 10, 15, and 20 h of growth in either LB or LBG media at initial OD_{600nm} of 0.1 and 0.3 and at 37 °C. The pH values were deduced from the initial pH value (0 h) of each condition (medium and OD_{600nm}). G) Scheme depicting the cumate genetic switch controlling NucA expression, which can be quantified via the NucA assay. H) 10 μ L of $P_{23_cymR_NucA}$ bacteria, uninduced and induced for 8 h in MRS medium at both 30° and 37 °C, were spotted in a DNase agar plate and incubated at 37 °C for 40 h. “un” = uninduced condition, “in” = induced condition (5 μ M of cumate). I) Illustration showing the procedure of bacterial growth and subculturing. Bacteria were subcultured in MRS medium and grown at an OD_{600nm} of either 0.1, and then transferred to DNase medium with and without cumate. J) Plot showing the drop in the fluorescence intensity as a result of the NucA secretion ($n = 4$, mean $\pm$ SD). EV = empty vector, “un” = uninduced condition, “in” = induced condition (5 μ M of cumate).

cumate switch and increase the uninduced-induced state fold-change would add great value to the genetic programmability of *L. plantarum*.

3.2. Improving the cumate switch performance

From previous experience, we were aware that the strength of many promoters in *L. plantarum* is temperature-dependent [23]. Similarly, we observed that the P_{tec} promoter is two-fold stronger at 37 °C compared to 30 °C (Fig. S5A). Furthermore, temperature also affects the growth rate of bacteria, which is typically slower at lower temperatures. Based on these, we speculated that temperature could mechanistically aid the performance of the cumate genetic switch by offering lower leakiness at low temperatures and strong induction at higher temperatures. We termed this proof-of-concept approach “thermally-aided switching”, involving both temperature and cumate to regulate the performance of the switch (Fig. 2A). We selected 30 °C for the lower end, since *L. plantarum* can still grow well at this temperature, and 37 °C for the higher end, which is equivalent to the physiological temperature of the human body.

We first evaluated the influence of temperature on the $P_{23_cymR_mCherry}$ variant through flow cytometry to confirm that the responses to temperature and cumate occurred across the entire population. Cultures prepared at a bacterial density of 0.01 OD_{600nm} were induced with 5 μM of cumate and incubated at both 30 °C and 37 °C for 8 h. Results showed low leakiness of the switch at 30 °C without induction and prominent levels of mCherry expression at 37 °C (Fig. 2B). Besides, induction at 30 °C also worked very efficiently, with the entire population switching to higher levels of fluorescence intensity. Thermally-aided switching was even visible in the coloration of the bacterial pellets (Fig. 2B), which increased with both higher temperature and cumate induction.

We then estimated the kinetic performance of the genetic switch at both temperatures with and without cumate. The $P_{23_cymR_mCherry}$ variant exhibited a considerable delay in the start of leaky expression when grown at 30 °C (>11 h) instead of 37 °C (>8 h) while maintaining notable induced expression in the presence of cumate by these time points (Fig. 2C). These time points corresponded to a higher bacterial density toward the end of the log phase at 30 °C (~0.7 OD_{600nm}) compared to 37 °C (~0.4 OD_{600nm}) (Fig. S5B and S5C). This suggested that at 30 °C, leakiness is suppressed until a slightly higher biomass compared to 37 °C, although induced protein production at 30 °C is lower. Accordingly, after 10 h of induction, we calculated an 80-fold difference between uninduced and induced states at 30 °C, and a 208-fold difference between the uninduced state at 30 °C and the induced state at 37 °C (Fig. 2C).

Alternatively, we tested the cumate switch in a non-optimal medium to limit the growth of the bacteria while maintaining its protein production capability. Bacteria were grown until an OD_{600nm} of either 0.1 or 0.3 in MRS medium and then transferred into either LB medium or LB medium supplemented with 0.1 M of glucose (LBG) (Fig. 2D). Minimal leakiness was detected in LB medium after 15 h for both 0.1 and 0.3 initial OD_{600nm}, whereas strong leaky expression started after 6 and 4 h for the 0.1 OD_{600nm} and 0.3 OD_{600nm} bacteria grown in LBG medium, respectively (Fig. S6A). When glucose was added, bacterial growth was significantly higher (Fig. S6B), confirming that the leakiness of the cumate switch is linked to the exponential growth of the bacteria. Since lactobacilli growth and metabolism are typically associated with acid formation, we suspected that pH may play a role in disrupting the activity of the repressor. Accordingly, we measured changes in pH in both LB and LBG media after 20 h of bacterial growth. As suspected, while both media had an initial pH of nearly 6.8, this value dropped much less in LB (5.6–5.7) than LBG (3.5–3.6) (Fig. S6C and S6D). Consequently, we observed an inverse correlation between the fold-change of the cumate switch and the drop in pH mediated by bacterial growth (Fig. 2E and F). The same inverse correlation between a pH drop and fold-change was observed when the bacteria were grown in the optimal MRS medium, at

30 °C and 37 °C (Fig. S7A, S7B, and S7C). These results suggested a strong correlation between bacterial growth, pH drop, and leakiness of the cumate switch. We speculate that a drop in pH could alter the conformation of the repressor protein and/or its binding affinity with the operator sequence [48–50]. This may be a key factor affecting the performance of other poorly performing genetic switches in lactobacilli that have been previously reported in the literature [30,31]. It should be noted that *L. plantarum* has been reported to maintain a higher cytoplasmic pH compared to surrounding acidic media by 0.5–1 pH units using proton pumps [51]. Thus, the pH we measured in the medium can only be considered as an indication of a pH change in the cytoplasm, but cannot precisely indicate cytoplasmic pH values. Nevertheless, our results indicated that the performance of the cumate switch can be improved by growing bacteria in an environment that limits growth to prevent a drop in pH.

Since the cumate switch functions best under growth-limited conditions, intracellular protein production capacity would be limited by the amount of biomass in a culture. To overcome this limitation, we tested the possibility of using the switch to mediate protein secretion, which would enable more protein to accumulate in the culture from a given biomass. Protein secretion is also of great interest for industrial and medical applications since it allows for easier protein purification and programming bacteria to exert extracellular bioactivity [52–54]. For this, we used NucA, a nuclease from *Staphylococcus aureus*, as a reporter protein for secretion since there is a simple assay to measure its activity [55–57]. The assay is based on the degradation of fluorescently labeled DNA by NucA. Using this method, we previously reported the *Lp_3050* signal peptide to be highly efficient for secreting NucA with the P_{tec} promoter [25]. Accordingly, we cloned the *Lp_3050_NucA* gene under the control of the cumate switch, generating the $P_{23_cymR_NucA}$ variant (Fig. 2G).

Based on the results with the mCherry expression, we expected that at 30 °C without cumate, there would be low levels of NucA secretion, whereas at 37 °C with cumate, secretion of NucA would be higher. We evaluated thermally-aided switching in both DNase agar and liquid medium, which contained fluorescently labeled DNA. In the agar experiment, 10 μL of induced and uninduced bacteria grown in MRS until an OD_{600nm} of 1 at both 30 °C and 37 °C were spotted on DNase agar and incubated at 37 °C for roughly 40 h. We could not discern a halo around the spotted area of bacteria grown at 30 °C without cumate. On the other hand, there were clearly visible dark halos for the other three conditions, with the darkest and widest ones for the bacteria grown at 37 °C with and without cumate (Fig. 2H and S8A). These results indicate that at 37 °C, the cumate switch is also leaky for NucA, which matches the results obtained with mCherry. Image analysis of the fluorescence intensity drops in the halos revealed that, at 37 °C, the halos were slightly darker (less fluorescence) with cumate than without (Fig. S8B). For better quantification, the bacteria were grown and induced in the same way but then transferred into liquid DNase medium with or without cumate and incubated at either 30 °C or 37 °C. After 24 h, the fluorescence of the medium was measured, and the drop in fluorescence was correlated with a standard curve to estimate the concentration of secreted NucA. At 30 °C without cumate, NucA levels were below the detection limit, while over 1 nM of cumate was secreted from the bacteria with cumate. At 37 °C, roughly 9 and 11 nM were detected in the absence and presence of cumate, respectively (Fig. S8C). In contrast, the performance of the cumate switch greatly improves under low bacterial density conditions. When the bacteria were grown to an OD_{600nm} of 0.1 in MRS medium and then transferred to DNase medium (Fig. 2I), secretion of NucA under uninduced conditions was negligible for 20 h (Fig. 2J). On the other hand, a prominent and continuous drop in fluorescence was detected upon the addition of cumate over 20 h, suggesting steady production and secretion of NucA (Fig. 2J).

Overall, the results with both mCherry and NucA proved that thermally-aided switching and the use of nutrient-limiting medium can be efficient approaches to tightly regulate gene expression under the

cumate switch in *L. plantarum* when grown in culture.

3.3. High-performance encapsulated genetic switches

In our previously reported alginate core-shell encapsulation format for genetically engineered *L. plantarum* (PEARL) [25], we observed steady protein production and secretion for fourteen days without outgrowth of the bacteria. The results from that study suggested that the encapsulated bacteria stopped growing after a day or two but remained active to produce and secrete P_{lec} -driven recombinant proteins at a rate corresponding to log-phase in liquid cultures. From the results of the current study, which suggest the best performance of the cumate switch in the early to mid-log phase, we speculated that encapsulation in the PEARL format could prolong this performance by restricting the bacteria both mechanically and metabolically. Since cumate is a small-molecule inducer, it was expected to diffuse inside the PEARLs and activate protein production in the encapsulated bacteria (Fig. 3A). After fabricating PEARLs with the $P_{23\text{-cymR}}$ mCherry variant, cumate was added to the medium surrounding them, and fluorescence was measured over three days. The expression of mCherry in these induced PEARLs was detected after 4 h, slowly increased to ~12 h, then rapidly increased to nearly 24 h (Fig. 3B). The initial slow increase in expression may be due to the bacterial metabolism simultaneously supporting growth and mCherry production during this time. This is supported by our previously

reported observation that *L. plantarum* growth in PEARLs plateaus around 12 h [25]. Beyond this point, the metabolic resources in the bacteria are forced predominantly toward mCherry production. The slight drop in fluorescence after 24 h suggests a saturation of the expressed protein within the bacteria and possibly bacterial death due to it (Fig. 3B). More striking was the observation that leaky mCherry expression in the uninduced PEARLs was only observed after 15 h, increased a little to ~20 h, and plateaued beyond this time point. On calculating the fold-change between the induced and uninduced PEARLs, the highest value was surprisingly observed at 10.5 h ($92.5\times$), possibly due to negligible leaky expression, and after 20 h, it stabilized between $25\text{--}35\times$ (Fig. S9A). These results confirmed our hypothesis that encapsulation can help to reduce the leakiness of the cumate switch.

One possible explanation is due to the stable maintenance of the pH. We measured the pH in the medium surrounding the PEARLs over three consecutive days of incubation at 37 °C, and the drop was 0.80, 0.81, and 0.93 after one, two, and three days of incubation, respectively (Fig. 3C). Similar drops, 0.77 and 0.82, were measured when the media were refreshed each day after the first 24 h (Fig. 3C). These magnitudes of pH drops are similar to those seen with non-encapsulated cells in LB medium, where the highest fold-changes were recorded (Fig. 2E). While this is the most apparent physiological parameter we were able to observe, it is possible that encapsulation maintains the bacteria in a metabolic state similar to that of early log-phase or low bacterial density

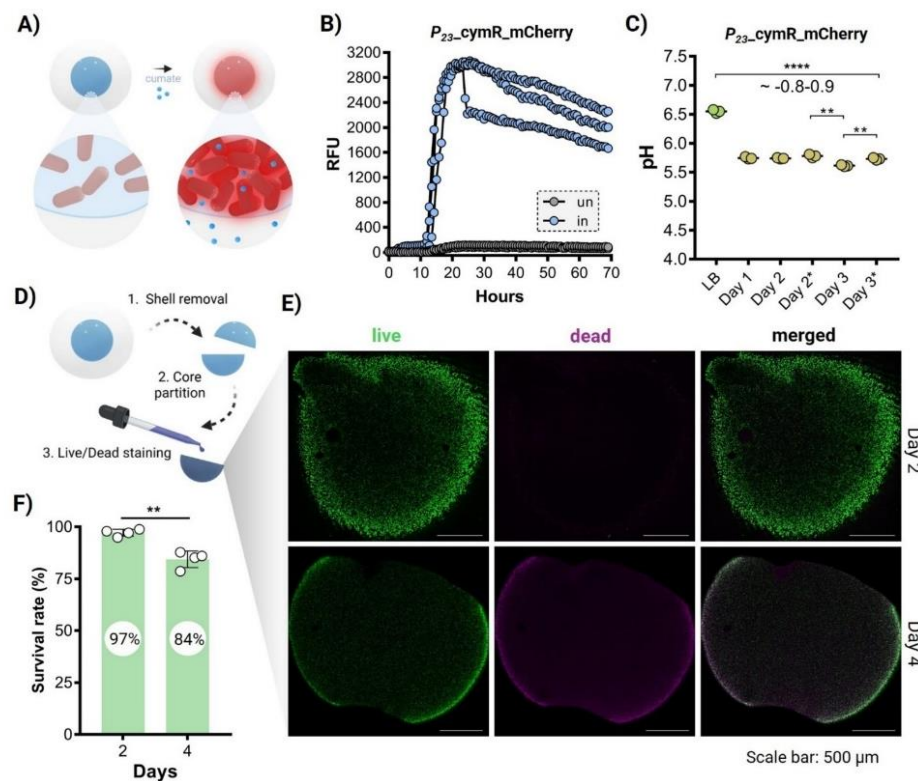


Fig. 3. A) Scheme showing the induction of *L. plantarum* bacteria harboring the cumate switch when encapsulated in PEARLs. Upon induction, the core of the PEARLs turns red due to bacterial activation and subsequent mCherry intracellular production. B) RFU for the $P_{23\text{-cymR}}$ mCherry variant when encapsulated in PEARLs and incubated at 37 °C for 24 h ($n = 3$, mean $\pm$ SD). “un” = uninduced condition, “in” = induced condition (100 μ M of cumate). C) Plot showing the significant pH drop in the LB medium when PEARLs were incubated for one to three days at 37 °C. * = fresh LB medium was added every 24 h. D) Illustration showing how PEARLs were processed before the live/dead imaging. The shell was carefully detached from the core, which was portioned in half prior to being subjected to the live/dead staining for 30 min at room temperature in static conditions. The stained cores were washed with DPBS before imaging. E) Representative images of the live/dead staining of the cores of PEARLs after two and four days of incubation. F) Plot showing the percentage of bacterial survival in the PEARLs after two and four days ($n = 4$, mean $\pm$ SD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conditions in culture. Further studies involving transcriptomics of the encapsulated cells could offer insights into this, although methods for reliable transcriptomic measurements from PEARLs are under development and not trivial. Over four days, the viability of the bacteria, determined by live/dead staining and confocal microscopy (Fig. 3D and E), revealed a slight drop from an average of 97 % viable on day 2 to 84 % on day 4 (Fig. 3F). This could be contributing to the drop in fluorescence seen after day 1 (Fig. 3B).

When comparing the behavior of $P_{23_cymR_mCherry}$ with $P_{rec_cymR_P_mCherry}$ in the PEARL format, we found that both performed very similarly, with $P_{rec_cymR_P_mCherry}$ being a bit leakier (Fig. S9B and S9C). In comparison, the pSIP_409 variant in the PEARL format exhibited expectedly negligible levels of uninduced leaky expression and low levels of induced mCherry production - ~ 37 and ~ 50 times lower than the $P_{23_cymR_mCherry}$ and the $P_{rec_cymR_P_mCherry}$ variants, respectively (Fig. S9D). Thus, encapsulation enhances the performance of the cumate switch by suppressing leaky expression but does not improve the performance of the pSIP_409 switch, since it has no influence over the promoter strength.

As an additional demonstration, we tested the performance of the cumate switch in a mammalian cell culture medium, DMEM. This is a sub-optimal medium for *L. plantarum* growth, and we have previously shown the possibility of secreting therapeutic proteins in it using PEARLs [25]. As anticipated, the cumate switch also performed really well in this medium with strong inducible expression at 24 h and poor uninduced expression (Fig. S9E). That indicates the possibility of using switchable PEARLs to regulate the production of proteins under conditions suitable for mammalian cells. Altogether, these results demonstrated that it is possible to further develop the PEARLs by becoming switchable on demand.

3.4. Switchable protein secretion from PEARLs

Since the PEARL format supported stable switching of mCherry production, we proceeded with the encapsulation of the $P_{23_cymR_NucA}$ variant to test switchable protein secretion. For this, we incubated the PEARLs in liquid DNase medium and measured changes in the fluorescence intensity over time (Fig. 4A). Over nine days, negligible leaky expression was observed in uninduced conditions, whereas a prominent fluorescence drop was detected on all days, indicating steady secretion and release of NucA from the PEARLs (Fig. 4B). The magnitude of the fluorescence intensity drop reduced from ~ 60 % to ~ 40 % over the first five days, after which it remained steady till day 9. This may be due to a loss in bacterial viability over the first five days and then stabilization of the encapsulated population. Next, we tested four different switching scenarios over two days in the absence and presence of cumate: i) OFF-OFF - PEARLs incubated in DNase medium without cumate for one day, then washed with sterile Milli-Q water, followed by another entire day incubation with fresh DNase medium without cumate. ii) OFF-ON - day 1 without cumate and day 2 with cumate. iii) ON-OFF - day 1 with cumate and day 2 without cumate. iv) ON-ON - day 1 with cumate and day 2 with cumate. The OFF-OFF scenario showed that the genetic switch remained in the OFF state on both days with no detectable NucA secretion (same as control), which means that encapsulation effectively prevented leaky expression (Fig. 4C, top left). The OFF-ON scenario also worked well, with encapsulated bacteria being activated only on the second day upon the addition of cumate in the fresh DNase medium (Fig. 4C, bottom left). This finding is particularly interesting from the ELM perspective since it highlights that encapsulated bacteria could remain inactive for hours and then be activated on demand. For the ON-ON scenario, as expected, we detected a strong drop in fluorescence for the first and second day as well (Fig. 4C, top right). Finally, in the ON-OFF scenario, while there was strong secretion on the first day, switching did not effectively occur on the second day. There was a slight decrease in the magnitude to which fluorescence in the DNase medium dropped (53 % and 38 % drop in fluorescence compared to the control

on the first and second day, correspondingly), but the inactivation was not as sharp as the activation in the OFF-ON scenario (Fig. 4C, bottom right). This could be due to retention of cumate within the PEARLs, keeping the switch active, slow release of accumulated NucA from the alginate, or continued secretion of NucA that was already produced in the bacteria. Most likely, all three phenomena influenced this outcome. Nevertheless, the result indicates that encapsulation can prolong the ON state beyond the period when the inducer is present. This is desirable in scenarios like living therapeutics for prolonged drug release with a short activation signal.

Based on these results, we studied the OFF-ON and the ON-OFF switching scenarios in more detail over seven days. It was possible to activate NucA secretion from encapsulated bacteria using cumate after leaving them in the OFF state for two and five days (Fig. 4D and E). Notably, the level of NucA release was lower on the later days, with fluorescence intensity drops of the DNase medium reaching 30–40 % on day 7. As for the ON-OFF switching condition, we observed that PEARLs activated on day 0 and turned OFF after day 1 kept releasing NucA for up to four days. Importantly, by day 5, almost no NucA was observed in the medium, indicating that the cumate switch retained its tight control over expression even after being activated once. Moreover, the switch could be activated once more on day 6, resulting in a ~ 30 % drop in DNase medium fluorescence intensity by day 7 (Fig. 4F). These results indicated the possibility of switching between different states in PEARLs, which is highly desirable for developing smart ELMs [58].

3.5. Dual-mode PEARLs

Bacterial encapsulation within PEARLs enhanced the performance of the cumate switch by reducing the non-induced state leakiness while maintaining activity in the induced state. Therefore, we tested whether it would work for the highly leaky vanillate switch (Fig. 1B, C and S1C). Accordingly, we observed a considerably enhanced performance of the vanillate switch when bacteria were encapsulated within PEARLs, with fold-changes close to $15\times$ after 20 h of induction (Fig. 5A and S10A). These results were validated by fluorescence imaging, where red fluorescence was observed only in the cores of the induced PEARLs (Fig. 5B). On the other hand, kinetic analysis of mCherry production in non-encapsulated bacteria with the vanillate switch yielded very poor fold-changes that were consistently below $1.5\times$ (Fig. S10A and S10B). Thus, alginate bead encapsulation enhanced the performance of the vanillate switch by an order of magnitude.

Based on these promising results, we attempted co-encapsulation of the two *L. plantarum* strains engineered with cumate ($P_{23_cymR_NucA}$) and vanillate ($P_{23_vanR_mCherry}$) switches (Fig. 5C). Encapsulation of bacteria in hydrogels offers the unique opportunity to establish stable co-cultures and thereby create multi-functional ELMs [59–61]. The dual-mode PEARLs were induced individually with vanillate or cumate and with both inducers simultaneously. Fluorescence imaging revealed that only the PEARLs that were induced with vanillate showed detectable levels of mCherry expression (Fig. 5D). In parallel, their NucA secretion was assessed by monitoring the drop in fluorescence in DNase medium, which was observed only in the PEARLs induced with cumate (Fig. 5E). Furthermore, both populations can also be induced simultaneously when both inducers are added to the medium (Fig. 5D and E). When comparing the levels of secretion between single-mode (whole population being $P_{23_cymR_NucA}$) and dual-mode PEARLs (half population being $P_{23_cymR_NucA}$), we estimated a drop of 40 % for the dual-mode PEARLs. Such an expected drop indicates that only with half the population responding to induction can lead to readily detectable levels of secreted protein (Fig. S10C). These experiments demonstrated that multi-switching and multi-functional PEARLs can be created by encapsulating different engineered *L. plantarum* strains.

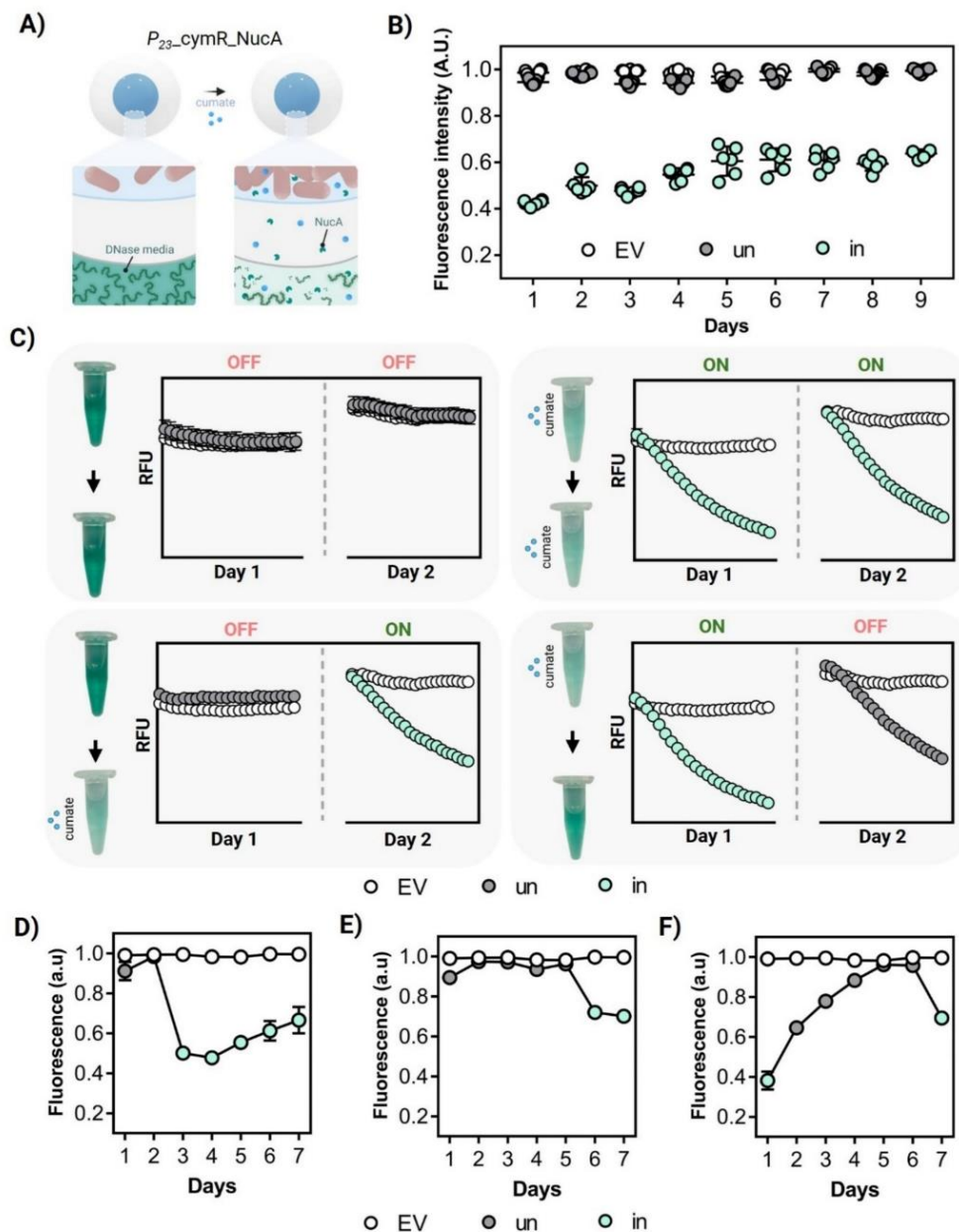


Fig. 4. A) Scheme showing the NucA production regulated by the cumate switch when bacteria are encapsulated in PEARLS. The cumate diffuses inside the core and activates the bacteria, triggering the secretion of NucA into the shell. The diffusion of NucA into the DNase medium results in the discoloration of the medium due to NucA activity. B) Plot showing the detection of secreted NucA (as a drop in fluorescence intensity) from the supernatant of PEARLS from day 1 to day 9 ($n = 6$, mean $\pm$ SD). EV = empty vector, "un" = uninduced condition, "in" = induced condition (100 μ M of cumate). C) $P_{23_cymR_NucA}$ ON and OFF switches depending on the incubation of the PEARLS in DNase medium with or without cumate on day 1 and day 2. PEARLS were washed between days 1 and 2, and measurements are shown as RFUs ($n = 4$, mean $\pm$ SD). The images of the tubes with DNase medium are only displayed for better clarity of the switches. D) Extended OFF-ON switch where PEARLS were incubated for two days in cumate-free DNase medium before induction with cumate for five consecutive days. The PEARLS were washed every day before adding fresh medium ($n = 4$, mean $\pm$ SD). E) Extended OFF-ON switch where PEARLS were incubated for five days in cumate-free DNase medium before induction with cumate for two consecutive days. The PEARLS were washed every day before adding fresh medium ($n = 4$, mean $\pm$ SD). F) Extended ON-OFF switch where PEARLS were incubated with DNase medium with cumate for one day, followed by five consecutive days in cumate-free DNase medium and a final incubation day in DNase medium with cumate. The PEARLS were washed every day before adding fresh medium ($n = 4$, mean $\pm$ SD). EV = empty vector, "un" = uninduced condition, "in" = induced condition (100 μ M of cumate).

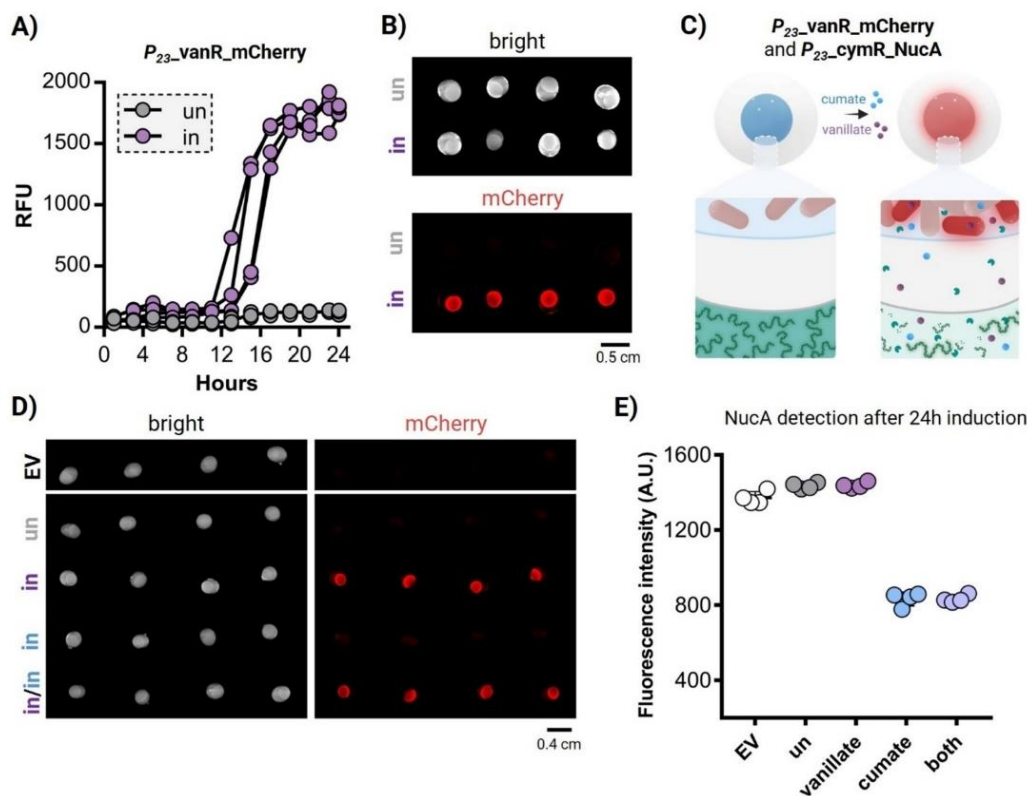


Fig. 5. A) RFU values for the induced and uninduced states of the *P23_vanR_mCherry* variant when encapsulated in PEARLs and grown at 37 °C for 23 h ($n = 4$, mean $\pm$ SD). "un" = uninduced condition, "in" = induced condition (100 μ M of vanillate). B) BioRad Gel Documentation System images of the induced and uninduced *P23_vanR_mCherry*-based PEARLs after 23 h of growth at 37 °C ($n = 4$, mean $\pm$ SD). "un" = uninduced condition, "in" = induced condition (100 μ M of vanillate). C) Scheme depicting the co-encapsulation of bacteria producing mCherry via the vanillate switch and secreting NucA via the cumate switch. Both inductions can be detected from the PEARLs by assessing the red fluorescence increase in the core of the PEARLs and the drop in the fluorescence in the DNase medium surrounding the PEARLs. D) Red fluorescence captured from the PEARLs (*P23_cymR_NucA* and *P23_vanR_mCherry*) that were incubated without any inducer, only with vanillate, only with cumate, and with both inducers at 37 °C for 24 h ($n = 4$, mean $\pm$ SD). Images were taken with the BioRad Gel Documentation System. EV = empty vector, "un" = uninduced condition, "in" = induced condition (100 μ M of cumate, 100 μ M of vanillate, or both). E) Drop in fluorescence triggered by NucA secretion into the DNase medium from the PEARLs. Empty Vector PEARLs were kept as a negative control ($n = 4$, mean $\pm$ SD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Cumate genetic switch in micro-PEARLs

Encouraged by these results, we proceeded to develop the PEARL system into a more compact format better suited for different applications. Furthermore, the PEARL format faces other limitations since it is a time-consuming manual approach that can lead to fabrication errors and has poor scalability. Therefore, we adopted a more automated, time-saving, up-scalable approach to produce a higher number of beads with smaller sizes [62,63]. For this, we used an electrospray micro-encapsulator B-390/B-395 (Buchi) to generate core-shell alginate microbeads (micro-PEARLs) that ranged in size from 100 μ m to 500 μ m. In these micro-PEARLs, we encapsulated a single *L. plantarum* strain that constitutively produces a red fluorescent protein, mScarlet3, and secretes NucA (Fig. 6A). Hence, we could simultaneously monitor gene expression within the micro-PEARLs by tracking red fluorescence production and assessing protein secretion through the DNase assay. After incubating the micro-PEARLs for 24 h on a DNase agar plate, we detected a prominent dark halo around the set of micro-PEARLs and red fluorescence in their core. Furthermore, the halo increased in size after five days, suggesting continued secretion of NucA over time (Fig. 6B and S11A). Moreover, bacterial growth within the cores could be observed

even by eye after incubating the micro-PEARLs in 2-mL tubes with MRS medium for 24 h (Fig. 6C).

Next, we evaluated whether these micro-PEARLs could be induced upon the addition of cumate in the medium. For that, we used the *P23_cymR_mcherry* variant, which would ideally produce mCherry in the core of the micro-PEARLs when cumate is added to the medium (Fig. 6D). As expected, it was possible to track the induction over time with a clear increase in red fluorescence only in the presence of cumate (Fig. 6E). Kinetic analysis over 19 h clearly revealed that even in the micro-PEARLs format, encapsulation ensured almost completely uninduced repression of mCherry expression, and the highest fold-change, >16, was achieved after 13 h of induction (Fig. S11B). This difference was visible through macroscopic fluorescence imaging (Fig. 6F). Microscopic analysis of the micro-PEARLs revealed mCherry fluorescence predominantly in the cores and only in those that were induced by cumate (Fig. 6G, S11C, and S11D). These results validate that the more automated, time-saving, effective, and compact encapsulation method for *L. plantarum* allows the bacteria to grow efficiently while maintaining them in an active and cumate-switchable state.

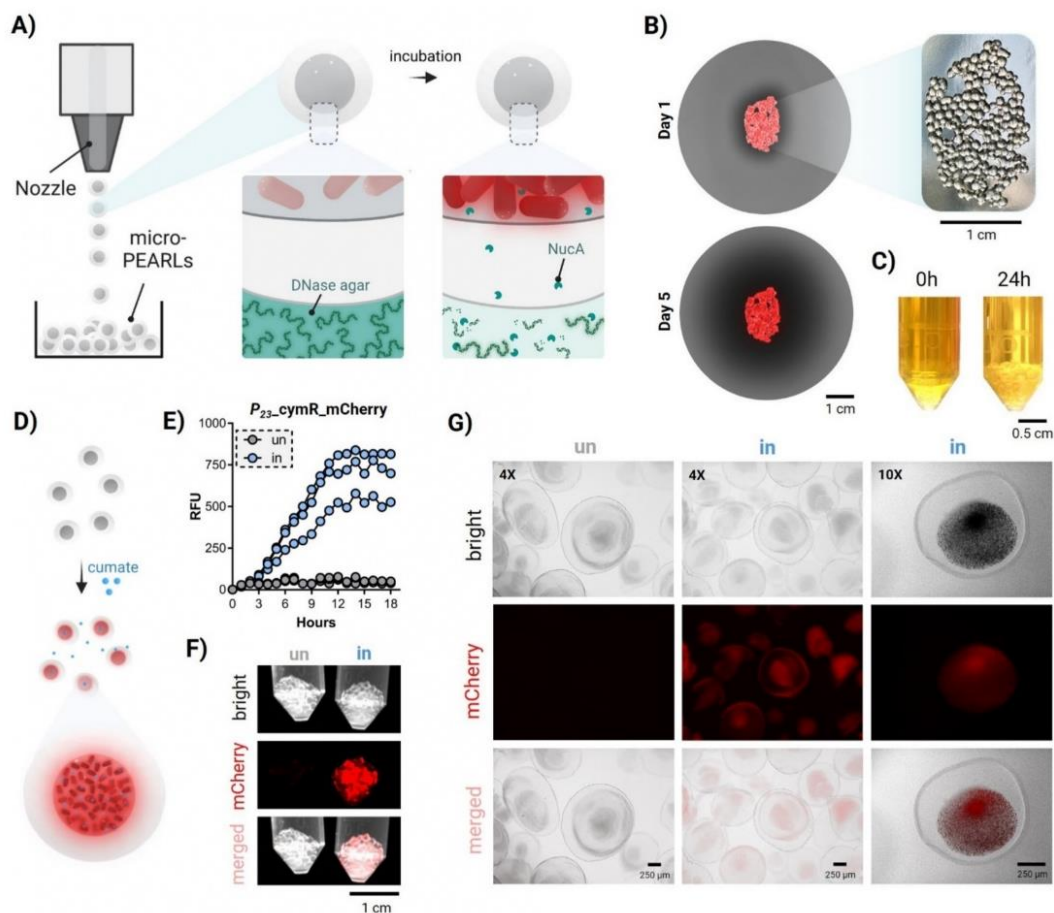


Fig. 6. A) Scheme showing micro-PEARL fabrication with the microencapsulator. The micro-PEARLS contain bacteria that produce mScarlet3 and secrete NucA. After incubation, the core of the micro-PEARLS turns red, and the fluorescence of the DNase agar fades away due to the degradation of fluorescently labeled DNA. B) DNase agar plates with micro-PEARLS after one and five days of incubation at 37 °C. Images taken using the BioRad Gel Documentation System. C) 2-mL tubes with micro-PEARLS incubated in MRS medium after 0 and 24 h. D) Scheme of the cumate induction in micro-PEARLS. Upon induction, the core of the micro-PEARLS turns red due to mCherry intracellular production. E) RFU values of the $P_{23}\text{-cymR-mCherry}$ variant when encapsulated in micro-PEARLS and induced with cumate for 19 h ($n = 3$, mean $\pm$ SD). “un” = uninduced condition, “in” = induced condition (100 μM of cumate). F) BioRad Gel Documentation System images of uninduced (un) and induced (in) micro-PEARLS after 24 h of incubation at 37 °C. Induction was done with 100 μM of cumate. G) Microscopy images, at both 10 $\times$ and 4 $\times$, of uninduced and induced micro-PEARLS after 24 h of incubation at 37 °C. “un” = uninduced condition, “in” = induced condition (100 μM of cumate). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

In this study, we have successfully modified *L. plantarum*'s currently strongest constitutive promoter, P_{tec} , into genetic switches responsive to cumate and vanillate. However, the switches suffered from leakiness of gene expression associated with bacterial growth and a drop in pH, especially when the bacteria entered the late log phase. Due to the natural capacity of P_{tec} to be more active at higher temperatures, we explored a workaround to reduce the leakiness of the cumate switch through thermally-aided switching, in which the uninduced state was maintained at 30 °C and cumate induction was performed at 37 °C. The performance of the cumate switch was also increased by subculturing the bacteria in a non-optimal medium that limited growth but supported protein production and secretion. However, a more promising strategy to enhance the performance of the switch was discovered through the encapsulation of the engineered bacteria in alginate beads to form PEARLS. This surprisingly suppressed leaky expression of the cumate switch, while supporting high levels of protein production and secretion,

thereby stably maintaining high fold-changes of induction. This effect was not observed for unencapsulated bacteria grown in MRS, LBG, and LB, where fold-changes were either low or rapidly decreased over time (Fig. 7).

This enhanced performance extended beyond the cumate switch to the much leakier vanillate switch and even sustained the performance of both switches in a co-encapsulation setting. Furthermore, the encapsulation-enhanced switching was not dependent on the size of the encapsulation matrix, as the effect persisted even in micro-PEARLS, which can be rapidly produced in an automated manner. While the functionality of these switches has been demonstrated through the expression of a fluorescent protein and secretion of a nuclease as reporter proteins, their functionality can be expected to apply to other proteins of interest. In particular, therapeutic proteins like Elafin and α -MSH that were previously shown to be constitutively secreted from the PEARL system can now be expressed in a switchable manner [25]. However, it must be noted that the rate of expression and secretion will still depend on the physicochemical and biochemical properties of each

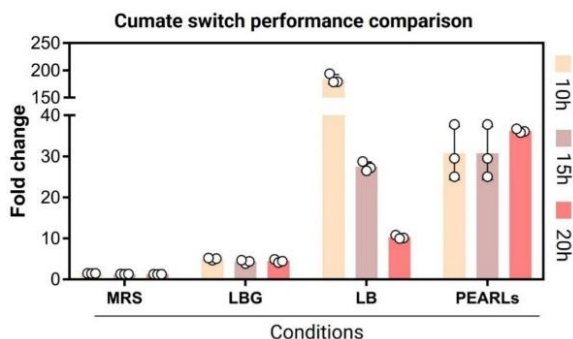


Fig. 7. Fold-change comparison of after 10, 15, and 20 h of induction for cultures grown in MRS, LBG, and LB, and for PEARL-encapsulated bacteria. Fold-changes were calculated from the kinetics shown in Fig. S4A, S6A, S6A, and S9A for the MRS, LBG, LB, and the PEARLs conditions, respectively. All conditions were incubated at 37 °C and at a starting OD_{600nm} of 0.1 (n = 3, mean ± SD).

protein. That said, these switches will offer more versatility in controlling the expression rates of recombinant proteins, especially under encapsulated conditions. Thus, these encapsulation-enhanced switches are promising for the development of lactobacilli-based ELMs toward stimuli-responsive therapeutic platforms for medical applications [64,65].

CRediT authorship contribution statement

Marc Blanch-Asensio: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Varun Sai Tadimarri:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Roberto Martinez:** Methodology. **Gurvinder Singh Dahiya:** Writing – review & editing, Conceptualization. **Cao Nguyen Duong:** Methodology, Investigation, Data curation. **Rahmi Lale:** Writing – review & editing, Conceptualization. **Shrikrishnan Sankaran:** Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization.

Funding

This work was supported by a Deutsche Forschungsgemeinschaft (DFG) Research grant (Project # 455063657), a DFG Collective Research Center (SFB1027) subproject grant (Project #200049484), and the Leibniz Association through the Leibniz Science Campus on Living Therapeutic Materials (LifeMat).

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

We thank Prof. Geir Mathiesen (Norwegian University of Life Sciences) for sharing the pLp_3050sNuc plasmid (Addgene plasmid # 122030) with us. The *L. plantarum* WCFS1 strain was a kind gift from Prof. Gregor Fuhrmann (Friedrich-Alexander-Universität, Erlangen-Nürnberg). The authors used Biorender for the illustrations of this study. We thank the group of Prof. Wilfried Weber for granting access to their Buchi B-390 microencapsulator device for the fabrication of the micro-PEARLs and providing initial training for the device.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2025.114264>.

Data availability

Data will be made available on request.

References

- [1] A. Cubillos Ruiz, T. Guo, A. Sokolovska, P.F. Miller, J.I. Collins, T.K. Lu, J.M. Lora, Nat. Rev. Drug Discov. 20 (12) (2021) 941–960, <https://doi.org/10.1038/s41573-021-00285-3>.
- [2] M.R. Charbonneau, V.M. Isabella, N. Li, C.B. Kurtz, Nat. Commun. 11 (1) (2020) 1738, <https://doi.org/10.1038/s41467-020-15508-1>.
- [3] V.W. Kelly, B.K. Liang, S.J. Sirk, ACS Synth. Biol. 9 (12) (2020) 3184–3201, <https://doi.org/10.1021/acssynbio.0c00444>.
- [4] E. Frutos-Grilo, Y. Ana, J. Gonzalez-de Miguel, M. Cardona-i-Collado, I. Rodriguez-Arce, L. Serrano, Mol. Syst. Biol. 20 (12) (2024), <https://doi.org/10.1038/s44320-024-00067-0>, 1261–1281.
- [5] D.B. Pedrolli, N.V. Ribeiro, P.N. Squizzato, V.N. de Jesus, D.A. Cozetto, R.B. Tuma, A. Gracindo, M.B. Cesar, P.J.C. Freire, A.F.M. da Costa, M.R.C.R. Lins, G.G. Correa, M.O. Cerri, Trends Biotechnol. 37 (1) (2019) 100–115, <https://doi.org/10.1016/j.tibtech.2018.09.005>.
- [6] Q. Yan, S.S. Fong, Front. Microbiol. volume 8–2017 (2017), <https://doi.org/10.3389/fmicb.2017.02060>.
- [7] M. Blanch-Asensio, S. Dey, V.S. Tadimarri, S. Sankaran, Microb. Biotechnol. 17 (1) (2024) e14335, <https://doi.org/10.1111/1751-7915.14335>.
- [8] J. Hahn, S. Ding, J. Im, T. Harimoto, K.W. Leong, T. Danino, Nat. Rev. Bioeng. 2 (2) (2024) 120–135, <https://doi.org/10.1038/s44222-023-00119-4>.
- [9] S. Dey, S. Sankaran, Trends Biotechnol. 42 (12) (2024) 1663–1676, <https://doi.org/10.1016/j.tibtech.2024.06.011>.
- [10] M. Afzaal, A.U. Khan, F. Saeed, A. Ahmed, M.H. Ahmad, A.A. Maan, T. Tufail, F. M. Anjum, S. Hussain, Food Sci. Nutr. 7 (12) (2019) 3931–3940, <https://doi.org/10.1002/fsn3.1254>.
- [11] M. Chávarri Hueda, I. Marañón, M.C. Villarán, Encapsulation technology to protect probiotic bacteria, in: E. Rigobelo (Ed.), Probiotics, IntechOpen, Rijeka, 2012.
- [12] R.I. Corona-Hernandez, E. Álvarez-Parrilla, J. Lizardi-Mendoza, A.R. Islas Rubio, L. A. de la Rosa, A. Wall-Medrano, Compr. Rev. Food Sci. Food Saf. 12 (6) (2013) 614–628, <https://doi.org/10.1111/1541-4337.12030>.
- [13] A.C. Anselmo, K.J. McHugh, J. Webster, R. Langer, A. Jaklenec, Adv. Mater. 28 (43) (2016) 9486–9490, <https://doi.org/10.1002/adma.201603270>.
- [14] R. Rajani, P. Subramanian, Beni-Suef Univ. J. Basic Appl. Sci. 11 (1) (2022) 46, <https://doi.org/10.1186/s43088-022-00228-w>.
- [15] A. Xie, M. Gao, H. Du, X. Pan, Curr. Opin. Food Sci. 61 (2025) 101234, <https://doi.org/10.1016/j.cofs.2024.101234>.
- [16] R. Omer, M.Z. Mohsin, A. Mohsin, B.S. Mushtaq, X. Huang, M. Guo, Y. Zhuang, J. Huang, Fron. Bioeng. Biotechnol. 10–2022 (2022), <https://doi.org/10.3389/fbioe.2022.870675>.
- [17] X. Dong, W. Wu, P. Pan, X. Z. Zhang, Adv. Mater. 37 (2025) 2304963, <https://doi.org/10.1002/adma.202304963>.
- [18] A. Cebeci, C. Güllükan, Food Microbiol. 20 (5) (2003) 511–518, [https://doi.org/10.1016/S0740-0020\(02\)00174-0](https://doi.org/10.1016/S0740-0020(02)00174-0).
- [19] S.D. Todorov, B.D.G.D.M. Franco, Food Rev. Int. 26 (3) (2010) 205–229, <https://doi.org/10.1080/87559129.2010.484113>.
- [20] A. Aljohani, N. Rashwan, S. Vasani, A. Alkhawashki, T.T. Wu, X. Lu, D.A. Castillo, J. Xiao, Prob. Antimicrobial Proteins (2024), <https://doi.org/10.1007/s12602-024-10287-3>.
- [21] H.A. Seddik, F. Bendali, F. Gancel, I. Fliss, G. Spano, D. Drider, Prob. Antimicrobial Proteins 9 (2) (2017) 111–122, <https://doi.org/10.1007/s12602-017-9264-z>.
- [22] S.S. Behera, R.C. Ray, N. Zdolec, Biomed. Res. Int. 2018 (1) (2018) 9361614.
- [23] S. Dey, M. Blanch-Asensio, S. Balaji Kuttac, S. Sankaran, Microb. Biotechnol. 16 (6) (2023) 1264–1276, <https://doi.org/10.1111/1751-7915.14228>.
- [24] M. Blanch-Asensio, V.S. Tadimarri, A. Wilk, S. Sankaran, Microb. Cell Factories 23 (1) (2024) 42, <https://doi.org/10.1186/s12934-024-02302-7>.
- [25] V.S. Tadimarri, M. Blanch-Asensio, K. Deshpande, J. Baumann, C. Baumann, R. Müller, S. Trujillo, S. Sankaran, Small (2025) 2408316, <https://doi.org/10.1002/smll.202408316>.
- [26] J. De Baets, B. De Paep, M. De Mey, Microb. Cell Factories 23 (1) (2024) 249, <https://doi.org/10.1186/s12934-024-02523-w>.
- [27] T. Brautaset, R. Lale, S. Valla, Microb. Biotechnol. 2 (1) (2009) 15–30, <https://doi.org/10.1111/j.1751-7915.2008.00048.x>.
- [28] E. Sørvig, G. Mathiesen, K. Naterstad, V.G.H. Eijsink, L. Axelsson, Microbiology 151 (7) (2005) 2439–2449, <https://doi.org/10.1099/mic.0.28084.0>.
- [29] L.L. Karlskäs, K. Maudal, L. Axelsson, I. Rud, V.G.H. Eijsink, G. Mathiesen, PLoS One 9 (3) (2014) e91125, <https://doi.org/10.1371/journal.pone.0091125>.
- [30] S. Heiss, A. Hörmann, C. Tauer, M. Sonnleitner, E. Egger, R. Grabherr, S. Heindl, Microb. Cell Factories 15 (1) (2016) 50, <https://doi.org/10.1186/s12934-016-0448-0>.
- [31] S. Zhang, Z. Xu, L. Qin, J. Kong, Biochem. Eng. J. 151 (2019) 107316, <https://doi.org/10.1016/j.bej.2019.107316>.

- [32] J.A. Martínez-Fernández, D. Bravo, Á. Peirotén, J.L. Arqués, J.M. Landete, *Appl. Microbiol. Biotechnol.* 103 (9) (2019) 3819–3827, <https://doi.org/10.1007/s00253-019-09743-w>.
- [33] M. Blanch-Asensio, S. Dey, S. Sankaran, *PLoS One* 18 (2) (2023) e0281625, <https://doi.org/10.1371/journal.pone.0281625>.
- [34] X.R. Bina, E.A. Wong, T.F. Bina, J.E. Bina, *Plasmid* 76 (2014) 87–94, <https://doi.org/10.1016/j.plasmid.2014.10.004>.
- [35] L. Zhang, W. Zou, M. Ni, Q. Hu, L. Zhao, X. Liao, Q. Huang, R. Zhou, *Microbiol. Spectrum* 10 (4) (2022), <https://doi.org/10.1128/spectrum.00363-22> e00363–22.
- [36] R. Lutz, H. Bujard, *Nucleic Acids Res.* 25 (6) (1997) 1203–1210, <https://doi.org/10.1093/nar/25.6.1203>.
- J.W. Kotula, S.J. Kems, L.A. Shakel, L. Siraj, J.J. Collins, J.C. Way, P.A. Silver, *Proc. Natl. Acad. Sci.* 111 (13) (2014) 4838–4843.
- [38] A. Klotz, A. Kaczmarczyk, U. Jenal, *Appl. Environ. Microbiol.* 89 (6) (2023) e00211–e00223, <https://doi.org/10.1128/aem.00211.23>.
- [39] S. O. Seo, C. Schmidt Dannert, *Appl. Microbiol. Biotechnol.* 103 (1) (2019) 303–313, <https://doi.org/10.1007/s00253-018-9485-4>.
- [40] A.J. Meyer, T.H. Segall-Shapiro, E. Glassey, J. Zhang, C.A. Voigt, *Nat. Chem. Biol.* 15 (2) (2019) 196–204, <https://doi.org/10.1038/s41589-018-0168-3>.
- [41] A. Mullick, Y. Xu, R. Warren, M. Koutroumanis, C. Guilbault, S. Broussau, F. Malenfant, L. Bourget, L. Lamoureux, R. Lo, A.W. Caron, A. Pilote, B. Massie, *BMC Biotechnol.* 6 (1) (2006) 43, <https://doi.org/10.1186/1472-6750-6-43>.
- [42] A.M. Kunjapur, K.L.J. Prather, *ACS Synth. Biol.* 8 (9) (2019) 1958–1967, <https://doi.org/10.1021/acssynbio.9b00071>.
- [43] A. Kaczmarczyk, J.A. Vorholt, A. Francez-Charlot, *Sci. Rep.* 4 (1) (2014) 6453, <https://doi.org/10.1038/srep06453>.
- [44] M. Thanbichler, A.A. Iniesta, L. Shapiro, *Nucleic Acids Res.* 35 (20) (2007) e137, <https://doi.org/10.1093/nar/gkm818>.
- [45] Q. Meng, Y. Yuan, Y. Li, S. Wu, K. Shi, S. Liu, *ACS Synth. Biol.* 10 (7) (2021) 1728–1738, <https://doi.org/10.1021/acssynbio.1c00123>.
- [46] C. Tan, P. Marguet, L. You, *Nat. Chem. Biol.* 5 (11) (2009) 842–848, <https://doi.org/10.1038/nchembio.218>.
- [47] R. Daniel, J.R. Rubens, R. Sarpeshkar, T.K. Lu, *Nature* 497 (7451) (2013) 619–623, <https://doi.org/10.1038/nature12148>.
- [48] A.-M. Dri, P.L. Moreau, *Mol. Microbiol.* 12 (4) (1994) 621–629, <https://doi.org/10.1111/j.1365-2958.1994.tb01049.x>.
- [49] K.A. Krukenberg, D.R. Southworth, T.O. Street, D.A. Agard, *J. Mol. Biol.* 390 (2) (2009) 278–291, <https://doi.org/10.1016/j.jmb.2009.04.080>.
- [50] L.R. Prost, M.E. Dacey, V. Le Sage, M.W. Bader, H. Le Moual, R.E. Klevit, S.J. Miller, *Mol. Cell* 26 (2) (2007) 165–174, <https://doi.org/10.1016/j.molcel.2007.03.008>.
- [51] C.L. Ramos, L. Thorsen, M. Ryssel, D.S. Nielsen, H. Siegunfeldt, R.F. Schwan, L. Jespersen, *Res. Microbiol.* 165 (3) (2014) 215–225, <https://doi.org/10.1016/j.resmic.2014.02.005>.
- [52] F. Bai, Z. Li, A. Umezawa, N. Terada, S. Jin, *Biotechnol. Adv.* 36 (2) (2018) 482–493, <https://doi.org/10.1016/j.biotechadv.2018.01.016>.
- [53] J. Neef, J.M. van Dijk, G. Buist, *Essays Biochem.* 65 (2) (2021) 187–195, <https://doi.org/10.1042/EBC20200171>.
- [54] B. Reed, R. Chen, *Res. Microbiol.* 164 (6) (2013) 675–682, <https://doi.org/10.1016/j.resmic.2013.03.006>.
- [55] G. Mathiesen, A. Svein, M.B. Brurberg, L. Fredriksen, L. Axelsson, V.G.H. Eijsink, *BMC Genomics* 10 (1) (2009) 425, <https://doi.org/10.1186/1471-2164-10-425>.
- [56] Y. Le Loir, S. Nouaille, J. Commissaire, L. Brétigny, A. Gruss, P. Langella, *Appl. Environ. Microbiol.* 67 (9) (2001) 4119–4127, <https://doi.org/10.1128/AEM.67.9.4119-4127.2001>.
- [57] S. Nouaille, E. Morello, N. Cortez Peres, Y. Le Loir, J. Commissaire, J.J. Gratadoux, E. Poumerol, A. Gruss, P. Langella, *Appl. Environ. Microbiol.* 72 (3) (2006) 2272–2279, <https://doi.org/10.1128/AEM.72.3.2272-2279.2006>.
- [58] Y. Wang, Y. Liu, J. Li, Y. Chen, S. Liu, C. Zhong, *Curr. Opin. Chem. Biol.* 70 (2022) 102188, <https://doi.org/10.1016/j.cbpa.2022.102188>.
- [59] D.P. Birnbaum, A. Manjula Basavanna, A. Kan, B.L. Tardy, N.S. Joshi, *Adv. Sci.* 8 (11) (2021) 2004699, <https://doi.org/10.1002/advsc.202004699>.
- [60] C. Gilbert, T.-C. Tang, W. Ott, B.A. Dorr, W.M. Shaw, G.L. Sun, T.K. Lu, T. Ellis, *Nat. Mater.* 20 (5) (2021) 691–700, <https://doi.org/10.1038/s41563-020-00857-5>.
- [61] T.G. Johnston, S.-F. Yuan, J.M. Wagner, X. Yi, A. Saha, P. Smith, A. Nelson, H. S. Alper, *Nat. Commun.* 11 (1) (2020) 563, <https://doi.org/10.1038/s41467-020-14371-4>.
- [62] Y.V. Rojas Muñoz, M. de Jesús Perea Flores, M.X. Quintanilla Carvajal, *Polymers* 16 (17) (2024) 2492, <https://doi.org/10.3390/polym16172492>.
- [63] T. Berninger, B. Mitter, C. Preininger, *J. Microencapsul.* 33 (2) (2016) 127–136, <https://doi.org/10.3109/02652048.2015.1134690>.
- [64] Y. Lyu, H. Huang, Y. Su, B. Ying, W.-C. Liu, K. Dong, N. Du, R.S. Langer, Z. Gu, K. Nan, *Matter* 7 (4) (2024) 1440–1465, <https://doi.org/10.1016/j.matt.2024.01.031>.
- [65] T. Harimoto, J. Hahn, Y. Y. Chen, J. Im, J. Zhang, N. Hou, F. Li, C. Coker, K. Gray, N. Harr, S. Chowdhury, K. Pu, C. Nimura, N. Arpaia, K.W. Leong, T. Danino, *Nat. Biotechnol.* 40 (8) (2022) 1259–1269, <https://doi.org/10.1038/s41587-022-01244-y>.

Chapter 5.

Genetic modules for antibiotic-free plasmid retention

The genetic parts of all the previous chapters are cloned into plasmids that express an antibiotic resistance marker. Plasmid retention is then ensured by adding the corresponding antibiotic to the growth media. As previously mentioned, such a retention approach cannot be realistically applied due to the requirement for antibiotics.

In bacteria, the most common approach to ensure plasmid retention is by generating auxotrophic strains that rely on the presence of an essential gene in the plasmid for survival [159, 160]. However, this approach relies on knocking out the essential gene from the genome, thus generating a GMO with an irreversible modification. Instead of further exploring the auxotrophy-based plasmid retention approach and generating permanent GMOs. In this work, a new concept and perspective have been proposed: the creation of transient GMOs with tunable plasmid retention rates.

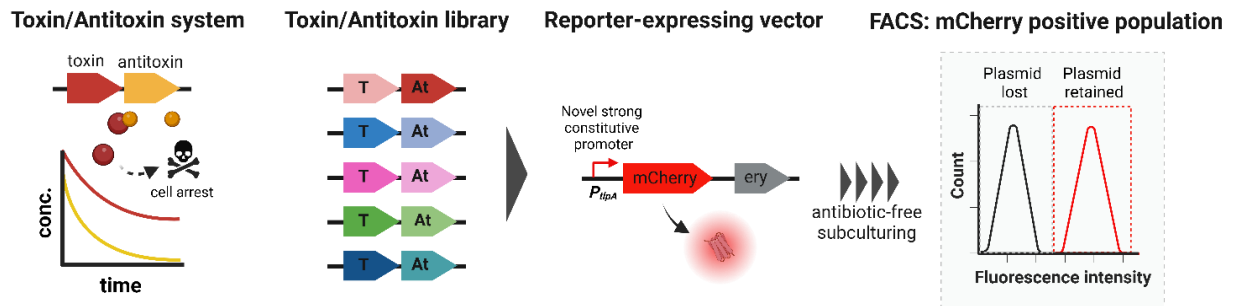
This chapter investigates whether lactobacilli are a promising source of genetic modules capable of favoring plasmid retention. Additionally, it demonstrates the applicability of these modules in mediating antibiotic-free plasmid retention without involving tampering with the genomic DNA in *L. plantarum*. To be precise, these modules are TA systems, whose mechanism of action to mediate plasmid maintenance has already been discussed.

First, type-II TA systems encoded in the genomes of lactobacilli species were identified using the TA Finder tool. A small library of TA systems was subsequently generated by integrating these systems into a plasmid vector based on the p256 ORI, a resistance marker, and the mCherry reporter gene under the constitutive promoter P_{tIpA} . The retention

or loss of the plasmid was quantified using fluorescence-activated cell sorting (FACS) by measuring the percentage of the bacterial population that was fluorescent after subsequent rounds of antibiotic-free subculturing. Non-fluorescent bacteria indicated a loss of plasmid due to the impossibility of expressing mCherry. All TA systems showed a positive impact on plasmid retention by slowing down the plasmid loss rate. More interestingly, the best results were obtained when two TA systems were combined in the same plasmid vector as two independent modules.

This publication reports on the use of TA systems to mediate plasmid retention in *L. plantarum* WCFS1. Notwithstanding, it is important to note that all the TA systems used here were not modified, meaning that the endogenous genetic architecture was preserved as it naturally exists in the bacterial hosts. That allows further optimization of each TA system for optimal performance or for generating additional combinations of two independent TA systems within a single plasmid. Additionally, the results demonstrate the functionality of both toxins and antitoxins in these bacteria, which can potentially unlock their use in other genetic scenarios in *L. plantarum*. For instance, as part of novel genetic kill switches to allow precise control of bacterial cell death by regulating the toxin production on demand [161].

Graphical Abstract



Insufficient amounts of antitoxin might lead to toxin-driven bacterial cell death in the case of plasmid loss. A small library of TA systems was generated and cloned into an mCherry-expressing plasmid to validate whether TA systems mediate plasmid retention in *L. plantarum*. Plasmid loss, quantified as non-fluorescent bacteria, or plasmid retention, as fluorescent bacteria, populations were determined using FACS after antibiotic-free subsequent rounds of subculturing.

Publication and contributions

Article Type: Research article.

Title: Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic lactobacilli ⁶.

Year Published: 2023.

Research Journal: Microbial Biotechnology.

Authors: Marc Blanch-Asensio, Sourik Dey, Sajana Balaji Kuttae, and Shrikrishnan Sankaran.

First authors: Marc Blanch-Asensio and Sourik Dey (joint co-authorship).

Author contributions: Marc Blanch-Asensio and Sourik Dey performed all the genetic engineering work. Marc Blanch-Asensio performed most of the experiments related to the TA systems. Sourik Dey performed most of the experiments concerning the promoter evaluation. Sajana Balaji Kuttae assisted in the genetic engineering experiments. Marc Blanch-Asensio made the illustrations. Sourik Dey analyzed and plotted the data. Marc Blanch-Asensio and Sourik Dey wrote the draft of the manuscript. Marc Blanch-Asensio, Sourik Dey, and Shrikrishnan Sankaran took part in the conceptualization. Shrikrishnan Sankaran led the supervision, reviewed, and edited the manuscript.

⁶ The supporting information of this publication is provided in Appendix II.5 at the end of this dissertation.

RESEARCH ARTICLE



Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic lactobacilli

Sourik Dey | Marc Blanch-Asensio | Sanjana Balaji Kuttae | Shrikrishnan Sankaran

Bioprogrammable Materials, INM - Leibniz Institute for New Materials, Saarbrücken, Germany

Correspondence

Shrikrishnan Sankaran, Bioprogrammable Materials, INM - Leibniz Institute for New Materials, Campus D2 2, 66123 Saarbrücken, Germany.
Email: shrikrishnan.sankaran@leibniz-inm.de

Funding information

Deutsche Forschungsgemeinschaft, Grant/Award Number: 455063657; Leibniz-Gemeinschaft

Abstract

Lactobacilli are ubiquitous in nature, often beneficially associated with animals as commensals and probiotics, and are extensively used in food fermentation. Due to this close-knit association, there is considerable interest to engineer them for healthcare applications in both humans and animals, for which high-performance and versatile genetic parts are greatly desired. For the first time, we describe two genetic modules in *Lactiplantibacillus plantarum* that achieve high-level gene expression using plasmids that can be retained without antibiotics, bacteriocins or genomic manipulations. These include (i) a promoter, P_{tlpA} , from a phylogenetically distant bacterium, *Salmonella typhimurium*, which drives up to 5-fold higher level of gene expression compared to previously reported promoters and (ii) multiple toxin-antitoxin systems as a self-contained and easy-to-implement plasmid retention strategy that facilitates the engineering of tuneable transient genetically modified organisms. These modules and the fundamental factors underlying their functionality that are described in this work will greatly contribute to expanding the genetic programmability of lactobacilli for healthcare applications.

INTRODUCTION

Lactobacilli are gram-positive rod-shaped lactic acid bacteria (LAB), typically found in humans and animals as commensals. Their stress tolerant phenotypic traits allow them to colonize a wide range of host microenvironments, like the gut, skin, vagina, nasal and oropharyngeal cavity (Ma et al., 2012; Turroni et al., 2014) often providing health benefits in the form of anti-inflammatory, anti-pathogenic and immunomodulatory activities (Bibalan et al., 2017; Darby & Jones, 2017). Due to this, they are one of the largest classes of probiotics and several species are being clinically tested for treating a variety of diseases like ulcerative colitis (Zocco et al., 2006), mastitis (Jiménez et al., 2008), atopic dermatitis (Rosenfeldt et al., 2003), bacterial

vaginosis (Mastromarino et al., 2009) and periodontitis (Teughels et al., 2013). Apart from their health benefits, lactobacilli are also vital for numerous fermentation processes in the food industry, for example, in the production of yogurt (Ashraf & Shah, 2011), cheese (Kasımoğlu et al., 2004), sourdough bread (Plessas et al., 2008), beer (Chan et al., 2019) and wine (du Toit et al., 2011). Due to this ubiquity in our lives, there is considerable interest to genetically enhance and expand the capabilities of these bacteria for healthcare applications (Pedrolli et al., 2019). For instance, lactobacilli are being engineered as live biotherapeutic products (LBPs) that produce and deliver drugs right at the site of diseases like ulcerative colitis (de Vos, 2011), human immunodeficiency virus (HIV) infection (Watterlot et al., 2010) and respiratory infections (Janahi et al., 2018). They

Sourik Dey and Marc Blanch-Asensio are contributed equally.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs License](https://creativecommons.org/licenses/by-nc-nd/4.0/), which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2023 The Authors. *Microbial Biotechnology* published by Applied Microbiology International and John Wiley & Sons Ltd.

are also prominent candidates for the development of mucosal vaccines in which they are engineered to either display heterologous antigens on their surface or to secrete them (LeCureux & Dean, 2018). These food-grade *Lactobacillus* vaccine vectors would be cheap to produce and can be easily administered orally or intranasally, improving the ability to deploy them both in humans and animals. Examples of infectious diseases against which such vaccines are under development include anthrax, infantile diarrhoea, pneumonia and viral infections like HIV, HPV, influenza and coronavirus (LeCureux & Dean, 2018; Wang et al., 2020). Finally, to track these therapeutic bacteria within the body and study their colonization and clearance profiles, there is considerable interest to make them express reporter proteins that can be imaged *in situ* (Landete et al., 2015; Salomé-Desnoullez et al., 2021).

Despite such potential, the main limitations for engineering lactobacilli are the scarcity of well-characterized genetic parts and insufficient understanding of biochemical pathways required to build the type of genetic circuits that have been demonstrated in *E. coli* (Elowitz & Leibler, 2000; Wang et al., 2011) and *B. subtilis* (Castillo-Hair et al., 2019; Courbet et al., 2015). Over two decades of painstaking investigation and screening across phylogenetically close bacteria have generated a handful of reliable parts for use in lactobacilli such as constitutive and inducible promoters, operators, replicons, retention-modules, signal peptides. Most of these have been developed in a few species that were found to be amenable to genetic modification, among which *Lactiplantibacillus plantarum* (Zheng et al., 2020) is widely reported (Siezen & van Hylckama Vlieg, 2011). While genomic integration of genes has been demonstrated in these bacteria, the greatest versatility of functions has been achieved using plasmids. Excellent progress has been made in establishing plasmid backbones with low, medium and high copy number replicons (Tauer et al., 2014), constitutive promoters with a wide range of expression strengths (Rud et al., 2006), a few inducible promoters that can be triggered by peptides (Halbmayer et al., 2008) or sugars (Heiss et al., 2016), signal peptides sequences enabling protein secretion (Mathiesen et al., 2009) or surface display (Mathiesen et al., 2020) and food-grade plasmid retention systems based on resistance to external stressors (e.g. bacteriocins) (Allison & Klaenhammer, 1996; Takala & Saris, 2002) or auxotrophy complementation requiring genomic knockout of a metabolic gene and providing it in the plasmid (Chen et al., 2018; Nguyen et al., 2011). However, the available set of well-characterized genetic parts is still minuscule compared to the toolbox of *E. coli* and needs to be expanded in order to improve the performance and versatility of *Lactobacillus* engineering for healthcare applications.

In this work, we introduce 2 new versatile and powerful genetic parts to expand the capabilities of

Lactobacillus engineering – (i) a novel constitutive promoter from a phylogenetically distant *Salmonella* species that drive protein expression at levels considerably higher than previously reported strong *L. plantarum* promoters and (ii) toxin-antitoxin systems as an alternative strategy for plasmid retention that does not require manipulating the bacterial genome. Unique features of the novel promoter sequence are discussed, which can lead to new design criteria for improving promoter strengths in lactobacilli. The toxin-antitoxin systems introduce a thus-far unexplored modality of plasmid retention in lactobacilli that enables the generation of temporary Genetically Engineered Microorganisms (GEMs), desirable for medical and food-grade applications. These parts and the fundamental insights gained in their characterization will strongly aid in expanding the genetic programmability of lactobacilli.

EXPERIMENTAL PROCEDURES

Strain, media and plasmids

Lactiplantibacillus plantarum WCFS1 was used as the parent strain for promoter strength and plasmid retention characterization. The strain was maintained in the De Man, Rogosa and Sharpe (MRS) media. The culture media, antibiotics and complementary reagents were purchased from Carl Roth GmbH, Germany. Growth media was supplemented with 10 µg/mL of erythromycin to culture engineered *L. plantarum* WCFS1 strains. The plasmids pSIP403 and pLp_3050sNuc used in this study were a kind gift from Prof. Lars Axelsson (Addgene plasmid # 122028) (Sørvig, Mathiesen, et al., 2005) and Prof. Geir Mathiesen (Addgene plasmid # 122030) (Mathiesen et al., 2009), respectively. The plasmid pTlpA39-Wasabi was a kind gift from Prof. Mikhail Shapiro (Addgene plasmid # 86116) (Piraner et al., 2017). The plasmid pUC-GFP-AT was a kind gift from Prof. Chris Barnes (Addgene plasmid # 133306) (Fedorec et al., 2019). The sequence verified genetic constructs created in this study have been maintained in *E. coli* DH5α.

Molecular biology

The genetic constructs developed in this study are based on the pLp3050sNuc/pSIP403 vector backbone. The HiFi Assembly Master Mix, Quick Blunting Kit and the T4 DNA Ligase enzyme were purchased from New England BioLabs (NEB, Germany). PCR was performed using Q5 High Fidelity 2× Master Mix (NEB) with primers purchased from Integrated DNA Technologies (IDT) (Leuven, Belgium). Oligonucleotide gene fragments were purchased as eBlocks from IDT (Coralville, USA). These were codon-optimized for

maximal expression in the host strain using the IDT Codon Optimization Tool (Coralville, USA). Plasmid extraction and DNA purification were performed using kits purchased from Qiagen GmbH (Hilden, Germany) and Promega GmbH (Walldorf, Germany), respectively. The general schematic of plasmid construction for this study is shown in Figure S1. The promoter sequences used in this study are provided in Table S1, and the nucleotide sequences of the toxin-antitoxin modules are highlighted in Table S2.

***L. plantarum* WCFS1 competent cell preparation and DNA transformation**

A single colony of *L. plantarum* WCFS1 was inoculated in 5 mL of MRS media and cultured overnight at 37°C with shaking (250 rpm). The primary culture was diluted in a 1:50 (v/v) ratio in a 25 mL secondary culture composed of MRS media and 1% (w/v) glycine premixed in a 4:1 ratio. The secondary culture was incubated at 37°C, 250 rpm until OD₆₀₀ reached 0.8, following which the cells were pelleted down by centrifuging at 4000 rpm (3363 g) for 10 min at 4°C. The pellet was washed twice with 5 mL of ice-cold 10 mM MgCl₂ and then washed twice with 5 mL and 1 mL of ice-cold Sac/Gly solution [10% (v/v) glycerol and 1 M sucrose mixed in a 1:1 (v/v) ratio], respectively. Finally, the residual supernatant was discarded, and the pellet resuspended in 500 µL of Sac/Gly solution. The competent cells were then dispensed in 60 µL aliquots for DNA transformation. For all transformations, 1 µg of dsDNA was added to the competent cells and then transferred to chilled 2 mm gap electroporation cuvettes (Bio-Rad Laboratories GmbH, Germany). Electroporation transformation was done with a single pulse at 1.8 kV, after which 1 mL of luke-warm MRS media was immediately added. The mixture was kept for incubation at 37°C, 250 rpm for a recovery period of 3 h. Following the recovery phase, the cells were centrifuged at 4000 rpm (3363 g) for 5 min, 800 µL of the supernatant discarded, and 200 µL of the resuspended pellet was plated on MRS Agar supplemented with 10 µg/mL of Erythromycin. The plates were incubated at 37°C for 48 h to allow the growth of distinct single colonies.

Direct cloning in *L. plantarum* WCFS1

To obtain sufficient plasmid quantities (~1 µg) for transformation in *L. plantarum* WCFS1, a modified direct cloning method (Spath et al., 2012) involving PCR-based amplification and circularization of recombinant plasmids was used. Plasmids were constructed and transformed directly in *L. plantarum* WCFS1 strain using a DNA assembly method. Complementary overhangs for HiFi Assembly were either created using PCR

primers or synthesized as custom-designed eBlocks. Purified overlapping DNA fragments were mixed with the HiFi DNA Assembly Master Mix and assembled as recommended in the standard reaction protocol from the manufacturer. The assembled DNA product was then exponentially amplified by another round of PCR using a pair of primers annealing specifically to the insert segment; 5 µL of the HiFi assembly reaction was used as a template for this PCR amplification of the assembled product (100 µL final volume). The purified PCR product was then subjected to phosphorylation using the Quick Blunting Kit. Then 2000 ng of the purified PCR product was mixed with 2.5 µL of 10× Quick blunting buffer and 1 µL of Enzyme Mix (Milli-Q water was added up to 25 µL). The reaction was incubated first at 25°C for 30 min and then at 70°C for 10 min for enzyme inactivation. Next, phosphorylated products were ligated using the T4 ligase enzyme; 6 µL of the phosphorylated DNA was mixed with 2.5 µL of 10× T4 Ligase Buffer and 1.5 µL of T4 Ligase enzyme (Milli-Q water was added up to 25 µL). Two ligation reactions were performed per cloning (25 µL each). The respective reactions were incubated at 25 °C for 2 hours and then at 70°C for 30 min for enzyme inactivation. The ligated reactions were mixed together and purified. In order to concentrate the final purified product, three elution rounds were performed instead of one. Each elution was based on 10 µL of Milli-Q water. The concentration of the ligated purified product was measured using the NanoDrop Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific GmbH, Germany). Finally, 1000 ng of the ligated product was transformed into *L. plantarum* WCFS1 electrocompetent cells, resulting in a transformation efficiency of 2–3 × 10² cfu/µg.

Notably, since *L. plantarum* harbours 3 endogenous plasmids (Van Kranenburg et al., 2005), sequencing was performed on PCR-amplified sections. In detail, colonies of interest were inoculated in MRS supplemented with 10 µg/mL of Erythromycin and grown overnight at 37 °C. The following day, 1 mL of the culture was pelleted down, and the supernatant was discarded. Next, a tip was used to collect a tiny part of the pellet, which was used as a template for PCR (100 µL final volume). Finally, PCR products were purified and sent for Sanger sequencing to Eurofins Genomics GmbH (Ebersberg, Germany) by opting for the additional DNA purification step.

Microplate reader setup for thermal gradient analysis

Bacterial cultures were cultivated in 5 mL of MRS media (supplemented with 10 µg/mL erythromycin) at 30°C with continuous shaking (250 rpm). The following day, cultures were diluted to 0.1 OD₆₀₀ in 3 mL of antibiotic supplemented fresh MRS media and propagated at

30°C, 250rpm. At $OD_{600} = 0.3$, the cultures were dispensed into Fisherbrand™ 0.2 mL PCR Tube Strips with Flat Caps (Thermo Electron LED GmbH, Germany) and placed in the Biometra Thermocycler (Analytik Jena, GmbH, Germany). For the P_{spp} -mCherry construct, 25 ng/mL of the 19 amino acid Sakacin P inducer peptide (Spplp) with the sequence NH_2 - MAGNSSNFIHK IKQIFTHR-COOH (GeneCust, France) was added to the culture and thoroughly vortexed before preparing the aliquots. The thermal assay was set at a temperature gradient from 31°C to 41°C with regular increment of 2°C. The lid temperature was set at 50°C to prevent the evaporation of the liquid and maintain a homogeneous temperature in the spatially allocated PCR tubes. After a time interval of 18 h, the PCR strips were centrifuged in a tabletop minicentrifuge (Biozym GmbH, Germany) to pellet down the cells and discard the supernatant. The cells were then resuspended in 200 μ L of 1× PBS and added to the clear bottom 96-well microtitre plate (Corning® 96 well clear bottom black plate, USA). The samples were then analysed in the Microplate Reader Infinite 200 Pro (Tecan Deutschland GmbH, Germany), and both the absorbance (600 nm wavelength) and mCherry fluorescence intensity ($Ex_{\lambda} / Em_{\lambda} = 587 \text{ nm} / 625 \text{ nm}$) were measured. The z-position and gain settings for recording the mCherry fluorescent intensity were set to 19,442 μ m and 136, respectively. Fluorescence values were normalized with the optical density of the bacterial cells to calculate the Relative Fluorescence Units (RFU) using the formula $RFU = \text{Fluorescence} / OD_{600}$.

Fluorescence microscopy analysis

Bacterial cultures were grown overnight in 5 mL of MRS media (supplemented with 10 μ g/mL erythromycin) at 37°C with continuous shaking (250 rpm). The following day, the OD_{600} of the P_{spp} -mCherry construct was measured and subcultured at $OD_{600} = 0.01$. When the P_{spp} -mCherry bacterial culture reached $OD_{600} = 0.3$, it was induced with 25 ng/mL of Spplp and the remaining constructs were subcultured in fresh media at 0.01 OD_{600} . All the cultures were then allowed to grow for 18 h under the same growth conditions (37°C, 250 rpm) to prevent any heterogeneity in promoter strength expression due to differential growth parameters. Later, 1 mL of the cultures were harvested by centrifugation (15,700 g, 5 min, 4°C), washed twice with Dulbecco's 1× PBS (Phosphate Buffer Saline) and finally resuspended in 1 mL of 1× PBS. And 10 μ L of the suspensions was placed on glass slides of 1.5 mm thickness (Paul Marienfeld GmbH, Germany) and 1.5H glass coverslips (Carl Roth GmbH, Germany) were placed on top of it. The samples were then observed under the Plan Apochromat 100× oil immersion lens (BZ-PA100, NA 1.45, WD 0.13 mm) of the Fluorescence Microscope BZ-X800 (Keyence

Corporation, Illinois, USA). The mCherry signal was captured in the BZ-X TRITC filter (model OP-87764) at excitation wavelength of 545/25 nm and emission wavelength of 605/70 nm with a dichroic mirror wavelength of 565 nm. The images were adjusted for identical brightness and contrast settings and were processed with the Fiji ImageJ2 software.

Flow cytometry analysis

Quantification of fluorescent protein expression levels of the strains was performed using Guava easyCyte BG flow-cytometer (Luminex, USA). Bacterial cultures subjected to the same treatment conditions mentioned above were used for Flow Cytometry analysis; 1 mL of the bacterial suspensions was harvested by centrifugation at $15,700 \times g$. The supernatant was discarded and the pellet was resuspended in 1 mL of sterile Dulbecco's 1× PBS. The samples were then serially diluted by a 10^4 Dilution Factor (DF) and 5000 bacteria events were recorded for analysis. Experiments were performed in triplicates on three different days. During each analysis, the non-fluorescent strain carrying the empty vector was kept as the negative control. A predesigned gate based on forward side scatter (FSC) and side scatter (SSC) thresholding was used to remove debris and doublets during event collection and analysis. mCherry fluorescence intensity was measured using excitation by a green laser at 532 nm (100 mW) and the Orange-G detection channel 620/52 nm filter was used for signal analysis. The gain settings used for the data recording were Forward Scatter (FSC) – 11.8, Side Scatter (SSC) – 4 and Orange-G Fluorescence – 1.68. The compensation control for fluorescence recording was set at 0.01 with an acquisition rate of 5 decades. Data analysis and representation were performed using the Luminex GuavaSoft 4.0 software for EasyCyte.

Toxin/antitoxin module-based plasmid construction

Similar to previous reports in *E. coli* (Fedorec et al., 2019), the effect of Txe/Axe (toxin/antitoxin) module from *E. faecium* (Grady & Hayes, 2003) was tested in *L. plantarum* WCFS1 to test its capability for antibiotic-free plasmid retention. TA Finder version 2.0 tool (Xie et al., 2018) was used to select further type-II TA (Toxin/Antitoxin) systems present in *Lactobacillus* genomes. *Lactobacillus acidophilus*, *L. crispatus*, *L. casei*, *L. reuteri* and *L. plantarum* WCFS1 genomes were retrieved from NCBI Genome. TA systems harboured within these genomes were mined using the default parameters of TA Finder. Only TA systems annotated by NCBI BlastP were selected as test candidates. The TA systems YafQ/DinJ, HicA/HicB, HigB/HigA and MazF/MazE from *L. casei*,

L. acidophilus and *L. plantarum* WCFS1 were selected for further testing and analysis.

Txe/Axe system was amplified by PCR from the plasmid pUC-GFP-AT (Fedorec et al., 2019). DinJ/YafQ and HicA/HicB systems were synthesized as custom-designed eBlocks. HigA/HigB and MazE/MazF were amplified from the genome of *L. plantarum* WCFS1. TA systems were inserted into the P_{tlpA} -mCherry plasmid, generating the plasmids P_{tlpA} -mCherry-Txe/Axe, P_{tlpA} -mCherry-YafQ/DinJ, P_{tlpA} -mCherry-HicA/HicB, P_{tlpA} -mCherry-HigB/HigA and P_{tlpA} -mCherry-MazF/MazE. For constructing the combinatorial TA module (P_{tlpA} -mCherry Combo), the best performing endogenous and non-endogenous TA systems recorded after 100 generations (MazF/MazE and YafQ/DinJ) were subcloned and integrated into the same plasmid in reverse orientations.

TA mediated plasmid retention analysis

The TA module containing constructs were inoculated in 5 mL cultures of 10 µg/mL erythromycin supplemented MRS media and incubated overnight at 37°C with continuous shaking (250 rpm). The following day, the constructs were subcultured at an initial $OD_{600} = 0.01$ in fresh MRS media (both with and without antibiotic supplementation). The bacterial cultures were incubated for 12 consecutive days with a daily growth period of 24 h ensuring an average of ~8 generations per day, until crossing the final threshold of 100 generations. Sample preparation for flow cytometry analysis was conducted according to the protocol mentioned before. The mCherry positive cell population directly correlated to the bacterial population retaining the engineered plasmid. The entire experiment was repeated in biological triplicates.

To cross-check the flow cytometry analysis, the bacterial cultures grown for 100 generations without antibiotic supplementation were centrifuged and resuspended in 1 mL of sterile Dulbecco's 1× PBS. The resuspended bacterial solutions were diluted ($df = 10^6$) and plated on MRS Agar plates supplemented without antibiotic and incubated in a static incubator for 48 h. The plates were then imaged using the GelDocumentation System Fluorchem Q (Alpha Innotech Biozym GmbH, Germany) both in the Ethidium Bromide channel ($Ex_{\lambda}/Em_{\lambda} = 300\text{ nm}/600\text{ nm}$) and Cy3 channel ($Ex_{\lambda}/Em_{\lambda} = 554\text{ nm}/568\text{ nm}$) to visualize the cell population producing mCherry fluorescence. The fluorescent bacterial subpopulation on the non-selective MRS agar medium correlated to the plasmid retention frequency of the respective TA systems in the absence of selection pressure.

Growth rate measurements

For studying the influence of the heterologous protein production and toxin-antitoxin modules on the bacterial

growth rate, bacterial cultures were cultivated overnight in antibiotic supplemented MRS media at 37°C with continuous shaking (250 rpm). Following day, the bacterial cultures were subcultured in secondary cultures at an initial $OD_{600} = 0.01$. After 4 h incubation at 37°C, the OD_{600} of the cultures reached 0.1 and 200 µL of the cultures were distributed in UV STAR Flat Bottom 96-well microtitre plates (Greiner BioOne GmbH, Germany). The 96-well assay plate was placed in the Microplate Reader with constant shaking conditions at an incubation temperature of 37°C. The kinetic assay was set to record the absorbance of the bacterial cultures at 600 nm wavelength with an interval of 10 min for an 18 h time duration. The experiment was conducted in triplicates on three independent days.

Bioinformatic analysis

All genome sequences included in the phylogenetic analysis were retrieved from NCBI Genome. The phylogenetic tree was built using the web server for genome-based prokaryote taxonomy 'Type (Strain) Genome Server' (TYGS), restricting the analysis only to the sequences provided (Meier-Kolthoff & Göker, 2019). The Genome BLAST Distance Phylogeny (GBDP) tree, based on 16S rDNA gene sequences, was obtained. The Interactive Tree of Life (iTOL) tool was used for the display, annotation and management of the phylogenetic tree (Letunic & Bork, 2007).

For the multiple sequence alignment, protein sequences of the $\sigma 70$ subunits from *L. plantarum*, *E. coli* and *S. typhimurium* RNA polymerases were first retrieved from Uniprot. Sequences were aligned using the tool MUSCLE (Edgar, 2004). Jalview was used to visualize and edit the multiple sequence alignment (Waterhouse et al., 2009).

SnapGene was used to identify DNA sequences similar to P_{tlpA} within the genome of *L. plantarum* WCFS1 using the feature 'Find Similar DNA Sequences'. The search allowed a mismatch or gap/insertion every 4 bases. BPROM, an online tool for predicting bacterial promoters, was used to identify the -35 and -10 boxes within this promoter (Madeira et al., 2022). BlastP was used to identify the protein encoded by the gene driven by this promoter. Promoter alignment was performed using MUSCLE (Edgar, 2004).

RESULTS AND DISCUSSIONS

P_{tlpA} promoter from *Salmonella* drives high-level constitutive expression

The strongest promoters in lactobacilli have been found by either screening the genome of the host strain (Bron et al., 2004; Rud et al., 2006) or adapting

those driving high-level protein expression in phylogenetically close lactic acid bacteria (Russo et al., 2015) (Figure 1A). In the few reports where promoters from phylogenetically distant species like *P. megaterium* (P_{xyIA}) or *E. coli* (P_{T7} from lambda phage) (Heiss et al., 2016) have been tested, expression levels were found to be comparatively low. Contrary to this trend, we serendipitously stumbled upon a promoter (P_{tIpA}) from the phylogenetically distant gram-negative *Salmonella typhimurium* (Figure 1A) capable of driving protein expression at levels higher than previously reported strong promoters in *L. plantarum* WCFS1. In *Salmonella*, P_{tIpA} along with its repressor is capable of thermo-responsively regulating gene expression and this functionality had been previously transferred to *E. coli* for therapeutic purposes (Hurme et al., 1997; Piraner et al., 2017). To test whether the P_{tIpA} promoter would be a suitable candidate for driving transcription in *L. plantarum*, a fluorescent reporter protein (mCherry) was cloned downstream of this promoter.

The promoter surprisingly seemed to constitutively drive a high-level of protein expression with a mild degree of thermal regulation (<5-fold increase from 31°C to 39°C) (Figure 1B). Next the repressor-based thermo-responsive functionality was tested in *L. plantarum*, by creating the pTIpA39 plasmid, with the P_{tIpA} promoter driving expression of mCherry and the codon optimized TIpA repressor being expressed constitutively by the P_{48} promoter (Rud et al., 2006). However, the pTIpA39 plasmid showed no significant repression of mCherry at lower temperature gradients in comparison to its repressor-free counterpart (Figure S2D). Most remarkably, flow cytometry and fluorescence spectroscopy analysis revealed that mCherry expression levels driven by the P_{tIpA} promoter significantly exceeded the levels driven by some of the strongest promoters previously reported in *L. plantarum* - P_{23} (Meng et al., 2021), P_{48} (Rud et al., 2006), P_{spp} (Sørvig et al., 2003) and P_{Tuf} (Spangler et al., 2019) (Figure 1C, Figure S3A). At 31°C, mCherry expression levels were

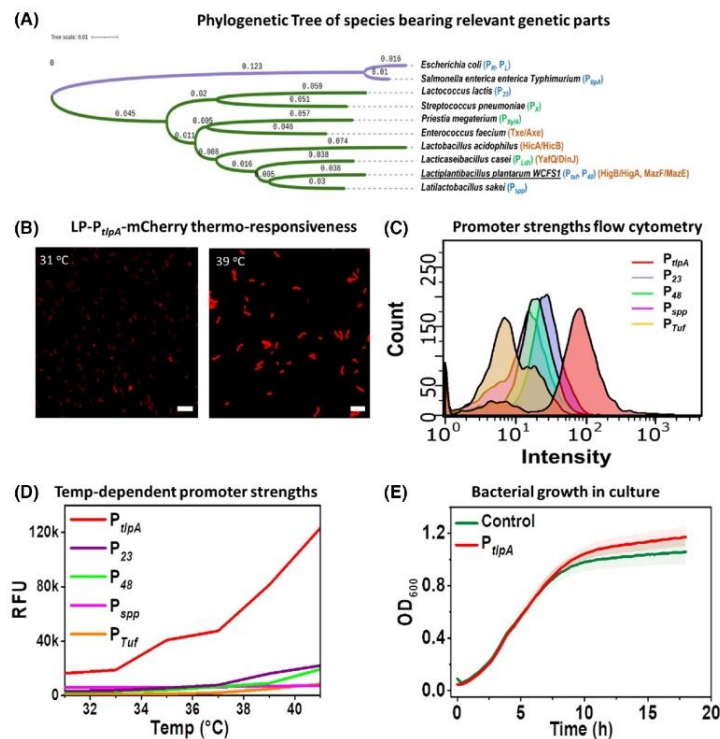


FIGURE 1 (A) Phylogenetic tree highlighting the distances between species from which various genetic parts have been tested in *Lactiplantibacillus plantarum*. Purple clade corresponds to Gram-negative bacteria. Green clade corresponds to Gram-positive bacteria. Promoters tested in this study are labelled in blue. Promoters tested by others in *L. plantarum* are labelled in green. Orange labels correspond to the TA systems tested in this study. (B) Fluorescence microscopy of P_{tIpA} driven mCherry expression in *L. plantarum* WCFS1 cultivated at 31°C and 39°C for 18 h. Scale bar = 10 µm. (C) Flow Cytometry analysis of P_{tIpA} , P_{23} , P_{48} , P_{spp} and P_{Tuf} driven mCherry expression in *L. plantarum* WCFS1 after 18 h incubation at 37°C. (D) Fluorescence spectroscopy analysis of the P_{tIpA} , P_{23} , P_{48} , P_{spp} and P_{Tuf} driven mCherry expression after 18 h incubation at temperatures ranging from 31°C to 41°C. (E) Growth rate (OD₆₀₀) measurement of *L. plantarum* WCFS1 strains containing a control plasmid and P_{tIpA} -mCherry for 18 h at 37°C. In (C) and (D), the solid lines represent mean values, and the lighter bands represents standard deviations calculated from three independent biological replicates.

at least 2-fold higher than these other promoters, while this increased to 5-fold at 39°C (Figure 1D, Figure S3B). All constitutive promoters (P_{23} , P_{48} , P_{Tuf}) were mildly thermo-responsive, while the inducible promoter (P_{spp}) was not (Figure S4A). To check whether such high gene expression can be driven by other phylogenetically distant thermo-responsive promoters, we tested the well-known heat inducible pR and pL promoters from *E. coli* lambda phage. However, only low levels of mCherry expression were observed with these promoters (Figure S2A, B). Fluorescence spectroscopy revealed that the strength of the P_{tlpA} promoter at 37°C was 26- and 39-fold higher than the pR and pL promoter, respectively (Figure S2C).

Another important factor for high-level gene expression driven by P_{tlpA} is that the spacer length between the ribosome binding site (RBS, 5'-AGGAGA-3') and the start codon needs to be different in *L. plantarum* compared to *E. coli*. In *E. coli* this spacer length of 6 bp has been previously reported (Chee et al., 2022; Kan et al., 2020; Piraner et al., 2017; Rottinghaus et al., 2022), whereas in *L. plantarum* a 9 bp spacer improves expression levels by 25-fold compared to a 6 bp spacer (Figure S4B), in accordance with previous reports (Tauer et al., 2014). Despite the high level of protein expression driven by P_{tlpA} with a 9 bp spacer, the growth rate of this strain at 37°C was similar to that of the empty vector control strain, suggesting that this protein overexpression did not metabolically burden the cell (Figure 1E).

To understand why P_{tlpA} drives gene expression in *L. plantarum*, we looked into its function in *Salmonella*, where it is a promoter of the $\sigma 70$ sigma factors

(Dawoud et al., 2017). This family of sigma factors is involved in regulating the expression of housekeeping genes in most prokaryotes, including lactobacilli (Todt et al., 2012). Multiple Sequence Alignment (MSA) among the major RNA polymerase $\sigma 70$ proteins (RpoD) of *E. coli*, *S. typhimurium* and *L. plantarum* strains (Figure 2A) revealed significant similarity between the domain-2 and domain-4 regions, responsible for binding to the -10 and -35 regions of the promoter during transcription initiation. Interestingly, Gaida et al. (2015) showed that when expressed in *E. coli*, the *L. plantarum* RpoD can recruit *E. coli*'s RNA polymerase to initiate transcription from a wide variety of heterologous promoters. Compared to sigma factors from six other bacteria, they found that the *L. plantarum* RpoD was the most promiscuous and helped to enlarge the genomic space that can be sampled in *E. coli*. These analyses explain why the P_{tlpA} promoter from a phylogenetically distant species functions in *L. plantarum* but does not necessarily reveal how it drives such high expression levels compared to previously reported promoters.

To understand this, we investigated aspects of P_{tlpA} 's sequence (Figure 2B). One unique characteristic is that it harbours the sigma70 consensus sequence at the -10 region (5'-TATAAT-3') but not at the -35 region (5'-TTGACA-3') (Todt et al., 2012). Based on the previous reports in gram positive bacteria like *B. subtilis* (Helmann, 1995), it is possible that the deviation from the consensus -35 sequence can be compensated for the presence of the conserved dinucleotide 'TG' sequence at the -14 and -15 position of the P_{tlpA} promoter. It has been shown that the presence of this sequence upstream of the -10 region in the promoter

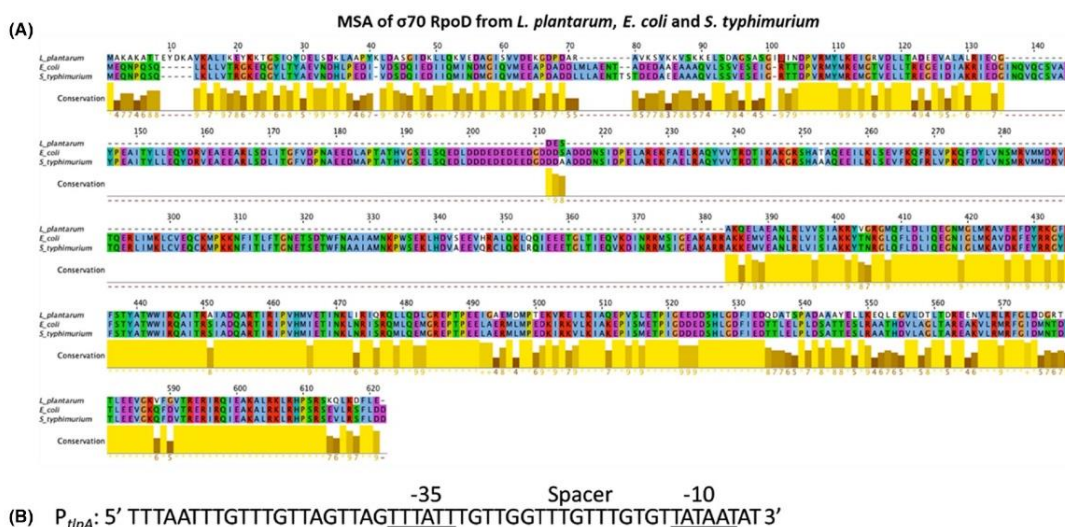


FIGURE 2 (A) Homology analysis of $\sigma 70$ RpoD genes from *Lactiplantibacillus plantarum*, *Escherichia coli* and *Salmonella typhimurium*. Height and brightness of the yellow bars indicate the extent to which individual residues are conserved across all 3 bacteria. (B) P_{tlpA} promoter sequence with -35, spacer and -10 regions labelled.

can mediate rapid promoter melting during transcription initiation and upregulate the transcription rate of corresponding genes. However, most of the promoters reported by Rud et al. (2006) for *L. plantarum* also have the conserved 'TG' dinucleotide at the -15 position of the promoter. When the strength of the strongest promoter in that library (P_{48}) was compared to the P_{tlpA} promoter, the mCherry production rate by the P_{tlpA} promoter was significantly higher. This suggests that the P_{tlpA} promoter must have additional reasons that contribute to its exceptional performance in *L. plantarum* WCFS1.

More interestingly, the whole promoter sequence contains no cytosine (C) bases, in contrast to previously reported in *L. plantarum* promoters, most of which contain 2 to 4 cytosine bases in the -35 to -10 region (Meng et al., 2021; Rud et al., 2006; Sørvig et al., 2003; Spangler et al., 2019). Additionally, the spacer between the -35 and -10 regions of the P_{tlpA} promoter contains no adenine (A) bases. Notably, A and C bases are susceptible to methylation in bacteria, which has been associated with epigenetic gene regulation (Beaulaurier et al., 2019; Casadesús & Low, 2006). However, on analysis of 34 constitutive promoter sequences from the synthetic promoter library reported by Rud et al. (2006) and those tested in this study (Table S3), no correlation could be derived between promoter strengths and number of C bases within the -35 to -10 region (Figure S6A) or the A bases in the spacer (Figure S6B). If methylation could be influencing promoter strengths, it would be necessary to identify the methyltransferase recognition sequences in *L. plantarum* to derive meaningful correlations. We then searched for DNA sequences similar to P_{tlpA} within the genome of *L. plantarum* WCFS1. Out of 6 hits (Figure S7A), only one of them was located upstream of a gene that encodes for a known protein (HAMP domain-containing histidine kinase - locus: lp_0282, complement: 255805.0.257181), with a percent identity score of 82.76 compared to P_{tlpA} . This sequence (GTTTATGTTGGTTATTTACGTAATAAAAT) was identified as a promoter (referred to as P_{HAMP}) using BPROM, with -35 and -10 regions (in bold) diverging from P_{tlpA} by single bases each (Figure S7B). Notably, P_{HAMP} also contains four A bases and one C base in the spacer. When the full promoter sequence (Table S1) was cloned upstream of mCherry, only weak expression was observed (Figure S7C), suggesting that one or more of these mismatches compared to P_{tlpA} are essential for driving high-level gene expression. These unique features of the P_{tlpA} promoter sequence provide interesting clues for understanding factors affecting promoter strengths in *L. plantarum*. To gain deeper insights into P_{tlpA} 's unprecedented strength, further studies analysing mutant libraries of the promoter and/or measuring DNA methylation patterns are required.

Toxin/antitoxin-based plasmid retention and transient GEMs

Apart from high expression levels, use of lactobacilli for healthcare applications requires strategies to retain heterologous genes in the engineered bacteria in a cheap and compatible manner. TA systems ensure plasmid retention in a bacterial population through a post-segregation killing mechanism. They constitutively express long-lasting toxins and short-lived antitoxins. As long as the plasmid is present, sufficient antitoxin is produced to neutralize the corresponding toxin. On bacterial division, if a daughter cell does not receive any plasmid copies, the antitoxin rapidly degrades, and the active toxin kills the cell. While TA systems have been investigated in the past for bioremediation and biotechnology purposes, their applicability was limited by the fact that their plasmid retention efficiency did not match that of antibiotic or auxotrophy-based retention systems (Stirling & Silver, 2020). However, interest in TA systems has re-emerged for living therapeutic applications because of two reasons: (i) better understanding of TA systems leading to improved efficiencies (Fedorec et al., 2019) and (ii) biosafety features they offer in reducing horizontal gene transfer (Wright et al., 2013). Accordingly, reports have recently emerged where TA systems are showing greater promise for bacteria engineered as live vaccines or drug delivery vehicles (Abedi et al., 2022; Kan et al., 2020). While these demonstrations have been done in *E. coli*, the use of TA system in lactobacilli for plasmid retention has not yet been systematically investigated. From literature reports and using the TA finder bioinformatics tool, we identified and selected 5 different type II TA system (all named as toxin/antitoxin): (i) Txe/Axe, from *Enterococcus faecium* that was shown to ensure long-term plasmid retention in *E. coli* (Fedorec et al., 2019), (ii) YafQ/DinJ from *L. casei* (Levante et al., 2019), (iii) HigB/HigA and (iv) MazF/MazE from *L. plantarum* WCFS1 and (v) HicA/HicB from *L. acidophilus* (Phylogeny in Figure 1A). In all these systems, the toxin is an endoribonuclease and the antitoxin is its corresponding inhibitory protein. These modules were added to the plasmid encoding P_{tlpA} -driven mCherry expression (Figure 3A), and the resultant strain was repeatedly sub-cultured for up to 100 generations. Plasmid retention was quantified by determining the proportion of the bacterial population expressing mCherry using flow cytometry and agar plate colony imaging analysis (Figure S5B). Notably, the sensitivity of this analysis was greatly improved by the high-level of expression driven by the P_{tlpA} promoter, which enabled clear demarcation of plasmid-retained and plasmid-lost cells (Figure 3B). Such a clear demarcation was not possible with the other promoters, like P_{23} since the fluorescent signal seemed to partially overlap with background signal from non-fluorescent cells (Figure S5A). In the absence of a TA system (P_{tlpA}

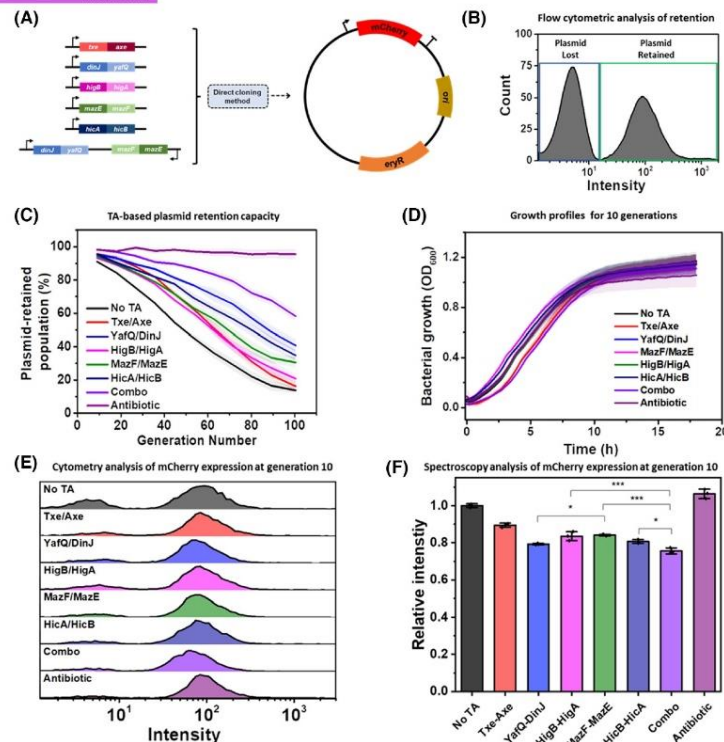


FIGURE 3 (A) Schematic representation of cloning the different TA genetic modules into the P_{tlpA}-mCherry plasmid. (B) Sample flow Cytometry histogram plot of the P_{tlpA}-mCherry plasmid containing strain without any TA module or selection pressure after 50 generations of serial passaging in the absence of antibiotic. The green box corresponds to the bacterial population retaining the plasmid and the blue box represents the population devoid of the plasmid. (C) Plasmid retention analysis of the TA module containing strains for 100 generations without antibiotics along with no TA and antibiotic selection pressure conditions for comparison. (D) Growth rate (OD₆₀₀) of strains with the TA modules, no TA and antibiotic retention over 10 generations at 37°C. In (C) and (D), the solid lines represent mean values and the lighter bands represent SD calculated from three independent biological replicates. Combo = MazF/MazE + YafQ/DinJ. (E) Flow cytometry plots of strains containing TA modules, no TA and antibiotic retention after 10 generations. The Y-axis for each plot represents counts with plot heights in the range of 450–500 (F) Fluorescence spectroscopy analysis of strains containing TA modules, no TA and antibiotic retention after 10 generations. The relative intensity has been plotted for all the TA strains by normalizing their respective fluorescence values against the 'No TA' strain. The data represent three independent biological replicates. *p*-values are calculated using one-way ANOVA with Tukey test on respective means (**p* < 0.05, ****p* < 0.001). The 'No TA', 'Txe-Axe' and 'Antibiotic' conditions are significantly different from other candidates, so their *p*-values have not been explicitly highlighted.

mCherry plasmid), the proportion of plasmid-bearing bacteria steadily declined by about 1%/ generation, ending with ~15% of the population retaining the plasmid after 100 generations (Figure 3C). Compared to this, the Txe/Axe system initially supported better retention with a plasmid loss of about 0.5%/generation for 40 generations, after which this loss accelerated to ~1.2%/generation, ending in ~18% of the population retaining the plasmid after 100 generations. HigB/HigA and MazF/MazE systems performed similarly for the most part but provided slightly better retention after 100 generations (20% and 30%, respectively). HicA/HicB slowed plasmid loss to 0.5%/generation for 50 generations and 0.8%/ generation, thereafter, resulting in retention level of ~35% after 100 generations. Finally, YafQ/DinJ was found to provide the best retention

capabilities with plasmid loss of 0.5%/generation for 70 generations and 1%/ generation thereafter, resulting in a retention level of ~40% after 100 generations (Figure 3C).

Previous studies have shown that combining different TA systems can cumulatively offer better plasmid retention capabilities (Torres et al., 2003; Bardaji et al., 2019), although this has not been tested in *Lactobacilli*. So, we combined the best-performing TA system endogenous to *L. plantarum* WCFS1 (MazF/MazE) with the best-performing non-endogenous system (YafQ/DinJ) and observed better plasmid retention capabilities with this combination, yielding a slow plasmid loss of 0.2%/generation for 50 generations and a gradual increase to 0.8%/generation thereafter, resulting in a considerably higher retention

of 60% over 100 generations. Comparatively, plasmids maintained under antibiotic selection pressure were steadily retained at >90% through 100 generations, as expected. In all strains harbouring TA modules, bacterial growth rates (Figure 3D) and mCherry expression levels (Figure 3E) were found to be minimally impacted compared to 'No TA' or antibiotic-retention conditions over the first 10 generations. These results suggest that the toxins did not drastically impede the regular functioning of the cells. Fluorescence spectroscopy analysis of the liquid cultures after 10 generations (Figure 3F) reveals that the TA modules showing higher efficiency in retaining plasmids in the absence of selection pressure (YafQ/DinJ and combo) have significantly lower intensities of mCherry production in comparison to the other TA candidates. The greatest drop in protein expression (~23%) was observed in the strain harbouring the TA combo and could be due to an increase in the plasmid size possibly burdening the cells and maybe even resulting in a minor drop in copy number. However, since the YafQ/DinJ construct also causes a drop of comparable magnitude (~20%), it is possible that the toxin in this system mildly interferes with protein expression, which becomes detectable with the overexpression of mCherry by P_{tlpA} but does not drastically affect growth. Further in depth investigation would be required to identify the specific cause of this effect. However, it must be noted that even with the drop in expression level caused by the combo TA system, P_{tlpA} -driven mCherry expression was at least 4-fold higher than that of the next strongest promoter, P_{23} .

It is important to note that a single generation corresponds to a bacterial duplication, so 10 generations = 2^{10} or $\sim 10^3$ bacteria and 100 generations = 2^{100} or $\sim 10^{30}$ bacteria from a single cell. Potential applications of lactobacilli for living therapeutics or engineered living materials are not expected to reach such high generation numbers either due to short application time periods (Janahi et al., 2018; LeCureux & Dean, 2018; Wang et al., 2020) or external growth restrictions (Bhusari et al., 2022). Thus, the >90% retention levels provided by the combo TA system for up to 40 generations should be more than sufficient for these applications. Furthermore, loss of the plasmid only reverts the bacteria to their non-GEM probiotic status, thus enabling the generation of transient GEMs that would be desirable for such applications. Accordingly, by varying the TA system used, the GEM lifetime of these organisms could be tuned. Based on this concept, we introduce a new metric, G_{50} , for characterizing such transient GEMs. The G_{50} value corresponds to the generation at which half the population of a strain has lost its plasmid. As shown in Figure 4, G_{50} can be tuned from 50 generations for the No TA condition up to 110 generations (extrapolated) for the combo system. Further exploration of additional TA systems in

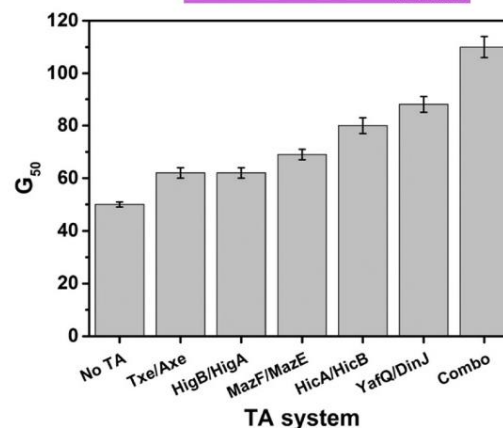


FIGURE 4 G_{50} values of the different TA systems tested in *Lactiplantibacillus plantarum*. Combo = MazF/MazE + YafQ/DinJ.

future studies will contribute to more fine tuning of retention lifetimes and possibly even lead to near-perfect retention as has been achieved in *E. coli* by the Txe/Axe system (Fedorec et al., 2019). These G_{50} values are expected to depend on culture parameters and environmental factors, due to which it could also become a useful metric for assessing natural and industrial conditions in which lactobacilli grow and function.

CONCLUSIONS

Lactobacilli as probiotics and commensals in humans and animals have immense potential to be developed for healthcare applications but as non-model organisms have very poorly equipped genetic toolboxes. Addressing this limitation, this study describes two new genetic modules, characterized in probiotic *L. plantarum*: an ultra-strong constitutive promoter (P_{tlpA}) and TA plasmid retention systems. Our results demonstrate that the promoter drives gene expression at levels over 5-fold higher than the strongest promoters previously reported in *L. plantarum* and the TA systems decelerate plasmid loss in a tuneable manner without the need for external selection pressures or genomic manipulations.

Apart from the impact, these modules will have in expanding the programmability of lactobacilli, the unique conceptual insights gained from this work will aid in the further development of genetic parts. For one, the unique features of the P_{tlpA} promoter sequence that originate from phylogenetically distant *Salmonella* provide clues to understanding what drives promoter strength. Second, both homologous and heterologous toxin/antitoxin systems can be used in *L. plantarum* for plasmid retention without considerably affecting bacterial growth rates or protein production levels. More

interestingly, the plasmid retention efficacy of these systems can be improved by combining two toxin-antitoxin systems, a phenomenon that has yet been tested only in *E. coli*. Finally, these systems provide the possibility to generate tuneable transient GEMs since plasmid loss reverts the cells to their non-GEM probiotic status, characterized by the new G_{50} metric.

AUTHOR CONTRIBUTIONS

Sourik Dey: Conceptualization (equal); data curation (equal); formal analysis (equal); methodology (equal); visualization (equal); writing – original draft (equal). **Marc Blanch-Asensio:** Conceptualization (equal); data curation (equal); formal analysis (equal); methodology (equal); visualization (equal); writing – original draft (equal). **Sanjana Balaji Kuttae:** Investigation (equal). **Shrikrishnan Sankaran:** Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); visualization (equal); writing – review and editing (equal).

ACKNOWLEDGMENTS

The *Lactiplantibacillus plantarum* WCFS1 strain was a kind gift from Prof. Gregor Fuhrmann (Helmholtz Institute for Pharmaceutical Research, Saarland). The plasmids pSIP403 and pLp_3050sNuc were a kind gift from Prof. Lars Axelsson (Addgene plasmid # 122028) and Prof. Geir Mathiesen (Addgene plasmid # 122030), respectively. The plasmid pTlpA39-Wasabi was a kind gift from Prof. Mikhail Shapiro (Addgene plasmid # 86116). The plasmid pUC-GFP-AT was a kind gift from Prof. Chris Barnes (Addgene plasmid # 133306). The authors thank Dr. Samuel Pearson at the INM – Leibniz Institute for New Materials for discussions that led to the G_{50} concept. This work was supported by the Deutsche Forschungsgemeinschaft's Research grant [Project # 455063657], Collaborative Research Centre, SFB 1027 [Project # 200049484] and the Leibniz Science Campus on Living Therapeutic Materials [LifeMat]. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

A patent application has been filed based on the results of this work (Application no. is DE 102022 119024.2).

DATA AVAILABILITY STATEMENT

All data are available from the corresponding authors upon reasonable request.

ORCID

Shrikrishnan Sankaran  <https://orcid.org/0000-0001-6251-585X>

REFERENCES

Abedi, M.H., Yao, M.S., Mittelstein, D.R., Bar-Zion, A., Swift, M.B., Lee-Gosselin, A. et al. (2022) Ultrasound-controllable

engineered bacteria for cancer immunotherapy. *Nature Communications*, 13, 1–11.

- Allison, G.E. & Klaenhammer, T.R. (1996) Functional analysis of the gene encoding immunity to lactacin F, lafl, and its use as a *Lactobacillus*-specific, food-grade genetic marker. *Applied and Environmental Microbiology*, 62, 4450–4460. Available from: <https://doi.org/10.1128/aem.62.12.4450-4460.1996>
- Ashraf, R. & Shah, N.P. (2011) Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt—A review. *International Journal of Food Microbiology*, 149, 194–208. Available from: <https://doi.org/10.1016/j.ijfoodmicro.2011.07.008>
- Bardaji, L., Añorga, M., Echeverría, M., Ramos, C. & Murillo, J. (2019) The toxic guardians—multiple toxin-antitoxin systems provide stability, avoid deletions and maintain virulence genes of *Pseudomonas syringae* virulence plasmids. *Mobile DNA*, 10, 1–17.
- Beaulaurier, J., Schadt, E.E. & Fang, G. (2019) Deciphering bacterial epigenomes using modern sequencing technologies. *Nature Reviews. Genetics*, 20, 157–172. Available from: <https://doi.org/10.1038/s41576-018-0081-3>
- Bhusari, S., Sankaran, S. & Del Campo, A. (2022) Regulating bacterial behavior within hydrogels of tunable viscoelasticity. *Advancement of Science*, 9, 2106026.
- Bibalan, M.H., Eshaghi, M., Rohani, M., Esghaei, M., Darban-Sarokhalil, D., Pourshafie, M.R. et al. (2017) Isolates of *Lactobacillus plantarum* and *L. reuteri* display greater antiproliferative and antipathogenic activity than other *Lactobacillus* isolates. *Journal of Medical Microbiology*, 66, 1416–1420. Available from: <https://doi.org/10.1099/jmm.0.000591>
- Bron, P.A., Hoffer, S.M., Van Swam, I.I., De Vos, W.M. & Kleerebezem, M. (2004) Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. *Applied and Environmental Microbiology*, 70, 310–317. Available from: <https://doi.org/10.1128/AEM.70.1.310-317.2004>
- Casadesús, J. & Low, D. (2006) Epigenetic gene regulation in the bacterial world. *Microbiology and Molecular Biology Reviews*, 70, 830–856. Available from: <https://doi.org/10.1128/MMBR.00016-06>
- Castillo-Hair, S.M., Baerman, E.A., Fujita, M., Igoshin, O.A. & Tabor, J.J. (2019) Optogenetic control of *Bacillus subtilis* gene expression. *Nature Communications*, 10, 1–11.
- Chan, M.Z.A., Chua, J.Y., Toh, M. & Liu, S.Q. (2019) Survival of probiotic strain *Lactobacillus paracasei* L26 during co-fermentation with *S. cerevisiae* for the development of a novel beer beverage. *Food Microbiology*, 82, 541–550. Available from: <https://doi.org/10.1016/j.fm.2019.04.001>
- Chee, W.K.D., Yeoh, J.W., Dao, V.L. & Poh, C.L. (2022) Highly reversible tunable thermal-repressible split-7 RNA polymerases (thermal-T7RNAPs) for dynamic gene regulation. *ACS Synthetic Biology*, 11(2), 921–937. Available from: <https://doi.org/10.1021/acssynbio.1c00545>
- Chen, Y., Qi, M., Xu, M., Huan, H., Shao, W. & Yang, Y. (2018) Food-grade gene transformation system constructed in *Lactobacillus plantarum* using a GImS-encoding selection marker. *FEMS Microbiology Letters*, 365, fny254.
- Courbet, A., Endy, D., Renard, E., Molina, F. & Bonnet, J. (2015) Detection of pathological biomarkers in human clinical samples via amplifying genetic switches and logic gates. *Science Translational Medicine*, 7, 289ra83.
- Darby, T.M. & Jones, R.M. (2017) Beneficial influences of *Lactobacillus plantarum* on human health and disease. In: Floch, M., Ringel, Y. & Walker, W.A. (Eds.) *The microbiota in gastrointestinal pathophysiology*. Boston: Academic Press, pp. 109–117.

- Dawoud, T.M., Davis, M.L., Park, S.H., Kim, S.A., Kwon, Y.M., Jarvis, N. et al. (2017) The potential link between thermal resistance and virulence in *Salmonella*: a review. *Frontiers in Veterinary Science*, 4, 93.
- de Vos, W.M. (2011) Systems solutions by lactic acid bacteria: from paradigms to practice. *Microbial Cell Factories*, 10, 1–13.
- du Toit, M., Engelbrecht, L., Lerm, E. & Krieger-Weber, S. (2011) *Lactobacillus*: the next generation of malolactic fermentation starter cultures—an overview. *Food and Bioprocess Technology*, 4, 876–906. Available from: <https://doi.org/10.1007/s11947-010-0448-8>
- Edgar, R.C. (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics*, 5, 1–19.
- Eloitz, M.B. & Leibler, S. (2000) A synthetic oscillatory network of transcriptional regulators. *Nature*, 403, 335–338. Available from: <https://doi.org/10.1038/35002125>
- Fedorec, A.-J., Ozdemir, T., Doshi, A., Ho, Y.K., Rosa, L., Rutter, J. et al. (2019) Two new plasmid post-segregational killing mechanisms for the implementation of synthetic gene networks in *Escherichia coli*. *iscience*, 14, 323–334. Available from: <https://doi.org/10.1016/j.isci.2019.03.019>
- Gaida, S.M., Sandoval, N.R., Nicolaou, S.A., Chen, Y., Venkataraman, K.P. & Papoutsakis, E.T. (2015) Expression of heterologous sigma factors enables functional screening of metagenomic and heterologous genomic libraries. *Nature Communications*, 6, 1–10.
- Grady, R. & Hayes, F. (2003) Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of *Enterococcus faecium*. *Molecular Microbiology*, 47, 1419–1432. Available from: <https://doi.org/10.1046/j.1365-2958.2003.03387.x>
- Halbmayer, E., Mathiesen, G., Nguyen, T.H., Maischberger, T., Peterbauer, C.K., Eijssink, V.G. et al. (2008) High-level expression of recombinant β -galactosidases in *Lactobacillus plantarum* and *Lactobacillus sakei* using a sakacin P-based expression system. *Journal of Agricultural and Food Chemistry*, 56, 4710–4719. Available from: <https://doi.org/10.1021/jf073260>
- Heiss, S., Hörmann, A., Tauer, C., Sonnleitner, M., Egger, E., Grabherr, R. et al. (2016) Evaluation of novel inducible promoter/repressor systems for recombinant protein expression in *Lactobacillus plantarum*. *Microbial Cell Factories*, 15, 1–17.
- Helmann, J.D. (1995) Compilation and analysis of *Bacillus subtilis* σ A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Research*, 23, 2351–2360. Available from: <https://doi.org/10.1093/nar/23.13.2351>
- Hurme, R., Berndt, K.D., Normark, S.J. & Rhen, M. (1997) A proteinaceous gene regulatory thermometer in *Salmonella*. *Cell*, 90, 55–64. Available from: [https://doi.org/10.1016/S0092-8674\(00\)80313-X](https://doi.org/10.1016/S0092-8674(00)80313-X)
- Janahi, E.M.A., Haque, S., Akhter, N., Wahid, M., Jawed, A., Mandal, R.K. et al. (2018) Bioengineered intravaginal isolate of *Lactobacillus plantarum* expresses algal lectin scytovirin demonstrating anti-HIV-1 activity. *Microbial Pathogenesis*, 122, 1–6. Available from: <https://doi.org/10.1016/j.micpath.2018.06.002>
- Jiménez, E., Fernández, L., Maldonado, A., Martín, R., Olivares, M., Xaus, J. et al. (2008) Oral administration of *Lactobacillus* strains isolated from breast milk as an alternative for the treatment of infectious mastitis during lactation. *Applied and Environmental Microbiology*, 74, 4650–4655. Available from: <https://doi.org/10.1128/AEM.02599-07>
- Kan, A., Gelfat, I., Emani, S., Praveschotinunt, P. & Joshi, N.S. (2020) Plasmid vectors for in vivo selection-free use with the probiotic *E. coli* Nissle 1917. *ACS Synthetic Biology*, 10(1), 94–106.
- Kasımoğlu, A., Göncüoğlu, M. & Akgün, S. (2004) Probiotic white cheese with *Lactobacillus acidophilus*. *International Dairy Journal*, 14, 1067–1073. Available from: <https://doi.org/10.1016/j.idairyj.2004.04.006>
- Landete, J.M., Langa, S., Revilla, C., Margolles, A., Medina, M. & Arqués, J.L. (2015) Use of anaerobic green fluorescent protein versus green fluorescent protein as reporter in lactic acid bacteria. *Applied Microbiology and Biotechnology*, 99, 6865–6877. Available from: <https://doi.org/10.1007/s00253-015-6770-3>
- LeCureux, J.S. & Dean, G.A. (2018) *Lactobacillus mucosae* vaccine vectors: immune responses against bacterial and viral antigens. *mSphere*, 3, e00061–e00018.
- Letunic, I. & Bork, P. (2007) Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics*, 23, 127–128.
- Levante, A., Folli, C., Montanini, B., Ferrari, A., Neviani, E. & Lazzi, C. (2019) Expression of DinJ-YafQ system of *Lactobacillus casei* group strains in response to food processing stresses. *Microorganisms*, 7, 438. Available from: <https://doi.org/10.3390/microorganisms7100438>
- Ma, B., Forney, L.J. & Ravel, J. (2012) The vaginal microbiome: rethinking health and diseases. *Annual Review of Microbiology*, 66, 371–389. Available from: <https://doi.org/10.1146/annurev-micro-092611-150157>
- Madeira, F., Pearce, M., Tivey, A., Basutkar, P., Lee, J., Edbali, O. et al. (2022) Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Research*, 50, W276–W279. Available from: <https://doi.org/10.1093/nar/gkac240>
- Mastromarino, P., Macchia, S., Meggiorini, L., Trinchieri, V., Mosca, L., Perluigi, M. et al. (2009) Effectiveness of *Lactobacillus*-containing vaginal tablets in the treatment of symptomatic bacterial vaginosis. *Clinical Microbiology and Infection*, 15, 67–74. Available from: <https://doi.org/10.1111/j.1469-0691.2008.02112.x>
- Mathiesen, G., Øverland, L., Kuczkowska, K. & Eijssink, V.G. (2020) Anchoring of heterologous proteins in multiple *Lactobacillus* species using anchors derived from *Lactobacillus plantarum*. *Scientific Reports*, 10, 1–10.
- Mathiesen, G., Sveen, A., Brurberg, M.B., Fredriksen, L., Axelsson, L. & Eijssink, V.G. (2009) Genome-wide analysis of signal peptide functionality in *Lactobacillus plantarum* WCFS1. *BMC Genomics*, 10, 1–13.
- Meier-Kolthoff, J.P. & Göker, M. (2019) TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nature Communications*, 10, 1–10.
- Meng, Q., Yuan, Y., Li, Y., Wu, S., Shi, K. & Liu, S. (2021) Optimization of electrotransformation parameters and engineered promoters for *Lactobacillus plantarum* from wine. *ACS Synthetic Biology*, 10, 1728–1738. Available from: <https://doi.org/10.1021/acssynbio.1c00123>
- Nguyen, T.T., Mathiesen, G., Fredriksen, L., Kittl, R., Nguyen, T.H., Eijssink, V.G. et al. (2011) A food-grade system for inducible gene expression in *Lactobacillus plantarum* using an alanine racemase-encoding selection marker. *Journal of Agricultural and Food Chemistry*, 59, 5617–5624. Available from: <https://doi.org/10.1021/jf104755r>
- Pedrolli, D.B., Ribeiro, N.V., Squizzato, P.N., de Jesus, V.N., Cozetto, D.A., Tuma, R.B. et al. (2019) Engineering microbial living therapeutics: the synthetic biology toolbox. *Trends in Biotechnology*, 37, 100–115. Available from: <https://doi.org/10.1016/j.tibtech.2018.09.005>
- Piraner, D.I., Abedi, M.H., Moser, B.A., Lee-Gosselin, A. & Shapiro, M.G. (2017) Tunable thermal bioswitches for in vivo control of microbial therapeutics. *Nature Chemical Biology*, 13, 75–80. Available from: <https://doi.org/10.1038/nchembio.2233>
- Plessas, S., Fisher, A., Koureta, K., Psarianos, C., Nigam, P. & Koutinas, A.A. (2008) Application of *Kluyveromyces marxianus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *L. helveticus* for sourdough bread making. *Food Chemistry*, 106, 985–990. Available from: <https://doi.org/10.1016/j.foodchem.2007.07.012>
- Rosenfeldt, V., Benfeldt, E., Nielsen, S.D., Michaelsen, K.F., Jeppesen, D.L., Valerius, N.H. et al. (2003) Effect of probiotic

- Lactobacillus* strains in children with atopic dermatitis. *The Journal of Allergy and Clinical Immunology*, 111, 389–395. Available from: <https://doi.org/10.1067/mai.2003.389>
- Rottinghaus, A.G., Ferreiro, A., Fishbein, S.R., Dantas, G. & Moon, T.S. (2022) Genetically stable CRISPR-based kill switches for engineered microbes. *Nature Communications*, 13(1), 1–17.
- Rud, I., Jensen, P.R., Naterstad, K. & Axelsson, L. (2006) A synthetic promoter library for constitutive gene expression in *Lactobacillus plantarum*. *Microbiology*, 152, 1011–1019. Available from: <https://doi.org/10.1099/mic.0.28599-0>
- Russo, P., Iturria, I., Mohedano, M.L., Caggianiello, G., Rainieri, S., Fiocco, D. et al. (2015) Zebrafish gut colonization by mCherry-labelled lactic acid bacteria. *Applied Microbiology and Biotechnology*, 99, 3479–3490. Available from: <https://doi.org/10.1007/s00253-014-6351-x>
- Salomé-Desnoullez, S., Poirer, S., Foligné, B., Muharram, G., Peucelle, V., Lafont, F. et al. (2021) Persistence and dynamics of fluorescent *Lactobacillus plantarum* in the healthy versus inflamed gut. *Gut Microbes*, 13, 1897374–1897316.
- Siezen, R.J. & van Hylckama Vlieg, J.E. (2011) Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. *Microbial Cell Factories*, 10, 1–13.
- Sørvig, E., Grönqvist, S., Naterstad, K., Mathiesen, G., Eijsink, V.G. & Axelsson, L. (2003) Construction of vectors for inducible gene expression in *Lactobacillus sakei* and *L. plantarum*. *FEMS Microbiology Letters*, 229, 119–126. Available from: [https://doi.org/10.1016/S0378-1097\(03\)00798-5](https://doi.org/10.1016/S0378-1097(03)00798-5)
- Sørvig, E., Mathiesen, G., Naterstad, K., Eijsink, V.G. & Axelsson, L. (2005) High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. *Microbiology*, 151, 2439–2449.
- Spangler, J.R., Caruana, J.C., Phillips, D.A. & Walper, S.A. (2019) Broad range shuttle vector construction and promoter evaluation for the use of *Lactobacillus plantarum* WCFS1 as a microbial engineering platform. *Synthetic Biology*, 4, ysz012.
- Spath, K., Heini, S. & Grabherr, R. (2012) Direct cloning in *Lactobacillus plantarum*: electroporation with non-methylated plasmid DNA enhances transformation efficiency and makes shuttle vectors obsolete. *Microbial Cell Factories*, 11, 1–8.
- Stirling, F. & Silver, P.A. (2020) Controlling the implementation of transgenic microbes: are we ready for what synthetic biology has to offer? *Molecular Cell*, 78(4), 614–623. Available from: <https://doi.org/10.1016/j.molcel.2020.03.034>
- Takala, T. & Saris, P. (2002) A food-grade cloning vector for lactic acid bacteria based on the nisin immunity gene *nisI*. *Applied Microbiology and Biotechnology*, 59, 467–471.
- Tauer, C., Heini, S., Egger, E., Heiss, S. & Grabherr, R. (2014) Tuning constitutive recombinant gene expression in *Lactobacillus plantarum*. *Microbial Cell Factories*, 13, 1–11.
- Teughels, W., Durukan, A., Ozcelik, O., Pauwels, M., Quirynen, M. & Haytac, M.C. (2013) Clinical and microbiological effects of *Lactobacillus reuteri* probiotics in the treatment of chronic periodontitis: a randomized placebo-controlled study. *Journal of Clinical Periodontology*, 40, 1025–1035. Available from: <https://doi.org/10.1111/jcpe.12155>
- Todt, T.J., Wels, M., Bongers, R.S., Siezen, R.S., Van Hijum, S.A. & Kleerebezem, M. (2012) Genome-wide prediction and validation of sigma70 promoters in *Lactobacillus plantarum* WCFS1. *PLoS One*, 7, e45097. Available from: <https://doi.org/10.1371/journal.pone.0045097>
- Torres, B., Jaenecke, S., Timmis, K.N., García, J.L. & Díaz, E. (2003) A dual lethal system to enhance containment of recombinant micro-organisms. *Microbiology*, 149(12), 3595–3601. Available from: <https://doi.org/10.1099/mic.0.26618-0>
- Turroni, F., Ventura, M., Buttó, L.F., Duranti, S., O'Toole, P.W., Metherway, M.O.C. et al. (2014) Molecular dialogue between the human gut microbiota and the host: a *Lactobacillus* and *Bifidobacterium* perspective. *Cellular and Molecular Life Sciences*, 71, 183–203. Available from: <https://doi.org/10.1007/s00018-013-1318-0>
- Van Kranenburg, R., Golic, N., Bongers, R., Leer, R.J., De Vos, W.M., Siezen, R.J. et al. (2005) Functional analysis of three plasmids from *Lactobacillus plantarum*. *Applied and Environmental Microbiology*, 71, 1223–1230. Available from: <https://doi.org/10.1128/AEM.71.3.1223-1230.2005>
- Wang, B., Kitney, R.I., Joly, N. & Buck, M. (2011) Engineering modular and orthogonal genetic logic gates for robust digital-like synthetic biology. *Nature Communications*, 2, 1–9.
- Wang, M., Fu, T., Hao, J., Li, L., Tian, M., Jin, N. et al. (2020) A recombinant *Lactobacillus plantarum* strain expressing the spike protein of SARS-CoV-2. *International Journal of Biological Macromolecules*, 160, 736–740. Available from: <https://doi.org/10.1016/j.ijbiomac.2020.05.239>
- Waterhouse, A.M., Procter, J.B., Martin, D.M., Clamp, M. & Barton, G.J. (2009) Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, 25, 1189–1191. Available from: <https://doi.org/10.1093/bioinformatics/btp033>
- Watterlot, L., Rochat, T., Sokol, H., Cherbuy, C., Bouloufa, I., Lefèvre, F. et al. (2010) Intragastric administration of a superoxide dismutase-producing recombinant *Lactobacillus casei* BL23 strain attenuates DSS colitis in mice. *International Journal of Food Microbiology*, 144, 35–41. Available from: <https://doi.org/10.1016/j.ijfoodmicro.2010.03.037>
- Wright, O., Stan, G.B. & Ellis, T. (2013) Building-in biosafety for synthetic biology. *Microbiology*, 159(Pt 7), 1221–1235. Available from: <https://doi.org/10.1099/mic.0.066308-0>
- Xie, Y., Wei, Y., Shen, Y., Li, X., Zhou, H., Tai, C. et al. (2018) TADB 2.0: an updated database of bacterial type II toxin–antitoxin loci. *Nucleic Acids Research*, 46, D749–D753. Available from: <https://doi.org/10.1093/nar/gkx1033>
- Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M., Harris, H.M., Mattarelli, P. et al. (2020) A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology*, 70, 2782–2858. Available from: <https://doi.org/10.1099/ijsem.0.004107>
- Zocco, M.A., Verme, L.Z.D., Cremonini, F., Piscaglia, A.C., Nista, E.C., Candelli, M. et al. (2006) Efficacy of *Lactobacillus GG* in maintaining remission of ulcerative colitis. *Alimentary Pharmacology & Therapeutics*, 23, 1567–1574. Available from: <https://doi.org/10.1111/j.1365-2036.2006.02927.x>

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Dey, S., Blanch-Asensio, M., Balaji Kuttae, S. & Sankaran, S. (2023) Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic *Lactobacilli*. *Microbial Biotechnology*, 16, 1264–1276. Available from: <https://doi.org/10.1111/1751-7915.14228>

Chapter 6.

Development of L. plantarum for in vivo imaging and therapeutic applications

Introduction

Lactobacilli are natural inhabitants of the human body that exhibit beneficial health-promoting and immunomodulating effects in the host [6]. Given these properties, lactobacilli have been extensively investigated in animal studies and clinical trials in humans for a wide range of medical applications, including their possible role as adjuvants in anticancer therapies, prevention of intestinal diseases, treatment of respiratory and liver diseases, and the maintenance of vaginal health [162-164].

The investigation of the effect of lactobacilli in these applications requires systemic or oral administration of these bacteria inside model organisms such as mice. For such experiments, a methodology to monitor the administered bacteria *in vivo* is commonly required. Bioluminescence remains the gold standard reporter technology for tracking and monitoring live bacteria inside mice in a non-invasive, real-time manner [165]. This method relies on a reaction catalyzed by an enzyme named luciferase, which emits light as a product [166].

Multiple luciferases have been expressed in *L. plantarum* for *in vivo* imaging purposes. For example, Daniel et al. in 2013 engineered *L. plantarum* to intracellularly produce the luciferase Gluc from a copepod and luciferase CBRluc from the red click beetle. The latter showed high levels of detection in mice, resulting in a promising *in vivo* reporter [167]. Two years later, the same authors additionally tested the green click beetle luciferase

mutant CBGluc, which showed results comparable to CBRluc [168]. In 2018, Van Zyl et al. expressed the firefly luciferase Fluc in *L. plantarum*, which also worked *in vivo* [169].

Despite promising outcomes using these luciferases, improvements were needed in terms of higher luminosity, reduced size, and increased stability [170]. For this, Hall et al. engineered a smaller luciferase, nanoLuc (nLuc), that exhibited higher levels of luminescence than other widely used luciferases [171]. In 2020, Zhao et al. engineered *L. plantarum* to express nLuc and observed significantly higher luminescence compared to CBRluc and Fluc [172]. Despite these positive results, in this last study, a moderate promoter (P_{ldh}) was used to drive the expression of the nLuc-coding gene, which provides room for enhanced performance from a genetic engineering perspective.

Apart from applications inside the body, lactobacilli have also shown great potential on the body's surface. Reports have demonstrated that lactobacilli play a role in inhibiting skin pathogens such as *S. aureus* and *Cutibacterium acnes*, and reducing skin inflammation upon topical application [173, 174]. The eye is another body surface where lactobacilli have been tested either as live therapeutics, cell lysates, or supernatants with promising results. The administration of *L. acidophilus* as eye-drop treatment significantly improved the symptoms associated with conjunctivitis [175]. Another example is the application of an ophthalmic formulation based on *L. sakei* cell lysate, which remarkably alleviated the symptoms of dry eye disease [176]. Ophthalmic formulations of *L. plantarum* supernatants have also exerted effective results in mediating anti-inflammatory properties and attenuating uveitis [26, 27].

By means of genetic engineering, *L. plantarum* can also be further developed to produce and secrete proteins relevant to treating eye or skin diseases and be used as probiotic living contact lenses or wearable living devices [177, 178]. The first therapeutic recombinant protein approved by the U.S. Food and Drug Administration as an eye drop drug for treating neurotrophic keratitis was a nerve growth factor (NGF). NGF is considered a therapeutic protein due to its crucial role in the growth, maintenance, and proliferation of neurons, and in other non-neuronal pathways. These include the regulation of epithelial cells during hair growth and wound healing, and in the regulation

and differentiation of hematopoietic stem cells and immune cells [179]. Apart from neurotrophic keratitis, NGF has been extensively investigated as a therapeutic protein for treating a wide range of brain, eye, skin, and lung diseases, including Alzheimer's disease and peripheral neuropathies, skin and corneal ulcers, glaucoma, retinitis pigmentosa, and pediatric optic gliomas [179-181].

The structure of NGF consists of three subunits: α , β , and γ , with the dimeric β -NGF form responsible for the biological activity of the protein [182, 183]. Due to the clinical relevance of this protein, attention has turned towards the recombinant production of β -NGF using bacteria, yeast, plants, mammalian cells, and even insect cells as factories for a high level of production [184]. Regarding bacteria, to my knowledge, recombinant β -NGF production has only been reported in *E. coli*, and, to date, there are no reports of either secretion or production of β -NGF from lactobacilli bacteria [185-187].

This chapter further focuses on the question of whether the novel genetic parts, namely the P_{tec} promoter and the SP3, can drive high levels of expression and secretion, respectively, of other POIs. This work presents preliminary results addressing both the reporting and therapeutic capabilities that can be engineered in lactobacilli. The first part describes the development of engineered *L. plantarum* strains towards enhanced *in vivo* detection capacities. To this end, luciferase-expressing and multi-reporter-expressing strains were developed for multimodal reporting of *L. plantarum* in mice. The second part explores the generation of an NGF-secreting *L. plantarum* strain suitable for ELMs.

Materials and Methods

Strains, media, and plasmid vector.

The *L. plantarum* WCFS1 strain was used in this study. The strain was maintained in De Man, Rogosa, and Sharpe (MRS) media (Carl Roth GmbH, Germany) and incubated at 37°C and 250 revolutions per minute (rpm). For the engineered strains, the MRS medium was supplemented with 10 μ L/mL of erythromycin (Carl Roth GmbH, Germany). The P_{tec} -mCherry plasmid (Addgene plasmid # 229145) was the backbone vector for the nLuc

and the NGF molecular cloning. The plasmid pLp_ P_{tec} _3050Nuc_mScarlet3 was the backbone for the double and triple reporter clonings [188]. The CBRLuc-encoding plasmid was a kind gift from Catherine Daniel (Institute Pasteur, Lille, France).

Molecular cloning reagents

Primers were synthesized by Eurofins Genomics GmbH (Köln, Germany). All the primers used in this study are listed in **Table S1**. All the gene fragments in this study were ordered as eBlocks from Integrated DNA Technologies (Coralville, USA). Q5 High Fidelity 2X Master Mix (NEB GmbH, Germany) was used for the polymerase chain reaction (PCR). The Wizard® SV Gel and PCR Clean-Up System (Promega GmbH, Germany) was used for PCR product purification. The eBlocks were assembled using the HiFi Assembly Master Mix (NEB GmbH, Germany).

Primer name	Sequence
Vector NGF/nLuc fw	5'- gaagataaatcccataagg-3'
Vector NGF/nLuc rev	5'- aagaactctattgaagccc-3'
Direct cloning fw	5'- ccgtagcgtagtagc-3'
Direct cloning rev	5'- cttcaaagggtaacagc-3'
CBRLuc rev	5'- tcacgttaagggattttgaaaccacctctaaaaggt-3'
CBRLuc fw	5'- atgacttattccatggtg-3'
P _{Idh} fw	5'- catcgtaatagagcatagacgcaactctgaagtaacgata-3'
Double reporter fw	5'- tggacctgtgccgttaa-3'
Double reporter rev	5'- ttattgacctgaatcagcg-3'
Triple reporter vector fw	5'- aataactagcataaccccttg-3'
Triple reporter vector rev	5'- ttagcttcacctgagcc-3'
Triple reporter insert fw	5'- gcggctcaggtggaagctaattggacctgtgccgttaa -3'
Triple reporter insert rev	5'- ccaaggggtatgctagtatt -3'

Table S1: List of primers used in this study.

L. plantarum WCFS1 competent cell preparation and DNA transformation

L. plantarum WCSF1 electrocompetent cells were prepared as described [160]. DNA transformation was done by electroporation, following the procedure described in [158].

***In vitro* bioluminescence detection**

The recombinant bacteria were grown in MRS medium overnight. After overnight growth, bacteria were subcultured to an optical density at 600 nm (OD_{600}) = 0.01 and grown for 16 hours at 37°C and 250 rpm. After that, 50 μ l of the bacterial cultures were adjusted to an OD_{600} = 2. Then, 50 μ L of the cultures were added to a white 96-well plate (μ CLEAR®, Greiner). The expression of CBRLuc in bacterial cultures was determined with the Bright-Glo® Luciferase Assay System (Promega, Germany), following the manufacturer's instructions. The expression of nLuc in bacterial cultures was determined with the Nano-Glo® Luciferase Assay System (Promega, Germany), following the manufacturer's instructions. The microplate reader was used to assess the levels of chemiluminescence (1000 ms, attenuation = automatic). *L. plantarum* carrying an empty vector was used as a control. The BioRad Gel Documentation System was used to capture the luminescence of bacteria grown in agar plates. The settings were as follows: chemiluminescence option with auto optimal exposure.

NucA secretion and mScarlet3 production detection

The recombinant bacteria were grown in MRS medium overnight. The following day, bacteria were subcultured to an OD_{600} = 0.1 and grown to an OD_{600} = 1 at 37°C and 250 rpm. Next, 10 μ L of the bacteria were spotted on DNase agar (Altmann Analytik GmbH, Germany) plates supplemented with 10 μ g/mL of erythromycin and incubated at 37 °C for 24 h. The detection of NucA secretion and intracellular mScarlet3 production was done by capturing the agar plates in RGB settings (auto optimal exposure) in the BioRad Gel Documentation System. The ImageJ software was used to measure the decrease in fluorescence intensity of the halos due to NucA secretion into the extracellular space. The drop in mScarlet3 expression was quantified as follows: overnight bacterial cultures were resuspended in 1 mL of DPBS, and 200 μ L of the mixture was added to a 96-well plate. The microplate reader was used to measure the levels of fluorescence (excitation/emission values: $Ex\lambda$ / $Em\lambda$ = 569 nm/600 nm, gain = 75, and Z-position = 19000 μ m). The values were divided by the OD_{600} values of the cultures (relative

fluorescence units (RFU)). Non-fluorescent *L. plantarum* bacteria were kept as a reference.

Detection and quantification of NGF secretion

NGF secretion was verified and quantified via the split nano luciferase assay described in [188]. The exact amount of secreted β -NGF in cell culture media (DMEM) was calculated using the standard curve for the split nano luciferase assay described in the same publication.

PC-12 cells passage

PC-12 cells were purchased from Cytion (Germany). Cells were grown in T75 cell culture flasks and in RPMI 1640 media supplemented with 10% FBS, 1% glutamine, and 2% NaHCO_3 . Cells were incubated at 37°C and 5% CO_2 . 4 mL of 1% trypsin was used for detaching the cells before cell passaging. Trypan Blue in a 1:1 ratio was used to stain the cells for cell counting using the CellDrop. The tabletop microscope Leica DMIL LED + MotiCamS3 was used for monitoring cell growth.

NGF treatment of PC-12 cells

The recombinant bacteria were grown in MRS medium overnight. The following day, bacteria were subcultured to an $\text{OD}_{600} = 0.1$ and grown to an $\text{OD}_{600} = 1$ at 37°C and 250 rpm. Next, 5 mL of the cultures were pelleted down at 4000 rpm for 5 minutes, and the bacteria were resuspended in 5 mL of RPMI media supplemented with 10% FBS, 1% glutamine, 2% NaHCO_3 , and 10 $\mu\text{g/mL}$ of erythromycin, and incubated at 37°C and 250 rpm for 20 hours. After that, 1 mL of the culture was pelleted at 8000 rpm for 3 minutes. The supernatant was collected and filter-sterilized before being used to treat PC-12 cells. *L. plantarum* carrying an empty vector was used as a negative control. Pure β -NGF was purchased from BioTeche (Wiesbaden, Germany) and used as a positive control for cell differentiation.

For the treatment, PC-12 cells were seeded in a transparent 96-well plate (Greiner, Germany) (20.000 cells per well, approximately). 100 μL of fresh RPMI media

supplemented with 10% FBS, 1% glutamine, and 2% NaHCO₃ was added to each well, and cells were incubated at 37°C and 5% CO₂ for 4 hours to allow cell attachment. After 4 hours, cells were treated with 100 µL of the bacterial supernatants (either NGF-secreting bacteria or empty vector-carrying bacteria) or 100 µL of pure NGF (100 ng/mL) and incubated at 37°C and 5% CO₂ for 4 days. The tabletop microscope Leica DMIL LED + MotiCamS3 was used for capturing the images after 4 days of incubation (96 hours).

Statistical, imaging, and bioinformatics analysis

The statistical analysis of the results was performed using GraphPad Prism 7.0 software. Student T-tests were performed to identify significant differences between the means of the groups: no statistically significant difference (ns), *p*-values <0.05 (*), *p*-values <0.01 (**), *p*-values <0.005 (***), *p*-values <0.001 (****). Images were processed using the software ImageJ (version 1.53). AlphaFold3 was used to predict the 3D structure of the secreted NGF protein. Biorender was used for the creation of the illustrations.

Results

Development of *L. plantarum* towards enhanced *in vivo* detection

We speculated that the strong novel *P_{tec}* promoter could be used to improve the levels of luciferase production and the luminescence signal generated by engineered *L. plantarum* WCFS1, thereby improving imaging quality for *in vivo* studies [189, 190]. To validate that, we generated the variant based on the commonly used CBRLuc reporter driven by the *P_{tec}* promoter. As a control, we cloned the CBRLuc gene under the *P_{ldh}* promoter in the same plasmid vector. Additionally, we cloned the nLuc coding gene under the *P_{tec}* promoter to confirm if higher luminescence can be detected with this more novel luciferase (**Figure 1A**). We observed that the CBRLuc-derived luminescence is 5.6 times higher when this luciferase is under *P_{tec}* compared to *P_{ldh}* (**Figure 1B**), validating that higher reporter production can be achieved when selecting a stronger constitutive promoter. On the other hand, the luminescence levels detected for the *P_{tec}*_nLuc strain were very high, 560 times higher than the *P_{tec}*_CBRLuc strain (**Figure 1B**). Notably, different substrates are needed

to generate luminescence from CBRLuc (beetle luciferin) and nLuc (furimazine). Nevertheless, a majority of the improved luminescence is expected to arise from nLuc being a more efficient enzyme [170]. Interestingly, the luminescence production of the P_{tec} _nLuc strain could even be seen by the eye in the dark, which was not possible with any of the other constructs (**Figure 1C**).

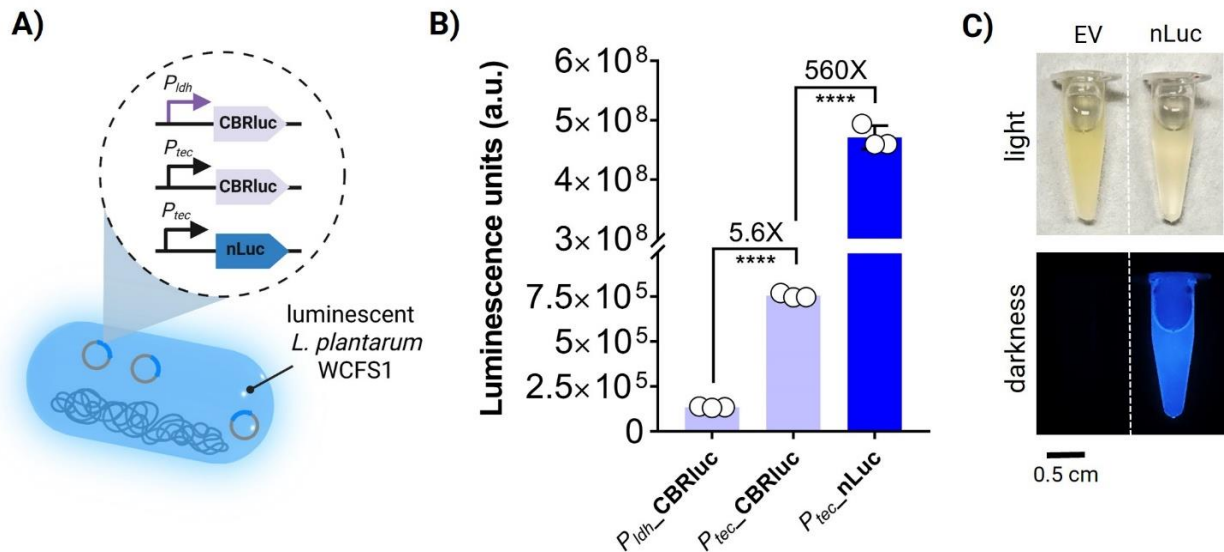


Figure 1. **A)** Illustration showing the luciferase-producing *L. plantarum* recombinant strains that were engineered in this work. **B)** Plot showing the absolute values of luminescence obtained with the P_{ldh} _CBRLuc, P_{tec} _CBRLuc, and P_{tec} _nLuc strains ($n = 3$, mean $\pm$ SD). **C)** Pictures captured in the light (exposure time = 1/100 sec) and darkness (exposure time = 1/15 sec) of tubes containing empty vector (EV) and nLuc bacteria, both at an $OD_{600} = 3$. All bacteria were grown in MRS media for 24 hours at 37 °C.

While nLuc offers a way to image and track the biodistribution of bacteria within an animal, encoding protein secretion would enable the delivery of therapeutics at disease sites or provide a non-invasive way to assess the *in vivo* activity of the engineered bacteria. A previous report used a nuclease from *S. aureus* as a reporter protein that could be detected in the urine and serve as a urinary tract infection biomarker [191]. In order to accomplish both simultaneously, I aimed to engineer a double reporter strain for *in vivo* mouse experiments based on the co-expression of nLuc and Ip_3050NucA by the P_{tec}

promoter (Lp_double), following a similar polycistronic approach already reported by us in *L. plantarum* (**Figure 2A**) [188]. Additionally, I wanted to investigate if not only two but three reporters (including mScarlet3) can be simultaneously expressed by the P_{tec} promoter following the polycistronic approach and, for the first time, develop an *L. plantarum* strain that expresses three reporter proteins at once (Lp_triple) (**Figure 2A**). The generation of both multi-reporter strains was successfully confirmed by the detection of both NucA and nLuc for the Lp_double strain and NucA, nLuc, and mScarlet3 for the Lp_triple strain (**Figure 2B**). Yet, as already observed previously, the co-expression of two genes under the same promoter comes with a cost [188]. For the Lp_double, the expression of NucA and nLuc genes dropped 52% and 46%, respectively, compared to the strains producing only one reporter (Lp_NucA and Lp_nLuc)(**Figure 2C**). Concerning the Lp_triple strain, the expression of NucA, nLuc, and mScarlet3 dropped by 10%, 52%, and 71%, respectively, when compared to the corresponding single reporter expressing strains (**Figure 2C**)⁷.

⁷ It is to be noted that even with these 50 to 70% drops in gene expression, the production of all the reporters could readily be visible to the naked eye.

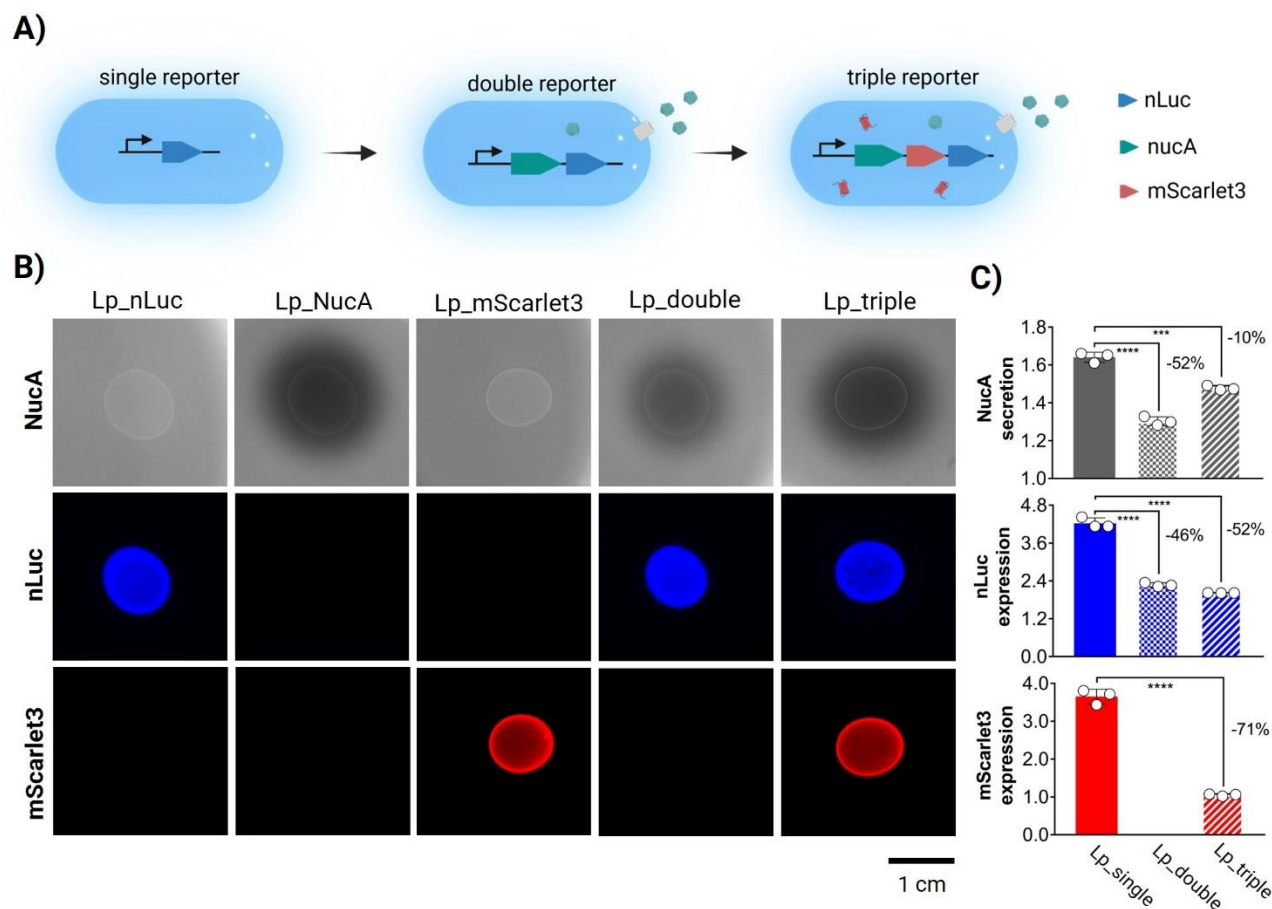


Figure 2. A) Further genetic modification of the nLuc-expressing *L. plantarum* strain (single reporter) by incorporating either the NucA-encoding gene upstream of the nLuc gene, thus generating a double reporter strain, or both the NucA-encoding gene and the mScarlet3 gene upstream of the nLuc gene, thereby generating the triple reporter strain. **B)** Bright, luminescent, and fluorescent pictures of DNase agar plates containing bacteria expressing nLuc (Lp_nLuc), NucA (Lp_NucA), mScarlet3 (Lp_mScarlet3), nLuc and NucA (Lp_double), and nLuc, NucA, and mScarlet3 (Lp_triple). Bacteria were incubated in DNase plates for 24 hours at 37 °C. **C)** Plots showing the drop in gene expression as a consequence of incorporating one or two additional genes as part of the polycistronic approach ($n = 3$, mean $\pm$ SD).

The *in vivo* evaluation of the multi-reporter strains is currently underway in mouse tumor models by the Hao lab at Boston University as part of a scientific collaboration.

Development of *L. plantarum* towards a nerve growth factor secretion

For programming *L. plantarum* to continuously secrete β -NGF to the extracellular environment, I designed a coding gene composed of three parts: i) the SP3 sequence for ensuring protein secretion, ii) the β -NGF coding sequence, and iii) the smBit sequence for secreted protein quantification. The gene was expressed under the P_{tlpA} strong constitutive promoter reported by us [192]. In this way, the levels of NGF secretion can be quantified with luminescence via the split-nano luciferase assay upon the addition of IgBit and substrate (**Figure 3A**). AlphaFold3 predicted the 3D protein structure of β -NGF with smBit at the C-terminal and the SP3 at the N-terminal, which will be cleaved by a signal peptidase during secretion (**Figure 3B**) [193]. The split nano-luciferase assay described in [188] was performed to validate the presence of β -NGF in the supernatant of β -NGF-secreting bacteria (Ns strain). Luminescence was only detected for the β -NGF-secreting bacteria upon the addition of substrate (IgBit and Nano-Glo® substrate (furimazine)) (**Figure 3C**). The exact amount of secreted β -NGF in cell culture media (DMEM) was calculated using the standard curve for the split nano luciferase assay described in [188] as well. After 24 hours of growth, >65 nM of β -NGF was detected in the supernatant, whereas negligible levels of luminescence were detected for EV bacteria (**Figure 3D**).

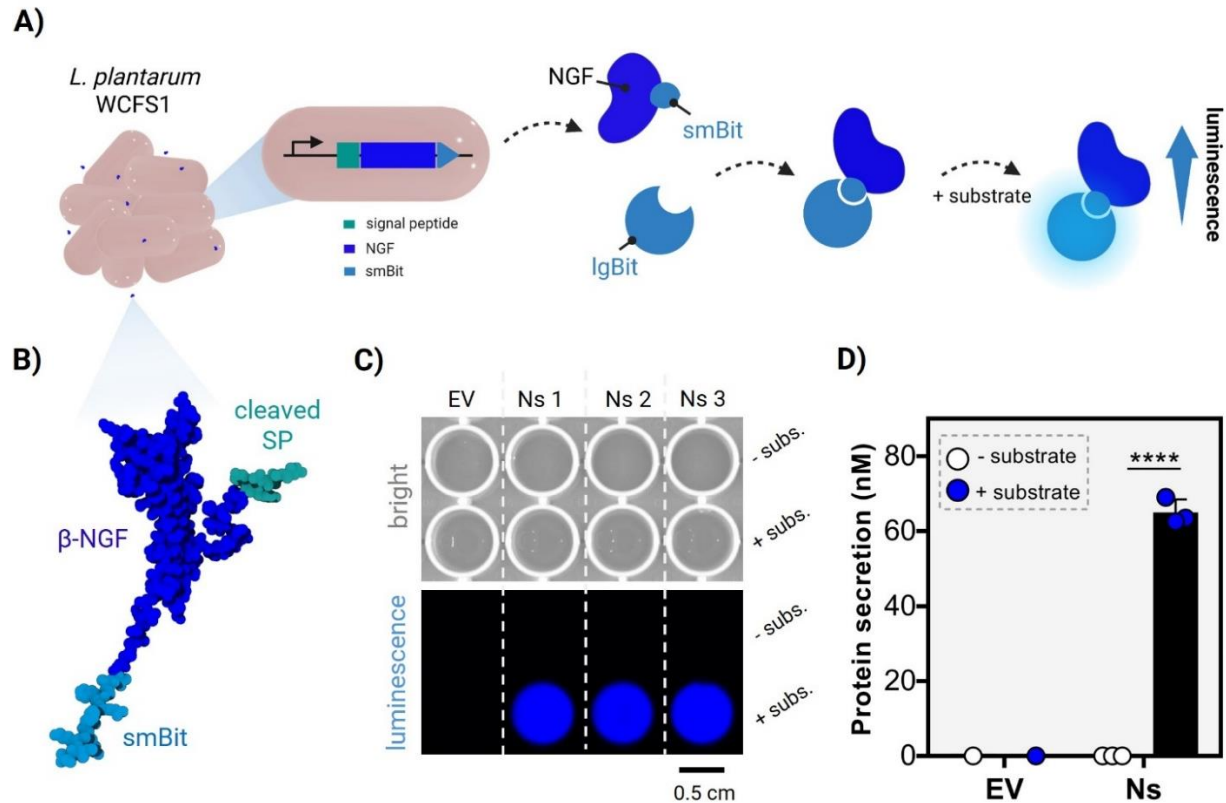


Figure 3. **A)** Illustration showing *L. plantarum* WCFS1 bacteria encoding for the β -NGF-secreting coding gene, composed of the SP3, the β -NGF coding sequence, and the smBit tag. The secretion of β -NGF was confirmed and quantified via the split-nano luciferase assay. The smBit tag in the β -NGF protein and the purified IgBit protein can interact with each other and, upon the addition of substrate, catalyze a chemical reaction that results in the production of bioluminescent light. **B)** AlphaFold3 3D protein structure of the cleaved SP3- β -NGF-smBit protein. **C)** Bright and luminescent pictures of a 96-well plate containing EV bacteria and three replicates of the β -NGF-secreting bacteria (Ns), with and without substrate addition. Bacteria were grown in MRS media for 16 hours. Bacteria with an $OD_{600} = 3$ were used for the assay in the 96-well plate. **D)** Plot showing the levels of β -NGF secretion (in nM) after growing the bacteria in DMEM media for 24 hours. The nM values were obtained after interpolating the luminescent values obtained in the microplate reader into the standard curve described in [188] ($n = 3$, mean $\pm$ SD).

Next, the bioactivity of the secreted β -NGF had to be confirmed. PC-12 cells are typically used as a model for neuronal differentiation and are widely employed for NGF-related studies [194-197]. Upon treatment with β -NGF, these cells start the process of

neurogenesis by developing neurites [198]. To validate this, pure β -NGF was added to PC-12 cell cultures, and their differentiation into neuron-like cells was assessed. Clear neurite formation was observed when PC-12 cells were treated with pure β -NGF after 96 hours of incubation, establishing a reliable experimental setup for assessing the bioactivity of β -NGF secreted by *L. plantarum* (**Figure 4**).

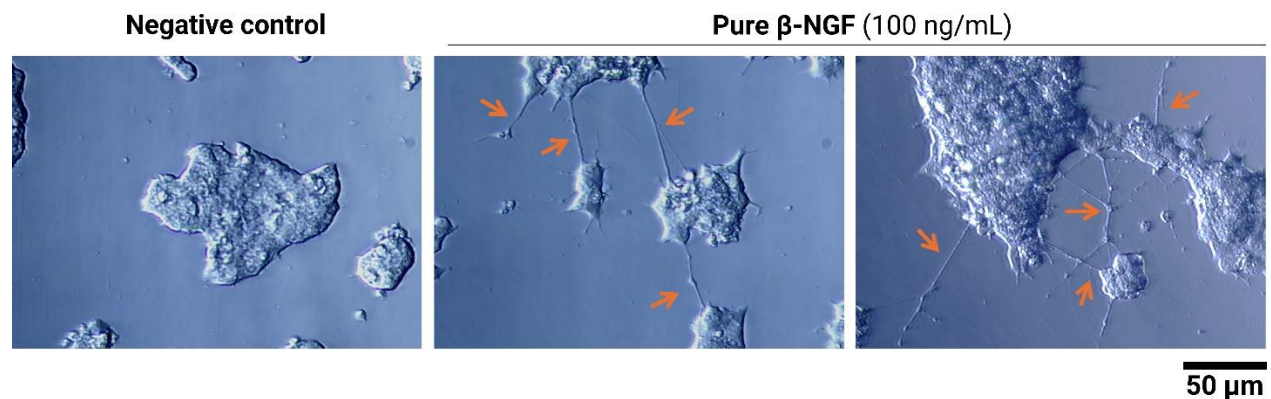


Figure 4. Images of PC-12 cells grown in optimal media (negative control) and in optimal media supplemented with 100 ng/mL of pure β -NGF. 20x magnification images were taken after 96 hours of incubation at 37°C and 5% CO₂. Neurite formation (indicated in orange arrows) only took place when PC-12 cells were treated with pure β -NGF.

The supernatant of β -NGF-secreting *L. plantarum* grown overnight in the PC-12 cells' growth media was used to treat PC-12 cells. The supernatant of EV-carrying *L. plantarum* and pure β -NGF were kept as negative and positive controls, respectively. After 96 hours, prominent neurite formation could only be visualized for the PC-12 cells treated with pure β -NGF (**Figure 5**). Interestingly, the cells treated with either EV or β -NGF *L. plantarum* supernatants showed neurite formation in a few cells, a phenotype that was not observed in PC-12 cells grown in just growth media, and was slightly more accentuated for β -NGF supernatants than EV supernatants (**Figure 5**). This observation suggested that native soluble factors released by *L. plantarum* seem to promote certain neurite formation in PC-12 cells.

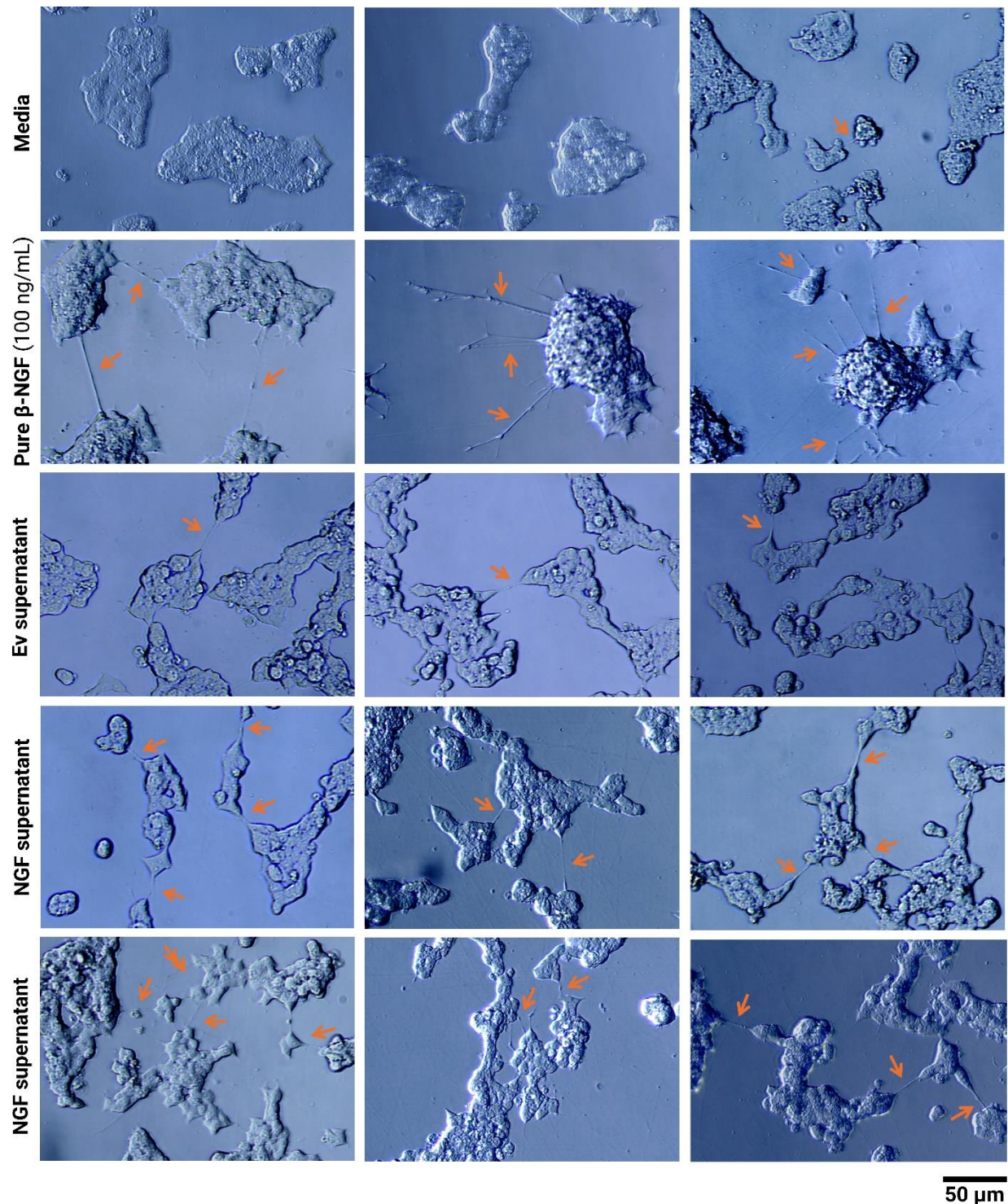


Figure 5. Images of PC-12 cells grown in optimal media (negative control) and in optimal media supplemented with 100 ng/mL of pure β -NGF, supernatant of EV-carrying *L. plantarum*, and supernatant of β -NGF-secreting *L. plantarum*. 20x magnification images were taken after 96 hours of incubation at 37°C and 5% CO₂. Neurite formation is indicated in orange arrows.

This preliminary data suggested that β -NGF *L. plantarum* supernatants promoted the formation of more neurites compared to EV supernatants. Yet, further optimization of the experimental conditions and repetitions would help to conclusively verify the bioactivity of the secreted β -NGF.

Discussion and Conclusions

In this work, I have generated valuable *L. plantarum* strains for *in vivo* multi-reporting in mice. The *in vitro* characterization of the engineered strains showed promising results, as high levels of gene expression were detected simultaneously. Moreover, an interesting gene architecture design for *L. plantarum* is presented, which can be adopted by other researchers in the field to produce at least three proteins in parallel through a single promoter. These results form part of a manuscript currently in preparation in collaboration with the Hao Lab at Boston University.

Promising yet inconclusive results were obtained in the experiments assessing β -NGF secretion by *L. plantarum*. While β -NGF secretion could be detected and quantified, the assay to validate the protein bioactivity failed to provide a conclusive outcome. A follow-up experiment is required to eliminate any soluble factor naturally secreted by *L. plantarum* and determine whether β -NGF is responsible for inducing neurogenesis. One option is to incorporate a protein purification tag (Histidine-tag or streptavidin tag) at the N-terminal of the β -NGF protein and perform an additional protein purification step before treating PC-12 cells. Alternatively, size exclusion chromatography can be performed for desalting and buffer exchange, and to remove native molecules from *L. plantarum*. Concerning PC-12 cell differentiation, quantitative PCR can also be used to examine gene up- and down-regulation due to β -NGF-mediated signalling pathway activation. This technique might be more sensitive than the neurite formation assay and might identify significant differences between the EV and β -NGF supernatants. Altogether, despite the interesting observations, further investigation is required to verify the development of a β -NGF-secreting *L. plantarum* strain suitable for biomedical applications.

Conclusions and Outlook

At the very beginning of this doctoral study, genetic engineering of *L. plantarum* was beset with several hurdles and significant limitations. That led me to *identify the factors limiting plasmid transformation in L. plantarum* and suggest solutions to overcome them, resulting in the development of the **direct cloning method** presented in Chapter 1. This direct cloning protocol can be widely adopted by the lactobacilli community as an attractive alternative to the conventional method involving an intermediate host.

After establishing a method to facilitate the transformation of these bacteria, the focus was switched to overcoming the limitations hindering effective protein production and secretion in *L. plantarum*. That required expanding the genetic toolbox of these bacteria. Toward that end, I explored the question: *What biological source can offer high-performance genetic parts for such expansion?* As accomplished with other bacteria, bacteriophages proved to be a promising source of functional genetic parts for *L. plantarum*, leading to the discovery of, to my knowledge, the **strongest reported transcriptional repressor** and **promoter** system in *L. plantarum*, as described in Chapter 2. The repressor, Rep, holds great potential for developing inverter circuits where the output of a given genetic switch can be reversed. Furthermore, the DNA-binding domain of Rep can be used to generate a hybrid repressor when it is fused to different stimulus-responsive dimerization domains and generate additional genetic switches [90]. That said, the strong promoter P_{tec} is anticipated to attract greater attention. The reason is that promoters are the most commonly used genetic part required for basic genetic engineering, meaning that P_{tec} can be part of both simple and complex genetic circuits. As of now, no promoter has been reported that matches the levels of expression driven by P_{tec} , making this promoter a potential game-changer in the genetic programmability of these bacteria.

Nonetheless, we have experienced challenges when attempting to drive the expression of challenging proteins (e.g., elafin or NGF) with this strong promoter. *L. plantarum*

responded with the deliberate introduction of mutations in key parts of these genes. That is one of the drawbacks of using a strong constitutive promoter that is constantly active. In Chapter 4, this was addressed by successfully turning constitutive P_{tec} into a **small-molecule-responsive promoter**, yielding promising results, especially in nutrient-deficient media, with the thermally-aided approach, and under material encapsulation conditions. In addition, further modification of this novel promoter can lead to the generation of a mutant library with varying strengths, which could further aid the production of certain challenging proteins. I have been part of a current ongoing collaboration with Dr. Rahmi Lale (Norwegian University of Science and Technology, Norway) aimed at generating a rationally designed library of P_{tec} -derived promoters in *L. plantarum* with inferior, known transcriptional rates. The goal is to select one variant or another depending on the anticipated difficulty of the cloning or the complexity/toxicity of the translated protein of interest.

As shown by others, endogenous genetic parts of *L. plantarum* and other lactobacilli can also be promising sources of genetic parts. In Chapter 3, a novel signal peptide (**SP3**⁸) was identified from the genome of *L. plantarum*. In combination with the strong promoters identified in this thesis, this SP can offer improved protein secretion performance compared to standard parts used so far by the research community. Under the P_{tlpA} promoter, reporter protein secretion levels were higher than lp_3050, the gold standard SP for protein secretion in *L. plantarum*. Furthermore, the secretion of elafin protein (Chapter 3) and NGF (Chapter 6) was also confirmed using SP3, suggesting that this SP operates well at driving the secretion of structurally different proteins. Hence, along with the strong promoter and repressor, SP3 might be established as a standardized SP for protein secretion in lactobacilli.

In Chapter 5, **TA systems** proved effective at mediating plasmid retention. These results validated the functionality of these systems in *L. plantarum*, which may lead to applications in other directions, like creating genetic biocontainment (as already reported in other bacteria [161, 199]). For that, a genetic switch such as the cumate switch

⁸ Note that this genetic part was identified by a co-worker, Varun Sai Tadimarri.

generated in Chapter 4 or the commonly used pSIP system would be required to control the expression of the toxin on demand. Bacterial death would be triggered upon the addition of the inducer, thereby generating a genetic biocontainment scenario in which bacterial growth is limited to inducer-free environments. The rep repressor (Chapter 2) could be integrated into this genetic circuit to invert the output and limit cell growth in inducer-containing environments.

Altogether, in this thesis, a collection of relevant genetic parts has been identified, tested, and *successfully applied to enhance the genetic programmability of *L. plantarum**. All these parts can be readily used by other researchers when designing their functional genes. During the testing and validation of these genetic parts, multiple valuable strains have been engineered, which can be of great interest to the community. For example, the **highly red fluorescent *L. plantarum*** strains from Chapters 2 (mCherry) and 3 (mScarlet3) can be used for imaging applications. The successful secretion of the therapeutically relevant proteins elafin and α -MSH (Chapter 3) also resulted in the development of potentially **clinically relevant engineered *L. plantarum* strains**, which are currently being assessed in mammalian cell experiments for anti-inflammatory studies. Additional promising strains are the incredibly **bioluminescent** and the **multi-reporter *L. plantarum*** bacteria presented in the first part of Chapter 6. These strains are currently being assessed for multimodal reporting of tumors in mice. In addition, the successful design and application of the **polycistronic genetic** approach (based on the P_{tec} promoter) can be an attractive gene architecture that other researchers can adopt when overexpression of multiple genes is intended in *L. plantarum*.

Another research focus of this thesis was the investigation of *whether these genetic parts remained functional while *L. plantarum* was encapsulated within materials*. The alginate core-shell encapsulation format, as both **PEARLs** (Chapter 3) and **micro-PEARLs** (Chapter 4), effectively contains *L. plantarum* for several days, while it fails to do so with *E. coli* for even a day. For the first time, we reported the development of lactobacilli-based **responsive** and **multi-functional ELMs**. There are very few studies on the encapsulation of engineered *L. plantarum* within materials, and given the positive results, it is expected

to promote the expansion of *L. plantarum*-based ELMs. Instead of using alginate beads, these studies could rely on more advanced techniques for bacterial encapsulation, including 3D printing, electrospinning, and microfluidics-based encapsulation.

As a conclusion, all the studies in this thesis contributed to improving the tractability, performance, and versatility of genetic programming in probiotic *L. plantarum*. These developments are expected to lead to a broader adoption of these probiotic bacteria by research groups as bacterial chassis for synthetic biology, biotechnology, and biomedical applications, either in culture or in the form of ELMs.

References

1. Tannock Gerald, W., *Applied and Environmental Microbiology* **2004**, 70 (6), 3189-3194. DOI 10.1128/AEM.70.6.3189-3194.2004.
2. Duar, R. M.; Lin, X. B.; Zheng, J.; Martino, M. E.; Grenier, T.; Pérez-Muñoz, M. E.; Leulier, F.; Gänzle, M.; Walter, J., *FEMS Microbiology Reviews* **2017**, 41 (Supp_1), S27-S48. DOI 10.1093/femsre/fux030.
3. Zheng, J.; Wittouck, S.; Salvetti, E.; Franz, C. M. A. P.; Harris, H. M. B.; Mattarelli, P.; O'Toole, P. W.; Pot, B.; Vandamme, P.; Walter, J.; Watanabe, K.; Wuyts, S.; Felis, G. E.; Gänzle, M. G.; Lebeer, S., *International Journal of Systematic and Evolutionary Microbiology* **2020**, 70 (4), 2782-2858. DOI <https://doi.org/10.1099/ijsem.0.004107>.
4. Giraffa, G.; Chanishvili, N.; Widyastuti, Y., *Research in Microbiology* **2010**, 161 (6), 480-487. DOI <https://doi.org/10.1016/j.resmic.2010.03.001>.
5. Yao, P.; Mohd Esah, E.; Zhao, C., *Frontiers in Microbiology* **2025**, 15. DOI 10.3389/fmicb.2024.1465373.
6. Zhang, Z.; Lv, J.; Pan, L.; Zhang, Y., *Applied Microbiology and Biotechnology* **2018**, 102 (19), 8135-8143. DOI 10.1007/s00253-018-9217-9.
7. Ding, Y.-H.; Qian, L.-Y.; Pang, J.; Lin, J.-Y.; Xu, Q.; Wang, L.-H.; Huang, D.-S.; Zou, H., *Oncotarget*; Vol 8, No 35 **2017**.
8. Di Cerbo, A.; Palmieri, B.; Aponte, M.; Morales-Medina, J. C.; Iannitti, T., *Journal of Clinical Pathology* **2016**, 69 (3), 187. DOI 10.1136/jclinpath-2015-202976.
9. Bertuccini, L.; Russo, R.; Iosi, F.; Superti, F., *International Journal of Immunopathology and Pharmacology* **2017**, 30 (2), 163-167. DOI 10.1177/0394632017697987.
10. O'Callaghan, J.; O'Toole, P. W., Lactobacillus: Host–Microbe Relationships. In *Between Pathogenicity and Commensalism*, Dobrindt, U.; Hacker, J. H.; Svanborg, C., Eds. Springer Berlin Heidelberg: Berlin, Heidelberg, 2013; pp 119-154.
11. Heczko, P. B.; Gienza, M.; Ponikiewska, W.; Strus, M. Importance of Lactobacilli for Human Health *Microorganisms* [Online], **2024**.
12. Yilmaz, B.; Bangar, S. P.; Echegaray, N.; Suri, S.; Tomasevic, I.; Manuel Lorenzo, J.; Melekoglu, E.; Rocha, J. M.; Ozogul, F. The Impacts of Lactiplantibacillus plantarum on the Functional Properties of Fermented Foods: A Review of Current Knowledge *Microorganisms* [Online], **2022**.
13. Aguirre-Garcia, Y. L.; Nery-Flores, S. D.; Campos-Muzquiz, L. G.; Flores-Gallegos, A. C.; Palomo-Ligas, L.; Ascacio-Valdés, J. A.; Sepúlveda-Torres, L.; Rodríguez-Herrera, R. Lactic Acid Fermentation in the Food Industry and Bio-Preservation of Food *Fermentation* [Online], **2024**.
14. de Souza, E. L.; de Oliveira, K. Á.; de Oliveira, M. E. G., *Current Opinion in Food Science* **2023**, 49, 100981. DOI <https://doi.org/10.1016/j.cofs.2022.100981>.
15. Kang, C. H.; Kim, J. S.; Park, H. M.; Kim, S.; Paek, N. S., *3 Biotech* **2021**, 11 (5), 217. DOI 10.1007/s13205-021-02767-y.
16. Hu, J.; Wang, C.; Ye, L.; Yang, W.; Huang, H.; Meng, F.; Shi, S.; Ding, Z., *J Biosci* **2015**, 40 (2), 269-79. DOI 10.1007/s12038-015-9518-4.
17. Silva, L. A.; Lopes Neto, J. H. P.; Cardarelli, H. R., *Annals of Microbiology* **2019**, 69 (4), 321-328. DOI 10.1007/s13213-019-01456-9.
18. Yang, S.; Xu, X.; Peng, Q.; Ma, L.; Qiao, Y.; Shi, B., *Animal Nutrition* **2023**, 13, 78-89. DOI <https://doi.org/10.1016/j.aninu.2023.02.004>.

19. Abarquero, D.; Renes, E.; Fresno, J. M.; Tornadijo, M. E., *International Journal of Food Science and Technology* **2021**, 57 (1), 16-26. DOI 10.1111/ijfs.15227.
20. Arqués, J. L.; Rodríguez, E.; Langa, S.; Landete, J. M.; Medina, M., *BioMed Research International* **2015**, 2015 (1), 584183. DOI <https://doi.org/10.1155/2015/584183>.
21. Mills, S.; Serrano, L.; Griffin, C.; O'Connor, P. M.; Schaad, G.; Bruining, C.; Hill, C.; Ross, R.; Meijer, W. C. In *Inhibitory activity of Lactobacillus plantarum LMG P-26358 against Listeria innocua when used as an adjunct starter in the manufacture of cheese*, Microbial cell factories, Springer: **2011**; pp 1-11.
22. Ennahar, S.; Assobhei, O.; Hasselmann, C., *Journal of Food Protection* **1998**, 61 (2), 186-195.
23. Silva Lopes, M. F.; Cunha, A. E.; Clemente, J. J.; Teixeira Carrondo, M. J.; Barreto Crespo, M. T., *Applied Microbiology and Biotechnology* **1999**, 51 (2), 249-254. DOI 10.1007/s002530051389.
24. Scheirlinck, T.; De Meutter, J.; Arnaut, G.; Joos, H.; Claeysens, M.; Michiels, F., *Applied Microbiology and Biotechnology* **1990**, 33 (5), 534-541. DOI 10.1007/BF00172547.
25. Ramírez, A. M.; Rodríguez-López, A.; Ardila, A.; Beltran, L.; Patarroyo, C. A.; Melendez, A. D. P.; Sánchez, O. F.; Alméciga-Díaz, C. J., *European Journal of Pharmaceutical Sciences* **2017**, 109, 48-55. DOI <https://doi.org/10.1016/j.ejps.2017.07.033>.
26. Shen, H.; Zhang, C.; Li, S.; Liang, Y.; Lee, L. T.; Aggarwal, N.; Wun, K. S.; Liu, J.; Nadarajan, S. P.; Weng, C.; Ling, H.; Tay, J. K.; Wang, D. Y.; Yao, S. Q.; Hwang, I. Y.; Lee, Y. S.; Chang, M. W., *Nature Communications* **2024**, 15 (1), 4343. DOI 10.1038/s41467-024-48661-y.
27. Shen, H.; Aggarwal, N.; Cui, B.; Foo, G. W.; He, Y.; Srivastava, S. K.; Li, S.; Seah, M. Z. X.; Wun, K. S.; Ling, H.; Hwang, I. Y.; Ho, C. L.; Lee, Y. S.; Chang, M. W., *Cell* **2025**, 188 (6), 1545-1562.e16. DOI 10.1016/j.cell.2025.01.017.
28. Wang, M.; Fu, T.; Hao, J.; Li, L.; Tian, M.; Jin, N.; Ren, L.; Li, C., *International Journal of Biological Macromolecules* **2020**, 160, 736-740. DOI <https://doi.org/10.1016/j.ijbiomac.2020.05.239>.
29. Rigaux, P.; Daniel, C.; Hisbergues, M.; Muraille, E.; Hols, P.; Pot, B.; Pestel, J.; Jacquet, A., *Allergy* **2009**, 64 (3), 406-414. DOI <https://doi.org/10.1111/j.1398-9995.2008.01825.x>.
30. Villa, T. G.; Feijoo-Siota, L.; Sánchez-Pérez, A.; Rama, J. R.; Sieiro, C., Horizontal Gene Transfer in Bacteria, an Overview of the Mechanisms Involved. In *Horizontal Gene Transfer: Breaking Borders Between Living Kingdoms*, Villa, T. G.; Viñas, M., Eds. Springer International Publishing: Cham, 2019; pp 3-76.
31. Johnston, C.; Martin, B.; Fichant, G.; Polard, P.; Claverys, J.-P., *Nature Reviews Microbiology* **2014**, 12 (3), 181-196. DOI 10.1038/nrmicro3199.
32. Liu, D.; Siguenza, N. E.; Zarrinpar, A.; Ding, Y., *Engineering Microbiology* **2022**, 2 (4), 100048. DOI <https://doi.org/10.1016/j.engmic.2022.100048>.
33. Calvin, N. M.; Hanawalt, P. C., *Journal of Bacteriology* **1988**, 170 (6), 2796-2801. DOI 10.1128/jb.170.6.2796-2801.1988.
34. Wei, M.-Q.; Rush, C. M.; Norman, J. M.; Hafner, L. M.; Epping, R. J.; Timms, P., *Journal of Microbiological Methods* **1995**, 21 (1), 97-109. DOI [https://doi.org/10.1016/0167-7012\(94\)00038-9](https://doi.org/10.1016/0167-7012(94)00038-9).
35. Chassy, B. M.; Mercenier, A.; Flickinger, J., *Trends in Biotechnology* **1988**, 6 (12), 303-309.
36. Posno, M.; Leer, R. J.; van Luijk, N.; van Giezen, M. J. F.; Heuvelmans, P. T. H. M.; Lokman, B. C.; Pouwels, P. H., *Applied and Environmental Microbiology* **1991**, 57 (6), 1822-1828. DOI 10.1128/aem.57.6.1822-1828.1991.
37. Thompson, K.; Collins, M. A., *Journal of Microbiological Methods* **1996**, 26 (1), 73-79. DOI [https://doi.org/10.1016/0167-7012\(96\)00845-7](https://doi.org/10.1016/0167-7012(96)00845-7).

38. Buson, F.; Gao, Y.; Wang, B., *ACS Synthetic Biology* **2024**, 13 (3), 697-713. DOI 10.1021/acssynbio.3c00691.
39. Kent, R.; Dixon, N., *Trends in Biotechnology* **2020**, 38 (3), 316-333. DOI 10.1016/j.tibtech.2019.09.007.
40. del Solar, G.; Giraldo, R.; Ruiz-Echevarría María, J.; Espinosa, M.; Díaz-Orejas, R., *Microbiology and Molecular Biology Reviews* **1998**, 62 (2), 434-464. DOI 10.1128/mmbr.62.2.434-464.1998.
41. Del Solar, G.; Espinosa, M., *Molecular Microbiology* **2000**, 37 (3), 492-500. DOI <https://doi.org/10.1046/j.1365-2958.2000.02005.x>.
42. Spath, K.; Heini, S.; Egger, E.; Grabherr, R., *Molecular Biotechnology* **2012**, 52 (1), 40-48. DOI 10.1007/s12033-011-9471-x.
43. Sørvig, E.; Skaugen, M.; Naterstad, K.; Eijsink, V. G. H.; Axelsson, L., *Microbiology* **2005**, 151 (2), 421-431. DOI <https://doi.org/10.1099/mic.0.27389-0>.
44. Li, X.; Schönberg, P. Y.; Wucherpennig, T.; Hinze, C.; Sulaj, F.; Henle, T.; Mascher, T., *ACS Synthetic Biology* **2024**, 13 (9), 2764-2779. DOI 10.1021/acssynbio.4c00075.
45. Curtsinger, H. D.; Martínez-Absalón, S.; Liu, Y.; Lopatkin, A. J., *BioEssays* **2025**, 47 (2), 2400164.
46. Leslie, A. G.; Moody, P. C.; Shaw, W. V., *Proceedings of the National Academy of Sciences* **1988**, 85 (12), 4133-4137. DOI 10.1073/pnas.85.12.4133.
47. Weisblum, B., *Antimicrobial agents and chemotherapy* **1995**, 39 (3), 577-585.
48. Opal, S. M.; Pop-Vicas, A., *Molecular Mechanisms of Antibiotic Resistance in Bacteria*. In *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)*, Bennett, J. E.; Dolin, R.; Blaser, M. J., Eds. W.B. Saunders: Philadelphia, 2015; pp 235-251.e3.
49. Svetlov, M. S.; Syroegin, E. A.; Aleksandrova, E. V.; Atkinson, G. C.; Gregory, S. T.; Mankin, A. S.; Polikanov, Y. S., *Nature Chemical Biology* **2021**, 17 (4), 412-420. DOI 10.1038/s41589-020-00715-0.
50. Skinner, R.; Cundliffe, E.; Schmidt, F. J., *Journal of Biological Chemistry* **1983**, 258 (20), 12702-12706. DOI [https://doi.org/10.1016/S0021-9258\(17\)44232-3](https://doi.org/10.1016/S0021-9258(17)44232-3).
51. Unterholzner, S. J.; Brigitte, P.; and Rozhon, W., *Mobile Genetic Elements* **2013**, 3 (5), e26219. DOI 10.4161/mge.26219.
52. Jurénas, D.; Fraikin, N.; Goormaghtigh, F.; Van Melderen, L., *Nature Reviews Microbiology* **2022**, 20 (6), 335-350. DOI 10.1038/s41579-021-00661-1.
53. Hall, A. M. J.; Gollan, B.; Helaine, S., *Current Opinion in Microbiology* **2017**, 36, 102-110. DOI <https://doi.org/10.1016/j.mib.2017.02.003>.
54. Fraikin, N.; Goormaghtigh, F.; Van Melderen, L., *Journal of Bacteriology* **2020**, 202 (7), 10.1128/jb.00763-19. DOI 10.1128/jb.00763-19.
55. Fedorec, A. J. H.; Ozdemir, T.; Doshi, A.; Ho, Y.-K.; Rosa, L.; Rutter, J.; Velazquez, O.; Pinheiro, V. B.; Danino, T.; Barnes, C. P., *iScience* **2019**, 14, 323-334. DOI <https://doi.org/10.1016/j.isci.2019.03.019>.
56. Gerdes, K., *Bio/technology* **1988**, 6 (12), 1402-1405.
57. Petrov, K.; Arsov, A.; Petrova, P. Butanol Tolerance of *Lactiplantibacillus plantarum*: A Transcriptome Study *Genes* [Online], **2021**.
58. Ferrari, A.; Maggi, S.; Montanini, B.; Levante, A.; Lazzi, C.; Yamaguchi, Y.; Rivetti, C.; Folli, C., *Scientific Reports* **2019**, 9 (1), 7645. DOI 10.1038/s41598-019-44094-6.
59. Klimina, K. M.; Kjasova, D. K.; Poluektova, E. U.; Krügel, H.; Leuschner, Y.; Saluz, H. P.; Danilenko, V. N., *Anaerobe* **2013**, 22, 82-89. DOI <https://doi.org/10.1016/j.anaerobe.2013.05.007>.
60. Ferrer-Miralles, N.; Villaverde, A., *Microbial Cell Factories* **2013**, 12 (1), 113. DOI 10.1186/1475-2859-12-113.

61. Overton, T. W., *Drug Discovery Today* **2014**, 19 (5), 590-601. DOI <https://doi.org/10.1016/j.drudis.2013.11.008>.
62. Busby, S.; Ebright, R. H., *Cell* **1994**, 79 (5), 743-746.
63. Paget, M. S. B.; Helmann, J. D., *Genome Biology* **2003**, 4 (1), 203. DOI 10.1186/gb-2003-4-1-203.
64. Shine, J.; Dalgarno, L., *Proceedings of the National Academy of Sciences* **1974**, 71 (4), 1342-1346.
65. Ringquist, S.; Shinedling, S.; Barrick, D.; Green, L.; Binkley, J.; Stormo, G. D.; Gold, L., *Molecular Microbiology* **1992**, 6 (9), 1219-1229. DOI <https://doi.org/10.1111/j.1365-2958.1992.tb01561.x>.
66. Vellanoeweth, R. L.; Rabinowitz, J. C., *Molecular Microbiology* **1992**, 6 (9), 1105-1114. DOI <https://doi.org/10.1111/j.1365-2958.1992.tb01548.x>.
67. Rodnina, M. V., *Protein Science* **2016**, 25 (8), 1390-1406.
68. Yu, L.; Song, X.; Wang, G.; Xia, Y.; Song, Z.; Chen, G.; Ai, L.; Xiong, Z., *Journal of Biotechnology* **2025**.
69. Rytter, J. V.; Helmark, S.; Chen, J.; Lezyk, M. J.; Solem, C.; Jensen, P. R., *Applied Microbiology and Biotechnology* **2014**, 98 (6), 2617-2623. DOI 10.1007/s00253-013-5481-x.
70. Brewster, R. C.; Jones, D. L.; Phillips, R., *PLOS Computational Biology* **2012**, 8 (12), e1002811. DOI 10.1371/journal.pcbi.1002811.
71. Neves, D.; Liebal, U. W.; Nies, S. C.; Alter, T. B.; Pitzler, C.; Blank, L. M.; Ebert, B. E., *ACS Synthetic Biology* **2023**, 12 (7), 2029-2040. DOI 10.1021/acssynbio.3c00084.
72. Song, Y.; Nikoloff, J. M.; Fu, G.; Chen, J.; Li, Q.; Xie, N.; Zheng, P.; Sun, J.; Zhang, D., *PLOS ONE* **2016**, 11 (7), e0158447. DOI 10.1371/journal.pone.0158447.
73. Liu, D.; Mao, Z.; Guo, J.; Wei, L.; Ma, H.; Tang, Y.; Chen, T.; Wang, Z.; Zhao, X., *ACS Synthetic Biology* **2018**, 7 (7), 1785-1797. DOI 10.1021/acssynbio.8b00115.
74. Li, S.; Wang, J.; Li, X.; Yin, S.; Wang, W.; Yang, K., *Microbial Cell Factories* **2015**, 14 (1), 172. DOI 10.1186/s12934-015-0351-0.
75. Sohoni, S. V.; Fazio, A.; Workman, C. T.; Mijakovic, I.; Lantz, A. E., *PLOS ONE* **2014**, 9 (6), e99701. DOI 10.1371/journal.pone.0099701.
76. Peiróten, Á.; Landete, J. M., *Applied Microbiology and Biotechnology* **2020**, 104 (9), 3797-3805. DOI 10.1007/s00253-020-10426-0.
77. Rud, I.; Jensen, P. R.; Naterstad, K.; Axelsson, L., *Microbiology* **2006**, 152 (4), 1011-1019.
78. Meng, Q.; Yuan, Y.; Li, Y.; Wu, S.; Shi, K.; Liu, S., *ACS Synthetic Biology* **2021**, 10 (7), 1728-1738.
79. Tauer, C.; Heinl, S.; Egger, E.; Heiss, S.; Grabherr, R., *Microbial Cell Factories* **2014**, 13 (1), 150. DOI 10.1186/s12934-014-0150-z.
80. Spangler, J. R.; Caruana, J. C.; Phillips, D. A.; Walper, S. A., *Synthetic Biology* **2019**, 4 (1). DOI 10.1093/synbio/ysz012.
81. Rojo, F., *Current Opinion in Microbiology* **2001**, 4 (2), 145-151. DOI [https://doi.org/10.1016/S1369-5274\(00\)00180-6](https://doi.org/10.1016/S1369-5274(00)00180-6).
82. Lewis, M.; Chang, G.; Horton, N. C.; Kercher, M. A.; Pace, H. C.; Schumacher, M. A.; Brennan, R. G.; Lu, P., *Science* **1996**, 271 (5253), 1247-1254. DOI doi:10.1126/science.271.5253.1247.
83. Sprinzak, D.; Elowitz, M. B., *Nature* **2005**, 438 (7067), 443-448. DOI 10.1038/nature04335.
84. Hasty, J.; McMillen, D.; Collins, J. J., *Nature* **2002**, 420 (6912), 224-230. DOI 10.1038/nature01257.
85. Riglar, D. T.; Giessen, T. W.; Baym, M.; Kerns, S. J.; Niederhuber, M. J.; Bronson, R. T.; Kotula, J. W.; Gerber, G. K.; Way, J. C.; Silver, P. A., *Nature Biotechnology* **2017**, 35 (7), 653-658. DOI 10.1038/nbt.3879.

86. Johnson, A.; Meyer, B. J.; Ptashne, M., *Proceedings of the National Academy of Sciences* **1978**, 75 (4), 1783-1787. DOI 10.1073/pnas.75.4.1783.
87. Backman, K.; Ptashne, M.; Gilbert, W., *Proceedings of the National Academy of Sciences* **1976**, 73 (11), 4174-4178. DOI 10.1073/pnas.73.11.4174.
88. Voigt, C. A., *Current Opinion in Biotechnology* **2006**, 17 (5), 548-557. DOI <https://doi.org/10.1016/j.copbio.2006.09.001>.
89. Daber, R.; Stayrook, S.; Rosenberg, A.; Lewis, M., *Journal of Molecular Biology* **2007**, 370 (4), 609-619. DOI <https://doi.org/10.1016/j.jmb.2007.04.028>.
90. Dimas, R. P.; Jiang, X.-L.; Alberto de la Paz, J.; Morcos, F.; Chan, C. T Y., *Nucleic Acids Research* **2019**, 47 (10), 5449-5463. DOI 10.1093/nar/gkz280.
91. Li, X.; Zhang, C.; Xu, X.; Miao, J.; Yao, J.; Liu, R.; Zhao, Y.; Chen, X.; Yang, Y., *Nucleic Acids Research* **2020**, 48 (6), e33-e33. DOI 10.1093/nar/gkaa044.
92. Xiong, L. L.; Garrett, M. A.; Buss, M. T.; Kornfield, J. A.; Shapiro, M. G., *ACS Synthetic Biology* **2022**, 11 (7), 2518-2522. DOI 10.1021/acssynbio.2c00093.
93. Gardner, T. S.; Cantor, C. R.; Collins, J. J., *Nature* **2000**, 403 (6767), 339-342. DOI 10.1038/35002131.
94. Rosano, G. L.; Morales, E. S.; Ceccarelli, E. A., *Protein Science* **2019**, 28 (8), 1412-1422. DOI <https://doi.org/10.1002/pro.3668>.
95. De Baets, J.; De Paepe, B.; De Mey, M., *Microbial Cell Factories* **2024**, 23 (1), 249. DOI 10.1186/s12934-024-02523-w.
96. Saltepe, B.; Kehribar, E. Ş.; Su Yirmibeşoğlu, S. S.; Şafak Şeker, U. Ö., *ACS Sensors* **2018**, 3 (1), 13-26. DOI 10.1021/acssensors.7b00728.
97. Higashikuni, Y.; Chen, W. C. W.; Lu, T. K., *Current Opinion in Biotechnology* **2017**, 47, 133-141. DOI <https://doi.org/10.1016/j.copbio.2017.06.011>.
98. Heiss, S.; Hörmann, A.; Tauer, C.; Sonnleitner, M.; Egger, E.; Grabherr, R.; Heinl, S., *Microbial Cell Factories* **2016**, 15 (1), 50. DOI 10.1186/s12934-016-0448-0.
99. Zhang, S.; Xu, Z.; Qin, L.; Kong, J., *Biochemical Engineering Journal* **2019**, 151, 107316. DOI <https://doi.org/10.1016/j.bej.2019.107316>.
100. Guzman, L. M.; Belin, D.; Carson, M. J.; Beckwith, J., *Journal of Bacteriology* **1995**, 177 (14), 4121-4130. DOI 10.1128/jb.177.14.4121-4130.1995.
101. Khlebnikov, A.; Datsenko, K. A.; Skaug, T.; Wanner, B. L.; Keasling, J. D., *Microbiology* **2001**, 147 (12), 3241-3247. DOI <https://doi.org/10.1099/00221287-147-12-3241>.
102. Lloyd, G.; Landini, P.; Busby, S., *Essays in biochemistry* **2001**, 37, 17-31.
103. Rhodius, V. A.; Busby, S. J. W., *Current Opinion in Microbiology* **1998**, 1 (2), 152-159. DOI [https://doi.org/10.1016/S1369-5274\(98\)80005-2](https://doi.org/10.1016/S1369-5274(98)80005-2).
104. Olivera, B. C. L.; Ugalde, E.; Martínez-Antonio, A., *Journal of Theoretical Biology* **2010**, 264 (2), 560-569. DOI <https://doi.org/10.1016/j.jtbi.2010.02.008>.
105. Zschiedrich, C. P.; Keidel, V.; Szurmant, H., *Journal of Molecular Biology* **2016**, 428 (19), 3752-3775. DOI <https://doi.org/10.1016/j.jmb.2016.08.003>.
106. Papon, N.; Stock, A. M., *Current Biology* **2019**, 29 (15), R724-R725. DOI 10.1016/j.cub.2019.06.010.
107. Sørvig, E.; Mathiesen, G.; Naterstad, K.; Eijsink, V. G. H.; Axelsson, L., *Microbiology* **2005**, 151 (7), 2439-2449. DOI <https://doi.org/10.1099/mic.0.28084-0>.
108. Karliskås, I. L.; Maudal, K.; Axelsson, L.; Rud, I.; Eijsink, V. G.; Mathiesen, G., *PLOS one* **2014**, 9 (3), e91125.
109. Böhmer, N.; Lutz-Wahl, S.; Fischer, L., *Applied Microbiology and Biotechnology* **2012**, 96 (4), 903-912. DOI 10.1007/s00253-012-4212-z.
110. Vaidyanathan, H.; Kandasamy, V.; Gopal Ramakrishnan, G.; Ramachandran, K. B.; Jayaraman, G.; Ramalingam, S., *AMB Express* **2011**, 1 (1), 37. DOI 10.1186/2191-0855-1-37.

111. Schümann, C.; Michlmayr, H.; Eder, R.; Hierro, A.; Kulbe, K.; Mathiesen, G.; Nguyen, T.-H., *AMB Express* **2012**, 2, 19. DOI 10.1186/2191-0855-2-19.
112. Nguyen, T.-T.; Nguyen, T.-H.; Maischberger, T.; Schmelzer, P.; Mathiesen, G.; Eijssink, V. G. H.; Haltrich, D.; Peterbauer, C. K., *Microbial Cell Factories* **2011**, 10 (1), 46. DOI 10.1186/1475-2859-10-46.
113. Yang, W.-T.; Li, Q.-Y.; Ata, E. B.; Jiang, Y.-L.; Huang, H.-B.; Shi, C.-W.; Wang, J.-Z.; Wang, G.; Kang, Y.-H.; Liu, J.; Yang, G.-L.; Wang, C.-F., *Applied Microbiology and Biotechnology* **2018**, 102 (19), 8307-8318. DOI 10.1007/s00253-018-9238-4.
114. Halbmayer, E.; Mathiesen, G.; Nguyen, T.-H.; Maischberger, T.; Peterbauer, C. K.; Eijssink, V. G. H.; Haltrich, D., *Journal of Agricultural and Food Chemistry* **2008**, 56 (12), 4710-4719. DOI 10.1021/jf073260+.
115. Fredriksen, L.; Mathiesen, G.; Sioud, M.; Eijssink Vincent, G. H., *Applied and Environmental Microbiology* **2010**, 76 (21), 7359-7362. DOI 10.1128/AEM.01031-10.
116. Freudl, R., *Journal of Biotechnology* **1992**, 23 (3), 231-240. DOI [https://doi.org/10.1016/0168-1656\(92\)90072-H](https://doi.org/10.1016/0168-1656(92)90072-H).
117. Harwood, C. R.; Cranenburgh, R., *Trends in Microbiology* **2008**, 16 (2), 73-79. DOI 10.1016/j.tim.2007.12.001.
118. Degering, C.; Eggert, T.; Puls, M.; Bongaerts, J.; Evers, S.; Maurer, K.-H.; Jaeger, K.-E., *Applied and Environmental Microbiology* **2010**, 76 (19), 6370-6376. DOI doi:10.1128/AEM.01146-10.
119. Liu, X.; Zhang, W.; Zhao, Z.; Dai, X.; Yang, Y.; Bai, Z., *Critical Reviews in Biotechnology* **2017**, 37 (4), 541-551. DOI 10.1080/07388551.2016.1206059.
120. Schneewind, O.; Missiakas, D. M., *Philosophical Transactions of the Royal Society B: Biological Sciences* **2012**, 367 (1592), 1123-1139. DOI 10.1098/rstb.2011.0210.
121. Anné, J.; Economou, A.; Bernaerts, K., Protein Secretion in Gram-Positive Bacteria: From Multiple Pathways to Biotechnology. In *Protein and Sugar Export and Assembly in Gram-positive Bacteria*, Bagnoli, F.; Rappuoli, R., Eds. Springer International Publishing: Cham, 2017; pp 267-308.
122. Prabudiansyah, I.; Driessen, A. J. M., The Canonical and Accessory Sec System of Gram-positive Bacteria. In *Protein and Sugar Export and Assembly in Gram-positive Bacteria*, Bagnoli, F.; Rappuoli, R., Eds. Springer International Publishing: Cham, 2017; pp 45-67.
123. Tsukazaki, T.; Mori, H.; Echizen, Y.; Ishitani, R.; Fukai, S.; Tanaka, T.; Perederina, A.; Vassilyev, D. G.; Kohno, T.; Maturana, A. D.; Ito, K.; Nureki, O., *Nature* **2011**, 474 (7350), 235-238. DOI 10.1038/nature09980.
124. Driessen, A. J. M.; Nouwen, N., *Annual Review of Biochemistry* **2008**, 77 (Volume 77, 2008), 643-667. DOI <https://doi.org/10.1146/annurev.biochem.77.061606.160747>.
125. Peng, C.; Shi, C.; Cao, X.; Li, Y.; Liu, F.; Lu, F., *Frontiers in Bioengineering and Biotechnology* **2019**, Volume 7 - 2019. DOI 10.3389/fbioe.2019.00139.
126. Fu, G.; Liu, J.; Li, J.; Zhu, B.; Zhang, D., *Journal of Agricultural and Food Chemistry* **2018**, 66 (50), 13141-13151. DOI 10.1021/acs.jafc.8b04183.
127. Morello, E.; Bermúdez-Humarán, L. G.; Llull, D.; Solé, V.; Miraglio, N.; Langella, P.; Poquet, I., *Journal of Molecular Microbiology and Biotechnology* **2007**, 14 (1-3), 48-58. DOI 10.1159/000106082.
128. van Asseldonk, M.; de Vos, W. M.; Simons, G., *Molecular and General Genetics MGG* **1993**, 240 (3), 428-434. DOI 10.1007/BF00280397.
129. Borrero, J.; Jiménez, J. J.; Gútiérrez, L.; Herranz, C.; Cintas, L. M.; Hernández, P. E., *Applied Microbiology and Biotechnology* **2011**, 89 (1), 131-143. DOI 10.1007/s00253-010-2849-z.
130. Ng Daphne, T. W.; Sarkar Casim, A., *Applied and Environmental Microbiology* **2013**, 79 (1), 347-356. DOI 10.1128/AEM.02667-12.
131. Langella, P.; Le Loir, Y., *Brazilian journal of medical and biological research* **1999**, 32, 191-198.

132. Mathiesen, G.; Sveen, A.; Piard, J. C.; Axelsson, L.; Eijssink, V. G. H., *Journal of Applied Microbiology* **2008**, 105 (1), 215-226. DOI 10.1111/j.1365-2672.2008.03734.x.
133. Mathiesen, G.; Sveen, A.; Brurberg, M. B.; Fredriksen, L.; Axelsson, L.; Eijssink, V. G. H., *BMC Genomics* **2009**, 10 (1), 425. DOI 10.1186/1471-2164-10-425.
134. Tran, A.-M.; Unban, K.; Kanpiengjai, A.; Khanongnuch, C.; Mathiesen, G.; Haltrich, D.; Nguyen, T.-H., *Frontiers in Microbiology* **2021**, 12.
135. Rodrigo-Navarro, A.; Sankaran, S.; Dalby, M. J.; del Campo, A.; Salmeron-Sanchez, M., *Nature Reviews Materials* **2021**, 6 (12), 1175-1190.
136. An, B.; Wang, Y.; Huang, Y.; Wang, X.; Liu, Y.; Xun, D.; Church, G. M.; Dai, Z.; Yi, X.; Tang, T.-C.; Zhong, C., *Chemical Reviews* **2023**, 123 (5), 2349-2419. DOI 10.1021/acs.chemrev.2c00512.
137. Wang, Q.; Hu, Z.; Li, Z.; Liu, T.; Bian, G., *Advanced Materials* **2023**, n/a (n/a), 2305828. DOI <https://doi.org/10.1002/adma.202305828>.
138. Chen, Q.-W.; Zhang, X.-Z., *Accounts of Materials Research* **2024**, 5 (11), 1440-1452. DOI 10.1021/accountsmr.4c00258.
139. Omer, R.; Mohsin, M. Z.; Mohsin, A.; Mushtaq, B. S.; Huang, X.; Guo, M.; Zhuang, Y.; Huang, J., *Frontiers in Bioengineering and Biotechnology* **2022**, 10.
140. Seliktar, D., *Science* **2012**, 336 (6085), 1124-1128.
141. Liu, X.; Inda, M. E.; Lai, Y.; Lu, T. K.; Zhao, X., *Advanced Materials* **2022**, 34 (26), 2201326. DOI <https://doi.org/10.1002/adma.202201326>.
142. Yu, S.; Sun, H.; Li, Y.; Wei, S.; Xu, J.; Liu, J., *Materials Today Bio* **2022**, 16, 100435. DOI <https://doi.org/10.1016/j.mtbio.2022.100435>.
143. Liu, X.; Yuk, H.; Lin, S.; Parada, G. A.; Tang, T.-C.; Tham, E.; de la Fuente-Nunez, C.; Lu, T. K.; Zhao, X., *Advanced Materials* **2018**, 30 (4), 1704821. DOI <https://doi.org/10.1002/adma.201704821>.
144. Liu, X.; Tang, T.-C.; Tham, E.; Yuk, H.; Lin, S.; Lu, T. K.; Zhao, X., *Proceedings of the National Academy of Sciences* **2017**, 114 (9), 2200-2205. DOI 10.1073/pnas.1618307114.
145. Dong, Y.; Wang, S.; Ke, Y.; Ding, L.; Zeng, X.; Magdassi, S.; Long, Y., *Advanced Materials Technologies* **2020**, 5 (6), 2000034. DOI <https://doi.org/10.1002/admt.202000034>.
146. Augst, A. D.; Kong, H. J.; Mooney, D. J., *Macromolecular Bioscience* **2006**, 6 (8), 623-633. DOI <https://doi.org/10.1002/mabi.200600069>.
147. Sun, J.; Tan, H. Alginate-Based Biomaterials for Regenerative Medicine Applications *Materials* [Online], **2013**, p. 1285-1309.
148. Li, P.; Müller, M.; Chang, M. W.; Frettlöh, M.; Schönherr, H., *ACS Applied Materials & Interfaces* **2017**, 9 (27), 22321-22331. DOI 10.1021/acsami.7b07166.
149. Yang, C.; Cui, M.; Zhang, Y.; Pan, H.; Liu, J.; Wang, S.; Ma, N.; Chang, J.; Sun, T.; Wang, H., *Communications Biology* **2020**, 3 (1), 561. DOI 10.1038/s42003-020-01287-4.
150. Pan, H.; Sun, T.; Cui, M.; Ma, N.; Yang, C.; Liu, J.; Pang, G.; Liu, B.; Li, L.; Zhang, X.; Zhang, W.; Chang, J.; Wang, H., *ACS Nano* **2022**, 16 (4), 6049-6063. DOI 10.1021/acsnano.1c11536.
151. Ding, W. K.; Shah, N. P., *Journal of Food Science* **2009**, 74 (2), M100-M107. DOI <https://doi.org/10.1111/j.1750-3841.2009.01067.x>.
152. Huq, T.; Avik, K.; A., K. R.; Bernard, R.; and Lacroix, M., *Critical Reviews in Food Science and Nutrition* **2013**, 53 (9), 909-916. DOI 10.1080/10408398.2011.573152.
153. Spath, K.; Heinl, S.; Grabherr, R., *Microbial Cell Factories* **2012**, 11 (1), 141. DOI 10.1186/1475-2859-11-141.
154. Savijoki, K.; Kahala, M.; Palva, A., *Gene* **1997**, 186 (2), 255-262. DOI [https://doi.org/10.1016/S0378-1119\(96\)00717-2](https://doi.org/10.1016/S0378-1119(96)00717-2).
155. Hampton, H. G.; Watson, B. N. J.; Fineran, P. C., *Nature* **2020**, 577 (7790), 327-336. DOI 10.1038/s41586-019-1894-8.

156. Tabor, S.; Richardson, C. C., *Proceedings of the National Academy of Sciences* **1985**, 82 (4), 1074-1078.
157. Brady, A.; Felipe-Ruiz, A.; Gallego del Sol, F.; Marina, A.; Quiles-Puchalt, N.; Penadés, J. R., *Annual Review of Microbiology* **2021**, 75 (Volume 75, 2021), 563-581. DOI <https://doi.org/10.1146/annurev-micro-033121-020757>.
158. Blanch-Asensio, M.; Dey, S.; Sankaran, S., *PLOS one* **2023**, 18 (2), e0281625.
159. Vidal, L.; Pinsach, J.; Striedner, G.; Caminal, G.; Ferrer, P., *Journal of Biotechnology* **2008**, 134 (1), 127-136. DOI <https://doi.org/10.1016/j.jbiotec.2008.01.011>.
160. Dong, W.-R.; Xiang, L.-X.; Shao, J.-Z., *Applied and Environmental Microbiology* **2010**, 76 (7), 2295-2303. DOI 10.1128/AEM.02462-09.
161. Chan, C. T. Y.; Lee, J. W.; Cameron, D. E.; Bashor, C. J.; Collins, J. J., *Nature Chemical Biology* **2016**, 12 (2), 82-86. DOI 10.1038/nchembio.1979.
162. Vélez, M. P.; De Keersmaecker, S. C. J.; Vanderleyden, J., *FEMS Microbiology Letters* **2007**, 276 (2), 140-148. DOI 10.1111/j.1574-6968.2007.00908.x.
163. Yu, Y.; Zong, M.; Lao, L.; Wen, J.; Pan, D.; Wu, Z., *Food & Function* **2022**, 13 (6), 3098-3109. DOI 10.1039/D1FO04328E.
164. Buck, B. L.; Altermann, E.; Svingerud, T.; Klaenhammer Todd, R., *Applied and Environmental Microbiology* **2005**, 71 (12), 8344-8351. DOI 10.1128/AEM.71.12.8344-8351.2005.
165. Andreu, N.; Zelmer, A.; Wiles, S., *FEMS Microbiology Reviews* **2011**, 35 (2), 360-394. DOI 10.1111/j.1574-6976.2010.00252.x.
166. Syed, A. J.; Anderson, J. C., *Chemical Society Reviews* **2021**, 50 (9), 5668-5705.
167. Daniel, C.; Poiret, S.; Dennin, V.; Boutillier, D.; Pot, B., *Applied and Environmental Microbiology* **2013**, 79 (4), 1086-1094. DOI 10.1128/AEM.03221-12.
168. Daniel, C.; Poiret, S.; Dennin, V.; Boutillier, D.; Lacorre Delphine, A.; Foligné, B.; Pot, B., *Applied and Environmental Microbiology* **2015**, 81 (16), 5344-5349. DOI 10.1128/AEM.01042-15.
169. Van Zyl, W. F.; Deane, S. M.; Dicks, L. M. T., *BMC Microbiology* **2018**, 18 (1), 171. DOI 10.1186/s12866-018-1315-4.
170. England, C. G.; Ehlerding, E. B.; Cai, W., *Bioconjugate Chemistry* **2016**, 27 (5), 1175-1187. DOI 10.1021/acs.bioconjchem.6b00112.
171. Hall, M. P.; Unch, J.; Binkowski, B. F.; Valley, M. P.; Butler, B. L.; Wood, M. G.; Otto, P.; Zimmerman, K.; Vidugiris, G.; Machleidt, T.; Robers, M. B.; Benink, H. A.; Eggers, C. T.; Slater, M. R.; Meisenheimer, P. L.; Klaubert, D. H.; Fan, F.; Encell, L. P.; Wood, K. V., *ACS Chemical Biology* **2012**, 7 (11), 1848-1857. DOI 10.1021/cb3002478.
172. Zhao, N.; Liu, J.-M.; Liu, S.; Ji, X.-M.; Lv, H.; Hu, Y.-Z.; Wang, Z.-H.; Lv, S.-W.; Li, C.-Y.; Wang, S., *RSC advances* **2020**, 10 (22), 13029-13036.
173. Delanghe, L.; Spacova, I.; Van Malderen, J.; Oerlemans, E.; Claes, I.; Lebeer, S., *Biochemical Society Transactions* **2021**, 49 (2), 617-627. DOI 10.1042/BST20200329.
174. Lebeer, S.; Oerlemans, E. F. M.; Claes, I.; Henkens, T.; Delanghe, L.; Wuyts, S.; Spacova, I.; van den Broek, M. F. L.; Tuyaerts, I.; Wittouck, S.; De Boeck, I.; Allonsius, C. N.; Kiekens, F.; Lambert, J., *Cell Reports Medicine* **2022**, 3 (2). DOI 10.1016/j.xcrm.2022.100521.
175. Iovieno, A.; Lambiase, A.; Sacchetti, M.; Stampachiacchiere, B.; Micera, A.; Bonini, S., *Graefe's Archive for Clinical and Experimental Ophthalmology* **2008**, 246 (3), 435-441. DOI 10.1007/s00417-007-0682-6.
176. Heydari, M.; Kalani, M.; Ghasemi, Y.; Nejabat, M., *Probiotics and Antimicrobial Proteins* **2024**, 16 (3), 1026-1035. DOI 10.1007/s12602-023-10079-1.
177. Puertas-Bartolomé, M.; Gutiérrez-Urrutia, I.; Teruel-Enrico, L. L.; Duong, C. N.; Desai, K.; Trujillo, S.; Wittmann, C.; Del Campo, A., *Advanced Materials* **2024**, 36 (27), 2313848.
178. Liu, X.; Yuk, H.; Lin, S.; Parada, G. A.; Tang, T. C.; Tham, E.; de la Fuente-Nunez, C.; Lu, T. K.; Zhao, X., *Advanced Materials* **2018**, 30 (4), 1704821.

179. Maria Luisa, R.; Marzia, S.; Luigi, M.; Luigi, A., *Current Neuropharmacology* **2018**, 16 (10), 1455-1465. DOI <http://dx.doi.org/10.2174/1570159X16666180412092859>.
180. Tuszynski, M. H.; Yang, J. H.; Barba, D.; Bakay, R. A.; Pay, M. M.; Masliah, E.; Conner, J. M.; Kobalka, P.; Roy, S.; Nagahara, A. H., *JAMA neurology* **2015**, 72 (10), 1139-1147.
181. Bonini, S.; Lambiase, A.; Rama, P.; Sinigaglia, F.; Allegretti, M.; Chao, W.; Mantelli, F.; Bonini, S.; Lambiase, A.; Rama, P.; Messmer, E.; Aragona, P.; Geerling, G.; Mastropasqua, L.; Mencucci, R.; Dart, J.; Leonardi, A.; Montero, J.; Rolando, M.; Reinhard, T.; Cursiefen, C.; Ettxebarria, J.; Gabison, E.; Szaflik, J. P.; Bouheraoua, N.; De La Paz, M.; Sainz de la Maza, M.; Wylegala, E.; Figueiredo, F.; Fogagnolo, P.; Hossain, P.; Lorenz, K.; Robert, P.-Y.; Benitez del Castillo, J.; Creuzot-Garcher, C.; Kruse, F.; Malecaze, F.; Merayo-Lloves, J.; Rauz, S.; Alio, J.; Carley, F.; Kanna, R.; Koppen, C.; Nemeth, J.; Murta, J. N.; Torrao, L., *Ophthalmology* **2018**, 125 (9), 1332-1343. DOI <https://doi.org/10.1016/j.ophtha.2018.02.022>.
182. Wiesmann, C.; de Vos, A. M., *Cellular and Molecular Life Sciences CMLS* **2001**, 58 (5), 748-759. DOI 10.1007/PL00000898.
183. McDonald, N. Q.; Lapatto, R.; Rust, J. M.; Gunning, J.; Wlodawer, A.; Blundell, T. L., *Nature* **1991**, 354 (6352), 411-414. DOI 10.1038/354411a0.
184. Zhou, N.; Gu, T.; Xu, Y.; Liu, Y.; Peng, L., *Journal of Biological Engineering* **2023**, 17 (1), 75. DOI 10.1186/s13036-023-00392-2.
185. Fujimori, K.; Shinichi, F.; Naoe, K.; Norihito, K.; and Shimizu, N., *Bioscience, Biotechnology, and Biochemistry* **1992**, 56 (12), 1985-1990. DOI 10.1271/bbb.56.1985.
186. Negro, A.; Martini, I.; Bigon, E.; Cazzola, F.; Minozzi, C.; Skaper, S. D.; Callegaro, L., *Gene* **1992**, 110 (2), 251-256. DOI [https://doi.org/10.1016/0378-1119\(92\)90657-B](https://doi.org/10.1016/0378-1119(92)90657-B).
187. Dicou, E., *Neurochemistry International* **1992**, 20 (1), 129-134. DOI [https://doi.org/10.1016/0197-0186\(92\)90136-F](https://doi.org/10.1016/0197-0186(92)90136-F).
188. Tadimarri, V. S.; Blanch-Asensio, M.; Deshpande, K.; Baumann, J.; Baumann, C.; Müller, R.; Trujillo, S.; Sankaran, S., *Small* **2025**, n/a (n/a), 2408316. DOI <https://doi.org/10.1002/sml.202408316>.
189. Blanch-Asensio, M.; Tadimarri, V. S.; Wilk, A.; Sankaran, S., *Microbial Cell Factories* **2024**, 23 (1), 42. DOI 10.1186/s12934-024-02302-7.
190. Blanch-Asensio, M.; Tadimarri, V. S.; Martinez, R.; Dahiya, G. S.; Duong, C. N.; Lale, R.; Sankaran, S., *Journal of Controlled Release* **2025**, 387, 114264. DOI <https://doi.org/10.1016/j.jconrel.2025.114264>.
191. Flenker, K. S.; Burghardt, E. L.; Dutta, N.; Burns, W. J.; Grover, J. M.; Kenkel, E. J.; Weaver, T. M.; Mills, J.; Kim, H.; Huang, L.; Owczarzy, R.; Musselman, C. A.; Behlke, M. A.; Ford, B.; McNamara, J. O., *Molecular Therapy* **2017**, 25 (6), 1353-1362. DOI <https://doi.org/10.1016/j.ymthe.2017.03.015>.
192. Dey, S.; Blanch-Asensio, M.; Balaji Kuttae, S.; Sankaran, S., *Microbial Biotechnology* **2023**, 16 (6), 1264-1276. DOI <https://doi.org/10.1111/1751-7915.14228>.
193. Abramson, J.; Adler, J.; Dunger, J.; Evans, R.; Green, T.; Pritzel, A.; Ronneberger, O.; Willmore, L.; Ballard, A. J.; Bambrick, J.; Bodenstein, S. W.; Evans, D. A.; Hung, C.-C.; O'Neill, M.; Reiman, D.; Tunyasuvunakool, K.; Wu, Z.; Žemgulytė, A.; Arvaniti, E.; Beattie, C.; Bertolli, O.; Bridgland, A.; Cherepanov, A.; Congreve, M.; Cowen-Rivers, A. I.; Cowie, A.; Figurnov, M.; Fuchs, F. B.; Gladman, H.; Jain, R.; Khan, Y. A.; Low, C. M. R.; Perlin, K.; Potapenko, A.; Savy, P.; Singh, S.; Stecula, A.; Thillaisundaram, A.; Tong, C.; Yakneen, S.; Zhong, E. D.; Zielinski, M.; Židek, A.; Bapst, V.; Kohli, P.; Jaderberg, M.; Hassabis, D.; Jumper, J. M., *Nature* **2024**, 630 (8016), 493-500. DOI 10.1038/s41586-024-07487-w.
194. Hecker, G.; Lewis, D. L.; Rausch, D. M.; Jelsema, C. L., *Biochemical Society Transactions* **1991**, 19 (2), 385-386. DOI 10.1042/bst0190385.

195. Kongsamut, S.; Miller, R. J., *Proceedings of the National Academy of Sciences* **1986**, 83 (7), 2243-2247.
196. Koizumi, S.; Contreras, M. L.; Matsuda, Y.; Hama, T.; Lazarovici, P.; Guroff, G., *The Journal of Neuroscience* **1988**, 8 (2), 715. DOI 10.1523/JNEUROSCI.08-02-00715.1988.
197. Westerink, R. H. S.; Ewing, A. G., *Acta Physiologica* **2008**, 192 (2), 273-285. DOI <https://doi.org/10.1111/j.1748-1716.2007.01805.x>.
198. Greene, L. A.; Tischler, A. S., *Proceedings of the National Academy of Sciences* **1976**, 73 (7), 2424-2428. DOI 10.1073/pnas.73.7.2424.
199. Halvorsen, T. M.; Ricci, D. P.; Park, D. M.; Jiao, Y.; Yung, M. C., *ACS Synthetic Biology* **2022**, 11 (11), 3785-3796. DOI 10.1021/acssynbio.2c00386.

Publications, scientific communications and patents

Peer-reviewed publications

1. Dey, S*, **Blanch-Asensio, M***, Balaji Kuttae, S. and Sankaran, S. 2023. Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic lactobacilli. *Microbial Biotechnology*.
2. **Blanch-Asensio, M***, Dey, S*. and Sankaran, S. 2023. In vitro assembly of plasmid DNA for direct cloning in *Lactiplantibacillus plantarum* WCSF1. *PLOS one*
3. **Blanch-Asensio, M.**, Dey, S., Tadimarri, V.S. and Sankaran, S. 2024. Expanding the genetic programmability of *Lactiplantibacillus plantarum*. *Microbial Biotechnology*
4. **Blanch-Asensio, M.**, Tadimarri, V.S., Wilk, A. and Sankaran, S. 2024. Discovery of a high-performance phage-derived promoter/repressor system for probiotic lactobacillus engineering. *Microbial Cell Factories*.
5. Tadimarri, V.S*, **Blanch-Asensio, M***, Deshpande, K., Baumann, J., Baumann, C., Mueller, R., Trujillo, S. and Sankaran, S. 2025. PEARL: Protein Eluting Alginate with Recombinant Lactobacilli. *Small*.
6. **Blanch-Asensio, M.**, Tadimarri, V.S., Martinez, R., Dahiya, G.S., Duong, C.N., Lale, R., and Sankaran, S. 2025. Encapsulation-enhanced switchable protein release from engineered probiotic lactobacilli. *Journal of Controlled Release*.
7. Van Heest, A.E., **Blanch-Asensio, M.**, Sankaran, S., and Hao, L. 2025. Multimodal reporting of tumors in mice with engineered lactobacilli. *Manuscript in preparation*.
8. Van Tente, I., **Blanch-Asensio, M.**, Eilers, T., Vandenhuevel, D., Lebeer, S., Spacova, I., and Sankaran, S. 2025. Genetic toolbox expansion enables constitutively fluorescent *Lactocaseibacillus rhamnosus* for functional microbiome research. *Submitted to Microbial Biotechnology*.

9. Tadimarri, V.S., **Blanch-Asensio, M.**, and Sankaran, S. 2025. Exploiting the probiotic potential of LAB for Engineered Living Materials. *Manuscript in preparation*.

Scientific talks

1. German Conference on Synthetic Biology, Online Event, Germany (September 2021). **Poster presentation:** *Light-regulated release of recombinant Nerve Growth Factor from Bacterial Hydrogels*.
2. 3rd Engineered Living Materials 2022, Saarbrücken, Germany (June 2022). **Poster presentation:** *Building an optogenetic circuit in probiotic bacteria as a novel living therapeutic*.
3. Symposium on Synthetic and Systems Biology, Paris, France (September 2022). **Oral presentation:** *Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic Lactobacillus*.
4. 6th Applied Synthetic Biology in Europe, Edinburgh, UK, (November 2022). **Poster presentation:** *In vitro assembly of plasmid DNA for direct cloning in Lactiplantibacillus plantarum WCSF1*. Awarded the poster prize.
5. LSC Summer School on Engineered Living Materials, Nonnweiler, Germany (July 2023). **Poster presentation:** *Stimuli-responsive release of Nerve Growth Factor from probiotic bacteria*.
6. 14th International Symposium on Lactic Acid Bacteria, Egmond aan Zee, The Netherlands (August 2023). **Poster presentation:** *Discovery of a high-performance phage-derived promoter/repressor system for probiotic lactobacillus engineering*.
7. Biomedical Engineering Student Seminar, Boston University, Boston, USA (May 2024). **Oral presentation:** *Programming probiotic lactobacilli towards next-generation probiotics*.
8. Engineered Living Materials 2024, Saarbrücken, Germany (September 2024). **Oral presentation.** *“PEARL”: Protein Eluting Alginate Beads with Recombinant Lactobacilli*.

9. Synthetic Biology for Health and Sustainability, Hinxton, UK (October 2024). **Flash oral presentation** and **poster presentation**: *"PEARL": Protein Eluting Alginate Beads with Recombinant Lactobacilli*.

Patent applications

1. S. Sankaran, S. Dey and **M. Blanch Asensio**; *"Novel genetic tools for Lactobacillus"/ "Neuartige genetische Werkzeuge"*, Publication No.: WO2024022791; International application - Date 01.02.2024.
2. S. Sankaran and **M. Blanch Asensio**; *"Novel genetic tools for Lactobacillus"/ "Neuartige genetische Werkzeuge"* Publication No.: WO 2025109081; International application - Date 30.05.2025.

Abbreviations

LAB: Lactic Acid Bacteria

ELM: Engineered Living Materials

SCFA: Short-Chain Fatty Acids

EPS: Extracellular Polysaccharides

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

mRNA: Messenger Ribonucleic Acid

ORI: Origin of replication

TA: Toxin/Antitoxin

GOI: Gene of Interest

POI: Protein of Interest

PCR: Polymerase Chain Reaction

GFP: Green Fluorescent Protein

SP: Signal Peptide

RBS: Ribosome-Binding Site

NucA: Nuclease A

GMO: Genetically Modified Organism

ng: nanograms

α -MSH: α -Melanocyte Stimulating Hormone

PEARL: Protein Eluting Alginate with Recombinant Lactobacilli

FACS: Fluorescence-Activated Cell Sorting

nLuc: nanoLuc (luciferase)

RPM: Revolutions Per Minute

OD₆₀₀: Optical Density at 600 nanometers

RFU: Relative Fluorescence Units

MRS: De Man, Rogosa and Sharpe

EV: Empty Vector

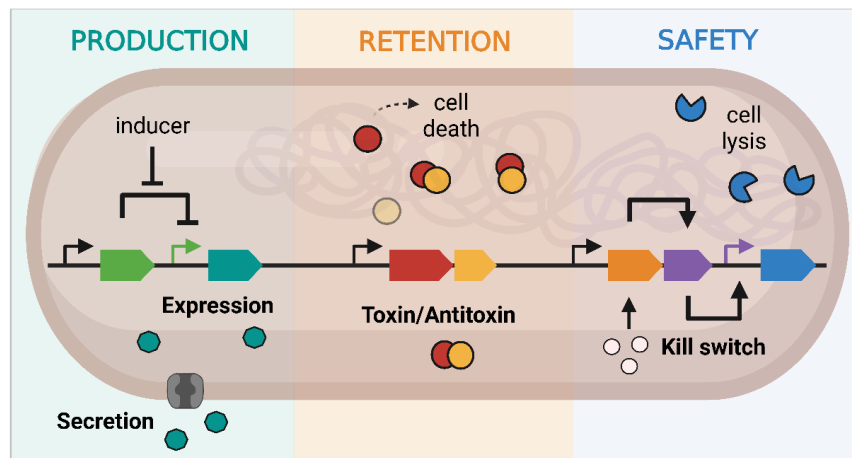
NGF: Nerve Growth Factor

Appendix I

*Expanding the genetic programmability of *L. plantarum**

Appendix A contains a peer-reviewed Mini-Review. In this review, we compiled the work related to the genetic programmability of *L. plantarum* that has been done in the past decades. The review covers gene expression and regulation, protein secretion, plasmid retention and biocontainment modules. Furthermore, we propose potential new steps the community can take to move forward the expansion of the genetic toolbox of these bacteria. Some of the suggestions or perspectives of this review have been investigated in this thesis.

Graphical Abstract



Bacteria encoding three genetic modules. The first one is the production modules, which comprise inducible gene expression and protein secretion. The second is the retention module, based on a TA system that assists in the retention of the genetic parts. The last is the safety module, based on a kill switch that triggers cell lysis as a biocontainment measurement.

Publication and contributions

Article Type: Mini-review.

Title: Expanding the genetic programmability of *Lactiplantibacillus plantarum*.

Year Published: 2023.

Research Journal: Microbial Biotechnology.

Authors: Marc Blanch-Asensio, Sourik Dey, Varun Sai Tadimarri and Shrikrishnan Sankaran.

First author: Marc Blanch-Asensio.

Author contributions: Marc Blanch-Asensio made all the illustrations. Marc Blanch-Asensio, Sourik Dey and Varun Sai Tadimarri wrote the draft of the manuscript. All authors took part in the conceptualization. Shrikrishnan Sankaran led the supervision and reviewed and edited the manuscript.

MINI REVIEW

Expanding the genetic programmability of *Lactiplantibacillus plantarum*

Marc Blanch-Asensio | Sourik Dey | Varun Sai Tadimarri | Shrikrishnan Sankaran 

Bioprogrammable Materials, INM—Leibniz Institute for New Materials, Saarbrücken, Germany

Correspondence

Shrikrishnan Sankaran, Bioprogrammable Materials, INM—Leibniz Institute for New Materials, Campus D2 2, 66123 Saarbrücken, Germany.
Email: shrikrishnan.sankaran@leibniz-inm.de

Funding information

Deutsche Forschungsgemeinschaft, Grant/Award Number: 455063657; Leibniz Association

Abstract

Lactobacilli are ubiquitous in nature and symbiotically provide health benefits for countless organisms including humans, animals and plants. They are vital for the fermented food industry and are being extensively explored for health-care applications. For all these reasons, there is considerable interest in enhancing and controlling their capabilities through the engineering of genetic modules and circuits. One of the most robust and reliable microbial chassis for these synthetic biology applications is the widely used *Lactiplantibacillus plantarum* species. However, the genetic toolkit needed to advance its applicability remains poorly equipped. This mini-review highlights the genetic parts that have been discovered to achieve food-grade recombinant protein production and speculates on lessons learned from these studies for *L. plantarum* engineering. Furthermore, strategies to identify, create and optimize genetic parts for real-time regulation of gene expression and enhancement of biosafety are also suggested.

INTRODUCTION

In recent decades, advancements in bacterial synthetic biology have opened more doors to bacteria-based applications beyond bioprocessing, like bioremediation, biosensing and living therapeutics. Among the many diverse types of bacteria that exist in nature, lactobacilli occupy a central beneficial role for humans, supporting human, animal and plant health as commensals and probiotics (Di Cerbo et al., 2016) and driving fermentation in the food industry (Dewi & Kollanoor Johny, 2022). Due to this, there has been considerable interest in genetically programming these bacteria for enhancing their role as probiotics, increasing pharmaceutical protein production and establishing their role as living drug delivery vectors and mucosal vaccine candidates (Wu et al., 2021). The application fields where genetically engineered lactobacilli play a very important role have been highlighted in Figure 1. This is in line with the growing

development of several lactic acid bacteria (e.g. lactococci, pediococci, streptococci etc.) for such applications through the steady expansion of their genetic toolboxes (Bosma et al., 2017; Landete, 2017; Plavec & Berlec, 2020; Wu et al., 2021).

Due to steady growth in the variety of species discovered as lactobacilli, this genus was split into 25 genera in 2020 based on whole genome sequences (Zheng et al., 2020). Among this vast variety, *Lactiplantibacillus plantarum* is the most extensively studied and engineered species as exemplified by the number of publications in the last 25 years (Figure 2A) and an increasing number of reports to date (Figure 2B).

This is due to a combination of multiple reasons such as:

- (i) *Lactiplantibacillus plantarum* strains have been qualified with a 'generally recognized as safe' (GRAS) status by the US Food and Drug Administration (USFDA).

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2023 The Authors. *Microbial Biotechnology* published by Applied Microbiology International and John Wiley & Sons Ltd.

- (ii) They have proven to be effective probiotics for human health with beneficial effects such as lowering cholesterol levels (Naruszewicz et al., 2002), attenuation of inflammatory bowel diseases (Schultz et al., 2002) and prevention of *Clostridium difficile* infections (Kujawa-Szewieczek et al., 2015).
- (iii) They are extensively used in the food industry as natural alternatives for chemical additives in dairy products (Arena et al., 2016) and as supplements to increase the shelf-life of fermented food products (Behera et al., 2018).

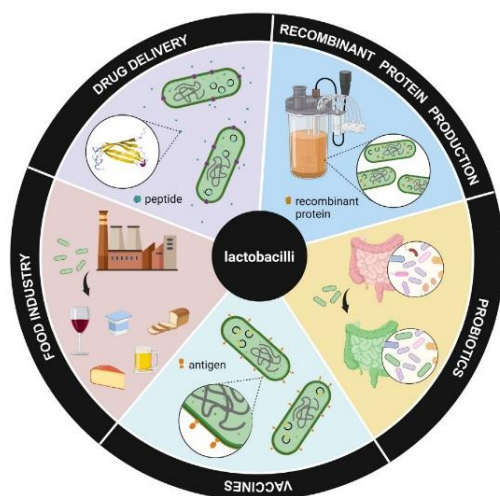
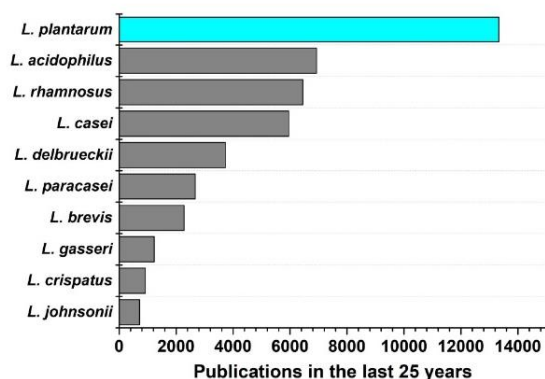


FIGURE 1 Schematic showing the potential applications of genetically engineered lactobacilli in drug delivery, food fermentation, vaccine development, probiotic enhancement and recombinant protein production.

- (iv) They are among the few lactobacilli species that are considered to be nomadic in nature, capable of occupying a wide variety of niches (Duar et al., 2017). For instance, they have been found to thrive in the oral cavity, guts and vaginas of vertebrates, in the guts of fish and insects, in dairy products, fermented fruits and vegetables, etc. That is because they encode diverse metabolic pathways, like those for uptake and utilization of a large spectrum of sugars as carbon sources, for exopolysaccharide synthesis and protein secretion. Also, unlike many other lactobacilli species, they can synthesize most amino acids and are auxotrophic to only a few, namely valine, leucine, isoleucine and glutamate.
- (v) Most strains have high-stress tolerance to acid, alkaline and osmotic stresses, while some are additionally tolerant to heat, oxidative and starvation stresses (Parente et al., 2010). Due to this, they can survive in gastric juices and bile acids, apart from diverse environmental conditions.
- (vi) They are facultatively anaerobic or microaerophilic and can be easily cultured in the lab and in bioreactors.
- (vii) The genomes of multiple strains such as WCFS1, JDM1, NC8, ST-III and many more have been sequenced, leading to an improved understanding of their metabolic, biochemical and physical characteristics (Siezen & van Hylckama Vlieg, 2011).
- (viii) They are genetically tractable and several genetic parts including plasmid replicons, promoters, signal peptides for secretion, plasmid retention systems (antibiotic resistance, auxotrophic complementation, toxin–antitoxin) and gene editing tools have been demonstrated to work reliably and stably (Zhou et al., 2019).

(A)



(B)

L. plantarum publications

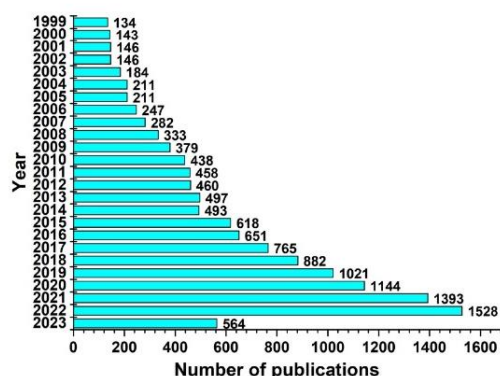


FIGURE 2 (A) Publication counts of different pre-2020 *Lactobacillus* species over the last 25 years. (B). Yearly publication counts of *Lactiplantibacillus plantarum* from 1999 to 2023 (June). The statistics were gathered using the Web of Science by performing topic searches for each species using their pre-2020, post-2020 and abbreviated phylogenetic names.

- (ix) Inducible gene expression has been demonstrated using the class II bacteriocin sakacin peptide-inducible two-component system (TCS) (from *Latilactobacillus sakei*; Sørvig, Mathiesen, et al., 2005; Sørvig, Skaugen, et al., 2005). This, along with their protein secretion capability, has led to the exploration of *L. plantarum* strains for the heterologous expression of recombinant proteins.

Multiple genetic parts developed in *L. plantarum* have been shown to function across other lactobacilli genera and species (Sørvig et al., 2003). Thus, *L. plantarum* has been used as a gateway host for developing cross-genera compatible genetic parts.

Despite these advances, the synthetic biology toolbox of *L. plantarum* pales in comparison to model microbes like *Escherichia coli*, *Bacillus subtilis* or *Lactococcus lactis*. This is exemplified by the lack of strong promoters, reliable repressors and orthogonal polymerases. Such tools are essential for the development of genetic circuits in *L. plantarum* and lactobacilli as a whole, which will allow them to be programmed with enhanced functions for various applications as already described.

In this minireview, we have addressed this technological gap and suggested unexplored or poorly explored options to fill it. We cover four crucial aspects concerning the genetic programmability of *L. plantarum* and the possibility to establish this species as a model *Lactobacillus* chassis for synthetic biology applications. The first part covers gene expression modules, with a focus on existing and potential genetic parts that enable tuning or regulation of gene expression in *L. plantarum*. The second part highlights advances in programming protein secretion from *L. plantarum* and offers strategies to accelerate signal peptide screening. The third part involves plasmid bioretention, particularly focusing on approaches that do not require antibiotic selection pressures. In the last part, we cover biocontainment strategies to ensure the programmable death of these genetically modified bacteria and prevent their release into the environment.

Tuning and regulation of gene expression

Promotion and control of gene expression can be encoded and tuned using promoters, repressors, ribosome binding site (RBS) sequences, RNA polymerases, sigma factors etc. The greatest advances in *L. plantarum* have been in the development of constitutive and inducible gene expression systems through promoter screening, the adaptation of TCSs and repressor engineering attempts. These aspects have been briefly covered in this section, followed by suggestions to identify new parts.

CONSTITUTIVE GENE EXPRESSION SYSTEMS

A repertoire of promoters is crucial for establishing synthetic genetic circuits in a microbial chassis (Mays & Nair, 2018; Mugwanda et al., 2023). Different strategies have been pursued to increase the available set of promoters for *L. plantarum*, like screening native promoters from the bacterial genome, creating synthetic promoter libraries by random or directed mutagenesis and employing promoter regions from both phylogenetically related and distant bacterial species. From the diverse set of native promoters tested in *L. plantarum*, P_{ldh} (Anbazhagan et al., 2013; Reveneau et al., 2002) and P_{tuf} (Spangler, Caruana, et al., 2019; Spangler, Dean, et al., 2019) have been used most frequently by researchers. Comparative studies have shown that these two promoters can drive the expression of heterologous genes at moderately high levels in the bacterial chassis (Peirotén & Landete, 2020). However, endogenous promoters can also be affected by native metabolic networks during cellular growth and division, which can affect the overall expression of a heterologous protein (Wang, Fu, et al., 2020; Wang, Liang, et al., 2020). To bypass promoter cross-reactivity and improve the overall transcription rate, synthetic promoter libraries were created and tested in *L. plantarum*. The first reported synthetic promoter library used the 16S rRNA promoter template to create an array of artificial promoters of varying degrees of strength in *L. plantarum* WCFS1 (Rud et al., 2006). Promoters P_{48} and P_{11} from this library showed the highest gene expression levels for multiple heterologous protein-encoding genes (Guo et al., 2019; Ma et al., 2016). Apart from native promoter templates, the constitutive P_{23} promoter of *L. lactis* was rationally mutagenized to create a promoter library of different strengths. From this library, the P_{OL2} promoter showed the highest expression strength in the *L. plantarum* strains XJ25, XJ14 and XJA2 isolated from Chinese red wine and traditional Chinese pickle (Meng et al., 2021). However, these reports relied on saturation mutagenesis of spacer regions in promoter sequences which predominantly leads to decreased promoter strength (Blazeck & Alper, 2013). To increase the overall transcription rate, efforts are being made to develop hybrid promoters with potent enhancer elements fused to conserved core promoter regions as successfully demonstrated in unicellular yeasts (Cazier & Blazeck, 2021). Apart from the transcription rate, the translational efficiency of heterologous proteins has also been seen to be affected by spacer lengths between the RBS and the start codon (Tauer et al., 2014). Significant down-regulation in the protein yield is expected when the spacer length is not in the range of 7–11 base pairs (bp). Yet, it is still unclear whether the nucleotide composition of the spacer also plays a role in regulating the protein yield.

Promoters from non-endogenous sources have also shown considerable potential for protein production in *L. plantarum*, *L. lactis* and *L. acidophilus*, and have been extensively used for orthogonal promoter analysis from phylogenetically related bacteria. These isolated promoters have played an important role in enhancing the performance of *L. plantarum* in industrially relevant processes. For example, the strong lactococcal promoter P_{59} was used for the recombinant expression of catalase in *L. plantarum* TISTR850, which helped significantly reduce the lipid oxidation level in fermented meat (Noonpakdee et al., 2004). The P_{32} promoter from *L. lactis* was used for the overexpression of the stress response regulator protein (ctsR) to increase the ethanol tolerance of *L. plantarum* WCFS1 during wine fermentation (Zhao et al., 2019). Promoters P_{pgm} and P_{SlpA} isolated from *L. acidophilus* strains were used for surface anchoring of the β -mannanase protein in *L. plantarum*, which showed enzyme activities of 1200 and 3500 U per gram of biomass (Nguyen et al., 2019).

Apart from related genera, constitutive promoters from phylogenetically distant bacteria have been also tested in *L. plantarum* strains. The constitutive promoter P_X , isolated from the Gram-positive bacteria *Streptococcus pneumoniae*, was able to drive high expression of the red fluorescent (mRFP5) protein in probiotic *L. plantarum* 90 and B2 strains (Russo et al., 2015). The strong fluorescent protein expression allowed

monitoring of bacterial colonization in the intestinal tract of zebrafish larvae. The P_X promoter-driven mRFP5 expression also helped assess the stress tolerance and adhesion capability of the riboflavin overproducer *L. plantarum* M5MA1-B2 strain in the mouse digestive tract (Mohedano et al., 2019). In line with these findings, alternate promoters from Gram-negative bacteria were tested in *L. plantarum* WCFS1 (Dey et al., 2023). Although the promoters P_R and P_L (strong promoters reported in *E. coli*) gave very low levels of fluorescent protein expression, the promoter P_{tlpA} (strong promoter in *Salmonella typhimurium*) surpassed the protein expression levels of previously described strong promoters like P_{48} and P_{23} by fivefold. Although the reason for the high expression strength is still unknown, it has previously been reported that the rpoD RNA polymerase of *L. plantarum* has an innate ability to recognize conserved regions of orthogonal promoters and initiate mRNA production irrespective of the expression host (Gaida et al., 2015).

These studies have yielded a collection of well-characterized constitutive promoters with strengths varying across several orders of magnitude, enabling tuning of protein expression levels (Table 1). However, even the strongest constitutive promoters in *L. plantarum* are weaker than inducible promoters in *E. coli*. One key disadvantage in increasing constitutive promoter strengths is that protein expression competes with the

TABLE 1 Examples of constitutive gene expression systems tested in *Lactiplantibacillus plantarum*.

Origin strain	Promoter	Relative expression strength ^a	Reference
<i>Constitutive expression systems</i>			
<i>Lactiplantibacillus plantarum</i>	P_{ldh}	—	Reveneau et al. (2002)
<i>Lactiplantibacillus plantarum</i>	P_{tuf}	3	Spangler, Caruana, et al. (2019), Spangler, Dean, et al. (2019)
<i>Lactiplantibacillus plantarum</i>	P_{48}	3	Rud et al. (2006)
<i>Lactiplantibacillus plantarum</i>	P_{11}	3	Rud et al. (2006)
<i>Lactiplantibacillus plantarum</i>	P_{21}	2	Rud et al. (2006)
<i>Lactiplantibacillus plantarum</i>	P_{44}	1	Rud et al. (2006)
<i>Lactiplantibacillus plantarum</i>	P_{23}	3	Meng et al. (2021)
<i>Lactococcus lactis</i>	P_{OL1}	2	Meng et al. (2021)
<i>Lactococcus lactis</i>	P_{OL2}	3	Meng et al. (2021)
<i>Lactococcus lactis</i>	P_{OL3}	1	Meng et al. (2021)
<i>Lactococcus lactis</i>	P_{69}	—	Noonpakdee et al. (2004)
<i>Lactococcus lactis</i>	P_{32}	—	Zhao et al. (2019)
<i>Lactobacillus acidophilus</i>	P_{pgm}	—	Nguyen et al. (2019)
<i>Lactobacillus acidophilus</i>	P_{SlpA}	—	Nguyen et al. (2019)
<i>Streptococcus pneumoniae</i>	P_X	—	Russo et al. (2015)
<i>Salmonella typhimurium</i>	P_{tlpA}	5	Dey et al. (2023)

^aExpression strengths have been computed in relation to the P_{tlpA} promoter based on comparative expression levels reported in the different studies. Since expression levels in different studies were tested under different conditions, these values are approximations on a scale from 1 to 5, with 5 being the highest expression level and each integer lower representing a twofold decrease. Rows without numbers were from studies where such relative expression strengths could not be estimated.



natural metabolism in the cell and decelerates growth. Inducible promoters help circumvent this condition by enabling temporal separation of the growth and production phases, thereby allowing overexpression to occur only in well-grown healthy biomass. The next section covers examples of such inducible gene expression systems in *L. plantarum*.

INDUCIBLE GENE EXPRESSION SYSTEMS

While constitutive promoters enable the tuneable expression of proteins, inducible systems provide temporal control of them. On the one hand, this is useful for optimizing bioprocess conditions to improve protein yields and on the other, it enables the construction of

layered genetic circuits for more advanced applications (Wong et al., 2015). The most widely used inducible systems reproducibly verified in *L. plantarum* are based on pSIP vectors (Sørvig et al., 2003). These vectors encode TCSs systems that are originally part of quorum sensing-based bacteriocin regulation operons in *L. sakei* and are induced by autoinducer peptides (AIP) (Figure 3A). With this AIP inducible system, several recombinant proteins have been expressed in a dose-dependent manner in *L. plantarum* at decent yields. The pSIP411 vector produced 1800 Miller Unit equivalents (MU) of β -glucuronidase (GusA) in *L. plantarum* NC8 post-induction with 50 ng/mL sakacin inducer peptide (Spplp), accounting for a fold induction of 87. On the other hand, 50 ng/mL of Spplp induction for the pSIP407 vector produced the protein aminopeptidase-N (PepN) at a specific activity of 3.5 U/mg of protein,

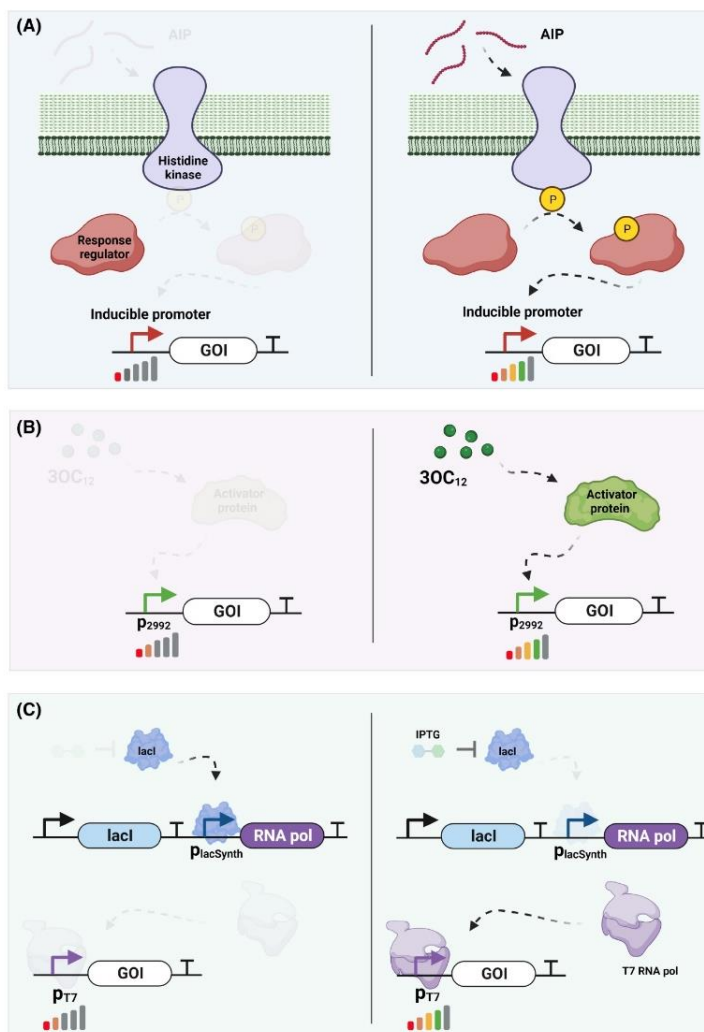


FIGURE 3 Scheme showing the inducible protein expression systems in *Lactiplantibacillus plantarum* (A) TCS pSIP system. The AIP interacts with the histidine kinase, which gets phosphorylated and transfers the phosphate to the response regulator protein. This protein then triggers the expression of the gene of interest (GOI) by activating the inducible promoter. (B) 3OC₁₂ system. The addition of 3OC₁₂ induces the expression of the GOI by activating the P₂₉₉₂ promoter through an unknown activator protein. (C) T7RNAP system. IPTG inhibits the lacI repressor, thus stopping the repression of P_{lacSynth} and promoting the production of the T7 RNA polymerase, which drives the expression of the GOI.

constituting up to 40% of the total intracellular protein content of the bacterial host (Mathiesen et al., 2004; Sørvig, Mathiesen, et al., 2005; Sørvig, Skaugen, et al., 2005). Notably, the AIP inducible pSIP system has enabled both the extracellular secretion of recombinant proteins such as nuclease (Karlskås et al., 2014; Tran et al., 2021) as well as cell-surface anchoring of proteins for applications ranging from biocatalysis (Nguyen et al., 2016) to mucosal vaccine development (Li et al., 2021; Wang, Fu, et al., 2020; Wang, Liang, et al., 2020). However, the heterologous protein yield can decrease significantly when AIP induction is conducted at growth phases other than the early exponential phase (personal communication). An alternative AIP inducible system commonly used in lactococci is based on the polycyclic inducer peptide, nisin and the nisK–nisR regulatory cascade (Mierau et al., 2005). This dual-plasmid system was originally unsuitable for the expression of the tetanus toxin (TTFC) in *L. plantarum* WCFS1 (Pavan et al., 2000). However, when the nisK–nisR regulatory modules were integrated into the bacterial genome, the TTFC yield constituted about 10% of the total intracellular protein content post 25 ng/mL of nisin induction.

Transcriptomic and proteomics analysis showed that when *L. plantarum* WCFS1 cultures were subjected to treatment with N-3-oxododecanoyl homoserine lactone (3OC₁₂), the predominant Acyl-homoserine lactone (AHL) derived from *Pseudomonas aeruginosa*, the gene expression levels of luxS and plantaricin were significantly upregulated (Spangler, Caruana, et al., 2019; Spangler, Dean, et al., 2019). Detailed analysis of the upregulated gene networks showed that 3OC₁₂ induction for 4 h was able to significantly increase the expression levels of promoters P₂₉₉₂ and P₃₂₁₄ (Spangler et al., 2022). A small set of promoters generated by modifying the –10 and –35 regions of the P₂₉₉₂ promoter resulted in a complete loss of 3OC₁₂-based induction, whereas the R8 mutant (8 bp spacer) retained the AHL induction ability even though the overall expression was severely compromised (~1000 fold). This study proved that the P₂₉₉₂ consensus promoter sequence can be a potential part of a TCS whose activation mechanisms are yet to be elucidated and expanded (Figure 3B).

In addition to AIP and AHL, natural sugars and related analogues have also been explored as inducer molecules for gene expression. An endogenous promoter (P_{lacA}) system showed an ~8-fold induction capability in response to 2% (w/v) lactose in *L. plantarum* 3NSH (Heiss et al., 2016). The system was completely repressed in the presence of monomeric sugars (glucose and galactose), indicating the presence of catabolite-responsive control in the promoter region (Marasco et al., 1998). The orthogonal xylose inducible promoter (P_{xyIA}), derived from *Bacillus megaterium*, was responsive to xylose supplementation but showed significant leaky expression in the absence of

the inducer, reducing the dynamic range to only ~2-fold. This fold change was only achievable when the sole carbon source for bacterial growth was replaced from glucose to galactose. This study also tested the ability of T7 RNA Polymerase (T7 RNAP) to drive protein expression in *L. plantarum* 3NSH. The reporter mCherry gene was encoded downstream of the P_{T7} promoter in the high copy plasmid pCDLbu1 and the codon optimized T7RNAP was expressed by the isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible P_{lacSynth} promoter on a low copy pCD256 vector (Figure 3C). Post IPTG induction, the dynamic range of the system accounted for an ~6-fold higher mCherry expression. The low induction fold changes of these systems were attributed to the weak promoters driving insufficient repressor protein expression, leading to a high basal level expression of reporter proteins. The maximum protein expression rate of all the inducible promoters showed ~6-fold lower expression in comparison to the synthetic constitutive promoter P₁₁. Rational modification of the lactose/galactose inducible P_{lacA} and P_{lacLM} promoters isolated from *L. plantarum* WCFS1 increased their expression strengths to ~10 fold and ~13 fold, respectively, in comparison to their native promoter sequences (Zhang et al., 2019). The β-galactosidase yield (45.72 ± 0.44 U/mL) of the modified P_{lacLM-35-10} promoter was significantly higher than the well-established expression systems driven by promoters P_{spp} and P_{ldh}.

Several stress-inducible systems, from metal starvation and bile stress to temperature fluctuations, have also been established in *L. plantarum*. The promoter P_{MntH2} showed an inverse relationship to manganese ion presence, producing β-glucosidase (CelB) at an enzyme activity of ~18 per mg of total protein in the complete absence of MnSO₄ (Böhmer et al., 2013). However, the overall protein yield was 60 times lower than the pSIP-based inducible system, suggesting low expression strength of promoter P_{MntH2}. Promoter P₁₆₀₉₀ isolated from *Lactobacillus casei* BL23 was tested for its bile stress induction capacity in other bacterial species and was observed to give the highest induction in *L. plantarum* WCFS1 (Martínez-Fernández et al., 2019). The strain was able to survive at high bile salt concentrations (>0.2% w/v) and showed no leaky expression of the fluorescent reporter protein, evoglow-Pp1. The promoter P_{ldh} originally encoding for the L-lactate dehydrogenase enzyme in *Lactobacillus johnsonii* PF01 was also shown to be responsive to bile supplementation in *L. plantarum*, with an induction fold change of ~1.8 (Chae et al., 2019). Finally, a temperature gradient shift from 27 to 8°C has been shown to activate the cold shock response promoter, P_{cspL}, and increase the expression of the reporter protein β-galactosidase by ~1.4 fold (Derzelle et al., 2002).

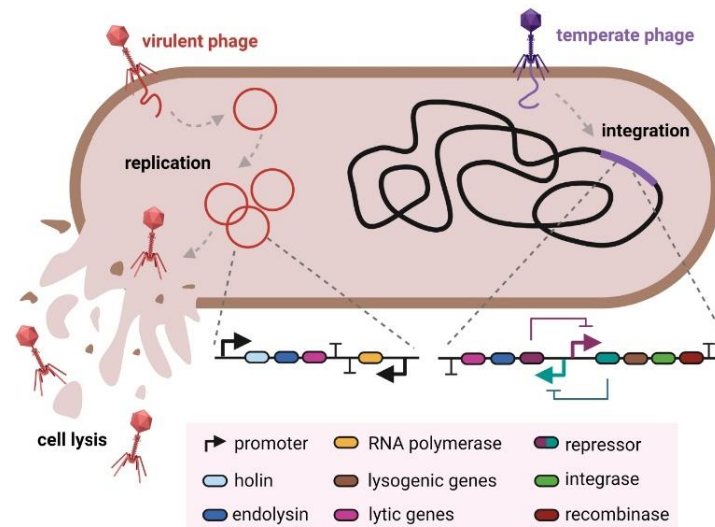
These few studies reveal that inducible protein expression (Table 2) in *L. plantarum* is severely limited,

TABLE 2 List of inducible gene expression systems tested in *Lactiplantibacillus plantarum*.

Origin species/strain	Promoter	Inducer	Dynamic range ^a	Reference
<i>Inducible expression systems</i>				
<i>Lactobacillus sakei</i>	P _{sppA}	Spplp (AIP)	87	Mathiesen et al. (2004)
<i>Lactococcus lactis</i>	P _{nisA}	Nisin (AIP)	20	Pavan et al. (2000)
<i>Lactiplantibacillus plantarum</i> WCFS1	P ₂₉₉₂	3OC ₁₂ (AHL)	10	Spangler et al. (2022)
<i>Lactiplantibacillus plantarum</i> WCFS1	P _{lacA}	Lactose	8	Heiss et al. (2016)
<i>Bacillus megaterium</i>	P _{xylA}	Xylose	2	Heiss et al. (2016)
T7 bacteriophage	P _{T7}	IPTG	6	Heiss et al. (2016)
<i>Lactiplantibacillus plantarum</i> WCFS1	P _{lacLM}	Galactose	13	Zhang et al. (2019)
<i>Lactobacillus johnsonii</i> PF01	P _{ldh}	Bile Salt	1.8	Chae et al. (2019)
<i>Lactiplantibacillus plantarum</i> NC8	P _{cspL}	Cold Shock	1.4	Derzelle et al. (2002)

^aDynamic ranges were calculated from data reported in each study as the ratio of protein expression level on induction by expression level without induction.

FIGURE 4 Schematic figure showing a bacterium being infected by both a virulent phage and a temperate phage. Relevant genes crucial for the lytic and the lysogenic cycles are highlighted in the genomes of the virulent and temperate phage respectively.



and additional robust and versatile systems are required. To develop such systems, genetic parts involved in gene regulation, like repressors, polymerases, etc., need to be identified. One crucially untapped source for gene regulatory parts includes bacteriophages that infect lactic acid bacteria. They naturally encode repressors in endogenous genetic switches that control the lysogenic and lytic cycles (Brady et al., 2021; Figure 4). Furthermore, phage-derived genetic parts are naturally adapted to their hosts due to the arms race between bacteriophages and bacteria and millions of years of coevolution (Hampton et al., 2020).

There are several publications based on the identification and characterization of repressors encoded in genetic switches of phages that infect *L. plantarum* and other species closely related to *L. plantarum* (Binishofer et al., 2002; Kakikawa et al., 2000; Ladero et al., 1998).

However, there are no reports on the experimental testing of these repressors in *L. plantarum*. Exploring and implementing these repressors in novel genetic circuits in *L. plantarum* could be an interesting approach to expanding the genetic programmability of these bacteria. Besides, there is evidence that phage-derived parts can be functional in *L. plantarum*. In 2015, Yang and colleagues employed phage recombinases encoded by a prophage within the genome of *L. plantarum* WCFS1 as a genetic tool to successfully manipulate the genome of these bacteria (Yang et al., 2015). Such recombinases are frequently found in the genomes of prophages since these are necessary to integrate the genome of the phage into the genome of the bacteria (Smith, 2015) and have extensively been used for recombineering, a technique for efficient genetic engineering (Li et al., 2023). If phage-derived repressors prove to be effective in blocking the expression

of the target gene in *L. plantarum*, they could be further modified to respond to external factors (e.g. temperature, light, chemicals), as it has previously been done for the Lambda phage repressor *ci* (Xiong et al., 2022).

Moreover, bacteriophage-encoded RNA polymerases (Figure 4) are greatly used as synthetic biology tools and are frequently part of complex genetic circuits. The most classic example is the T7 expression system. This system is based on a lactose-responsive repressor, *lacI* and the T7 RNA polymerase. Upon induction with lactose, the repressor stops blocking the expression of the polymerase, which starts driving the expression of the gene of interest (Dubendorf & Studier, 1991). This breakthrough prompted interest in generating T7 RNA polymerase mutant libraries that responded to different stimuli. For example, split polymerase versions were created that respond to temperature and light (Baumschlager et al., 2017; Chee et al., 2022). However, when the T7 RNA polymerase was encoded in *L. plantarum*, its activity was found to be surprisingly underwhelming (Heiss et al., 2016).

RNA polymerases have been identified in the genome of phages infecting *Lactobacillus* species (Grasdaschi et al., 2021; Kyrkou et al., 2019). Nevertheless, the specific promoters to which RNA polymerase drives expression are unknown. The identification of those could lead to the potential implementation of RNA polymerases and their specific promoters in genetic circuits in *L. plantarum*. Additionally, other RNA polymerases could be identified using bioinformatic tools such as PHASTEST (PHAge Search Tool with Enhanced Sequence Translation), which identify putative prophages within the genomes of bacteria (Wishart et al., 2023).

Thus, the genomes of phages infecting lactobacilli offer several unexplored parts to achieve efficient and versatile regulation of gene expression in *L. plantarum*.

Signal peptides driving protein secretion

Lactiplantibacillus plantarum is considered a safe and effective host for the recombinant production of enzymes and therapeutic proteins (Wells & Mercenier, 2008). Recombinant lactobacilli species can secrete high titre of protein into the growth medium which can be subjected to further downstream purification processes. Considerable interest has been shown in developing Living Biotherapeutic Products where engineered microbes can be used to deliver drugs in the host environment (Heavey et al., 2022). Successful translation of these products depends on robust yields of secreted proteins which mainly relies on the choice of signal peptides coupled to the protein. A study by and colleagues involving functional analysis of 647 hypothetical proteins of *Lactiplantibacillus plantarum* predicted by bioinformatic

tools, revealed 112 transporter protein families and 40 protein families comprised of homologous signal peptides (Davray & Kulkarni, 2023). Mathiesen and colleagues also tested the secretion capability of 76 native signal peptides in *L. plantarum* WCFS1 using staphylococcal nuclease (*nucA*) as the reporter protein (Mathiesen et al., 2009). Lp_3050 and Lp_2145, endogenous signal peptides were shown to secrete significant amounts of the reporter protein in comparison to its other counterparts. These signal peptides have also shown great promise in secreting recombinant proteins in related *L. plantarum* strains. Signal peptides Lp_0373 and Lp_2145 secreted 13.1 and 8.1 kU of amylase per litre of fermentation broth from *L. plantarum* S21, which was significantly higher than the yields produced using its native signal peptide SP_AmyL (Tran et al., 2021). However, it has also been seen that no single signal peptide can guarantee the maximum secretion of a heterologous protein from *L. plantarum*. Factors like protein hydrophobicity, the overall charge of the protein, the presence of the transmembrane helix and the length of anchoring motifs can significantly affect the secretion efficiency of a signal peptide (Mathiesen et al., 2009).

The greatest bottleneck in the applicability of heterologous protein secretion is the requirement to identify the set of signal peptides that can optimally work for a range of different proteins. So far, this involves experimentally screening multiple signal peptides and selecting the candidate that demonstrates significant protein secretion yield. Standard computational tools like SignalP 6.0 can partially expedite the signal peptide analysis, by suggesting suitable signal peptide combinations and their respective cleavage sites (Teufel et al., 2022). However, there are no generalized correlations identified between protein properties and signal peptide sequences that support strong secretion. To gain such understanding, studies are required that systematically test commonly reported signal peptides with multiple proteins having different sizes, hydrophobicity indices, structural repeats and functional activities. Regarding experimental screening, commonly used methods include detecting the proteins in the cell-free culture supernatant by Polyacrylamide gel electrophoresis (PAGE), Western blot and mass spectrometry analysis. While these techniques have been effective in many cases, limitations pertaining to the detection limit range (at least ~100 nanograms), complex sample preparation procedure and high temporal requirements reduce the wide-scale adoption of these methods. One strategy to overcome these limitations involves the fusion of a functional enzyme to the protein of interest and indirectly quantifying its yield by assessing the activity of the fused enzyme in the extracellular growth media. For example, the commonly used reporter protein *nucA*, which

is known to be efficiently secreted from *L. plantarum*, can be fused to the heterologous protein of interest. The secretion of nucA-fused proteins can be assessed by a well-characterized agar plate-based assay in which supernatant or bacterial colonies secreting the nucA-fused protein create a halo proportional to the amount of secreted protein (Langella & Le Loir, 1999). The main drawback of this method is that the fusion of such enzymes considerably increases the size of the protein to be secreted (nucA M.W ~17 kDa). Additionally, it is often not desired for a secreted protein to harbour an active enzyme, especially for applications like living therapeutics. Another approach to overcome this issue could be the fusion of short peptide tags/protein domains to the protein of interest that is part of split GFP/split Luciferase complementation assays. Such assays involve incomplete versions of GFP or luciferase, which are not fluorescent/luminescent until the complementary peptide unit or protein domain binds to them and completes their structure. These methods are relatively simple and fast (<30 min), providing quantitative results with high sensitivity and requiring simple spectroscopic devices (Knapp et al., 2017; Wang et al., 2021). Such methods can also be automated and have been recently reported with other bacteria for achieving higher throughput screening of signal peptides for secretion (Müller et al., 2022).

Plasmid retention systems

The most common approach to constructing genetically modified *L. plantarum* has been through plasmid engineering. Extrachromosomal plasmid DNA has allowed the expression of heterologous proteins without requiring tedious methodologies of gene integration into the bacterial genome. The choice of selecting vectors with multiple plasmid copy numbers, diverse antibiotic resistance markers and gene expression systems has increased the ease of genetic circuit construction. However, as with any plasmid-based engineered system, the presence of an active selective pressure is a prerequisite for maintaining the synthetic gene construct in the microbe (Van Zyl et al., 2019). This creates complexities while trying to use these modified bacteria for product upscaling, drug delivery or recombinant enzyme production. The plasmid retention systems developed in *L. plantarum* are highlighted in the following section, along with suggestions to further expand the current portfolio.

AUXOTROPHY-BASED PLASMID RETENTION

Auxotrophy-based plasmid retention employs the principle of deleting an essential gene from the bacterial

genome and reintroducing it into the plasmid vector. The essential protein is crucial for the bacterial host metabolism and without it, the bacteria will be unable to grow and divide. This complementation of the essential gene on the plasmid allows for efficient retention of the heterologous protein without any further external selection pressure. Multiple studies have reported creating auxotrophic strains of *L. plantarum* which have maintained the recombinant plasmid for several generations without compromising on growth and protein production. The most used auxotrophic system encodes for the deletion of the alanine racemase (*alr*) gene from the bacterial genome to inhibit the D-alanine biosynthesis pathway. Insufficient production of the D-alanine amino acid prevents bacterial cell wall formation (Palumbo et al., 2004). Earlier reports have shown that the *alr* gene-complemented plasmids can be stably maintained for 200 generations in auxotrophic *L. plantarum* in the absence of any selection pressure (Bron et al., 2002). To revalidate this claim, a pSIP609R vector expressing β -Galactosidase (β -Gal) was ligated to the endogenous *alr* gene and transformed in the Δ *alr* auxotroph of *L. plantarum* WCFS1 (TLG02). Post induction, the recombinant strain was able to show moderate segregational stability for 84 generations, with 17% of the population still harbouring the recombinant plasmid, yielding a net titre of 5 U/mg β -Gal (Nguyen et al., 2011). This vector was further used to secrete heterologous proteins, chitosanase and β -mannanase into the growth media at concentrations 79 and 50 kU/L, respectively, in the absence of any external selection pressure (Sak-Ubol et al., 2016). Paul et al. replaced the antibiotic resistance gene in the pSIP609 vector with the *alr* gene and encoded the constitutive secretion of the oxalate degrading enzyme (*oxdC*). A Δ *alr* auxotrophic *L. plantarum* WCFS1 strain harbouring this plasmid was shown to significantly reduce urinary oxalate excretion and calcium oxalate deposition in the kidneys of hyperoxaluric rats (Paul et al., 2018). The deletion of the glucosamine-6-phosphate synthase (*glmS*) gene has also been shown to disrupt the hexosamine biosynthetic pathway and inhibit the N-acetylglucosamine (GlcNAc) synthesis, which is an essential component of the *L. plantarum* cell wall (Rolain et al., 2012). Vector pSIPH497 was created by replacing the antibiotic-resistant gene with the P_{idh} promoter-driven *glmS1* gene and transforming it into the *glmS1*-deficient *L. plantarum* WCFS1 strain (NZ5333). The recombinant plasmid was stable for 100 generations without any selection pressure and was able to express mCherry when induced with the AIP for the pSIP system (Chen et al., 2018). In addition to these existing systems, natural auxotrophy of the *L. plantarum* strain 17-5 (ATCC 8014) towards essential vitamins like biotin can be complemented by the biotin synthase gene responsible for the conversion of desthiobiotin into biotin (Bowman & DeMoll, 1993). Auxotrophic

strains targeting the pyrimidine biosynthesis pathway can also be used for retaining plasmids in the bacterial hosts. The deletion of the thymidylate synthase (*thyA*) gene prevented DNA synthesis and cell division in *L. casei*, and was restored by *thyA* gene complementation in the plasmid (Zhou et al., 2018). The recombinant strain showed stable retention of the plasmid for over 50 generations and active production of the antimicrobial peptide, lactoferricin. Such strategies can also be extended to *L. plantarum* in the future depending on the intended application. The only major drawback of this promising strategy involves the generation of permanently modified microbes with altered genetic loci. This limits the versatility of plasmid-based engineering since the plasmids can then only be used in strains that are already genomically modified, restricting the options of testing recombinant plasmids in multiple strains or related genera.

TOXIN–ANTITOXIN–BASED PLASMID RETENTION

The adaptive evolution of bacteria under resource-limited conditions allows for selecting specific genes that provide a survival benefit to the host strain. These genes are primarily acquired from the environment through horizontal gene transfer and transposable elements and are retained in the bacteria as extrachromosomal DNA (Arnold et al., 2022). One of the most prominent ways to naturally retain these elements in the recipient organism includes a toxin–antitoxin (TA) gene pair in the acquired gene segment (Mruk & Kobayashi, 2014). These gene pairs have been classified into five major classes depending on the antitoxin's action mechanism, with the majority of TA pairs belonging to the Type I (antisense RNA antitoxin) or Type II (protein antitoxin) class (Singh et al., 2021). The wide diversity of TA pairs prompted researchers to test them for retaining engineered plasmids in bacteria without any external selection pressure. The low-copy number p256 plasmid in *L. plantarum* NC7 was seen to have high segregational stability during cellular division (Cosby et al., 1989). Detailed analysis of the p256 plasmid showed that the retention ability was correlated to the presence of the Type II TA system based on *pemK* (toxin) and *pemI* (antitoxin; Sørvig, Mathiesen, et al., 2005; Sørvig, Skaugen, et al., 2005). The recombinant plasmid, pLPV100 harbouring the chloramphenicol resistance gene cassette (*cat*) along with the *pemK*–*pemI* gene pair showed 100% plasmid retention after 80 generations in non-selective media. This TA system showed a higher stability function for the toxin protein than the antitoxin protein, causing a significant decrease in the viability of the bacterial cells that had been cured of the plasmid. This crucial observation resulted in further exploration of potential

type II TA candidates in related lactobacilli strains over the last decade. TA candidates have been found to be naturally existing in several cryptic plasmids, for example, the RelE (toxin)–RelB (antitoxin) pair was identified in the cryptic plasmids K25p1, pLU4 and pIR52-1 in *L. plantarum* K25 (Jiang et al., 2018), *L. reuteri* LU4 (Kim et al., 2017) and *L. helveticus* R0052 respectively (Hagen et al., 2010). Type II toxins like MazE have been annotated both in bacterial genomes (Yan et al., 2012) as well as in mobile plasmids (Abriouel et al., 2019). The prevalence of Type II toxins hinted towards their effective mechanism of action which involves targeting the bacterial RNA population due to its endoribonuclease activity. Fedorec et al. (2019) revalidated the claim showing that the Type II Txe (Toxin)–Axe (Antitoxin) system helped in the plasmid retention in *E. coli* for >30 days in comparison to the Type I hok (toxin)–sok (antitoxin) system. We decided to test the stability function of five different Type II TA systems in *L. plantarum* WCFS1 to achieve stable plasmid retention over multiple generations (Dey et al., 2023). The G50 value (generation number with 50% of plasmid cured population) was highest (~85 generations) for the YafQ (toxin)/DinJ (antitoxin) TA system in comparison to the other TA systems. This G50 was further increased to ~110 generations by combining two TA systems (YafQ/DinJ and MazF/MazE) in the same plasmid. This showed that the presence of multiple toxins targeting the bacterial RNA population can be used to increase the temporal duration of plasmid retention. However, none of the tested TA systems could retain the plasmid beyond ~150 generations. This can be attributed to the gradual development of toxin resistance or upregulation of molecular chaperones to counteract the toxin effect (Mutschler & Meinhart, 2011). This TA system-based approach is promising for plasmid retention despite such long-term instability. The results also indicate the opportunity to generate recombinant strains with temporary GMO status since they do not require permanent modification in the bacterial genome like the auxotrophy approach. The bacteria that lose the plasmid over time revert to their non-GMO probiotic forms, thereby offering the possibility to use such transient GMOs with inbuilt temporal biocontainment for medical or food applications (Figure 5). In addition to the plethora of Type II TA systems available, the focus should also be put on exploring the stability function of Type I TA systems (Fozo et al., 2010). The discovery of the Type I Lpt toxin in the plasmid of cheese ripening bacteria, *Lactobacillus rhamnosus*, demonstrated the key role of the TA system in plasmid segregational stability (Folli et al., 2017). Bioinformatic software like T1Tadb (Tourasse & Darfeuille, 2021) and TADB 2.0 (Xie et al., 2018) can help researchers in faster identification and subsequent stability function testing of Type I and Type II TA systems, respectively, in

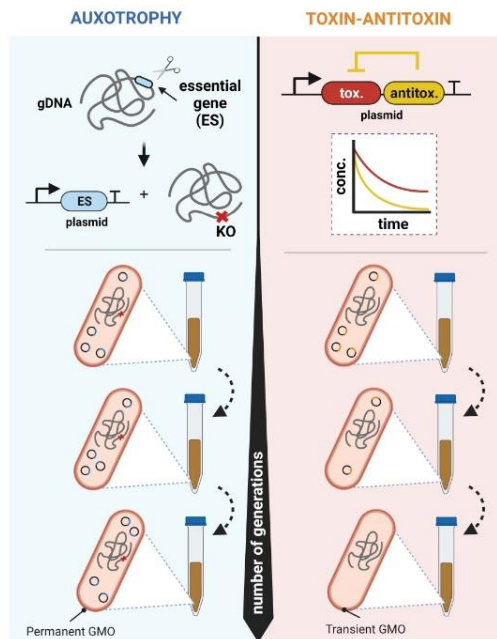


FIGURE 5 Comparison between the auxotrophy and the toxin–antitoxin-based plasmid retention approaches.

lactobacilli species. A combinatorial library of these different TA systems could then mediate stable plasmid retention over several generations in *L. plantarum* and other related species.

As an alternative to auxotrophy and TA systems, specific genetic parts from the plasmidome of *L. plantarum* strains could be explored as novel plasmid retention systems. A recent comparative genomic analysis on the plasmidome of 105 *L. plantarum* strains has identified 1141 protein families whose genes are uniquely encoded within plasmids and are not present in the chromosome (Davray & Bawane 2023). Such genes could potentially be promising candidates to positively contribute to plasmid retention in *L. plantarum* when cloned to a different vector. Furthermore, cryptic plasmids often contain highly stable plasmid retention strategies that are host-dependent and are not yet well understood. Thus, cryptic plasmids can be used as vector backbones themselves in which genes of interest can be encoded and stably retained in the host. Such a strategy has been recently demonstrated in the probiotic *E. coli* Nissle 1917 (Kan et al., 2020).

Biocontainment modules

Lastly, any genetically altered microbe must be carefully designed to prevent its escape and proliferation

into the natural environment. Specific guidelines have been set by regulatory agencies to control the release and disposal of genetically engineered microbes and transgenes post-application (Wilson, 1993). Abiding by these regulations, there have been several successful attempts to establish kill-switch-based genetic circuits in conventional microbial chassis like *E. coli* and *L. lactis*. Kill switches mainly rely on proteins that modulate the growth and survival of the microbe post-production (Wright et al., 2013). The inducer-regulated expression of this protein creates non-permissive growth conditions for the microbe. Although there has been no report of a kill-switch-based biocontainment strategy for *L. plantarum*, several candidates can be tested for creating such modules.

One of the most promising candidates for developing such 'kill-switches' can be the natural (endo)lysins which are an integral part of the bacteriophage genome (Figure 4). When the lytic cycle is induced, lysins are expressed and these enzymes start degrading the bacterial cell wall (Schmelcher et al., 2012). This results in the lysis of the cell and promotes the release of virions outside the bacterium. Despite these fundamental discoveries, not many of these lysins have progressed for translational applications. This provides an opportunity to explore potential kill switch candidates from multiple phages (Figure 6A).

Toxins from orthogonal toxin–antitoxin systems are additional interesting candidates for kill switches in *L. plantarum*. Toxins have been used as part of kill switches in other bacteria. Well-known examples are the 'Deadman' kill switch and the 'GeneGuard' system in *E. coli* (Chan et al., 2016; Wright et al., 2015). For *L. plantarum*, an interesting approach could be to integrate the toxin within the genome under a constitutive promoter and then transform these bacteria with a plasmid carrying the antitoxin under an inducible system. Therefore, the expression of the antitoxin would depend on the presence of an inducer (permissive conditions) and without the inducer (non-permissive conditions), the toxin would trigger cell death (Figure 6B).

CRISPR/Cas9 could also be an attractive alternative to toxic proteins that trigger cell death. The use of CRISPR/Cas9 in a kill switch was successfully employed in the probiotic *E. coli* Nissle 1917, where chemical and temperature-responsive switches were designed to induce the production of the Cas9 and several guide RNAs targeting different regions of the genome (Rottinghaus et al., 2022). The same approach could be implemented in *L. plantarum* (Figure 6C). Besides, CRISPR has been widely used in *L. plantarum* as a genome editing tool (Myrbråten et al., 2019; Zhou et al., 2019), which strengthens the potential use of CRISPR as a kill switch in these bacteria.

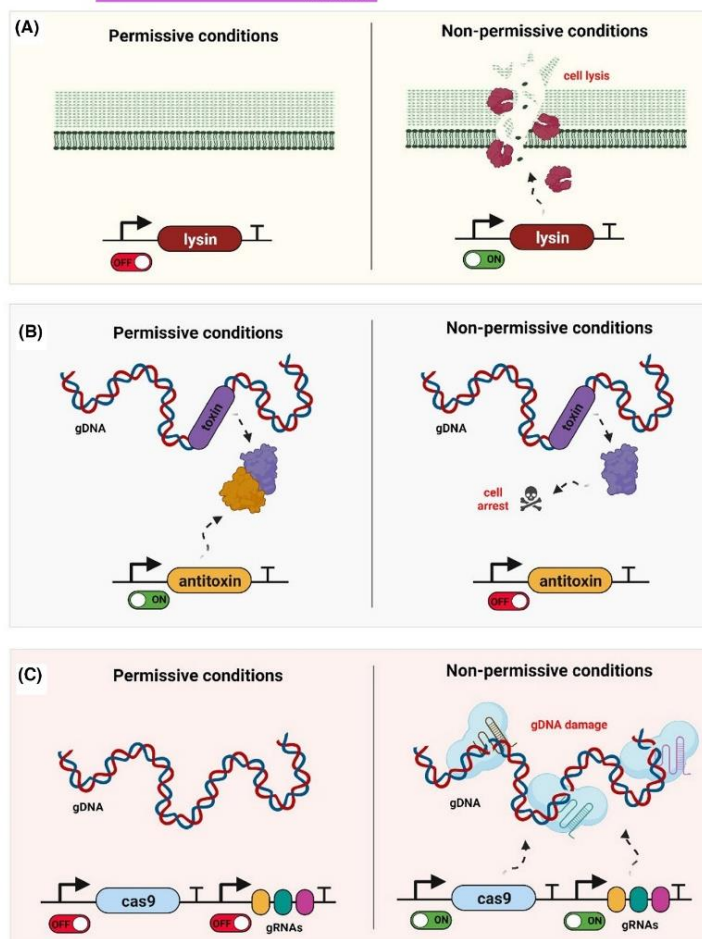


FIGURE 6 Schematic highlighting the different approaches to constructing kill-switch genetic circuits in *L. actiplantibacillusplantarum*. (A) Lysin-based kill switch. In non-permissive conditions, the expression of the lysin is induced, triggering the cell lysis of the bacteria. (B) Toxin-antitoxin-based kill switch. In non-permissive conditions, the expression of the antitoxin is stopped, and the toxin, which is constitutively produced, triggers cell death. (C) CRISPR/Cas9-based kill switch. In non-permissive conditions, both the Cas9 and genome-targeting gRNAs are expressed, which causes the cleavage of the genomic DNA and cell death.

CONCLUDING REMARKS

As the world is increasingly looking to synthetic biology for solving major global challenges, lactobacilli, with their numerous benefits and ubiquity in our lives, are the ideal chassis to lead this bioengineering revolution. However, the vast diversity within this bacterial family and their poorly understood biochemistry have greatly limited progress in expanding their genetic programmability. Nevertheless, a strong foundation has been laid in a few select species, among which *L. plantarum* is at the forefront. This mini-review makes the case for establishing *L. plantarum* as a model species among lactobacilli by (i) enumerating the genetic parts established to achieve food-grade recombinant protein production and (ii) suggesting novel strategies and sources to identify parts that will enable reliable regulation of gene expression and improved biosafety. There are numerous unexplored avenues to expand the genetic

toolkit in these bacteria, which have shown promising results in other model organisms like *E. coli* and *B. subtilis*. This knowledge combined with advances in computational tools and automation technologies promises the possibility for rapid development in lactobacilli engineering and the elevation of *L. plantarum* to a model synthetic biology host. In turn, this will expand their applicability in fields such as bioprocess engineering, agritechology, living therapeutics and engineered living materials.

AUTHOR CONTRIBUTIONS

Marc Blanch-Asensio: Conceptualization (lead); writing – original draft (lead); writing – review and editing (equal). **Sourik Dey:** Conceptualization (supporting); writing – original draft (equal). **Varun Sai Tadimarri:** Conceptualization (supporting); writing – original draft (supporting). **Shrikrishnan Sankaran:** Conceptualization (equal); writing – original draft (supporting); writing – review and editing (lead).

ACKNOWLEDGEMENTS

Biorender was used for generating all the figures of this mini-review. This work was supported by the Deutsche Forschungsgemeinschaft's Research grant (Project #455063657), Collaborative Research Centre, SFB 1027 (Project #200049484) and the Leibniz Science Campus on Living Therapeutic Materials [LifeMat]. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

ORCID

Shrikrishnan Sankaran  <https://orcid.org/0000-0001-6251-585X>

REFERENCES

- Abriouel, H., Pérez Montoro, B., de la Fuente Ordoñez, J.J., Lavilla Lema, L., Knapp, C.W. & Benomar, N. (2019) New insights into the role of plasmids from probiotic *Lactobacillus pentosus* MP-10 in Aloreña table olive brine fermentation. *Scientific Reports*, 9(1), 1–19.
- Anbazhagan, K., Sasikumar, P., Gomathi, S., Priya, H.P. & Selvam, G.S. (2013) In vitro degradation of oxalate by recombinant *Lactobacillus plantarum* expressing heterologous oxalate decarboxylase. *Journal of Applied Microbiology*, 115(3), 880–887.
- Arena, M.P., Silvain, A., Normanno, G., Grieco, F., Drider, D., Spano, G. et al. (2016) Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. *Frontiers in Microbiology*, 7, 464.
- Arnold, B.J., Huang, I.T. & Hanage, W.P. (2022) Horizontal gene transfer and adaptive evolution in bacteria. *Nature Reviews Microbiology*, 20(4), 206–218.
- Baumschlager, A., Aoki, S.K. & Khammash, M. (2017) Dynamic blue light-inducible T7 RNA polymerases (Opto-T7RNAPs) for precise spatiotemporal gene expression control. *ACS Synthetic Biology*, 6(11), 2157–2167.
- Behera, S.S., Ray, R.C. & Zdolec, N. (2018) *Lactobacillus plantarum* with functional properties: an approach to increase safety and shelf-life of fermented foods. *BioMed Research International*, 2018, 5.
- Binishofer, B., Moll, I., Henrich, B. & Bläsi, U. (2002) Inducible promoter-repressor system from the *Lactobacillus casei* phage ϕ FSW. *Applied and Environmental Microbiology*, 68(8), 4132–4135.
- Blazeck, J. & Alper, H.S. (2013) Promoter engineering: recent advances in controlling transcription at the most fundamental level. *Biotechnology Journal*, 8(1), 46–58.
- Böhmer, N., König, S. & Fischer, L. (2013) A novel manganese starvation-inducible expression system for *Lactobacillus plantarum*. *FEMS Microbiology Letters*, 342(1), 37–44.
- Bosma, E.F., Forster, J. & Nielsen, A.T. (2017) Lactobacilli and pediococci as versatile cell factories—evaluation of strain properties and genetic tools. *Biotechnology Advances*, 35(4), 419–442.
- Bowman, W.C. & DeMoll, E. (1993) Biosynthesis of biotin from de-thiobiotin by the biotin auxotroph *Lactobacillus plantarum*. *Journal of Bacteriology*, 175(23), 7702–7704.
- Brady, A., Felipe-Ruiz, A., Gallego del Sol, F., Marina, A., Quiles-Puchalt, N. & Penadés, J.R. (2021) Molecular basis of lysis-lysogeny decisions in Gram-positive phages. *Annual Review of Microbiology*, 75, 563–581.
- Bron, P.A., Benchimol, M.G., Lambert, J., Palumbo, E., Deghorain, M., Delcour, J. et al. (2002) Use of the *alc* gene as a food-grade selection marker in lactic acid bacteria. *Applied and Environmental Microbiology*, 68(11), 5663–5670.
- Cazier, A.P. & Blazeck, J. (2021) Advances in promoter engineering: novel applications and predefined transcriptional control. *Biotechnology Journal*, 16(10), 2100239.
- Chae, J.P., Pajarillo, E.A., Hwang, I.C. & Kang, D.K. (2019) Construction of a bile-responsive expression system in *Lactobacillus plantarum*. *Food Science of Animal Resources*, 39(1), 13–22.
- Chan, C.T., Lee, J.W., Cameron, D.E., Bashor, C.J. & Collins, J.J. (2016) 'Deadman' and 'Passcode' microbial kill switches for bacterial containment. *Nature Chemical Biology*, 12(2), 82–86.
- Chee, W.K.D., Yeoh, J.W., Dao, V.L. & Poh, C.L. (2022) Highly reversible tunable thermal-repressible split-T7 RNA polymerases (thermal-T7RNAPs) for dynamic gene regulation. *ACS Synthetic Biology*, 11(2), 921–937.
- Chen, Y., Qi, M., Xu, M., Huan, H., Shao, W. & Yang, Y. (2018) Food-grade gene transformation system constructed in *Lactobacillus plantarum* using a GlmS-encoding selection marker. *FEMS Microbiology Letters*, 365(21), p.fny254.
- Cosby, W.M., Axelsson, L.T. & Dobrogosz, W.J. (1989) Tn917 transposition in *Lactobacillus plantarum* using the highly temperature-sensitive plasmid pTV1Ts as a vector. *Plasmid*, 22(3), 236–243.
- Davray, D., Bawane, H. & Kulkarni, R. (2023) Non-redundant nature of *Lactiplantibacillus plantarum* plasmidome revealed by comparative genomic analysis of 105 strains. *Food Microbiology*, 109, 104153.
- Davray, D. & Kulkarni, R. (2023) In-silico functional analysis of hypothetical proteins from *Lactiplantibacillus plantarum* plasmids reveals enrichment of cell envelope proteins. *Plasmid*, 127, 102693.
- Derzelle, S., Hallet, B., Ferain, T., Delcour, J. & Hols, P. (2002) Cold shock induction of the *cspL* gene in *Lactobacillus plantarum* involves transcriptional regulation. *Journal of Bacteriology*, 184(19), 5518–5523.
- Dewi, G. & Kollanoor Johny, A. (2022) *Lactobacillus* in food animal production—a forerunner for clean label prospects in animal-derived products. *Frontiers in Sustainable Food Systems*, 6, 831195.
- Dey, S., Blanch-Asensio, M., Balaji Kuttae, S. & Sankaran, S., 2023. Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic lactobacilli. *Microbial Biotechnology*, 16(6), 1264–1276.
- Di Cerbo, A., Palmieri, B., Aponte, M., Morales-Medina, J.C. & Iannitti, T. (2016) Mechanisms and therapeutic effectiveness of lactobacilli. *Journal of Clinical Pathology*, 69(3), 187–203.
- Duar, R.M., Lin, X.B., Zheng, J., Martino, M.E., Grenier, T., Pérez-Muñoz, M.E. et al. (2017) Lifestyles in transition: evolution and natural history of the genus *Lactobacillus*. *FEMS Microbiology Reviews*, 41(Suppl_1), S27–S48.
- Dubendorf, J.W. & Studier, F.W. (1991) Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. *Journal of Molecular Biology*, 219(1), 45–59.
- Fedorec, A.J., Ozdemir, T., Doshi, A., Ho, Y.K., Rosa, L., Rutter, J. et al. (2019) Two new plasmid post-segregational killing mechanisms for the implementation of synthetic gene networks in *Escherichia coli*. *IScience*, 14, 323–334.
- Folli, C., Levante, A., Percudani, R., Amidani, D., Bottazzi, S., Ferrari, A. et al. (2017) Toward the identification of a type I toxin-antitoxin system in the plasmid DNA of dairy *Lactobacillus rhamnosus*. *Scientific Reports*, 7(1), 1–13.
- Fozo, E.M., Makarova, K.S., Shabalina, S.A., Yutin, N., Koonin, E.V. & Storz, G. (2010) Abundance of type I toxin-antitoxin systems

- in bacteria: searches for new candidates and discovery of novel families. *Nucleic Acids Research*, 38(11), 3743–3759.
- Gaida, S.M., Sandoval, N.R., Nicolaou, S.A., Chen, Y., Venkataramanan, K.P. & Papoutsakis, E.T. (2015) Expression of heterologous sigma factors enables functional screening of metagenomic and heterologous genomic libraries. *Nature Communications*, 6(1), 7045.
- Gradaschi, V., Payaslian, F., Dieterle, M.E., Rondón Salazar, L., Urdániz, E., Di Paola, M. et al. (2021) Genome sequence and characterization of *Lactobacillus casei* phage, vB_LcaM_Lbab1 isolated from raw Milk. *Therapy, Applications, and Research*, 2(1), 57–63.
- Guo, J., Xie, Y., Yu, Z., Meng, G. & Wu, Z. (2019) Effect of *Lactobacillus plantarum* expressing multifunctional glycoside hydrolases on the characteristics of alfalfa silage. *Applied Microbiology and Biotechnology*, 103, 7983–7995.
- Hagen, K.E., Tramp, C.A., Altermann, E., Welker, D.L. & Tompkins, T.A. (2010) Sequence analysis of plasmid pIR52-1 from *Lactobacillus helveticus* R0052 and investigation of its origin of replication. *Plasmid*, 63(2), 108–117.
- Hampton, H.G., Watson, B.N. & Fineran, P.C. (2020) The arms race between bacteria and their phage foes. *Nature*, 577(7790), 327–336.
- Heavey, M.K., Durmusoglu, D., Crook, N. & Anselmo, A.C. (2022) Discovery and delivery strategies for engineered live biotherapeutic products. *Trends in Biotechnology*, 40(3), 354–369.
- Heiss, S., Hörmann, A., Tauer, C., Sonnleitner, M., Egger, E., Grabherr, R. et al. (2016) Evaluation of novel inducible promoter/repressor systems for recombinant protein expression in *Lactobacillus plantarum*. *Microbial Cell Factories*, 15, 1–17.
- Jiang, Y., Zhang, J., Zhao, X., Zhao, W., Yu, Z., Chen, C. et al. (2018) Complete genome sequencing of exopolysaccharide-producing *Lactobacillus plantarum* K25 provides genetic evidence for the probiotic functionality and cold endurance capacity of the strain. *Bioscience, Biotechnology, and Biochemistry*, 82(7), 1225–1233.
- Kakikawa, M., Ohkubo, S., Syama, M., Taketo, A. & Kodaira, K.I. (2000) The genetic switch for the regulatory pathway of *Lactobacillus plantarum* phage ϕ g1: characterization of the promoter PL, the repressor gene cpg, and the cpg-encoded protein Cpg in *Escherichia coli*. *Gene*, 242(1–2), 155–166.
- Kan, A., Gelfat, I., Emani, S., Praveschotinunt, P. & Joshi, N.S. (2020) Plasmid vectors for in vivo selection-free use with the probiotic *E. coli* Nissle 1917. *ACS Synthetic Biology*, 10(1), 94–106.
- Karliskäs, I.L., Maudal, K., Axelsson, L., Rud, I., Eijssink, V.G. & Mathiesen, G. (2014) Heterologous protein secretion in lactobacilli with modified pSIP vectors. *PLoS One*, 9(3), e91125.
- Kim, D.H., Jeon, Y.J., Chung, M.J., Seo, J.G. & Ro, Y.T. (2017) Complete sequence and gene analysis of a cryptic plasmid pLU4 in *Lactobacillus reuteri* strain LU4 (KCTC 12397BP). *Applied Biological Chemistry*, 60(2), 145–153.
- Knapp, A., Rippahn, M., Volkenborn, K., Skoczinski, P. & Jaeger, K.E. (2017) Activity-independent screening of secreted proteins using split GFP. *Journal of Biotechnology*, 258, 110–116.
- Kujawa-Szewieczek, A., Adamczak, M., Kwiecień, K., Dudzicz, S., Gazda, M. & Więcek, A. (2015) The effect of *Lactobacillus plantarum* 299v on the incidence of *Clostridium difficile* infection in high risk patients treated with antibiotics. *Nutrients*, 7(12), 10179–10188.
- Kyrkou, I., Byth Carstens, A., Ellegaard-Jensen, L., Kot, W., Zervas, A., Djurhuus, A.M. et al. (2019) Expanding the diversity of Myoviridae phages infecting *Lactobacillus plantarum*—a novel lineage of *Lactobacillus* phages comprising five new members. *Viruses*, 11(7), 611.
- Ladero, V., García, P., Bascarán, V., Herrero, M., Alvarez, M.A. & Suárez, J.E. (1998) Identification of the repressor-encoding gene of the *Lactobacillus* bacteriophage A2. *Journal of Bacteriology*, 180(13), 3474–3476.
- Landete, J.M. (2017) A review of food-grade vectors in lactic acid bacteria: from the laboratory to their application. *Critical Reviews in Biotechnology*, 37(3), 296–308.
- Langella, P. & Le Loir, Y. (1999) Heterologous protein secretion in *Lactococcus lactis*: a novel antigen delivery system. *Brazilian Journal of Medical and Biological Research*, 32, 191–198.
- Li, L., Wang, M., Hao, J., Han, J., Fu, T., Bai, J. et al. (2021) Mucosal IgA response elicited by intranasal immunization of *Lactobacillus plantarum* expressing surface-displayed RBD protein of SARS-CoV-2. *International Journal of Biological Macromolecules*, 190, 409–416.
- Li, R., Li, A., Zhang, Y. & Fu, J. (2023) The emerging role of recombining in microbiology. *Engineering Microbiology*, 3, 100097.
- Ma, X., Wang, G., Li, D. & Hao, Y. (2016) Microcin V production in *Lactobacillus plantarum* LB-B1 using heterologous leader peptide from pediocin PA-1. *Current Microbiology*, 72, 357–362.
- Marasco, R., Muscarello, L., Varcamonti, M., De Felice, M. & Sacco, M. (1998) Expression of the bglH gene of *Lactobacillus plantarum* is controlled by carbon catabolite repression. *Journal of Bacteriology*, 180(13), 3400–3404.
- Martínez-Fernández, J.A., Bravo, D., Peiróten, Á., Arqués, J.L. & Landete, J.M. (2019) Bile-induced promoters for gene expression in *Lactobacillus* strains. *Applied Microbiology and Biotechnology*, 103, 3819–3827.
- Mathiesen, G., Sørvig, E., Blatny, J., Naterstad, K., Axelsson, L. & Eijssink, V.G.H. (2004) High-level gene expression in *Lactobacillus plantarum* using a pheromone-regulated bacteriocin promoter. *Letters in Applied Microbiology*, 39(2), 137–143.
- Mathiesen, G., Sveen, A., Brurberg, M., Fredriksen, L., Axelsson, L. & Eijssink, V.G.H. (2009) Genome-wide analysis of signal peptide functionality in *Lactobacillus plantarum* WCFS1. *BMC Genomics*, 10(1), 425. Available from: <https://doi.org/10.1186/1471-2164-10-425>
- Mays, Z.J. & Nair, N.U. (2018) Synthetic biology in probiotic lactic acid bacteria: at the frontier of living therapeutics. *Current Opinion in Biotechnology*, 53, 224–231.
- Meng, Q., Yuan, Y., Li, Y., Wu, S., Shi, K. & Liu, S. (2021) Optimization of electrotransformation parameters and engineered promoters for *Lactobacillus plantarum* from wine. *ACS Synthetic Biology*, 10(7), 1728–1738.
- Mierau, I., Olieman, K., Mond, J. & Smid, E.J. (2005) Optimization of the *Lactococcus lactis* nisin-controlled gene expression system NICE for industrial applications. *Microbial Cell Factories*, 4, 1–12.
- Mohedano, M.L., Hernández-Recio, S., Yépez, A., Requena, T., Martínez-Cuesta, M.C., Peláez, C. et al. (2019) Real-time detection of riboflavin production by *Lactobacillus plantarum* strains and tracking of their gastrointestinal survival and functionality in vitro and in vivo using mCherry labeling. *Frontiers in Microbiology*, 10, 1748.
- Mruk, I. & Kobayashi, I. (2014) To be or not to be: regulation of restriction-modification systems and other toxin-antitoxin systems. *Nucleic Acids Research*, 42(1), 70–86.
- Mugwanda, K., Hamese, S., Van Zyl, W.F., Prinsloo, E., Du Plessis, M., Dicks, L.M. et al. (2023) Recent advances in genetic tools for engineering probiotic lactic acid bacteria. *Bioscience Reports*, 43(1), BSR20211299.
- Müller, C., Bakkes, P.J., Lenz, P., Waffenschmidt, V., Helleckes, L.M., Jaeger, K.E. et al. (2022) Accelerated strain construction and characterization of *C. glutamicum* protein secretion by laboratory automation. *Applied Microbiology and Biotechnology*, 106(12), 4481–4497.
- Mutschler, H. & Meinhart, A. (2011) ϵ/ζ systems: their role in resistance, virulence, and their potential for antibiotic development. *Journal of Molecular Medicine*, 89, 1183–1194.
- Myrbråten, I.S., Wiull, K., Salehian, Z., Håvarstein, L.S., Straume, D., Mathiesen, G. et al. (2019) CRISPR interference for rapid

- knockdown of essential cell cycle genes in *Lactobacillus plantarum*. *Msphere*, 4(2), e00007-19.
- Naruszewicz, M., Johansson, M.L., Zapolska-Downar, D. & Bukowska, H. (2002) Effect of *Lactobacillus plantarum* 299v on cardiovascular disease risk factors in smokers. *The American Journal of Clinical Nutrition*, 76(6), 1249–1255.
- Nguyen, H.M., Mathiesen, G., Stelzer, E.M., Pham, M.L., Kuczkowska, K., Mackenzie, A. et al. (2016) Display of a β -mannanase and a chitosanase on the cell surface of *Lactobacillus plantarum* towards the development of whole-cell biocatalysts. *Microbial Cell Factories*, 15(1), 1–14.
- Nguyen, H.M., Pham, M.L., Stelzer, E.M., Plattner, E., Grabherr, R., Mathiesen, G. et al. (2019) Constitutive expression and cell-surface display of a bacterial β -mannanase in *Lactobacillus plantarum*. *Microbial Cell Factories*, 18, 1–12.
- Nguyen, T.T., Mathiesen, G., Fredriksen, L., Kittl, R., Nguyen, T.H., Eijssink, V.G. et al. (2011) A food-grade system for inducible gene expression in *Lactobacillus plantarum* using an alanine racemase-encoding selection marker. *Journal of Agricultural and Food Chemistry*, 59(10), 5617–5624.
- Noonpakdee, W., Sitthimonchai, S., Panyim, S. & Lertsiri, S. (2004) Expression of the catalase gene katA in starter culture *Lactobacillus plantarum* TISTR850 tolerates oxidative stress and reduces lipid oxidation in fermented meat product. *International Journal of Food Microbiology*, 95(2), 127–135.
- Palumbo, E., Favier, C.F., Deghorain, M., Cocconcelli, P.S., Grangette, C., Mercenier, A. et al. (2004) Knockout of the alanine racemase gene in *Lactobacillus plantarum* results in septation defects and cell wall perforation. *FEMS Microbiology Letters*, 233(1), 131–138.
- Parente, E., Ciocia, F., Ricciardi, A., Zotta, T., Felis, G.E. & Torriani, S. (2010) Diversity of stress tolerance in *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*: a multivariate screening study. *International Journal of Food Microbiology*, 144(2), 270–279.
- Paul, E., Albert, A., Ponnusamy, S., Mishra, S.R., Vignesh, A.G., Sivakumar, S.M. et al. (2018) Designer probiotic *Lactobacillus plantarum* expressing oxalate decarboxylase developed using group II intron degrades intestinal oxalate in hyperoxaluric rats. *Microbiological Research*, 215, 65–75.
- Pavan, S., Hols, P., Delcour, J., Geoffroy, M.C., Grangette, C., Kleerebezem, M. et al. (2000) Adaptation of the nisin-controlled expression system in *Lactobacillus plantarum*: a tool to study in vivo biological effects. *Applied and Environmental Microbiology*, 66(10), 4427–4432.
- Peiróten, Á. & Landete, J.M. (2020) Natural and engineered promoters for gene expression in *Lactobacillus* species. *Applied Microbiology and Biotechnology*, 104, 3797–3805.
- Plavec, T.V. & Berlec, A. (2020) Safety aspects of genetically modified lactic acid bacteria. *Microorganisms*, 8(2), 297.
- Reveneau, N., Geoffroy, M.C., Locht, C., Chagnaud, P. & Mercenier, A. (2002) Comparison of the immune responses induced by local immunizations with recombinant *Lactobacillus plantarum* producing tetanus toxin fragment C in different cellular locations. *Vaccine*, 20(13–14), 1769–1777.
- Rolain, T., Bernard, E., Courtin, P., Bron, P.A., Kleerebezem, M., Chapot-Chartier, M.P. et al. (2012) Identification of key peptidoglycan hydrolases for morphogenesis, autolysis, and peptidoglycan composition of *Lactobacillus plantarum* WCFS1. *Microbial Cell Factories*, 11, 1–15.
- Rottinghaus, A.G., Ferreira, A., Fishbein, S.R., Dantas, G. & Moon, T.S. (2022) Genetically stable CRISPR-based kill switches for engineered microbes. *Nature Communications*, 13(1), 672.
- Rud, I., Jensen, P.R., Naterstad, K. & Axelsson, L. (2006) A synthetic promoter library for constitutive gene expression in *Lactobacillus plantarum*. *Microbiology*, 152(4), 1011–1019.
- Russo, P., Iturria, I., Mohedano, M.L., Caggianiello, G., Rainieri, S., Fiocco, D. et al. 2015. Zebrafish gut colonization by mCherry-labelled lactic acid bacteria. *Applied microbiology and biotechnology*, 99, 3479–3490.
- Sak-Ubol, S., Namvijitr, P., Pechrichuang, P., Haltrich, D., Nguyen, T.H., Mathiesen, G. et al. (2016) Secretory production of a beta-mannanase and a chitosanase using a *Lactobacillus plantarum* expression system. *Microbial Cell Factories*, 15(1), 1–12.
- Schmelcher, M., Donovan, D.M. & Loessner, M.J. (2012) Bacteriophage endolysins as novel antimicrobials. *Future Microbiology*, 7(10), 1147–1171.
- Schultz, M., Veltkamp, C., Dieleman, L.A., Grentner, W.B., Wyrick, P.B., Tonkonogy, S.L. et al. (2002) *Lactobacillus plantarum* 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflammatory Bowel Diseases*, 8(2), 71–80.
- Siezen, R.J. & van Hylckama Vlieg, J.E. (2011) Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. *Microbial Cell Factories*, 10(1), 1–13.
- Singh, G., Yadav, M., Ghosh, C. & Rathore, J.S. (2021) Bacterial toxin-antitoxin modules: classification, functions, and association with persistence. *Current Research in Microbial Sciences*, 2, 100047.
- Smith, M.C. (2015) Phage-encoded serine integrases and other large serine recombinases. *Mobile DNA*, 3, 253–272.
- Sørvig, E., Grönqvist, S., Naterstad, K., Mathiesen, G., Eijssink, V.G. & Axelsson, L. (2003) Construction of vectors for inducible gene expression in *Lactobacillus sakei* and *L. plantarum*. *FEMS Microbiology Letters*, 229(1), 119–126.
- Sørvig, E., Mathiesen, G., Naterstad, K., Eijssink, V.G. & Axelsson, L. (2005) High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. *Microbiology*, 151(7), 2439–2449.
- Sørvig, E., Skaugen, M., Naterstad, K., Eijssink, V.G. & Axelsson, L. (2005) Plasmid p256 from *Lactobacillus plantarum* represents a new type of replicon in lactic acid bacteria, and contains a toxin-antitoxin-like plasmid maintenance system. *Microbiology*, 151(2), 421–431.
- Spangler, J.R., Caruana, J.C., Phillips, D.A. & Walper, S.A. (2019) Broad range shuttle vector construction and promoter evaluation for the use of *Lactobacillus plantarum* WCFS1 as a microbial engineering platform. *Synthetic Biology*, 4(1), p.ysz012.
- Spangler, J.R., Cooper, D.N., Malanoski, A.P. & Walper, S.A. (2022) Promoter identification and optimization for the response of *Lactobacillus plantarum* WCFS1 to the gram-negative pathogen-associated molecule N-3-Oxododecanoyl homoserine lactone. *ACS Biomaterials Science & Engineering*.
- Spangler, J.R., Dean, S.N., Leary, D.H. & Walper, S.A. (2019) Response of *Lactobacillus plantarum* WCFS1 to the Gram-negative pathogen-associated quorum sensing molecule N-3-oxododecanoyl homoserine lactone. *Frontiers in Microbiology*, 10, 715.
- Tauer, C., Heini, S., Egger, E., Heiss, S. & Grabherr, R. (2014) Tuning constitutive recombinant gene expression in *Lactobacillus plantarum*. *Microbial Cell Factories*, 13(1), 1–11.
- Teufel, F., Almagro Armenteros, J.J., Johansen, A.R., Gislason, M.H., Pihl, S.I., Tsirigos, K.D. et al. (2022) SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology*, 40(7), 1023–1025.
- Tourasse, N.J. & Darfeuille, F. (2021) T1TAdb: the database of type I toxin-antitoxin systems. *RNA*, 27(12), 1471–1481.
- Tran, A.M., Unban, K., Kanpiengjai, A., Khanongnuch, C., Mathiesen, G., Haltrich, D. et al. (2021) Efficient secretion and recombinant production of a lactobacillal α -amylase in *Lactiplantibacillus plantarum* WCFS1: analysis and comparison of the secretion using different signal peptides. *Frontiers in Microbiology*, 12, 689413.

- Van Zyl, W.F., Dicks, L.M. & Deane, S.M. (2019) Development of a novel selection/counter-selection system for chromosomal gene integrations and deletions in lactic acid bacteria. *BMC Molecular Biology*, 20, 1–16.
- Wang, L., Yu, G., Macho, A.P. & Lozano-Durán, R. (2021) Split-luciferase complementation imaging assay to study protein-protein interactions in *Nicotiana benthamiana*. *Bio-Protocol*, 11(23), e4237.
- Wang, M., Fu, T., Hao, J., Li, L., Tian, M., Jin, N. et al. (2020) A recombinant *Lactobacillus plantarum* strain expressing the spike protein of SARS-CoV-2. *International Journal of Biological Macromolecules*, 160, 736–740.
- Wang, T., Liang, C., Xing, W., Wu, W., Hou, Y., Zhang, L. et al. (2020) Transcriptional factor engineering in microbes for industrial biotechnology. *Journal of Chemical Technology & Biotechnology*, 95(12), 3071–3078.
- Wells, J.M. & Mercenier, A. (2008) Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. *Nature Reviews Microbiology*, 6(5), 349–362.
- Wilson, D.J. (1993) NIH guidelines for research involving recombinant DNA molecules. *Accountability in Research*, 3(2–3), 177–185.
- Wishart, D.S., Han, S., Saha, S., Oler, E., Peters, H., Grant, J.R. et al. (2023) PHASTEST: faster than PHASTER, better than PHAST. *Nucleic Acids Research*, 51(W1), gkad382.
- Wong, A., Wang, H., Poh, C.L. & Kitney, R.I. (2015) Layering genetic circuits to build a single cell, bacterial half adder. *BMC Biology*, 13(1), 1–16.
- Wright, O., Delmans, M., Stan, G.B. & Ellis, T. (2015) GeneGuard: a modular plasmid system designed for biosafety. *ACS Synthetic Biology*, 4(3), 307–316.
- Wright, O., Stan, G.B. & Ellis, T. (2013) Building-in biosafety for synthetic biology. *Microbiology*, 159(Pt_7), 1221–1235.
- Wu, J., Xin, Y., Kong, J. & Guo, T. (2021) Genetic tools for the development of recombinant lactic acid bacteria. *Microbial Cell Factories*, 20(1), 1–10.
- Xie, Y., Wei, Y., Shen, Y., Li, X., Zhou, H., Tai, C. et al. (2018) TADB 2.0: an updated database of bacterial type II toxin–antitoxin loci. *Nucleic Acids Research*, 46(D1), D749–D753.
- Xiong, L.L., Garrett, M.A., Buss, M.T., Kornfield, J.A. & Shapiro, M.G. (2022) Tunable temperature-sensitive transcriptional activation based on lambda repressor. *ACS Synthetic Biology*, 11(7), 2518–2522.
- Yan, X., Gurtler, J.B., Fratafico, P.M., Hu, J. & Juneja, V.K. (2012) Phylogenetic identification of bacterial MazF toxin protein motifs among probiotic strains and foodborne pathogens and potential implications of engineered probiotic intervention in food. *Cell & Bioscience*, 2, 1–13.
- Yang, P., Wang, J. & Qi, Q. (2015) Prophage recombinases-mediated genome engineering in *Lactobacillus plantarum*. *Microbial Cell Factories*, 14, 1–11.
- Zhang, S., Xu, Z., Qin, L. & Kong, J. (2019) Development of strong lactose/galactose-inducible expression system for *Lactobacillus plantarum* by optimizing promoter. *Biochemical Engineering Journal*, 151, 107316.
- Zhao, H., Yuan, L., Hu, K., Liu, L., Peng, S., Li, H. et al. (2019) Heterologous expression of *ctsR* from *Oenococcus oeni* enhances the acid-ethanol resistance of *Lactobacillus plantarum*. *FEMS Microbiology Letters*, 366(15), p.fnz192.
- Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M., Harris, H.M., Mattarelli, P. et al. (2020) A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *International Journal of Systematic and Evolutionary Microbiology*, 70(4), 2782–2858.
- Zhou, D., Jiang, Z., Pang, Q., Zhu, Y., Wang, Q. & Qi, Q. (2019) CRISPR/Cas9-assisted seamless genome editing in *Lactobacillus plantarum* and its application in N-acetylglucosamine production. *Applied and Environmental Microbiology*, 85(21), e01367–19.
- Zhou, H., Li, X., Wang, Z., Yin, J., Tan, H., Wang, L. et al. (2018) Construction and characterization of thymidine auxotrophic (Δ thyA) recombinant *Lactobacillus casei* expressing bovine lactoferricin. *BMC Veterinary Research*, 14(1), 1–11.

How to cite this article: Blanch-Asensio, M., Dey, S., Tadimarri, V.S. & Sankaran, S. (2024) Expanding the genetic programmability of *Lactiplantibacillus plantarum*. *Microbial Biotechnology*, 17, e14335. Available from: <https://doi.org/10.1111/1751-7915.14335>

Appendix II

Supporting Information of the publications

II.1. Supporting Information Chapter 1

***In vitro*-assembly of plasmid DNA for direct cloning in *Lactiplantibacillus plantarum* WCSF1**

Marc Blanch Asensio,^{1,†} Sourik Dey,^{1,†} Shrikrishnan Sankaran^{*1}

¹ Bioprogrammable Materials, INM - Leibniz Institute for New Materials Campus D2
2, 66123 Saarbrücken, Germany

*E-mail: Shrikrishnan.sankaran@leibniz-inm.de

[†] Authors contributed equally

Primer name	Sequence
Vector mCherry fw	5'-GAAGATAAATCCCATAAGGG-3'
Vector mCherry rev	5'-AAGAACTCTATTGAAGCCC-3'
Insert mCherry fw	5'-GGGGCTTCAATAGAGTTCTTGGTGATGTCGGCGATATAG-3'
Insert mCherry rev	5'-CCCTTATGGGATTTATCTTCGACTCGCACTGAGAGGAT-3'
mCherry full amp fw	5'-TTACAAGGCTAAGAAGCCAG-3'
mCherry full amp rev	5'-GTAGTCTTAACTTCAGCATCG-3'
Vector elafin fw	5'-AATAACTAGCATAACCCC-3'
Vector elafin rev	5'-CATGGTATATCTCCTTCTT-3'
Insert elafin fw	5'-TAAGAAGGAGATATACCATGATGCGTGCTTCATCATTC-3'
Insert elafin rev	5'-AAGGGGTTATGCTAGTTATTTTAGTGATGGTGATGGTG-3'
Elafin full amp fw	5'-CAAGAAGTGTTGTGAAGGT-3'
Elafin full amp rev / Elafin colony PCR rev	5'-ATACCTGGACAATCAGTATCC-3'
Elafin colony PCR fw	5'-CAATGTTCCAAATGCGTG-3'
Whole gene sequencing fw/ mCherry colony PCR fw	5'-CGTTACTAAAGGGAATGGAG-3'
Whole gene sequencing rev	5'-AGTGAACGAAAACCTCAC-3'
mCherry colony PCR rev	5'-AGTTCATAACACGTTCCAC-3'

Table S1. List of primers used in this study.

➤ mCherry

ATGGTTTCAAAGGGTGAAGAAGATAACATGGCTATCATCAAGGAATTCATGCGTTTCAAGGTTACATGGAAGGTTTCAGTT
AACGGTCACGAATTCGAAATCGAAGGTGAAGGTGAAGGTCGTCACATACGAAGGTACTCAAAGTGAAGTTAAAGGTTACT
AAGGGTGGTCCATTACCATTCGCTTGGGATATCTTATCACCACAATTCATGTACGGTTCAAAGGCTTACGTTAAGCACCCAG
CTGATATCCCAGATTACTTAAAGTTATCATTCCAGAAAGGTTTCAAGTGGGAACGTGTTATGAACTTCGAAGATGGTGGTGT
TGTTACTGTTACTCAAGATTCATCATTACAAGATGGTGAATTCATCTACAAGGTTAAGTTACGTGGTACTAACTTCCCATCAG
ATGGTCCAGTTATGCAAAAGAAGACTATGGGTTGGGAAGCTTCATCAGAACGTATGTACCCAGAAGATGGTGCTTTAAAGG
GTGAAATCAAGCAACGTTTAAAGTTAAAGGATGGTGGTCACTACGATGCTGAAGTTAAGACTACTTACAAGGCTAAGAAGC
CAGTTCAATTACCAGGTGCTTACAACGTTAACATCAAGTTAGATATCACTTCACACAACGAAGATTACACTATCGTTGAACA
ATACGAACGTGCTGAAGGTCGTCACTCAACTGGTGGTATGGATGAATTATACAAGTAA

➤ Elafin

ATGCGTGCTTCATCATTCTTAATCGTTGTTGTTTTCTTAATCGCTGGTACTTTAGTTTTAGAAGCTGCTGTTACTGGTGTTC
AGTTAAGGGTCAAGATACTGTTAAGGGTCGTGTTCCATTCAACGGTCAAGATCCAGTTAAGGGTCAAGTTTCAGTTAAGGG
TCAAGATAAGGTTAAGGCTCAAGAACCAGTTAAGGGTCCAGTTTCAACTAAGCCAGGTTTATGTCCAATCATCTTAATCCG
TTGTGCTATGTTAAACCCACCAAACCGTTGTTAAAGGATACTGATTGTCCAGGTATCAAGAAGTGTGTGAAGGTTTCATGT
GGTATGGCTTGTTCGTTCCACAAGGTGGTTCACATCACCATCACCATCACTAA

Table S2. Genetic Sequences of the mCherry and Elafin coding segments used in this study.

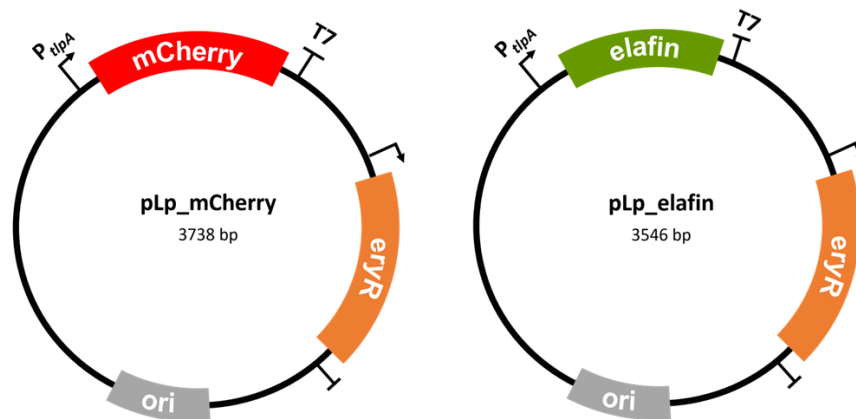


Figure S1. Schematic representation of the pLp_mCherry and pLp_elafin plasmids constructed in this study. The plasmid backbone is derived from the pLp3050sNuc plasmid (Mathiesen et al., 2009) and contains the p256 ORI and the erythromycin resistance gene cassette. The coding sequences of mCherry and elafin are under the strong P_{tlpA} promoter. The T7 terminator is downstream of both the coding gene segments.

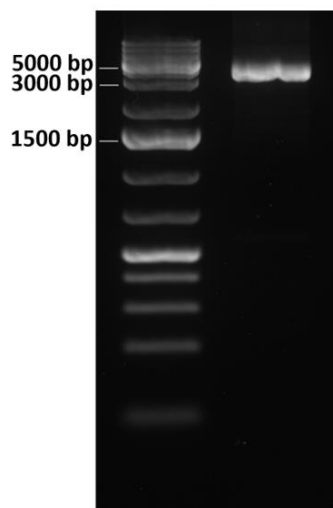


Figure S2. Agarose gel showing the PCR product (3728 bp) amplified from the assembled plasmid construct obtained after the Gibson Assembly Reaction. 1 kb Plus DNA ladder (ThermoFisher Scientific™) was used for the reference.

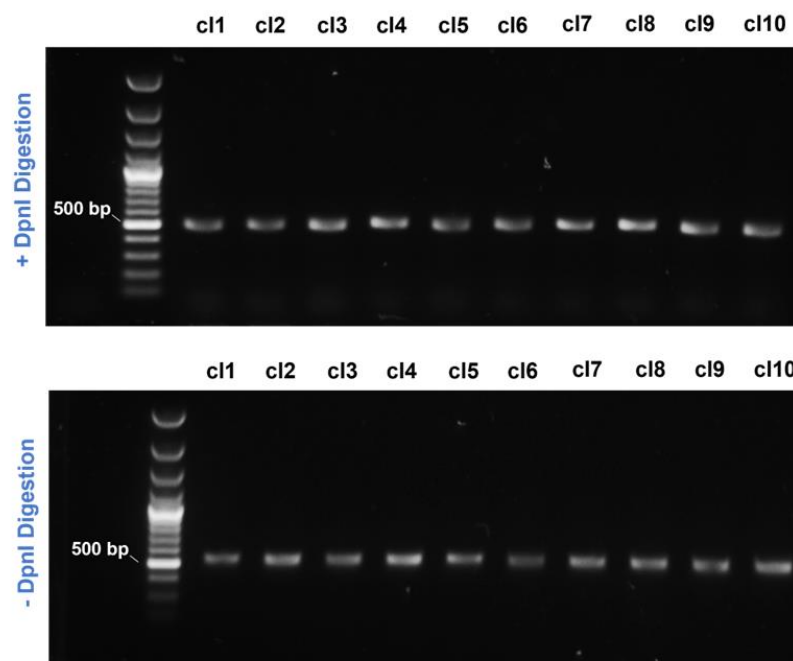


Figure S3. The agarose gel (top) is showing the colony PCR amplicon (558 bp) of 10 red *L. plantarum* colonies obtained through direct cloning with DpnI-treatment of the vector backbone. The agarose gel (bottom) is showing the colony PCR amplicon (558 bp) of 10 red *L. plantarum* colonies obtained through direct cloning without DpnI-treatment of the vector backbone. The PCR conditions were the same for both experimental setups. Generuler 100 bp Plus DNA Ladder (ThermoFisher Scientific™) was used for the reference.

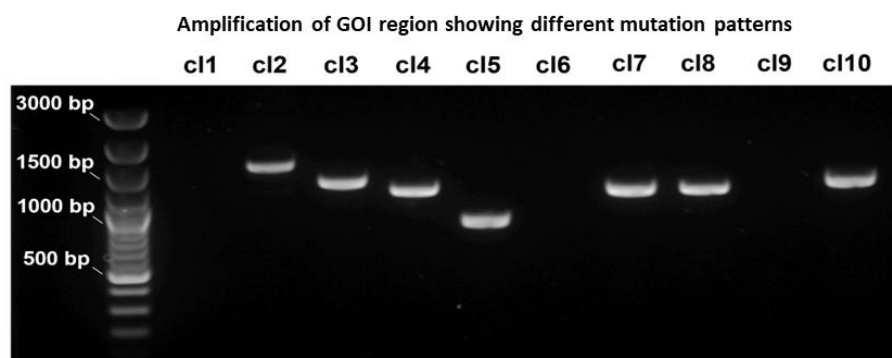


Figure S4. Agarose gel showing the colony PCR products corresponding to the GOI region from 10 non-red *L. plantarum* colonies obtained after direct cloning. The primer set used for amplification was the same as in **Figure 2C**. The different mutation patterns can be visualized for the respective colonies. Generuler 100 bp Plus DNA Ladder (ThermoFisher Scientific™) was used for the reference.

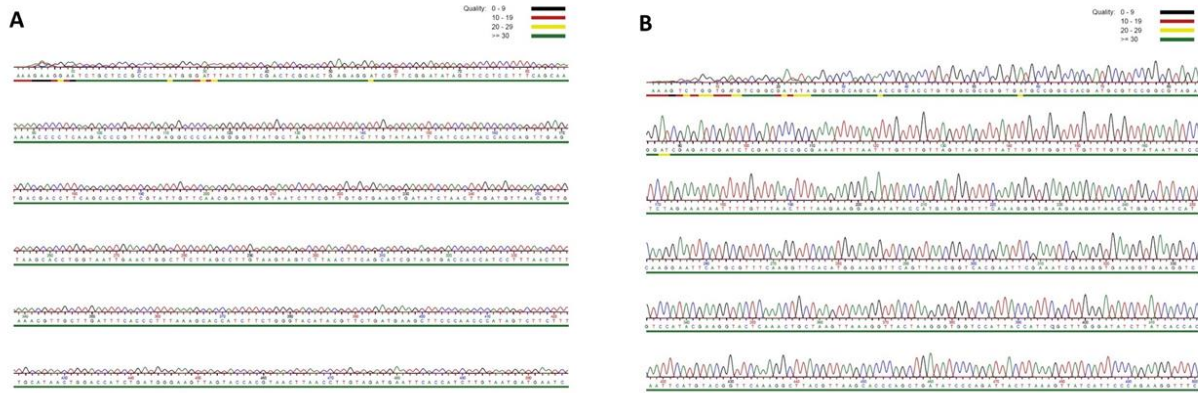


Figure S5. Sequencing chromatograms. A) Sequencing chromatogram without an initial purification step. B) Sequencing chromatogram with an initial purification step.

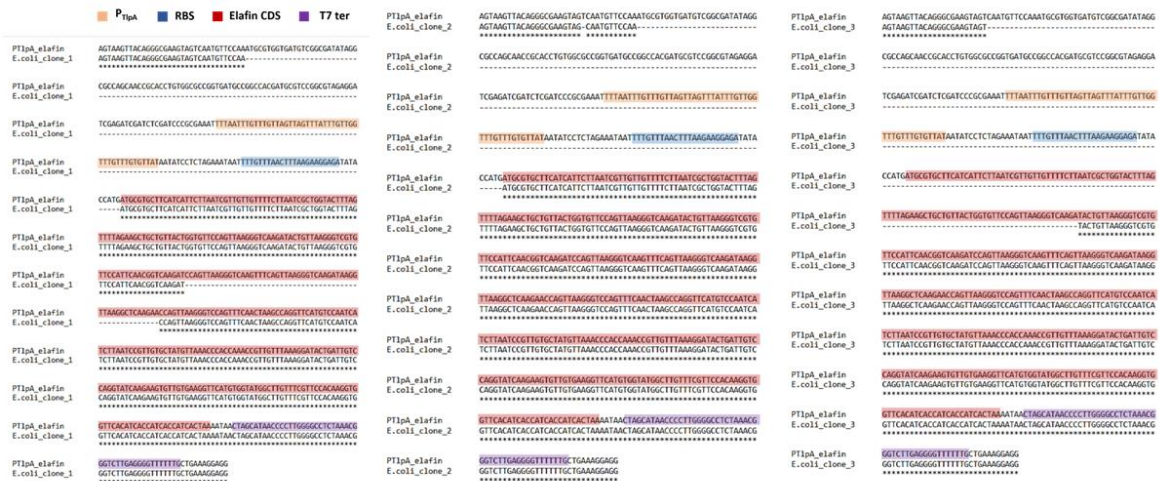


Figure S6. Multiple sequence alignments of the sequencing results (MSA). The genetic sequence of the elafin gene was aligned to the gene sequences of the three pLp_elafin clones obtained through *E. coli*. All three clones showed deletions in the P_{TlpA} promoter and RBS region. Clones 1 and 3 also showed additional deletions from the elafin coding sequence. The multiple sequence alignment tool (MUSCLE) was used for the comparative analysis of the Sanger Sequencing results.

II.2. Supporting Information Chapter 2

Discovery of a high-performance phage-derived promoter/repressor system for probiotic lactobacillus engineering

Marc Blanch-Asensio,^{1,2} Varun Sai Tadimarri,^{1,2} Alina Wilk,^{1,2} and Shrikrishnan Sankaran^{*1}

¹ Bioprogrammable Materials, INM - Leibniz Institute for New Materials Campus D2 2, 66123 Saarbrücken, Germany

² Saarland University, 66123 Saarbrücken, Germany

*E-mail: Shrikrishnan.sankaran@leibniz-inm.de

REPRESSOR	PHAGE	HOST	SEQUENCE	OPERATOR
cpg	øg1e	<i>Lactobacillus plantarum</i>	MASSGIGNRLKELRNMQGKTQDEVAKSIGISRARYS HLENERNEPDNELLKLLASYEVDYLLGNSEKSH KSPDWATEADRIDLDKLLQSNTPMGYGGMMSMAPE DKEKVRNVIEGIYWDRLKKLREEGKK	GATACGATATGTATC
cng	øg1e	<i>Lactobacillus plantarum</i>	MKRERLIAERNRNGWSQNSVAKLLDIAEITVRSIENG SRNPSSKLIAKFSYLFVKEILFPDIFLPDKDTKRIIS AKANKLTKEAAK	GATACACAATGTATC
rep	mv4	<i>Lactobacillus delbrueckii</i>	MPRANYTPQEKQLKSVIAGNLLNALLSKTAYKKADV RQTGISESTVYDYFNGKVLPSPKNVEKLADFFRVSN EEIDPRFATMPENMVPVDQSHLVKIPLIGHIACGEPI ADQNEGITYEYFPDHDVPSIFALKCEGDSMEPYIL DGDIAIRQQPEVEDGEIAAVLVDGDTRASLKRKVKV GNQVFLLPDNPYSPIVLDQDHPGKIIGKMIKMSRF Q	CCCGGTCTAGAACGG GG
tec	mv4	<i>Lactobacillus delbrueckii</i>	MPKISVRAARVNAGFSQDEAAKKLGISRFTLQRYES DPKQIRQGMLEKMRVYNMMDHDLFFKI	ACAAAAAGTCAACTT TTTTCAACTTTTGT
cl	A2	<i>Lactobacillus casei</i>	MKTNDEIITLNDLRNREGISISELARRVDMAKSSVS RYFNGTREFPLNYVDKFASALHTTPESLIGVSPVDPF KVKKLVHSYPYIPAEISAGILCNVDPLTSDDVETIQL PDSVMGRYAGDSSILMMHINGESMNQTIPDGLIAV KQYNDIQDLKGDIVVFADDGDYAVKYFYNDRQKQIV TFIPDSTDKRFSPIMYTYEDLEENIKIIGRVVYTVVL	CCCCAAAGGAAACG AAAGGA
cro	A2	<i>Lactobacillus casei</i>	MGTRMIFFRCSQKETKGGNPMTLNLKRLRAERIAK GMNQDEMAKAMGWHTRSSYAKRENGITTISATELV KMASILGYGTNQLDLFFTNVNPDRERKGMTV	CCCTATTGTGCACAA TCGGG

Table S1: List of all the repressors included in this study. The origin, host, sequence, and corresponding operator are indicated per repressor.

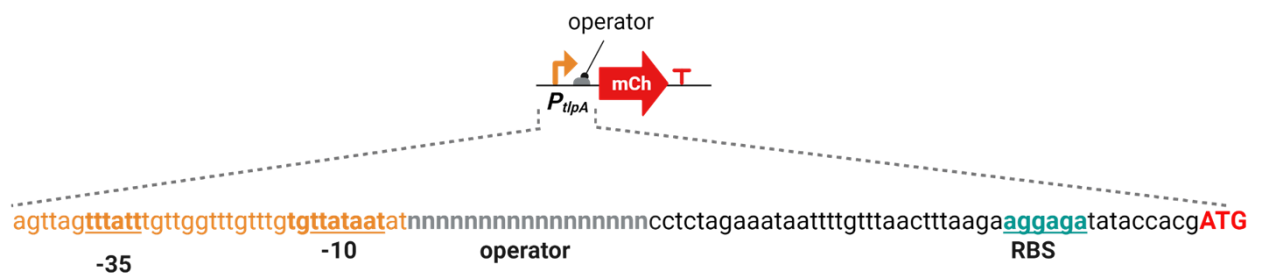


Figure S1: Placement of the operator in the promoter region. Each operator sequence was placed downstream of P_{tlpA} .

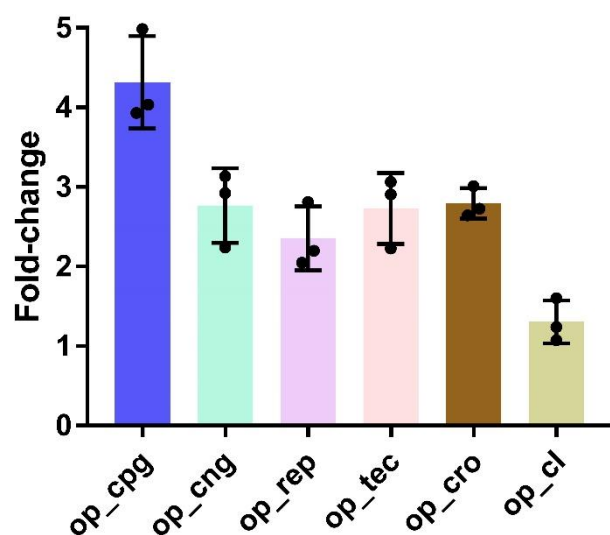


Figure S2: Fold-changes of P_{tlpA} (unmodified promoter) compared to P_{tlpA} operated with operators from each repressor tested in this study. This indicates the effect of placing each operator in P_{tlpA} . All the experiments were performed as experimental triplicates ($N = 3$). Column heights and error bars represent the means and SD.

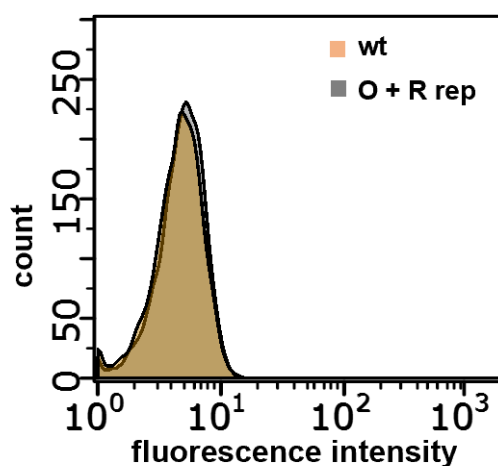


Figure S3: Flow cytometry data showing the comparison between bacteria carrying the rep repressor (repressing mCherry) and wild-type bacteria.

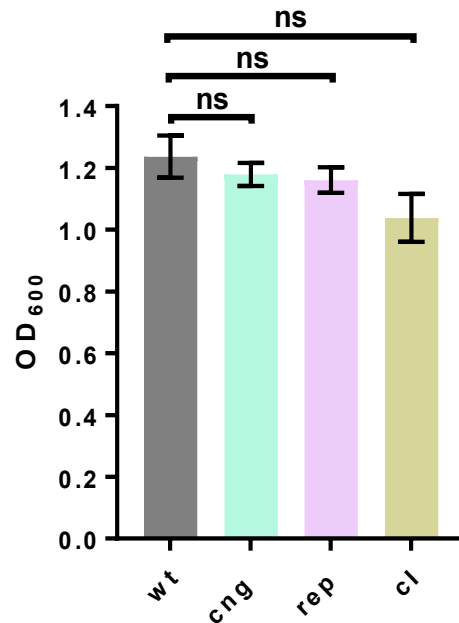


Figure S4: OD₆₀₀ values after 16 hours of growth in the microplate reader for wild-type bacteria and bacteria encoding for each repressor. Column heights and error bars represent the means and SD. Ns = not significant.

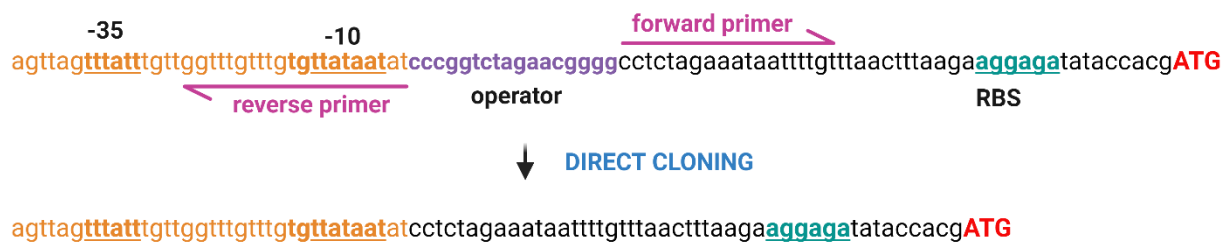


Figure S5: A) Scheme of the site-directed mutagenesis PCR to remove the operator sequence from the promoter region of the plasmid.

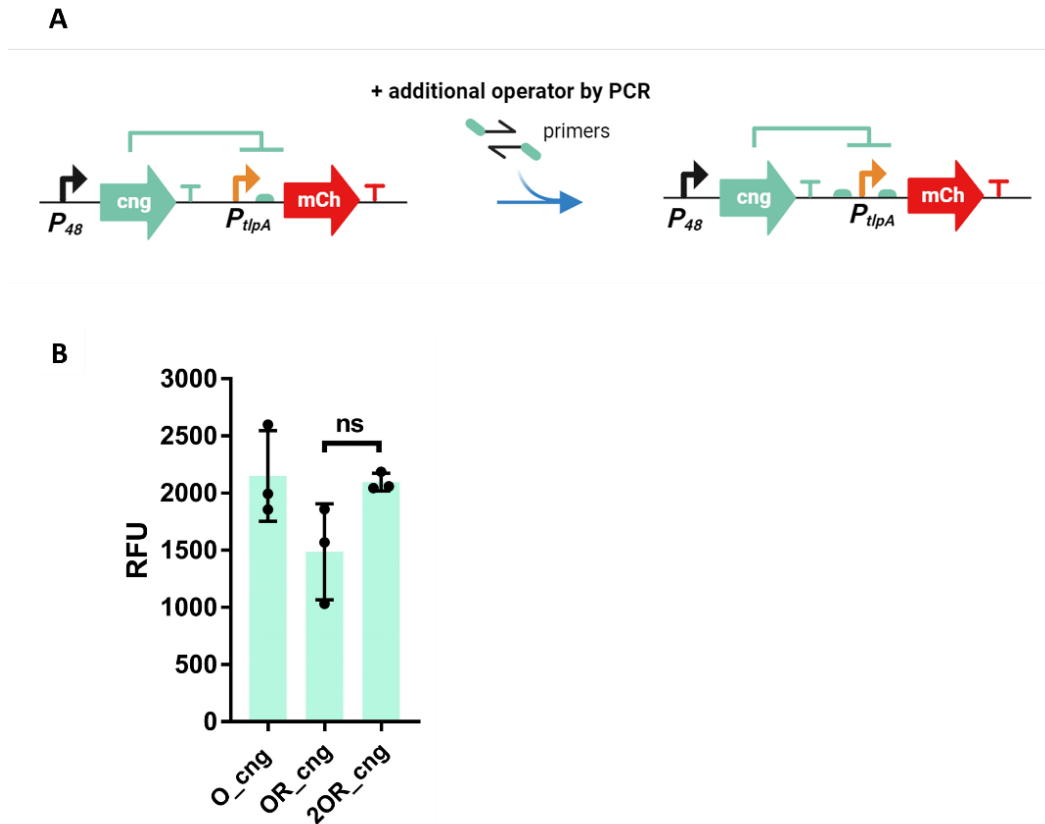


Figure S6: A) Scheme of the additional PCR to insert an additional operator sequence upstream of the promoter. **B)** Expression levels of mCherry in terms of RFU for the clones based on single cng operator (O_cng), cng operator plus repressor (OR_cng), and double cng operators plus repressor (2OR_cng). All the experiments were performed as experimental triplicates (N = 3). Column heights and error bars represent the means and SD. Ns = not significant.

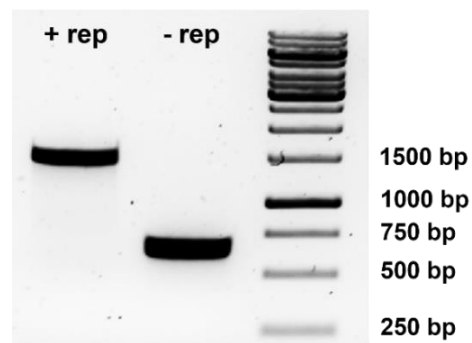


Figure S7: Agarose gel showing that the excision of rep from the plasmid by site-directed mutagenesis took place as expected. The “+ rep” product (1524 bp) is 891 bp bigger than the “- rep” product (633 bp) due to the excision of the *P₄₈_rep* gene. Bacterial pellets were used as a template for the PCR. Generuler 1 Kb DNA Ladder was used for the reference.

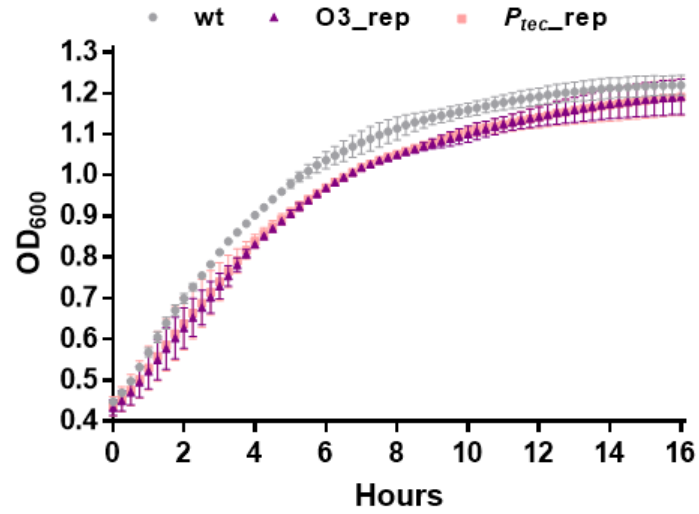


Figure S8: Growth curves of $P_{tipA_O3_rep_mCherry}$, $P_{tec_rep_mCherry}$, and wild-type bacteria over 16 hours. Experiments were performed as experimental duplicates, each with two technical replicates (N=2, n=2). Data points and error bars represent the means and SD.

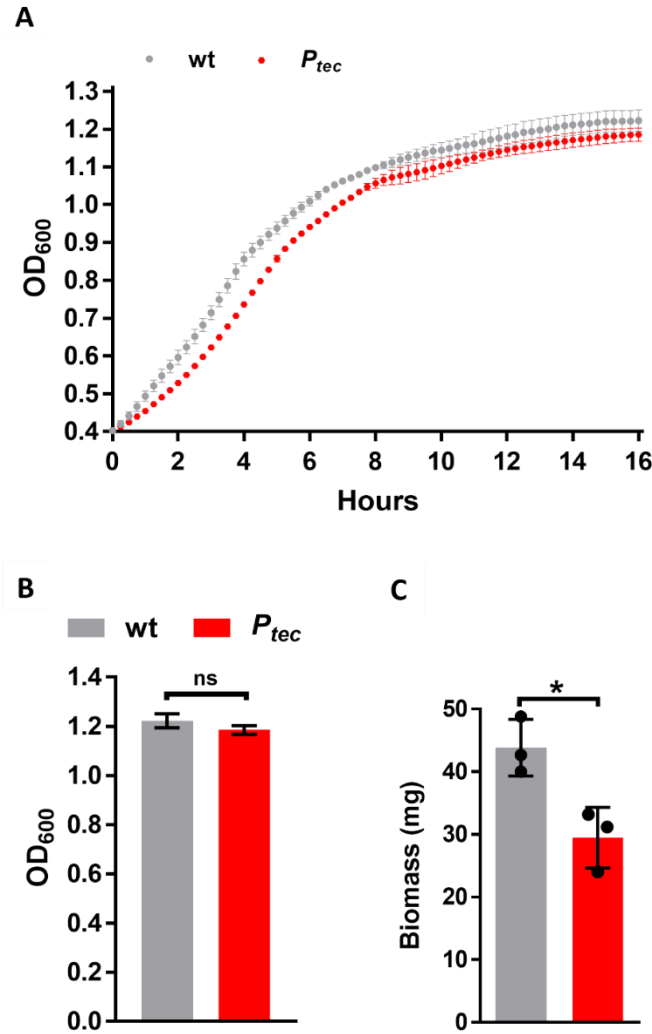
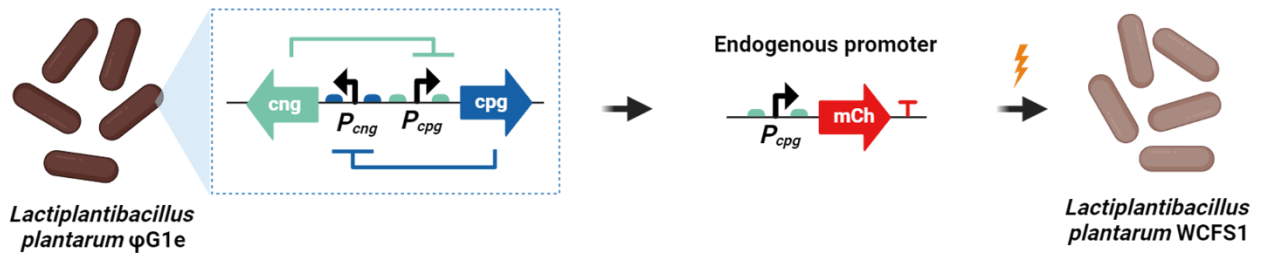
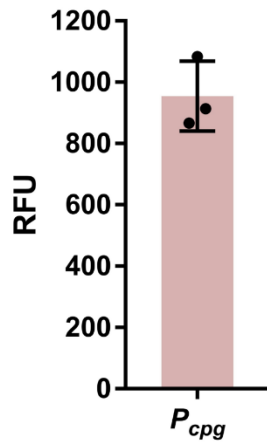


Figure S9: A) Growth curves of P_{tec} _mCherry and wild-type bacteria after 16 hours of growth in the microplate reader. **B)** OD₆₀₀ values for P_{tec} _mCherry and wild-type bacteria after 16 hours of growth in the microplate reader. **C)** Bacterial biomass of P_{tec} _mCherry and wild-type bacteria after overnight growth in the incubator. Each sample is based on a 5-mL culture. Experiments for Figures A and B were performed as experimental duplicates, each with two technical replicates (N=2, n=2). Experiments for Figure C were performed as experimental triplicates (N=3). Symbols or column heights and error bars represent the means and SD. ns = not significant, * $p < 0.05$.

A



B



C

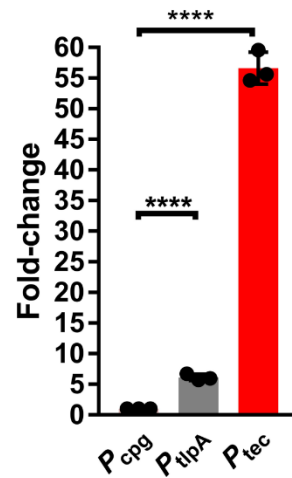


Figure S10: A) Scheme of the P_{cpg} cloning. The promoter was amplified from the genome of *L. plantarum* ϕ G1e and inserted into the mCherry-encoding plasmid. **B)** Expression levels of mCherry in terms of RFU for the P_{cpg} -mCherry bacteria. **C)** Fold changes between P_{cpg} and P_{tlpA} and P_{tec} promoters in *L. plantarum* WCFS1. All the experiments were performed in triplicate. Column heights and error bars represent the means and SD.

"O1" sequence: Modified clone, one operator sequence downstream of P_{tIpA} , shorter spacer.

agttagtttattgttggtttgttgtataatat**cccggtctagaacgggg**ttgtttaactttaaga**aggagata**taccacg**atg**

"O2" sequence: Modified clone, one operator sequence within P_{tIpA} .

agttagtttatt**cccggtctagaacgggg**ttgttataatatcctctagaaataattttgtttaactttaaga**aggagata**taccacg**atg**

"O3" sequence: Modified clone, two operators, upstream and downstream P_{tIpA} .

cccggtctagaacggggtagtttattgttggtttgttgtataatat**cccggtctagaacgggg**cctctagaaataattttgtttaactttaaga**aggagata**taccacg**atg**

" P_{tec} " sequence: Different promoter, endogenous P_{tec} promoter from *L. delbrueckii*

tttggtgttgact**cccggtctagaacgggg**tattattaaagcatcaagaacgaa**aggagaaa**acgaa**atg**

Figure S11: Relevant sequences generated in this study. P_{tIpA} promoter is shown in orange. P_{tec} promoter is shown in violet. The Rep operator is shown in violet and bold. The -35 and -10 boxes are underlined. The RBS sequence is shown in blue and in bold. The mCherry start codon is shown in red and bold.

NAME	SEQUENCE (5'-3')
Operator insertion fw	atggtttcaaaggggaagaag
Operator insertion rev	ttacgatgcagatctccg
Repressor insertion fw	ctttctgcgctgcattaagccggtagatcagcatagatct
Repressor insertion rev	ttacgatgcagatctccg
HiFi PCR repressors fw	catatctgacgacttagc
HiFi PCR repressors rev	gtctatgctctattacgat
Rep operator excision fw	cctctagaaataattttgt
Rep operator excision rev	atattataacacaaacaaacc
Rep optimization fw	atggtttcaaagggtgaa
Rep optimization rev	tctatctatatctctagcgag
HiFi PCR rep optimization fw	ccgtagcgtagtagtagc
HiFi PCR rep optimization rev	cttcaaagggtaacagc
Sequencing GOI fw	cgttactaaagggaatggag
Sequencing GOI rev	agtggaacgaaaactcac
Cng + operator fw	ataatatctagtttattgttggttg
Cng + operator rev	atgtatcactaacaacaaattaaacc
Cng ϕ g1e fw	tactaaagggaatggagaccccaactaaatcagcatgactc
Cng ϕ g1e rev	ttcttcaccctttgaaaccatagtaccgctcctt
P_{cpg} fw	tttgcacctccaattcc
P_{cpg} rev	ctagtagtgagattgccca

Table S2: List of all the primers used in this study.

II.3. Supporting Information Chapter 3

PEARL: Protein Eluting Alginate with Recombinant Lactobacilli

Varun Sai Tadimarri,^{1,4,†} Marc Blanch-Asensio,^{1,4,†} Ketaki Deshpande,^{1,4} Jonas Baumann,², Carole Baumann,² Rolf Müller,^{2,3} Sara Trujillo,¹ and Shrikrishnan Sankaran,¹

¹ INM - Leibniz Institute for New Materials,-Saarland University, Campus D2 2, 66123 Saarbrücken, Germany

² Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI),-Saarland University, Campus E8 1, 66123 Saarbrücken, Germany

³ German Centre for Infection Research (DZIF), Partner Site Hannover, Braunschweig, Germany

⁴ Saarland University, 66123 Saarbrücken, Germany

[†] Joint Authors

*E-mail: Shrikrishnan.sankaran@leibniz-inm.de

Primer name	Sequence
Vector mScarlet3/Nuc/Ela/ α -MSH fw	5'- gaagataaatcccataaggg -3'
Vector mScarlet3/Nuc/Ela/ α -MSH rev	5'- aagaactctattgaagccc -3'
Vector Operon fw	5'-aataactagcataacccc-3'
Vector Operon rev	5'-ttattgacctgaatcagcg-3'
Insert mScarlet3 Operon fw	5'-acgctgattcagggtcaataaaagaacgaaaggagaaaac -3'
Insert mScarlet3 Operon rev	5'-ggggttatgctagttatt-3'
Direct Protocol mScarlet3 fw	5'-gatcggtcgtagatcg-3'
Direct Protocol mScarlet3 rev	5'-cttgctcaacttggtg-3'
Direct Protocol Nuc/ Ela/ α -MSH/Operon fw	5'-ccgtagcgtagtagtagc-3'
Direct Protocol Nuc/ Ela/ α -MSH/Operon rev	5'-cttcaaagggtcaacagc-3'

Table S1. List of the primers used in this study



➤ **SP1_Nuc**

MKLLLVLLQQLLVHFEASSKALLCKHHHHHHPLSTKKLHKEPATLIKAIDGDTVKL MYKGQPM TFRLLL
V DTPETKHPKKGVEKYGPEASAFTKKMVENAKKIEVEFDKGQRTDKYGRGLAYIYADGKMVNEALVRQ
GLAKVAYVYKPNNTHEQHLRKSEAQA KKEKLN IWSEDNADSGQ

➤ **SP2_Nuc**

MQKKTVGLLLASAGPFLWGSSGTVAQH L FHHHHHHPLSTKKLHKEPATLIKAIDGDTVKL MYKGQPM
TFRLLLVDTPETKHPKKGVEKYGPEASAFTKKMVENAKKIEVEFDKGQRTDKYGRGLAYIYADGKMVNE
ALVRQGLAKVAYVYKPNNTHEQHLRKSEAQA KKEKLN IWSEDNADSGQ

➤ **SP3_Nuc**

MKKIVNWLLGSVLMIAAVTMLSSVSANASTYYHHHHHHPLSTKKLHKEPATLIKAIDGDTVKL MYKG
QPM TFRLLLVDTPETKHPKKGVEKYGPEASAFTKKMVENAKKIEVEFDKGQRTDKYGRGLAYIYADGK
MVNEALVRQGLAKVAYVYKPNNTHEQHLRKSEAQA KKEKLN IWSEDNADSGQ

➤ **SP4_Nuc**

MKKFMNSNWSMRL LALLCALVLFAYVNSLKTTHHHHHHPLSTKKLHKEPATLIKAIDGDTVKL MYKG
QPM TFRLLLVDTPETKHPKKGVEKYGPEASAFTKKMVENAKKIEVEFDKGQRTDKYGRGLAYIYADGK
MVNEALVRQGLAKVAYVYKPNNTHEQHLRKSEAQA KKEKLN IWSEDNADSGQ

➤ **SP5_Nuc**

MKKWSTYNKNTSHKIVELSSIVVMSSVLGILAKPVQGKADQTSHHHHHHHPLSTKKLHKEPATLIKAID
GDTVKL MYKGQPM TFRLLLVDTPETKHPKKGVEKYGPEASAFTKKMVENAKKIEVEFDKGQRTDKYGR
GLAYIYADGKMVNEALVRQGLAKVAYVYKPNNTHEQHRLKSEAQAKKEKLNWSEDNADSGQ

➤ **Lp_3050_Nuc**

MKKFNFKTM LLLVLASC VFGV VNVTTSLGPQTAITAQASKKLHHHHHHHPLSTKKLHKEPATLIKAID
GDTVKL MYKGQPM TFRLLLVDTPETKHPKKGVEKYGPEASAFTKKMVENAKKIEVEFDKGQRTDKYGR
GLAYIYADGKMVNEALVRQGLAKVAYVYKPNNTHEQHRLKSEAQAKKEKLNWSEDNADSGQ

➤ **Lp_3050_ela_smBit**

MKKFNFKTM LLLVLASC VFGV VNVTTSLGPQTAITAQASKKLEAAVTGVPVKGQDTVKG RVPFNGQ
DPVKGQVSVKGQDKVKAQEPVKGPVSTKPGSCPIILIRCAMLNPPNRCLKDTCPGIKKCCGSCGMA
CFVPQGGGGSGGGGSSVSGWRLFKKIS

➤ **SP3_ela_smBit**

MKKIVNWLLGSVLMIAAVTMLSSVSANASTYYEAAVTGVPVKGQDTVKG RVPFNGQDPVKGQVSVK
GQDKVKAQEPVKGPVSTKPGSCPIILIRCAMLNPPNRCLKDTCPGIKKCCGSCGMA CFVPQGGGG
GGGGSSVSGWRLFKKIS

➤ **Lp_3050_α-MSH_smBit**

MKKFNFKTM LLLVLASC VFGV VNVTTSLGPQTAITAQASKKLHHHHHHHGGSGGSSVSGWRLFKKISG
GSGGSSYSMEHFRWGKPV

➤ **SP3_α-MSH_smBit**

MKKIVNWLLGSVLMIAAVTMLSSVSANASTYYVSGWRLFKKISGGGGSGGGGSHHHHHHGGGGSG
GGGSSYSMEHFRWGKPV

Figure S1. Protein sequences from all the secretion modules used in this study. Signal peptides are shown in bold and red. The histidine tag is shown in purple. The NucA sequence is shown in green. The elafin sequence is shown in pink. The α-MSH sequence is shown in yellow. The smBit sequence is shown in orange. Linkers are shown in blue.

➤ ***P*_{tlpA} promoter**

TTTAATTTGTTTGTAGTTAGTTTATTTGTTGGTTTGTGTTATAATATCCTCTAGAAATAATTTT
GTTTAACTTTAAGA**AGGAGG**TATACCATG

➤ **P_{tec} promoter**

ACAAAAAGTTGAAAAAGTTGACTTTTTGTGTTGACTCCCGGTCTAGAACGGGGTATTATTAAAGC
ATCAAGAACGAA**AGGAG**AAAACGAA

Figure S2. DNA sequences of the promoters used in this study. Ribosome-binding sites are shown in bold.

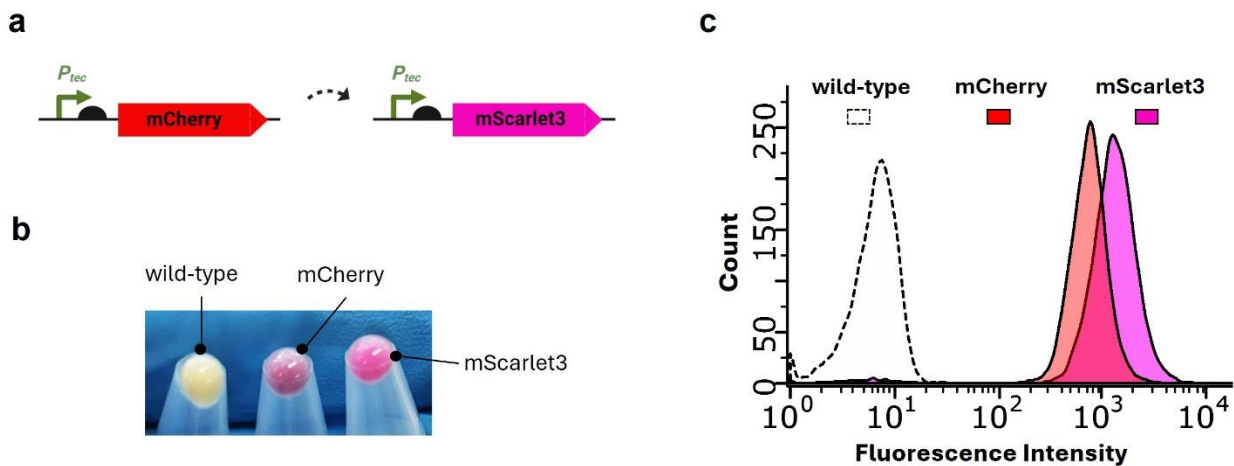


Figure S3. a) Scheme of the reporter change from mCherry to mScarlet3. b) Bacterial pellets from wild-type *L. plantarum* WCFS1, *L. plantarum* WCFS1 producing mCherry and *L. plantarum* WCFS1 producing mScarlet3. c) Flow cytometry plot showing the fluorescence intensity of wild-type *L. plantarum* WCFS1, *L. plantarum* WCFS1 producing mCherry, and *L. plantarum* WCFS1 producing mScarlet3.

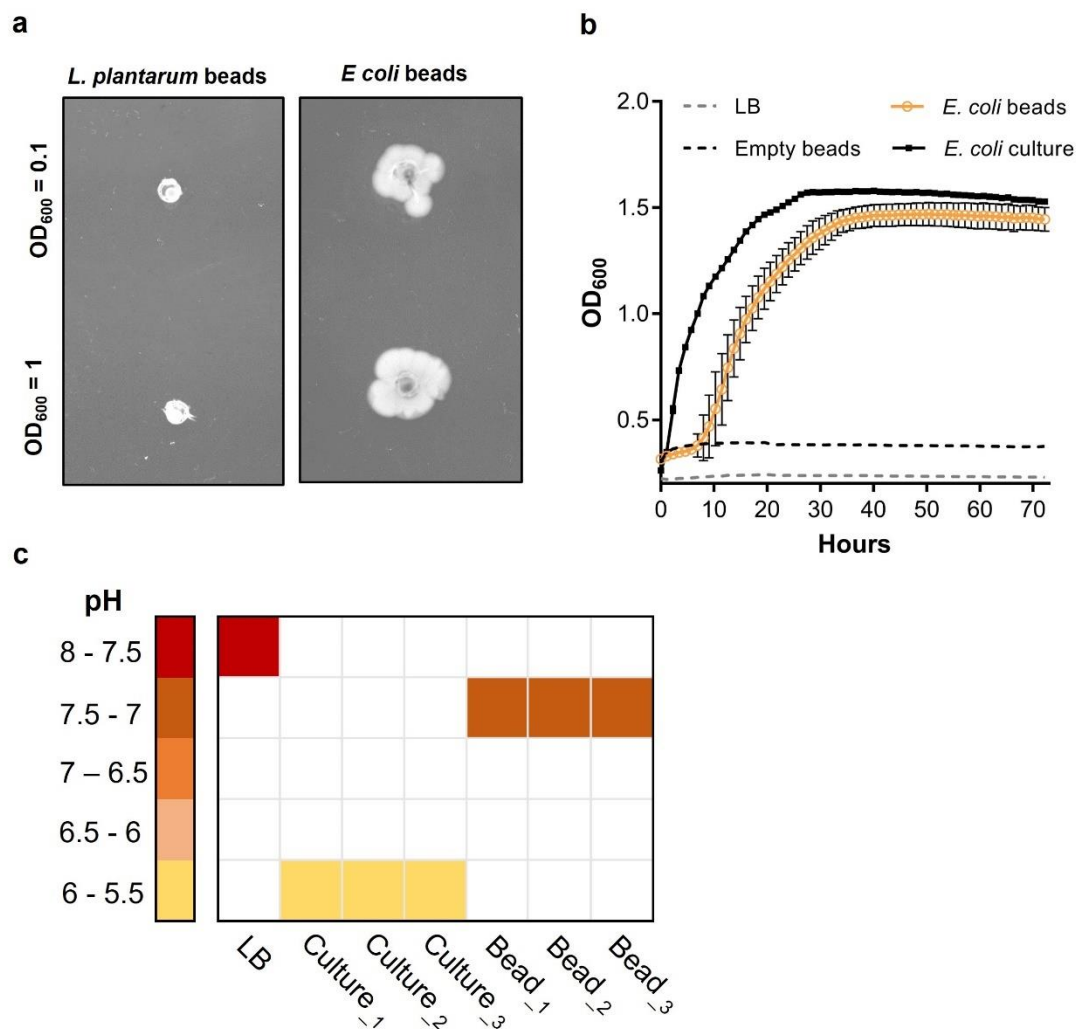


Figure S4. **a)** Alginate beads containing either wild-type *L. plantarum* or *E. coli* Nissle at an initial OD₆₀₀ of 0.1 and 1 were placed on an LB-kanamycin agar plate (for *E. coli* Nissle) and an MRS-erythromycin agar plate (for *L. plantarum*) after 24 hours of incubation. **b)** 72-hour growth kinetic measurements depicting OD₆₀₀ values of encapsulated *Escherichia coli* Nissle in PEARLs and non-encapsulated culture control (n=4, mean ± SD). **c)** pH values, measured with pH paper strips, from the supernatants (in LB media) of *L. plantarum* cultures and *L. plantarum* beads after 24 hours of growth at 37°C. Experiments were performed as triplicates (n = 3)

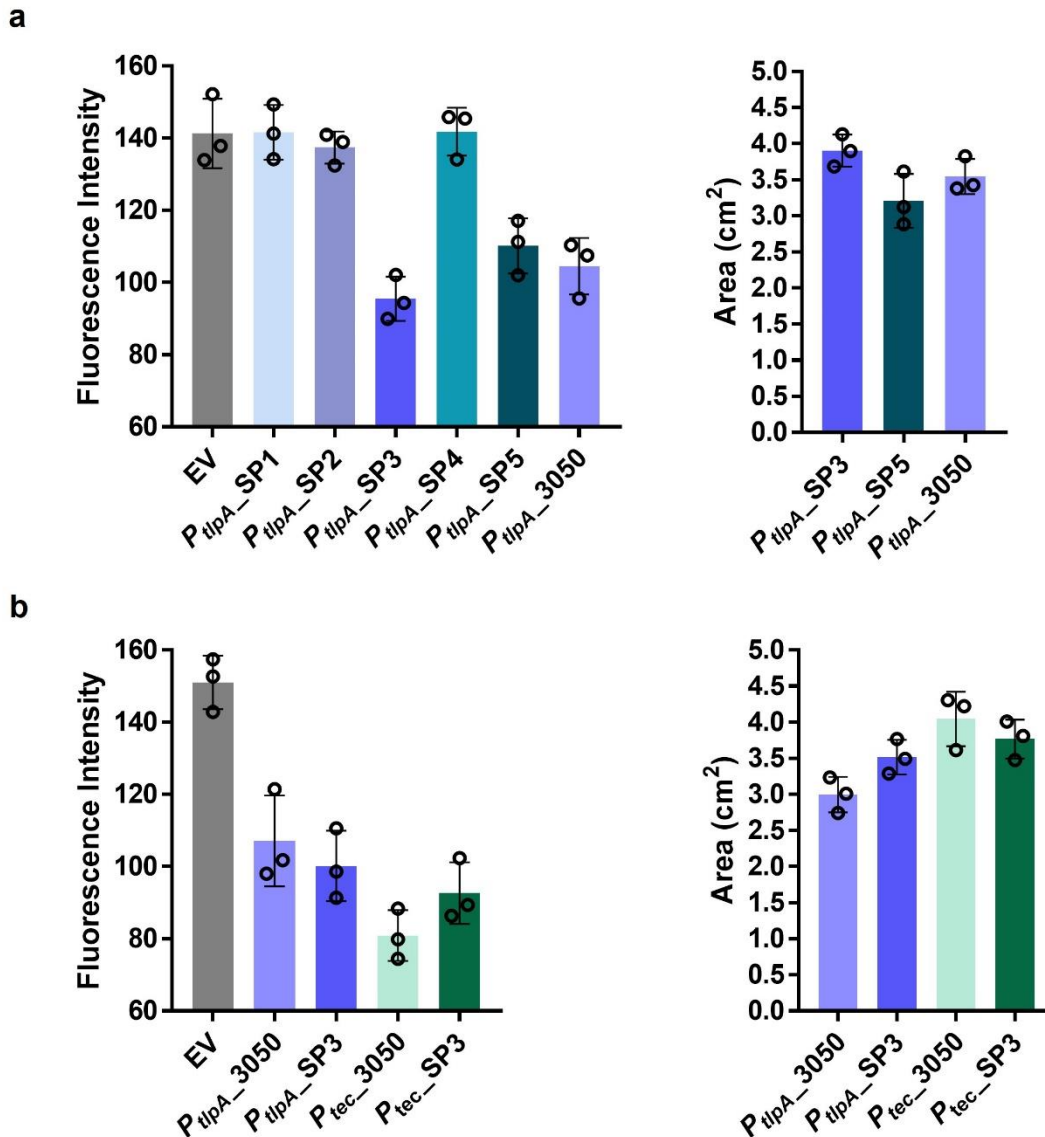


Figure S5. a) The left plot shows the decrease in fluorescence intensity caused by the discoloration of the DNA agar plate due to the halo formation of each NucA clone ($n=3$, mean $\pm$ SD). The right plot shows the area in cm^2 of the halos formed in the DNA agar plate by the three clones that secreted NucA ($n=3$, mean $\pm$ SD). **b)** The plot on the left shows the decrease in fluorescence intensity by each promoter/signal peptide combination ($n=3$, mean $\pm$ SD). The right plot shows the area in cm^2 by each promoter/signal peptide combination ($n = 3$, mean $\pm$ SD).

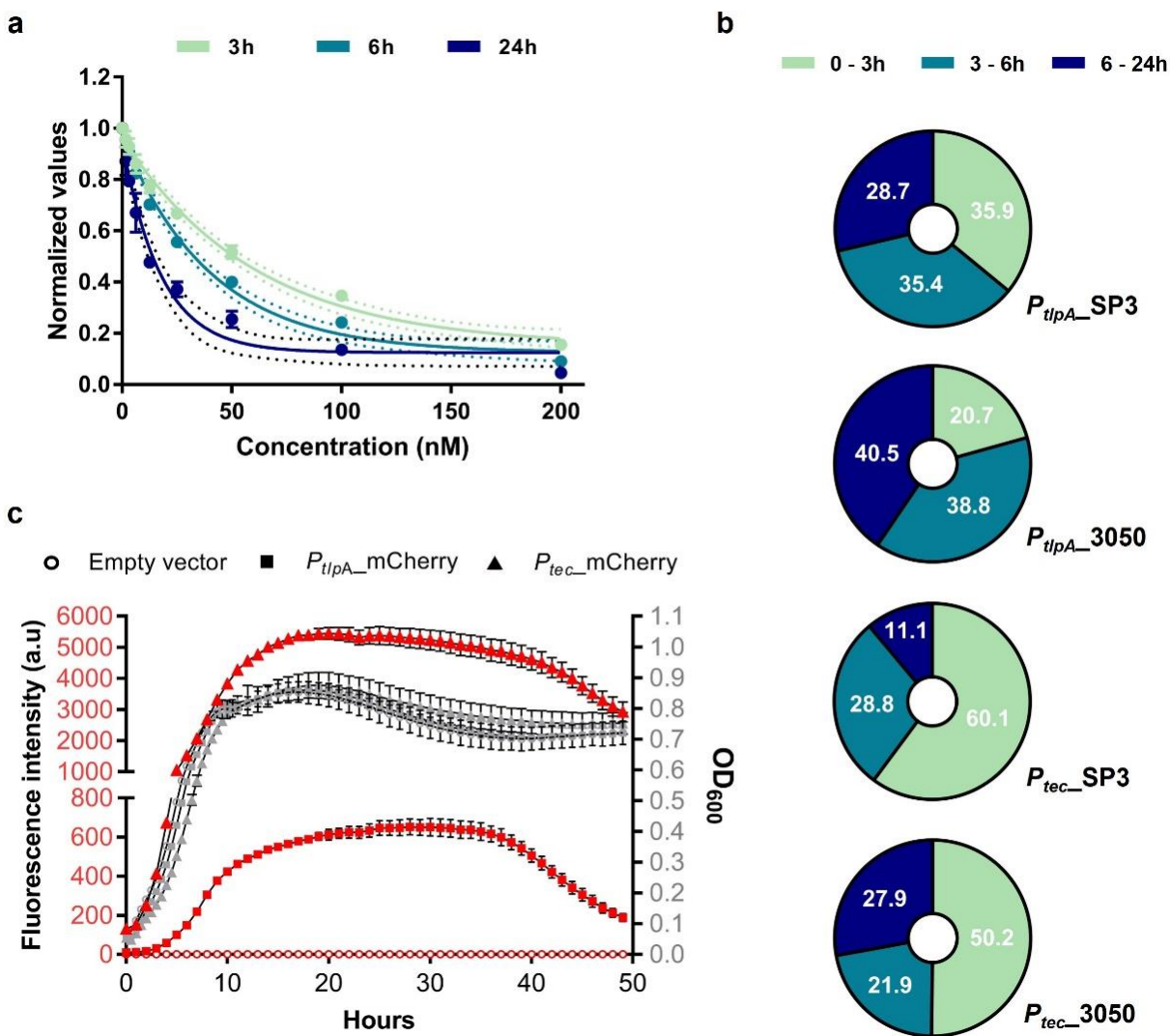


Figure S6. a) Standard curve for pure NucA after 3, 6, and 24 hours of incubation at 37 °C in shaking conditions (n=3, mean $\pm$ SD). **b)** Pie charts showing the percentage of the total NucA secreted after 3, 6, and 24 hours by each promoter/signal peptide combination. **c)** Plot showing both the differences in the levels of fluorescence (i.e., promoter activity) and growth (as OD₆₀₀) in MRS media for empty vector *L. plantarum*, $P_{tlpA_mCherry}$ *L. plantarum*, and $P_{tec_mCherry}$ *L. plantarum* (n=3, mean $\pm$ SD).

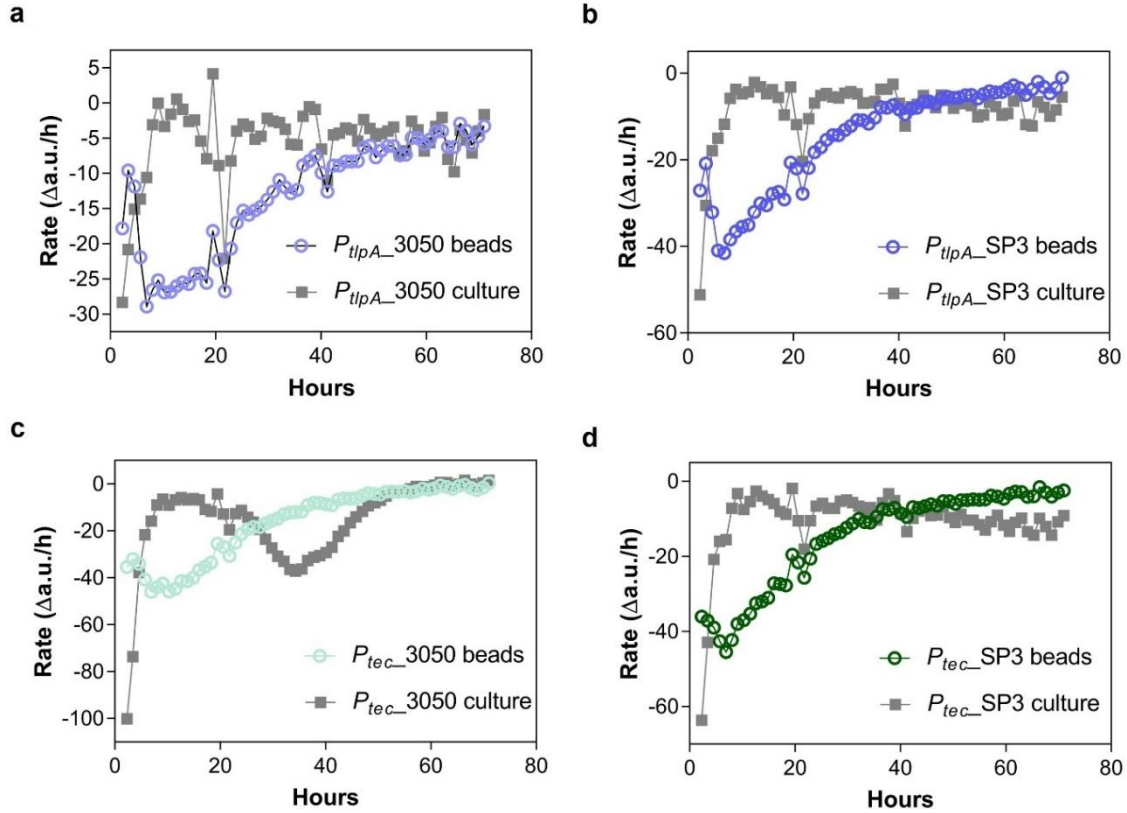


Figure S7. **a)** NucA secretion rate by P_{tlpA_3050} in culture and within PEARLs. **b)** NucA secretion rate by P_{tlpA_SP3} in culture and within PEARLs. **c)** NucA secretion rate by P_{tec_3050} in culture and within PEARLs. **d)** NucA secretion rate by P_{tec_SP3} in culture and within PEARLs. All the experiments were performed as experimental quadruplicates (in culture) ($n = 4$) or quintuplicates (in beads) ($n = 5$).

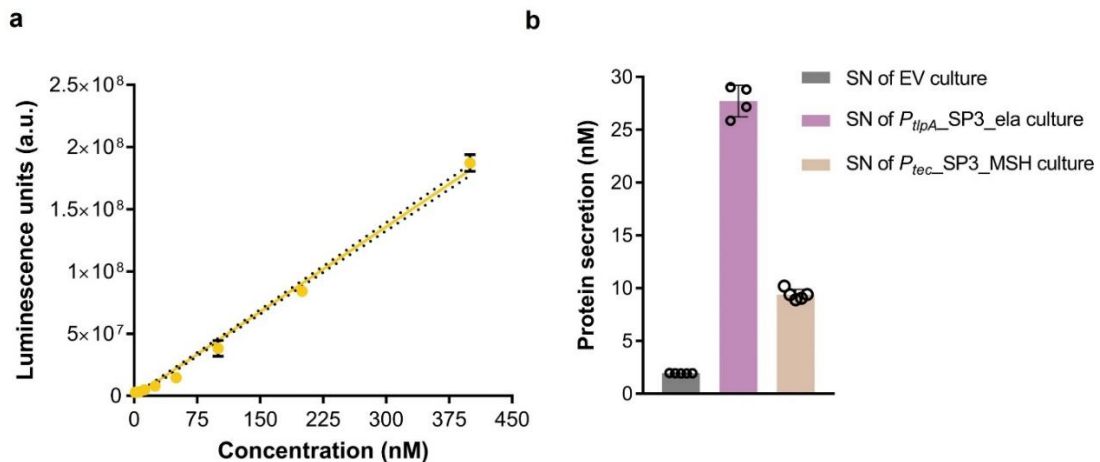


Figure S8. **a)** Standard curve of smBiT peptide and LgBiT (1 μM) with furimazine substrate. **b)** Quantification of elafin and α-MSH secreted by non-encapsulated bacteria incubated in DMEM media after 24 hours.

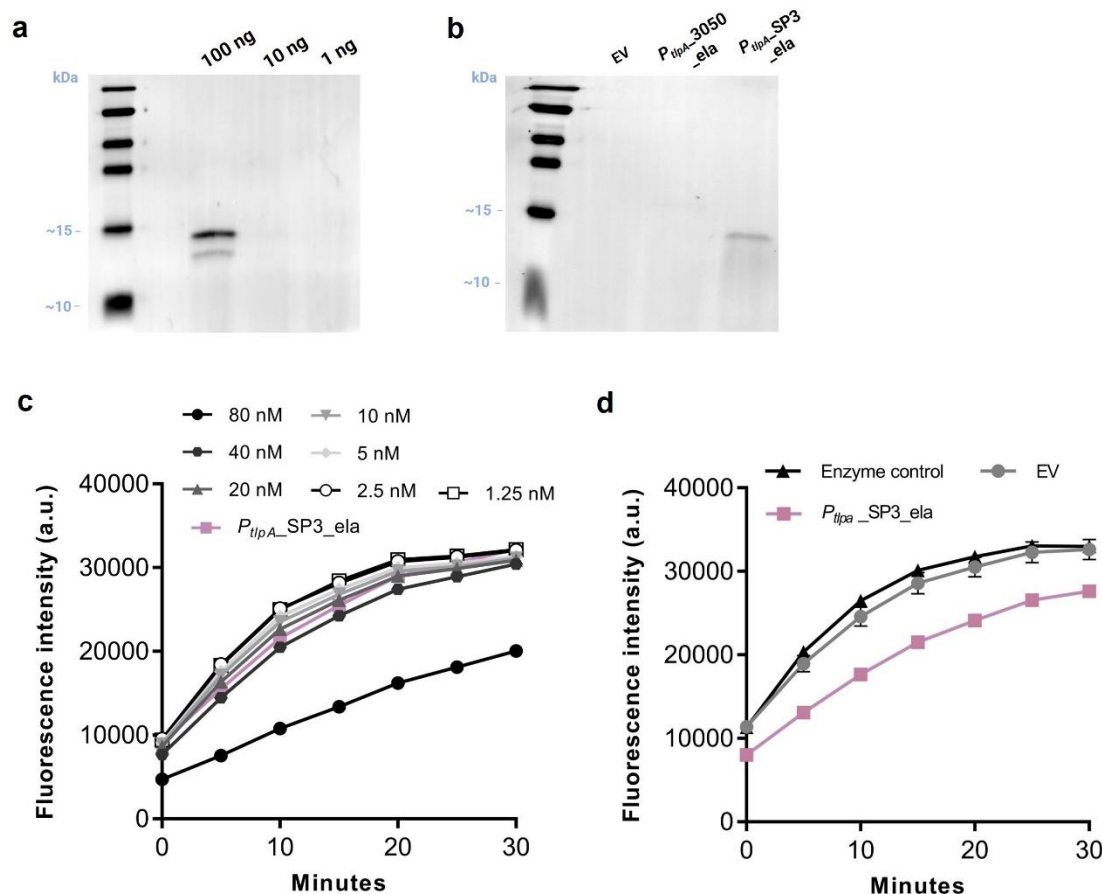


Figure S9. Elafin quantification and bioactivity assessment: a) Western blot of different concentrations of commercial elafin (purchased from R&D Biotechne GmbH) to check the sensitivity, using anti-elafin H2 antibody (Alexa fluor-647) (Santa Cruz Biotechnology) and Spectra Multicolor Broad range protein ladder (Cat 26634, Thermo Scientific). b) Western blot of culture supernatants of *wcfs1_EV*, *wcfs1_P_{tipa}-3050_ela*, and *wcfs1_P_{tipa}-sp3_ela* after incubation in DMEM at 37 °C incubator for 24 hours. c) Time-based kinetics of Neutrophil elastase inhibition (NE) by different concentrations of commercial elafin in DMEM media in the presence of fluorescent substrate. (n=3) D) Time-based kinetics of NE inhibition by culture supernatants of *wcfs1_EV* and *wcfs1_P_{tipa}-sp3_ela*, after incubation in DMEM media at 37 °C incubator for 24 hours; Significant decrease in the fluorescence of *wcfs1_P_{tipa}-sp3_ela* culture supernatant in comparison to *wcfs1_EV* and enzyme control (negative control) confirms the bioactivity of elafin.

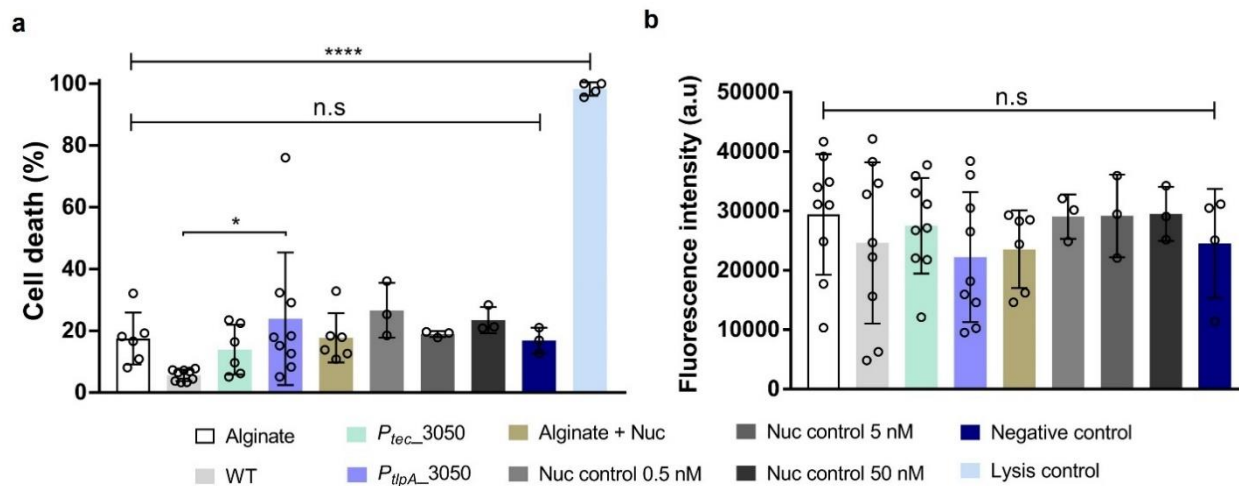


Figure S10. a) Percentage of cell death quantified after supernatant treatment via LDH assay (mean $\pm$ SD). b) Fluorescence intensity measured during alamarBlue assay after supernatant treatment (mean $\pm$ SD, arbitrary units (a.u)).

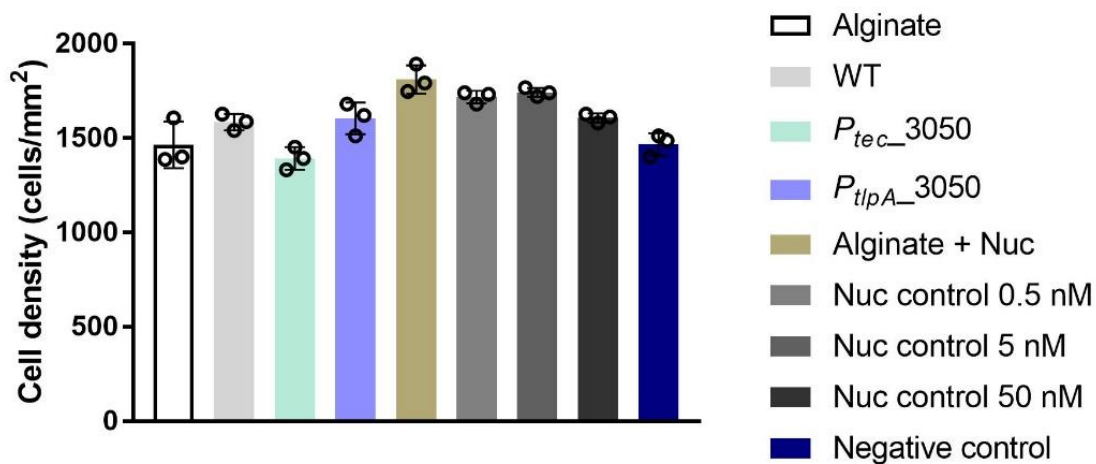


Figure S11. Plot showing the cell densities (as cells/mm²) (n=3, mean $\pm$ SD) of human fibroblasts obtained 24 hours after the supernatant treatments for all the different conditions.

Supernatant Test	n	mean pH	± SD
Negative control	20	7.5	0.107
SN EV (100 %)	20	6.1	0.110
SN EV (50 %)	20	6.0	0.188
SN EV (25 %)	20	6.9	0.604

Alginate Beads Test	n	mean pH	± SD
Negative control	8	7.6	0.005
Empty Beads	8	7.5	0.046
Beads + EV	8	7.6	0.045
Beads + P_{tec-} 3050	8	7.5	0.021
Beads + P_{tec-} 3050 (96h)	8	7.5	0.035

Table S2. pH values from the different conditions assessed during the zebrafish embryo toxicity tests.

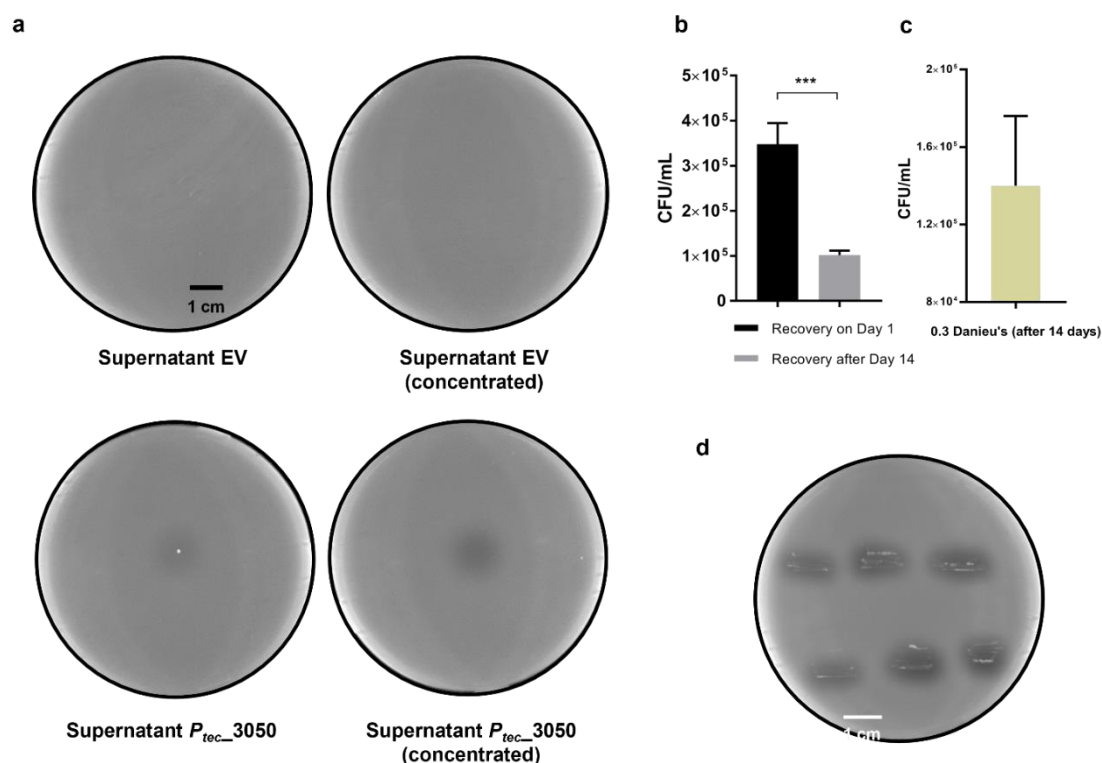


Figure S12. a) DNA agar plates showing NucA secretion in ZF medium by EV and *P_{tec}_3050*-Nuc secreting bacteria. 15 μ L of the SNs of bacteria OD₆₀₀ of 1 (growing in ZF medium for 24 hours) were spotted in DNA agar plates and incubated at 37 °C for 24 hours. The SNs were also concentrated, and 15 μ L were spotted on DNA agar plates and incubated under the same conditions. **b)** Plot showing recovery of bacteria (wcf1 *P_{tec}_3050* nuc) from two sets of PEARLs incubated in LB medium. One set (n=3, mean as column height) of PEARLs were lysed and recovered using 25 mM EDTA solution after 1 hour of encapsulation on Day 1, and another set (n=3, mean as column height) of PEARLs were lysed and recovered using 25 mM EDTA solution after 14 h days of encapsulation on Day 15 **c)** Plot showing recovery of bacteria (wcf1 *P_{tec}_3050* nuc) from PEARLs incubated in ZF medium for 14 days (n=3, mean as column height); on the 15th day, PEARLs were incubated in 25 mM EDTA solution for 30 minutes with shaking, followed by plating of spun down bacterial pellet resuspended in MRS nutrient broth; **d)** Images of DNase + methyl green agar plates with bacteria streaked from randomly selected colonies recovered from PEARLs; Halo formation for most of the recovered colonies indicates the active secretion of nuclease.

II.4. Supporting Information Chapter 4

Encapsulation-enhanced switchable protein release from engineered probiotic lactobacilli

Marc Blanch-Asensio,^{1,2} Varun Sai Tadimarri,^{1,2} Roberto Martinez,^{1,2} Gurvinder Singh Dahiya,³ Cao Nguyen Duong,¹ Rahmi Lale,^{3,4} and Shrikrishnan Sankaran¹

¹ INM - Leibniz Institute for New Materials, Saarland University, Campus D2 2, 66123 Saarbrücken, Germany

² Saarland University, 66123 Saarbrücken, Germany

³ Syngens AS, 7089 Norway

⁴ Department of Biotechnology and Food Science, Faculty of Natural Sciences, Norwegian University of Science and Technology, Trondheim, N-7491 Norway

*E-mail: Shrikrishnan.sankaran@leibniz-inm.de

Primer name	Sequence
Vector switch fw	5'- atggtttcaaaggggtgaa -3'
Vector switch rev	5'- tctatctatatctctagcgag -3'
Repressor removal fw	5'- gatcggtcgtagagatcg -3'
Repressor removal rev	5'- tctatctatatctctagcgag -3'
Operon CymR vector fw	5'- aataactagcataacccctt -3'
Operon CymR vector rev	5'- ttacttgataattcatccatacc -3'
Operon CymR insert fw	5'- tggatgaattatacaagtaatagaattaaataaaaagggagg -3'
Operon CymR insert rev	5'- aaggggttatgctagttatttagcgttgaatttagc -3'
Promoter swap vector fw	5'- ccaaatataatgtctccaaa -3'
Promoter swap vector rev	5'- tctatctatatctctagcgag -3'
Promoter swap P_{tec} fw	5'- tcgctagagatagatagag -3'
Promoter swap P_{tec} rev	5'- tttggagacattatattgggtctccttcgttcttgat -3'
Promoter swap P_{tlpA} fw	5'- tcgctagagatagatagaggcttcaatagagttcttag -3'
Promoter swap P_{tlpA} rev	5'- tttggagacattatattggcctccttcttaaagttaaac -3'
NucA CymR vector fw	5'- gtccgagactaattcatg -3'
NucA CymR vector rev	5'- ttcgtttctccttcgt -3'

Table S1: List of primers used in this study.

Plasmid	Bacteria	Promoter	Represso r	Protein
$P_{23_tetR_mCherry}$	<i>E. coli</i> DH5α	P_{23}	TetR	mCherry
$P_{23_tetR_mCherry}$	<i>L. plantarum</i> WCFS1	P_{23}	TetR	mCherry
$P_{23_vanR_mCherry}$	<i>E. coli</i> DH5α	P_{23}	VanR	mCherry
$P_{23_vanR_mCherry}$	<i>L. plantarum</i> WCFS1	P_{23}	VanR	mCherry
$P_{23_cymR_mCherry}$	<i>E. coli</i> DH5α	P_{23}	CymR	mCherry
$P_{23_cymR_mCherry}$	<i>L. plantarum</i> WCFS1	P_{23}	CymR	mCherry
$P_{tlpA_cymR_mCherry}$	<i>E. coli</i> DH5α	P_{tlpA}	CymR	mCherry
$P_{tlpA_cymR_mCherry}$	<i>L. plantarum</i> WCFS1	P_{tlpA}	CymR	mCherry
$P_{tec_cymR_mCherry}$	<i>E. coli</i> DH5α	P_{tec}	CymR	mCherry

<i>P_{tec}</i> _cymR_mCherry	<i>L. plantarum</i> WCFS1	<i>P_{tec}</i>	CymR	mCherry
<i>P_{tec}</i> _cymR_P_mCherry	<i>E. coli</i> DH5α	<i>P_{tec}</i>	CymR	mCherry
<i>P_{tec}</i> _cymR_P_mCherry	<i>L. plantarum</i> WCFS1	<i>P_{tec}</i>	CymR	mCherry
<i>P₂₃</i> _cymR_NucA	<i>E. coli</i> DH5α	<i>P₂₃</i>	CymR	NucA
<i>P₂₃</i> _cymR_NucA	<i>L. plantarum</i> WCFS1	<i>P₂₃</i>	CymR	NucA

Table S2: Plasmids and engineered strains generated in this study.

➤ **aTc genetic switch eBlock**

tcgctagagatatagatagataagcggttcggtcagtaaataatagaaataaaaaatcagacctaagactgatgacaaaaagagaaaaatttgataaaatagtccttagaattaaattaaaaagggaggccaaatataatgagtcgcttgacaaaagttaaagtcaatcagccttagaacttctaacgaggtaggcattgagggcttactactagaaaattagcacagaagttaggcgtagaaacacctacattatattggcatgtaaagaacaaacgtgcattattggatgcccttgcaatcgagatgcttgatcgtcaccacactcattttgcccttggaaggagagagttggcaagattttctcgcaataacgctaaatcatttcgatgtgctcttcttctcaccgtgatggagcaaaaagttcatttggaacccgaccaacagagaagcaatacgaacattagaaaatcagcttgcattttatgccaacagggattctcttgagaatgcactttacgctcttagtgacgtcgccatttcacattgggatgtgtattggaggaccaggagcaccaggctcgctaaagaagaacgtgagaccctaccaccgactcaatgccaccacttctcgccaagcaatcgagttatcgatcatcaagggtccgagcctgcattcctttcgcccttgaaacttatcatttcggccttgaaaaacaattaaaatgtgagtctggcagttaaaataaggctcaccttcgggtgggcctttctgcgctgcattaagctttaaccacaagttgagcaagagatcggttcgtagagatcgattgtttatttgtgcattttgttgacaaccctatcagtgatagagatttggtgactcccggtctagaaacgggggtattattaaaccctatcagtgatagagagcatcaagaacgaaaggagaaaaacgaaatggtttcaagggtgaaga

➤ **Cumate genetic switch eBlock (mCherry)**

tcgctagagatatagatagataagcggttcggtcagtaaataatagaaataaaaaatcagacctaagactgatgacaaaaagagaaaaatttgataaaatagtccttagaattaaattaaaaagggaggccaaatataatgtctccaaaacgccgacccaggcagaacgagctatggagacccagggcaaattgattgcagccgctttgggtgcttacgagagaaaaggatagcaggctttcgaattgcagacgtaccaggcgccgaggagttcacgaggtgccagagtcaccattttccaacaaagcttgaactttacttgcaacattcgagtgtgtatgaacaaatcaccgaacgctcacgtgcccgcttgcaaaattaaaaccagaagatgatgtcattcagcaaatgcttgatgacgctgctgagtttttagatgacgatttctcaattggcttgatttaatcgtagcagccgaccgtgatcctgcccttcgagaaggaatccaacgtactgtcgagcgcaaccgattcgtagttgaagatatgtggcttgagattggtaagtcgtggttatcacgtgatgatgcagaagatatcttgggtgatttttaatagtgtacgtggccttggtgtcagatcattgtggcaaaaagataaggaaagattcgagcgtgtccgcaactctactttagaaattgaagagaacgctacgctaaattcaaacgctaaaaataaggctcaccttcgggtgggcctttctgcgctgcattaagctttaaccacaagttgagcaagagatcggttcgtagatcgaaaacaaacagacaatctggtctgtttgtagaatagttgctcggaatttgagcaacaaacagacaatctggt

ctgtttgatattgtgttgactcccggtctagaacggggtattttaaaacacaacagacaatctggtctgttttgatgcatcaaga
acgaaaggagaaaaacgaaatgggttcaaagggtgaaga

➤ **Vanillate genetic switch eBlock**

tcgctagagatatagatagataagcgttcggtcagtaaataatagaaataaaaaatcagacctaagactgatgacaaaaag
agaaaattttgataaaatagctttagaattaaattaaaaaggaggccaaatataatggacatgcctcgattaaaccgggtc
agcgtgttatgatggcactgcgtaaaaatgattgcaagcggtgaaatcaaaagtgggaacgtattgcagaaattccgaccg
cagcagcactgggtgttagccgtatgccggttcgtatcgactgcgttcactggaacaagaaggctcgggtgttcgtctgggt
gcacgtggttatgcagcccgtggtgttagcagcgatcagattcgatgcaattgaagttcgtggtgttctggaagggtttgc
agcacgtcgtcggcagaacgtggtatgaccgcagaaacccatgcacgtttgtgtactgattgcagaagggtgaagcact
gtttgcagccgggtcgctgaatggtgaagatctggatcggtatgccgcatataatcaggcatttcagataccctggttagcg
cagcaggaatggtgcagttgaaagcgcactggcagcgaatggtttgaaccgtttgcagcagccgggtgcactggccctgg
atctgatggacgtgctgccgaatatgaacatctgctggcagcacatcgtcagcatcaggcagttctggatgcagttagctgt
ggtgatgccgaagggtgcagaacgtattatgcgtgatcagcactggcagcaattcgtaatgcaaaagttttgaagcagcag
caagcgcagggcgaccgctgggtgcagcatggtcaattcgtgcagattgataaaaataaggctcaccttcgggtgggccttt
ctgcgtgcattaagcttttaaccacaagttgagcaagagatcggtcgtagagatcgaagaatagttgctcgaatttgagca
attgatccaattttgtgttgactcccggtctagaacggggtattttaagcatcattgatccaataagaacgaaaggaga
aaacgaaatgggttcaaagggtgaaga

➤ **NucA genetic switch eBlock**

gaacgaaaggagaaaaacgaaatgaaaaaatttaactttaaaacatgttgctattagttttggctagttgtcttcggggctc
gtcgttaacgtgactactagctcttgaccacaaaccgcaatcaccgccaggccccaagaagctccatcatcaccatcat
caccattgtcaactaaaaaattacataaagaacctgcgactttaattaaagcgattgatggtgatacgggttaaattaatgtac
aaagggtcaaccaatgacattcagactatttgggtgatacacctgaaacaaagcatcctaaaaaagggtgtagagaaatag
gtcctgaagcaagtgcatttacgaaaaaaatggtagaaaaatgcaagaaaaattgaagtcgagttgacaaagggtcaaga
actgataaatatggacgtggcttagcgtatatttatgctgatggaaaaatggtaaacgaagctttagttcgtcaaggcttggt
aaagttgcttatgtttacaaacctaacatacacatgaacaacatttaagaaaaagtgaagcacaagcgaaaaagagaa
attaaatatttgagcgaagacaacgctgattcaggtcaataaaaataactagcataacccctggggcctctaaacgggtctt
gaggggtttttgctgaaaggaggaactatatccgaacgatcctctcagtcgagtcgtccgagactaatcatgac

Figure S0: List of synthetic genes (eBlocks) used in this study. The start of the mCherry gene is indicated in bold and red. The P_{23} and P_{tec} promoters are shown underlined and in green and orange, respectively. The tetR, cymR, and vanR genes are shown in bold and in yellow, blue, and purple, respectively. The tetR, cymR, and vanR operator sequences are underlined and in yellow, blue, and purple, respectively. NucA coding sequence is shown in bold and green. Lp_3050 signal peptide sequence is shown in bold and light grey.

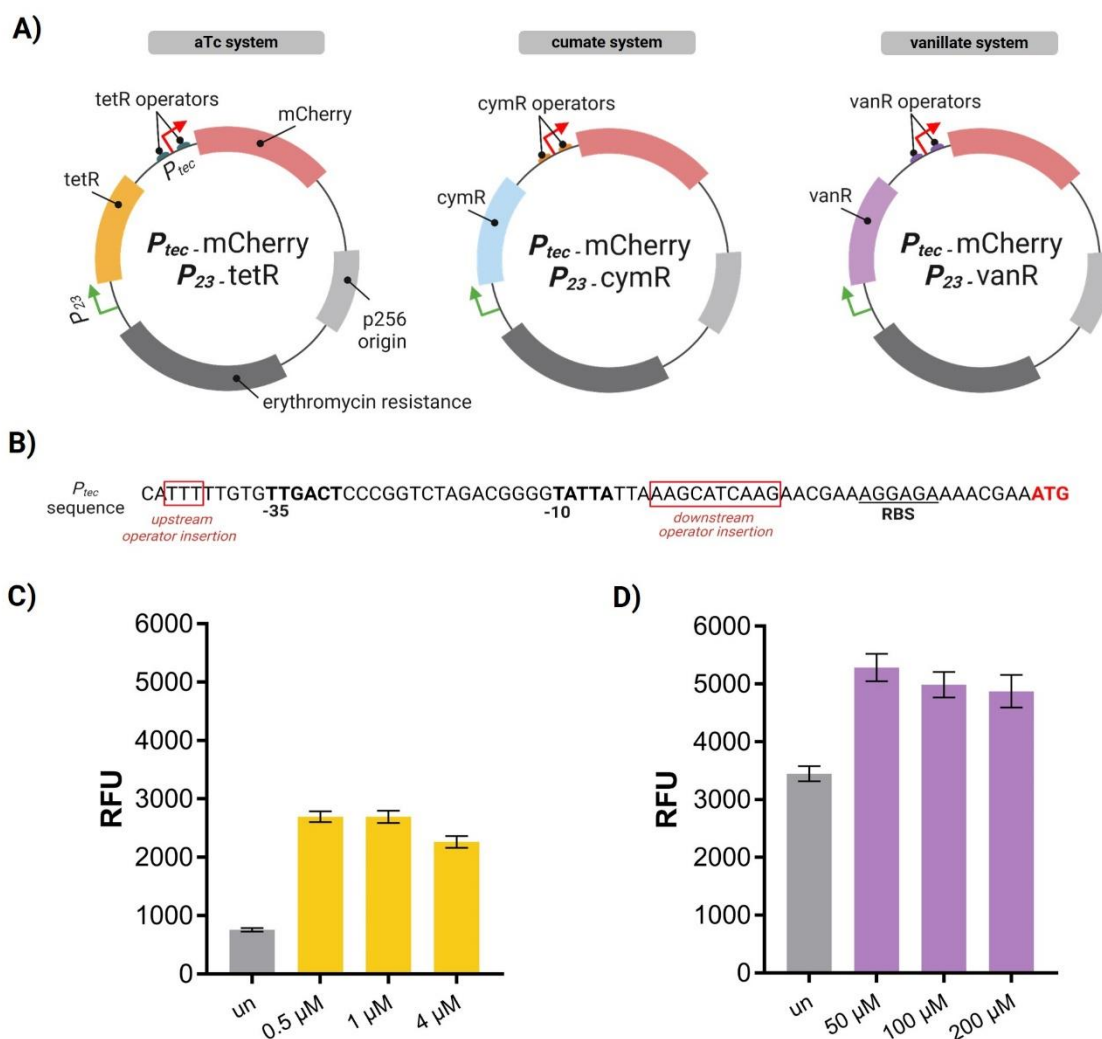


Figure S1: A) Plasmid maps for the aTc, cumate, and vanillate switches. All the relevant genetic parts are depicted. **B)** Insertion of the operator sequences into the P_{tec} promoter. The upstream and downstream operators were inserted within the areas marked in red. **C)** RFU values after inducing with different aTc concentrations for 6 hours (starting $OD_{600nm} = 0.05$, $n = 1$). **D)** RFU after inducing with different vanillate concentrations for 6 hours (starting $OD_{600nm} = 0.05$, $n = 1$).

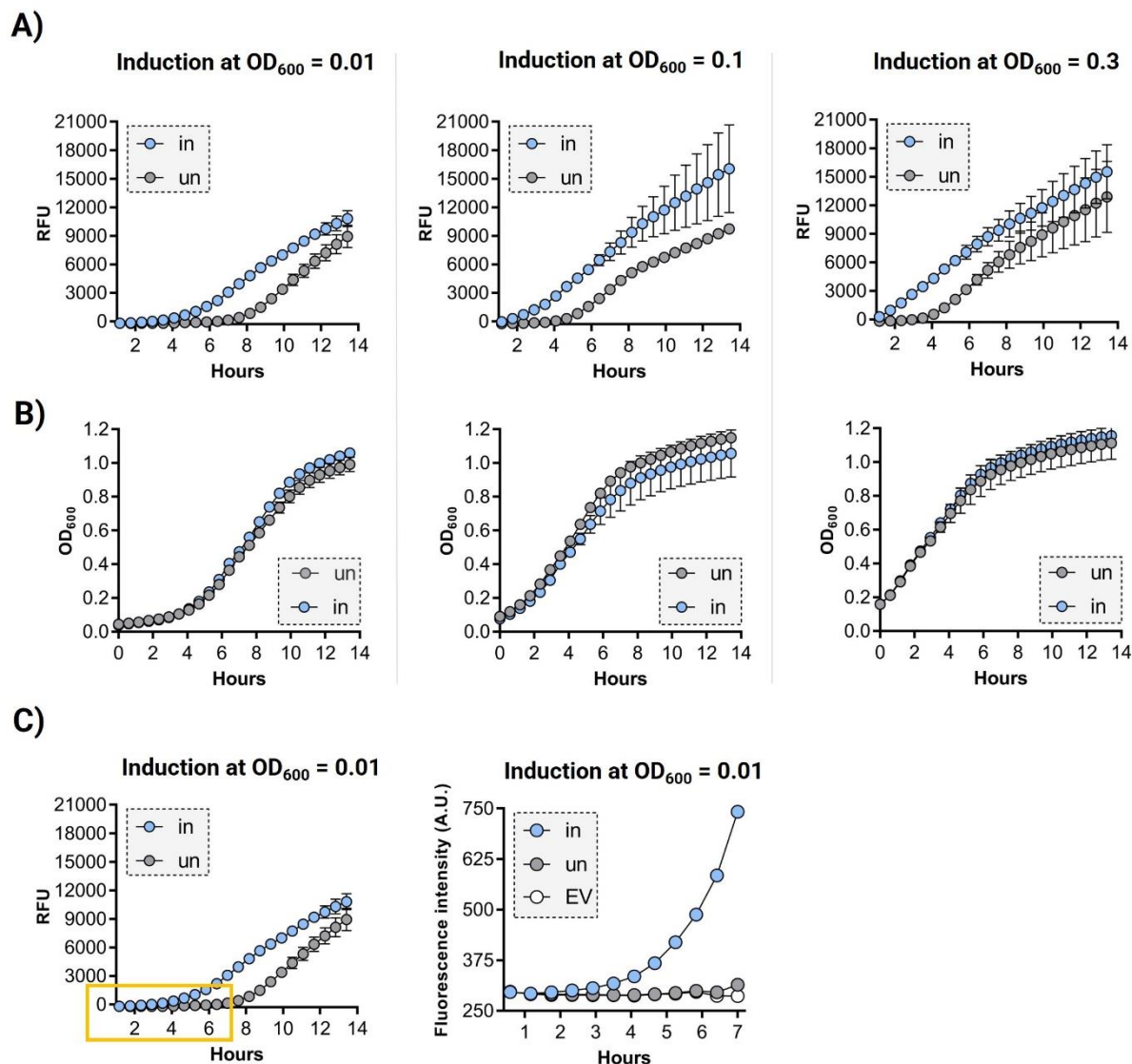


Figure S2: **A)** Kinetic plots showing the RFU values of the $P_{23_cymR_mCherry}$ variant when induced at different starting OD_{600nm} (0.3, 0.1, and 0.01) for 14 hours at 37°C ($n = 3$, mean $\pm$ SD). **B)** Plots showing the kinetic growth (as OD_{600nm}) of the $P_{23_cymR_mCherry}$ variant (uninduced and induced) at different starting OD_{600nm} (0.3, 0.1, and 0.01) for 14 hours at 37°C ($n = 3$, mean $\pm$ SD). “un” = uninduced condition, “in” = induced condition (5 μ M of cumate). **C)** The left plot is the same as shown in Fig. S2A. The right plot is based on the fluorescence intensity values of the yellow square shown in the left plot. The EV values are not subtracted from the “un” (uninduced) and “in” (induced) conditions. Induction of the induced conditions started after 2 hours of growth, whereas the leakiness for the uninduced conditions started after 6.5 hours of growth ($n = 3$, mean $\pm$ SD).

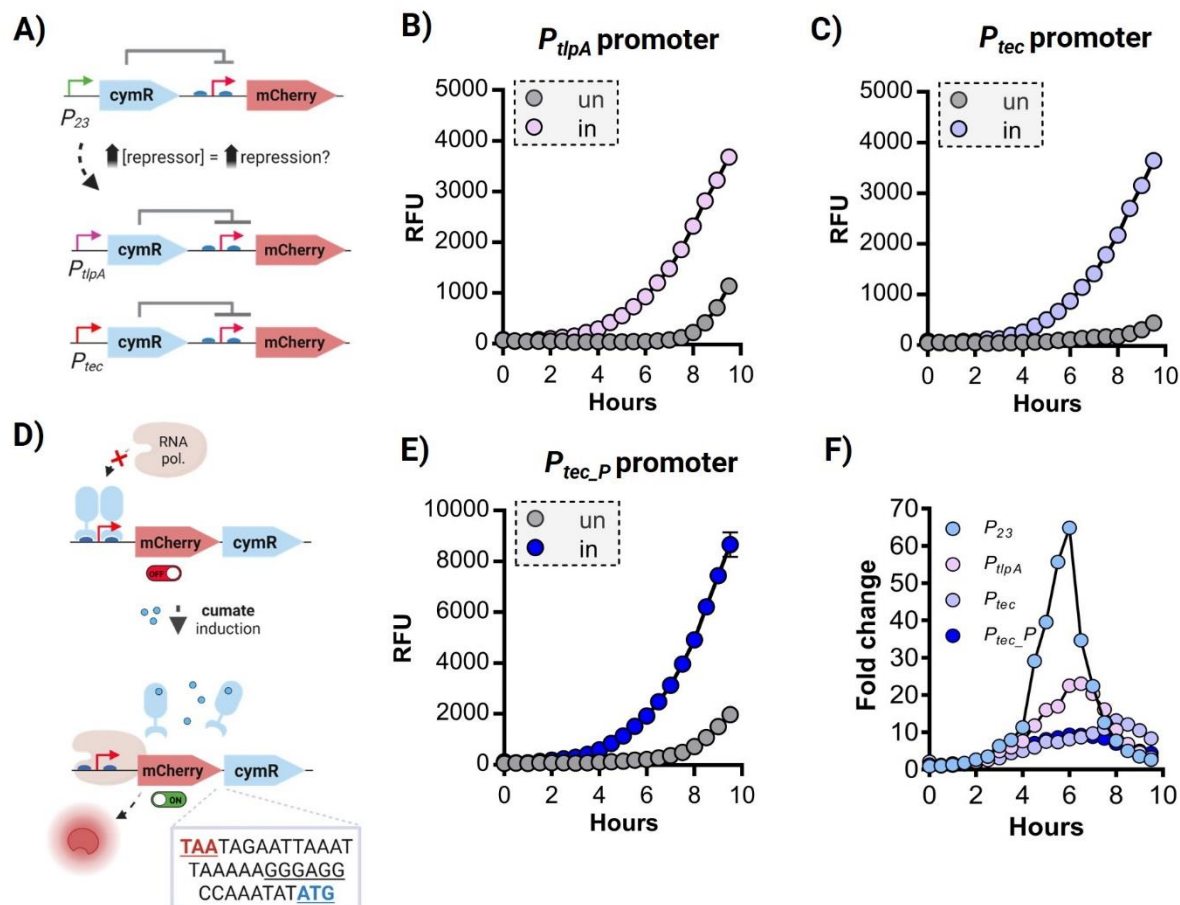


Figure S3: A) Scheme showing the P_{23} promoter exchange for P_{tlpA} and P_{tec} , thus generating the P_{tlpA} -cymR-mCherry and P_{tec} -cymR-mCherry variants, respectively. **B)** RFU values for the P_{tlpA} -cymR-mCherry variant after induction for ~10 hours (starting $OD_{600nm} = 0.01$, $n = 3$, mean $\pm$ SD). "un" = uninduced condition, "in" = induced condition (5 μ M of cumate). **C)** RFU values for the P_{tec} -cymR-mCherry variant after induction for ~10 hours (starting $OD_{600nm} = 0.01$, $n = 3$, mean $\pm$ SD). "un" = uninduced condition, "in" = induced condition (5 μ M of cumate). **D)** Scheme showing the feedback circuit approach based on cloning the *cymR* gene after the *mCherry* gene, both driven by the same (operated) promoter, P_{tec} (P_{tec} -cymR- $P_{mCherry}$ variant). The exact DNA sequence between the mCherry STOP codon (highlighted in red) and the *cymR* ATG codon (highlighted in blue) is shown at the bottom. An additional RBS (the one used for the P_{23} promoter) was included to ensure translation of the downstream gene (underlined). **E)** RFU values for the P_{tec} -cymR- $P_{mCherry}$ variant after induction for ~10 hours (starting $OD_{600nm} = 0.01$, $n = 3$, mean $\pm$ SD). "un" = uninduced condition, "in" = induced condition (5 μ M of cumate). **F)** Fold-changes for all the *cymR* variants when grown at 37°C and induced, and compared to 37°C and uninduced.

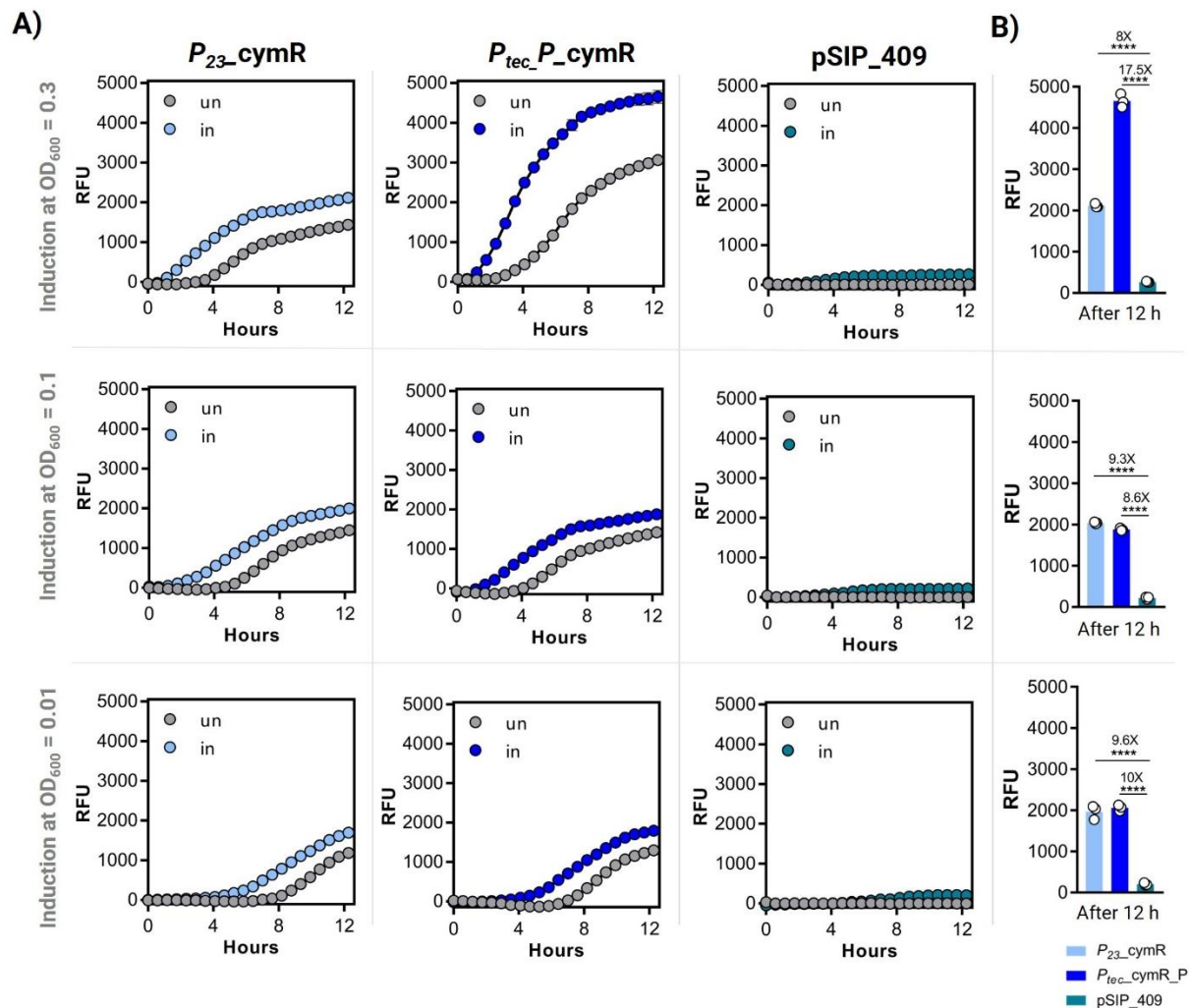


Figure S4: A) Comparison between the $P_{23_cymR_mCherry}$, the $P_{tec_cymR_P_mCherry}$, and the $pSIP_409_mCherry$ variant. Bacteria were induced at three different OD_{600nm} (0.01, 0.1, and 0.3) with 5 μM of cumate (for the cumate variants) and 100 ng/mL of IP673 (for the pSIP system) ($n = 3$, mean $\pm$ SD). "un" = uninduced condition, "in" = induced condition (5 μM of cumate). **B)** RFU values corresponding to the overall expression after 12 hours of induction for all three genetic switches ($n = 3$, mean $\pm$ SD). In these experiments, the microplate reader gain was set at 85 instead of 100 to avoid oversaturation of the detector by the mCherry expression of the $P_{tec_cymR_P_mCherry}$ variant.

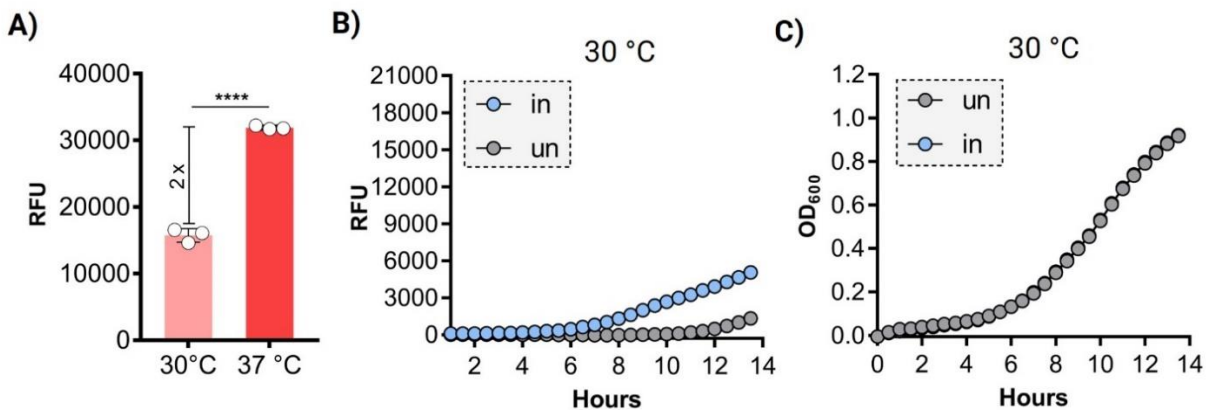


Figure S5: A) Thermo-responsiveness in terms of RFU for the P_{tec} promoter when grown at 30°C and 37°C for 16 hours (n = 3, mean ± SD). **B)** Kinetic plot showing the RFU values of the $P_{23_cymR_mCherry}$ variant (uninduced and induced) when grown at 30°C (starting $OD_{600nm} = 0.01$, n = 3, mean ± SD). **C)** Plot showing the kinetic growth (as OD_{600nm}) of the $P_{23_cymR_mCherry}$ variant (uninduced and induced) when grown at 30°C (starting $OD_{600nm} = 0.01$, n = 3, mean ± SD). “un” = uninduced condition, “in” = induced condition (5 μ M of cumate).

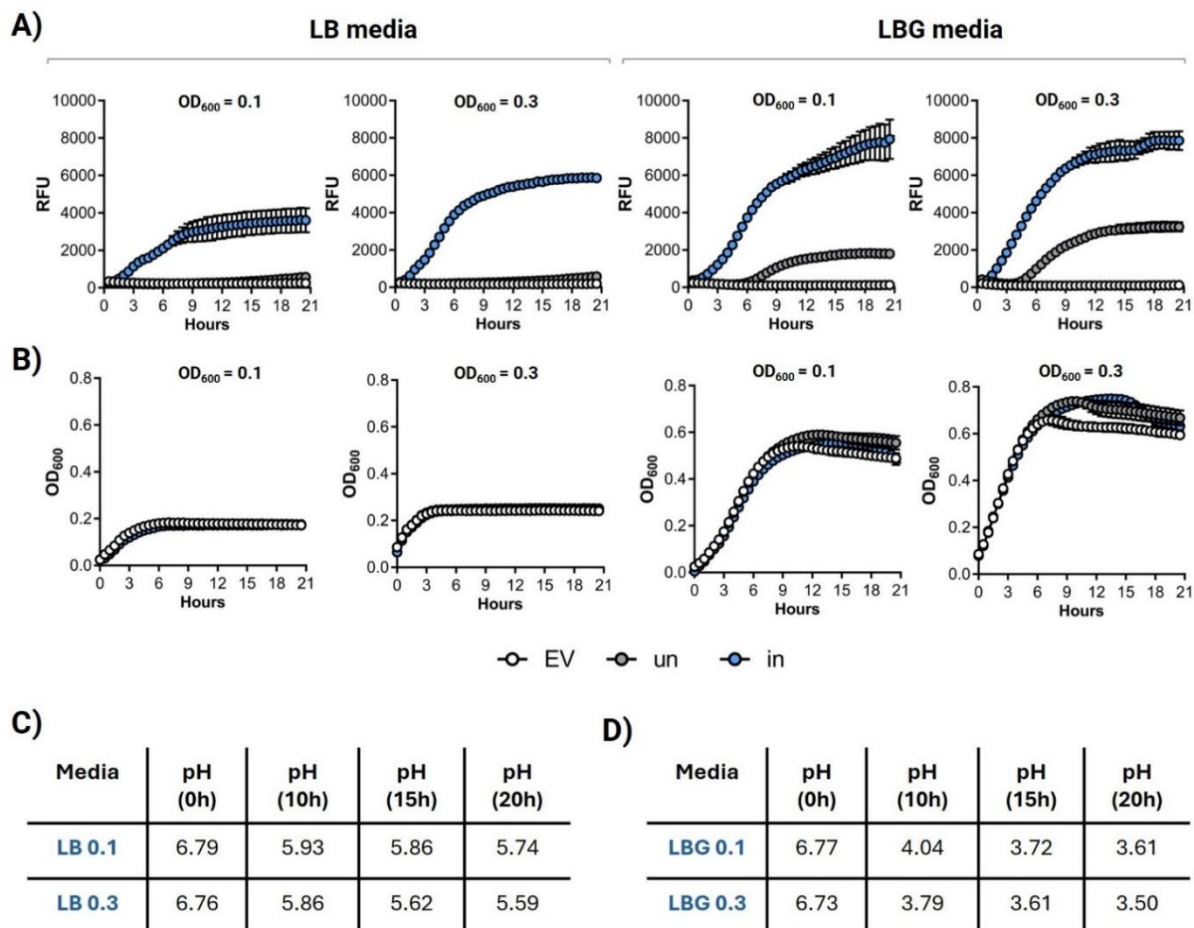


Figure S6: **A)** Kinetic plots showing the RFU values of the $P_{23_cymR_mCherry}$ variant when induced at different starting OD_{600nm} (0.3 and 0.1) for 21 hours at 37°C in LB or LBG media ($n = 3$, mean $\pm$ SD). **B)** Plots showing the kinetic growth (as OD_{600nm}) of the P_{23_cymR} variant (uninduced and induced) at different starting OD_{600nm} (0.3 and 0.1) for 21 hours at 37°C in LB or LBG media ($n = 3$, mean $\pm$ SD). “un” = uninduced condition, “in” = induced condition (5 μ M of cumate). **C)** Table showing the pH values after 0, 10, 15, and 20 hours of incubation at 37°C in LB medium and at starting OD_{600nm} of 0.1 and 0.3 ($n = 4$). **D)** Table showing the pH values after 0, 10, 15, and 20 hours of incubation at 37°C in LBG medium and at starting OD_{600nm} of 0.1 and 0.3 ($n = 4$).

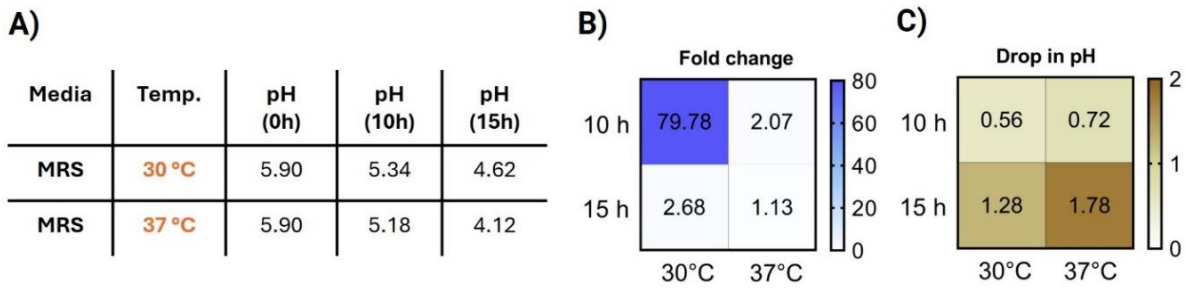


Figure S7: A) Table showing the pH values after 0, 10, and 15 hours of incubation at 30°C and 37°C in MRS medium at starting OD_{600nm} of 0.01 (n = 4). **B)** Heatmap plot depicting the fold-changes between the induced and uninduced conditions after 10 and 15 hours of growth at 30°C and 37°C in MRS medium and a starting OD_{600nm} of 0.01. **C)** Heatmap depicting the drop in pH after 10 and 15 hours of growth at 30°C and 37°C in MRS medium and a starting OD_{600nm} of 0.01. The pH values were deducted from the initial pH value (0 h) of each condition (30°C and 37°C).

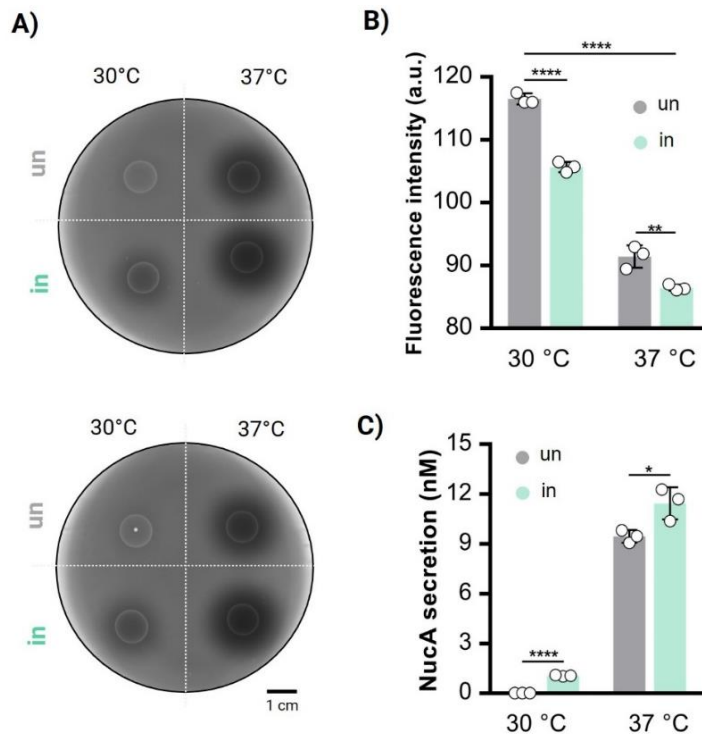


Figure S8: A) DNase agar plates with 10 µL of spotted *P₂₃-cymR-NucA* bacteria, previously uninduced and induced for 8 hours in MRS medium at both 30°C and 37°C, and incubated at 37°C for 40 hours. “un” = uninduced condition, “in” = induced condition (5 µM of cumate). **B)** Plot showing the levels of fluorescence intensity that were measured around the spotted area using ImageJ (n = 3, mean ± SD). **C)** Amounts of NucA secreted (in nM) by *P₂₃-cymR-NucA* bacteria after growing in DNase medium for 24 hours at both 30°C and 37°C and in the presence and absence of cumate (n = 3, mean ± SD). “un” = uninduced condition, “in” = induced condition (5 µM of cumate).

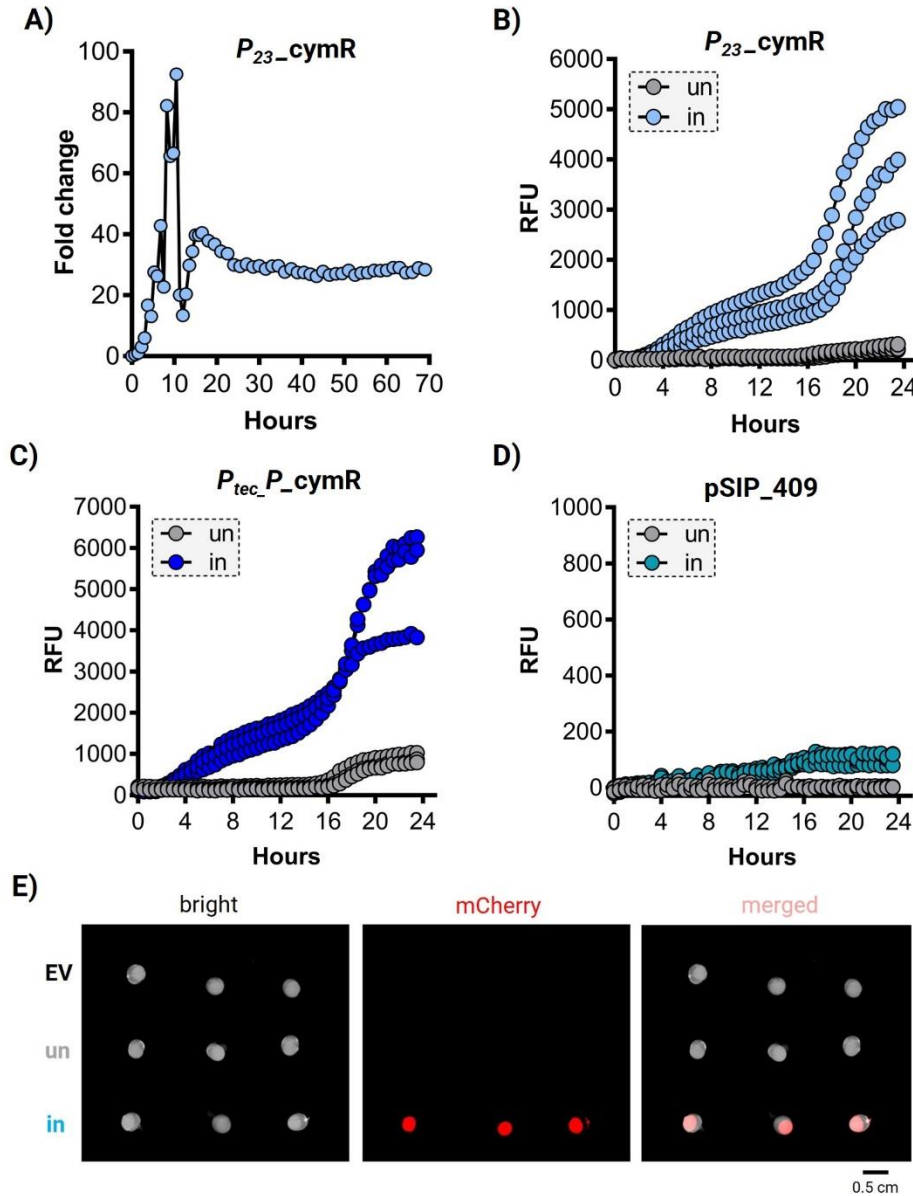


Figure S9: **A)** Fold-changes for the induced state compared to the uninduced state for the $P_{23_cymR_mCherry}$ variant over 70 hours of growth at 37°C. **B)** RFU values of P_{23_cymR} -based PEARLs after induction with cumate for 24 hours ($n = 3$, mean $\pm$ SD). **C)** RFU values of $P_{tec_cymR_P}$ -based PEARLs after induction with cumate for 24 hours ($n = 3$, mean $\pm$ SD). **D)** RFU for the mCherry-expressing pSIP409-based PEARLs when induced with IP673 for 24 hours ($n = 3$, mean $\pm$ SD). “un” = uninduced condition, “in” = induced condition (100 μ M of cumate). **E)** BioRad Gel Documentation System images of EV-based PEARLs and the induced and uninduced conditions of P_{23_cymR} -based PEARLs after 24 hours of growth in DMEM cell culture medium and at 37°C ($n = 3$, mean $\pm$ SD). “un” = uninduced condition, “in” = induced condition (100 μ M of cumate).

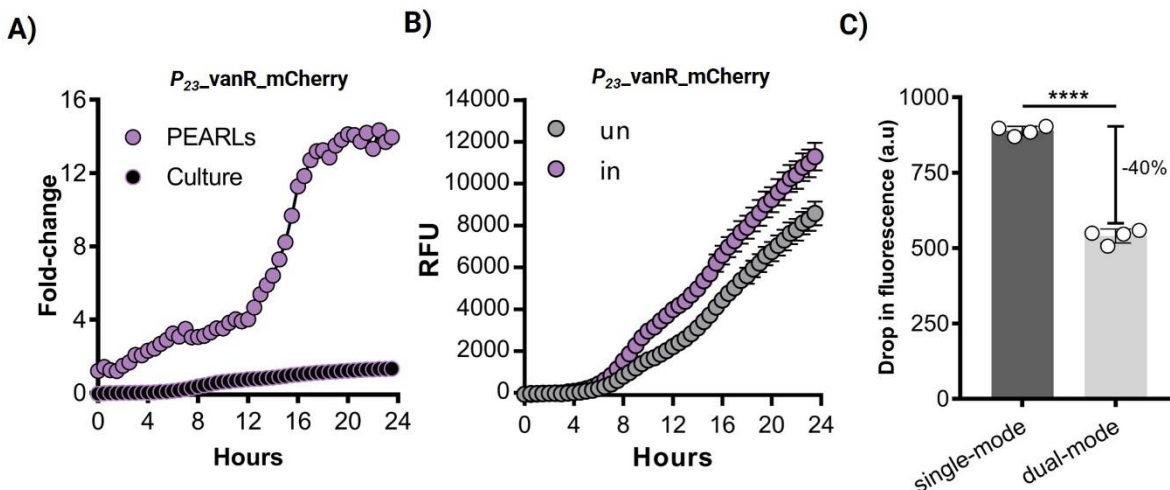


Figure S10: **A)** Fold-changes between the induced (with 100 μ M of vanillate) and uninduced states of the *P₂₃_vanR_mCherry* variant with and without encapsulation in PEARLs and grown at 37°C for 23 hours. **B)** RFU values corresponding to the induced and uninduced states of non-encapsulated *P₂₃_vanR* bacteria when grown at 37°C for 23 hours in culture (MRS medium) ($n = 4$, mean $\pm$ SD). “un” = uninduced condition, “in” = induced condition (100 μ M of vanillate). **C)** Comparison in the overall drop in fluorescence by single- and dual-mode PEARLs after 24 hours of incubation at 37°C ($n = 4$, mean $\pm$ SD).

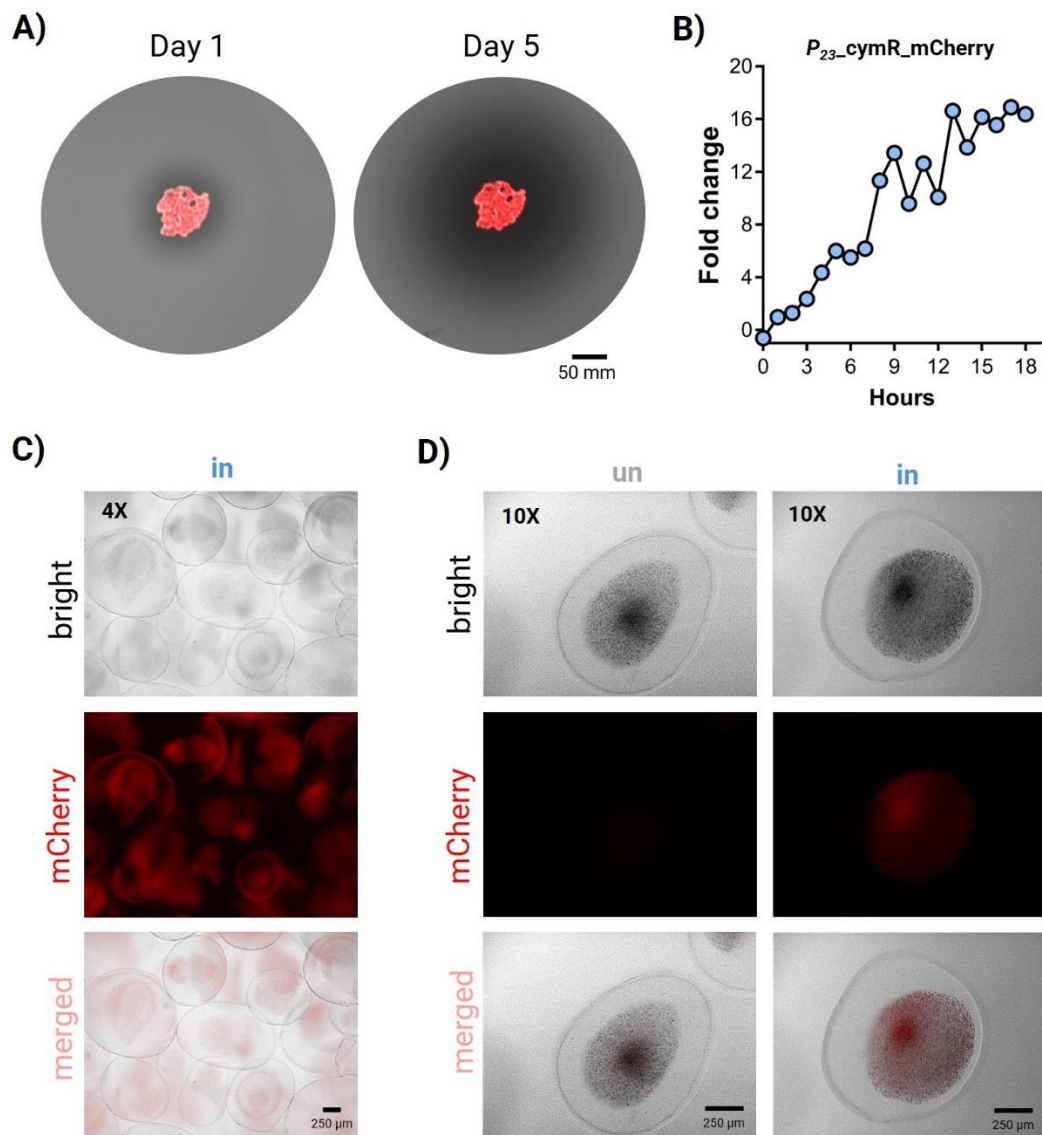


Figure S11: **A)** DNase agar plates with micro-PEARLs after 1 and 5 days of incubation at 37°C. Images taken using the BioRad Gel Documentation System. **B)** Fold-changes of the $P_{23_cymR_mCherry}$ variant when encapsulated in micro-PEARLs and induced with cumate for 19 hours (n = 3, mean ± SD). “un” = uninduced condition, “in” = induced condition (100 μM of cumate). **C)** Microscopy images at 4X of induced micro-PEARLs after 24 hours of incubation at 37°C in a 12-well plate. “in” = induced condition (100 μM of cumate). **D)** Microscopy images at 10X of uninduced and induced micro-PEARLs after 24 hours of incubation at 37°C in a 12-well plate. “un” = uninduced condition, “in” = induced condition (100 μM of cumate).

II.5. Supporting Information Chapter 5

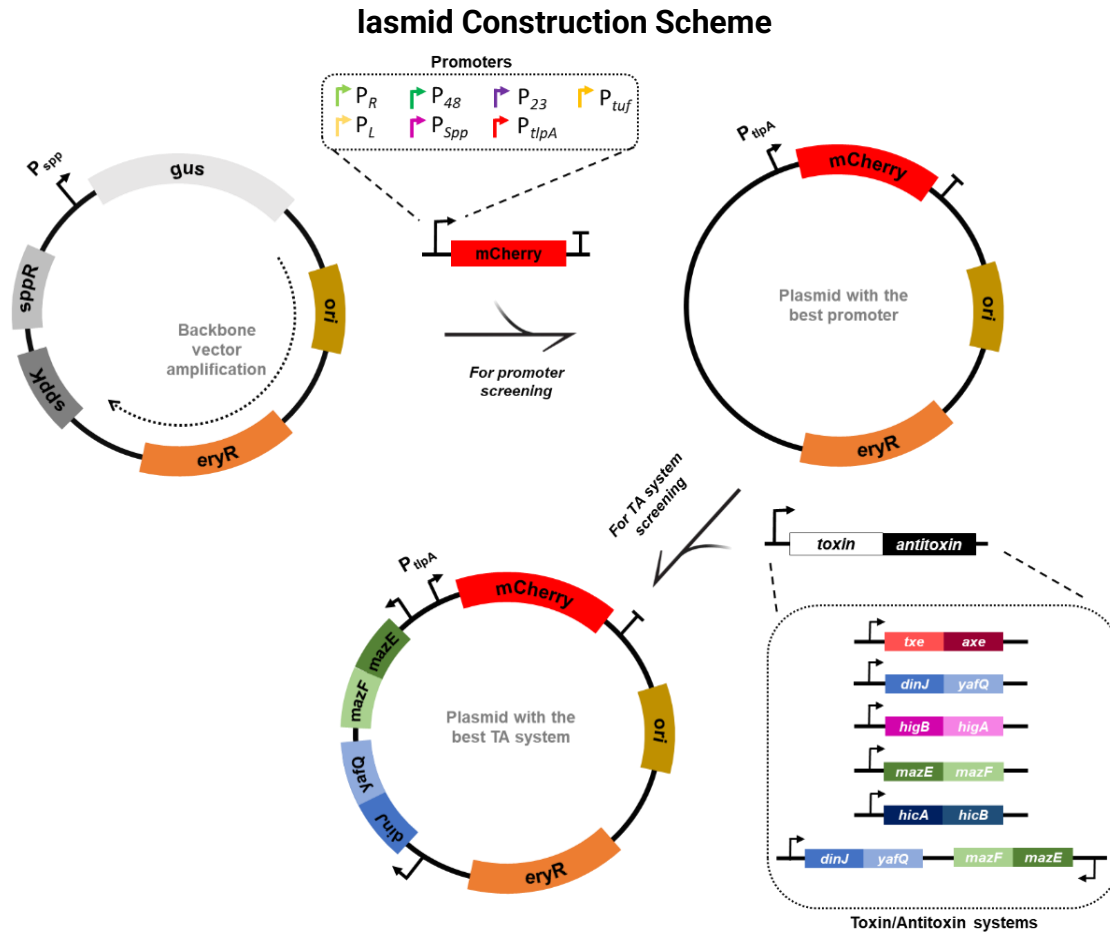
Novel genetic modules encoding high-level antibiotic-free protein expression in probiotic lactobacilli

Sourik Dey,^{1, †} Marc Blanch-Asensio,^{1, †} Sanjana Balaji Kuttae,¹ Shrikrishnan Sankaran^{*1}

¹ Bioprogrammable Materials, INM - Leibniz Institute for New Materials, Campus D2 2, 66123 Saarbrücken, Germany

*E-mail: Shrikrishnan.sankaran@leibniz-inm.de

[†] Joint Authors



Supplementary Figure S1. Construction of plasmid variants using the direct cloning approach developed in this study. The p256 ORI and the erythromycin resistance marker have been amplified from the pSIP403 vector backbone. The interchangeable promoter sequences were upstream of the mCherry reporter gene with P_{tlpA} promoter showing the best performance (highlighted). The activity of the toxin/antitoxin modules was checked in the pTlpA mCherry plasmid with the combo TA system, showing the best plasmid retention (highlighted).

➤ Origin p256

```

ttgagatccttttttctgcgcgtaatctgctgcttgcaaacaaaaaaccaccgctaccagcgggtggtttgttgccggatcaagagc
taccaactccttttccgaaggttaactggcttcagcagagcgcagataccaatactgttcttctagtgtagccgtagttaggccacca
cttcaagaactctgtagcaccgcctacatacctgctctgctaactcctgttaccagtggtgctgctgccagtgccgataagtcgtgtctt
accgggttgactcaagacgatagttaccggataaggcgcagcggctcgggtgaacggggggttcgtgcacacagcccagcttg
gagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaaggaggagaaaggcgg
acaggtatccggttaagcggcagggcggaacaggagagcgcacgaggggagctccaggggaaacgcctggtatctttatagtc
ctgtcgggttcgccacctgactgagcgtcgattttgtgatgctcgtcaggggggaggagcctatggaaa

```

➤ **eryR**

ttgaacaaaaatataaaatattctcaaaaactttttaacgagtgaaaaagtactcaaccaaataataaaacaattgaatttaaagaa
accgataccggtttacgaaattggaacaggtaaagggcatttaacgacgaaactggctaaaataagtaaacaggtaacgtctattga
attagacagtcatttcaacttatcgtcagaaaaattaaaactgaatactcgtgtcactttaattcaccaagatattctacagttcaa
ttccctaacaacagaggtataaaattgttggaatattccttacaatttaagcacacaaattataaaaaagtggttttgaaagccgt
gcgctcgacatctatctgactgttgaagaaggattctacaagcgtagcttgatattcaccgaacactagggttgctctgcacactc
aagtctcgattcagcaattgcttaagctgccagcgggaatgctttcatcctaaacaaaagtaaacagtgcttaataaaaacttacc
gccataccacagatgttcagataaatattggaagctatataagtagtcttttcaaaatgggtcaatcgagaatatcgtaactgttta
ctaaaaatcagtttcgtaagcaatgaaacacgccaaagtaacaatttaagtaccattacttatgagcaagtattgtctattttaata
gttatctatttttaacgggaggaaataa

➤ **mCherry**

atggtttcaaaggggtgaagaagataacatggctatcatcaaggaattcatgcgtttcaaggttcacatggaaggttcagttaacggt
cacgaattcgaaatcgaagggaaggtgaaggtcgccatacgaaggtagtcaaactgctaagttaaaggttactaaggggtggtc
cattaccattcgcttgggatatttatcaccacaattcatgtacggttcaaaggcttacgttaagcaccagctgatatcccagattact
taaagttatcattcccagaaggtttcaagtgggaacgtgttatgaacttcgaagatgggtggtgtgttactgttactcaagattcatcat
tacaagatgggtgaattcatctacaagggttaagttacgtgggtactaactcccatcagatgggtccagttatgcaaaagaagactatgg
gttggaagcttcacagaacgtatgtaccagaagatgggtgctttaaagggtgaaatcaagcaacgtttaagttaaaggatggt
gggtactacgatgctgaagttaagactacttacaaggctaagaagccagttcaattaccaggtgcttacaacgttaacatcaagtta
gatatacttcacacaacgaagattacactatcggtgaacaatacgaacgtgctgaaggtcgctactcaactgggtggtatggatgaa
ttatacaagtaa

➤ **RBS**

ttgtttaactttaagaaggaga

➤ **P_{tIpA}**

tttaattgtttgtagtagtttattgttggtttgttggttataatat

➤ **P_{spp}**

gcccatattaacgtttaaccgataaagttgaacgttaatatTTTTTT

➤ **P₄₈**

tcgtaagttgttgacatggaacgaggaatgtgataatctgtgagt

➤ **P₂₃**

ctgatgacaaaaagagaaaaatttgataaaatagtccttagaattaaattaaaaa

➤ **P_{tuf}**

tctgtttacaaatcagattaggctatatataatattaagga

➤ ***P_L***

ttgacataaataccactggcggtgatact

➤ ***P_R***

ttgactattttacctctggcggtgataa

➤ ***Phamp***

caaaatgctggtgacgtttatgttggtatttacgtaataaaatcacgac

➤ ***Phamp***

caaaatgctggtgacgtttatgttggtatttacgtaataaaatcacgac

➤ ***T7 ter***

ctagcataacccttggggcctctaaacgggtcttgaggggtttttg

Supplementary Table S1. Nucleotide Sequences of the common genetic parts and interchangeable promoter sequences tested in this study.

➤ ***YafQ/DinJ***

agatggcagttacgcttcatttgtgcaagaaaaagccagaaatccggtgaattcatctcacatgatgttatcccactgtgtttaca
ttgggataattcgtgatataattaggttgtagataggaaggagtggttagcaatggcagccacaaagaaagaaactcgcttgaata
ttcgtgttgatccggaattaaaaagtgtgctcaaatcgtagcaaatgatatgggcatcgacttgaccgcagctgttactatgttcat
gacgaaaatggtgaaagatcacgccctcccgtttaccccaacaagtctaccagttgaaaccttacaggcgctgaaagaagcaa
agcaccagagctgtcaaaaaatacagcacgcctgatgacatgtggagagacttgaatgtatagctctggttccgacgcctacat
ttaagcgcgatctaaaacgactctccaagaagcattggccgatggacgaactaaagacggctgtaatctcctagctgctggtac
aaatgctgaactattaagcaaaaagtatgcagatcatgcctgtcttcaagcagcgagtggaaaggatatcgtgaactacatgttg
acggccctcgtggcgactggttgctaatactataaaattaagcagcaagatctcattttgaccctggttagaactggatctcatcataa
ccttttgggtaaatagaactacgaaaggccgtccaaaaaaggcgcttttgtgttggtcaaccagcagattcattcgtcaatatac
acgtgacttaacgccaatctcaatcagagattagaaaccggaactcgtcggccagtaaaaatgttcagctatcgagatgagtt
gggtgatctggggaagttagcatcaataagatgccaatctttatcatgcagggttggaacgaaaatttaacccaatg

➤ ***MazF/MazE***

gaaggattacggtcaacgagtgctttcggacacgtccggtgatattgttatttgactcgtggtcttactccaatcaattatactgacaa
gcatcgcaaatataaaaccgccctacctaacctgagtaagttacagggcgaagtagtgctgaatagaatggaaagtcgaagttgta
atacaccggtttgcgcgacgtgataaaaatcggcactgcgcagtgtttcgtatgtatgtgatactccgtcctgctctgggggtcgcac
aacgaataaatttgggcagcagcaattgtccgtgggtattgtaaccattaagactaaagtagccgggcaaatcacgcattgtaga
cgtaattggtgaaacaattacaaaccgggtgttctgagtgtagagattactactaagaactacagctggccgacgtttctttatttcgc
ggccacgttggtgggtcaaaatcaatccagatgatgtctttttgttaggttaaataagtcattactcgacctcattccagtcgacatcgg
accatgcttcagtcattggccgtttcggcttgagccggcataaggattctcgatacgtggttacgagtgatatgactcaccatcact
tgatgggatgagtaaccatcgtgtcctttcttaaaacgaccgtcttaggttagacttaatgccaatgaattaccagactaactgtct

ttacctcaatcatatcaaggcctcctcgattataataaacacactatttatgcaacgagtataacacgtattacgaaataagacagcta
aagcaatcgcgataactaattgataagcacacggttttagtaattcatcaataaaggaagaga

➤ **HicA/HicB**

tactgatgatgaacatgccccagcctgtttagctctttgcgcaataaatcattttttgtctggattgaatagagcttgagcaaaatct
ttggtaaaatcattcatgaaggagttcttcttctgtatgtgtttttgttcaaaccaatcatacgagaaaggaactccttttctaattgtt
aaagaaataaatttaaggaatcattcatccttacacaaactattttacagtctcattcttcataaaccacatcctagaaaagaactatt
accatatcaaatcaaacaaattcaagataaaatcaaagagatgaataaagatgaaaaaactaatcaatatatgaagtataagg
gttatgaagggtcaattgaatatactttggaggataagattcttttggtaagggtcaggggattaagagctctatttcttacgaaggtaat
acaatagatgaattggagaaggattttcaaggagccattgatgattatctaagctgtaaggaagatggagttataccagagaaa
ccttttaaaggtaatttaattgtgcgcattgatccttcattacatgaaaaattggctaattatgctgcaacgaagcaccaatcattgaat
gctagtgtagaagaagctataaaaagattttggcttaggtgaaagacgcttggaattttcaagcgtttttcatgaaaacaattgct
aaattcattgaatgagtgactatttttagtataacaatagttgaacgggtgatgtcggcgatatagg

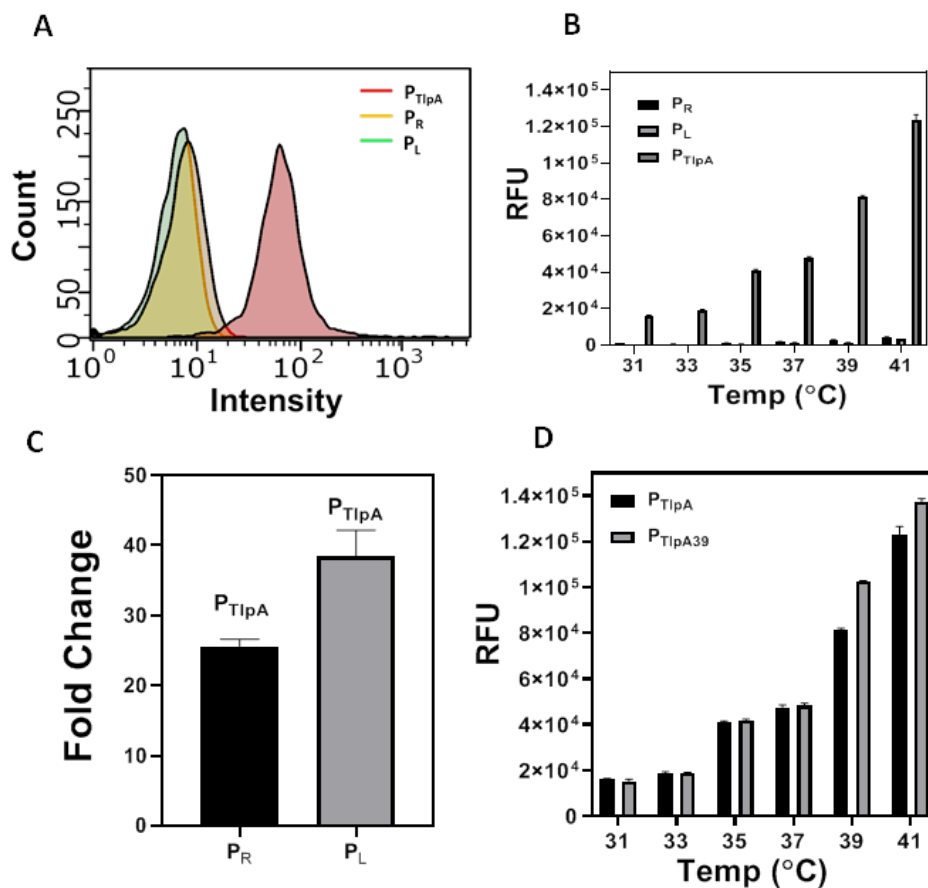
➤ **HigB/HigA**

tactgatgatgaacatgcccactctggttggttcctagctaaaaataggggtcgtactagtcgggtaatcagatgaattggatatttgg
gtgtggtcagctaataataggagcaagagtcggatggattgagatatacacgtgatacgggtataatcgattaagaattttgcagat
aaggaaactcacaaggtttaccaacaaaaatctctaaacaaatggccaccaaccattcagcaatttagcattgcgaaaattactaat
gattgatcatgcggaaacaattaatgatttgagcctaccgctgccaatcacttggaataaagtcagatgcgcaagggaat
atagcatcaggattaataatcagtatcggatatgtttgcaattcgaaatggtaatgagtttatgatgttgaaatagtcgattatcatca
tggttaggagacaaaaatgaatgaaatcccaacacctaataatgtagaattcttgaagaagagttatggctcccttgcatactc
tctgcatatttttggctcaacagattgggttacccacgtcacggattcaagacttactacatgatcgtcgacaggtgacggttgatac
atcgctgcggctaggacgattctttggagtgcggatcgattttcttggaaactcaaaatgatattgaaattcgtaatttgaacagat
acatggtgctgaatatgcacagataaaaaagtatcaagtcagttataaaggacgtagaccgtagttgcttgtagcgtactacgg
ttttgcaaatataacctaactgataagcagcagatcttaaatttaaggttatcaacaggtggataaagcagtgcggtttgtattatc
cacctgttgataatcattgagttggttaattgtacttactactgggttaactggctgtagaaaaatggtcaagcattagttcgtggt
gatgtcggcgatatagg

➤ **Txe/Axe**

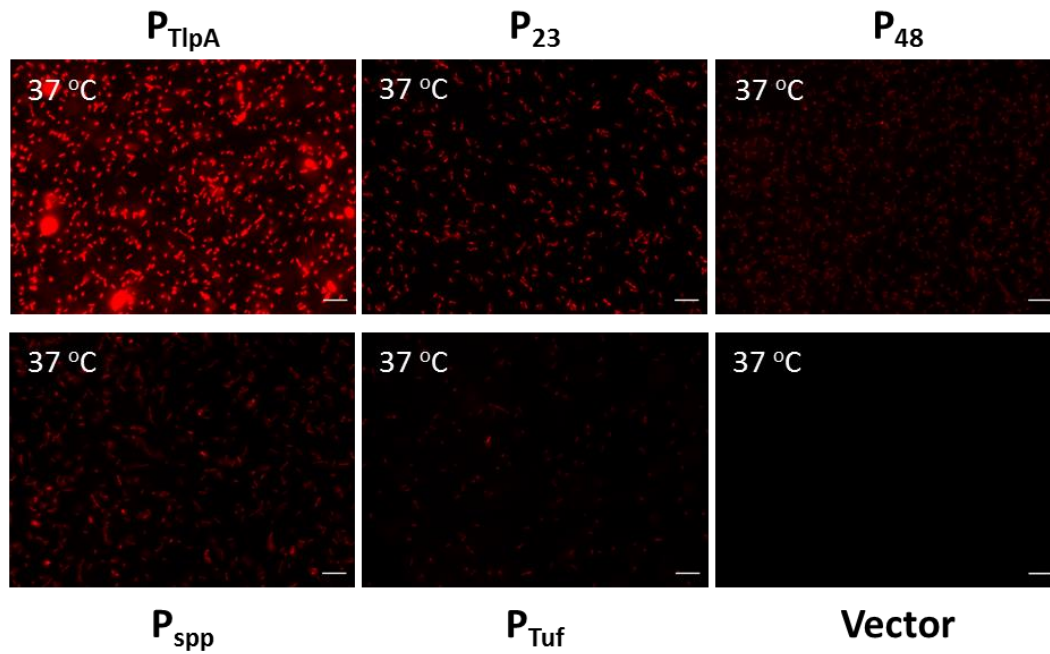
acgcgtaacaaacacattcattaataacaatcaacactaagctatattagcttttaaatgactagggtaataaaaataaggatcaa
aaacttttaagtttctgaccctttccttacttccgattggttaatagtgatcttttcgagaataaataaatatcgtttcattttcaactctata
tatcagtctatgttcattctgtaattcttctggaccattttccagataaatcatgctttaatggctcaggttttctaaccagcaaagggg
gaacgatcgatatcttttataacttgtaattctttttatattgcttttgttcttgcctcatgccaataaagataatcatccaagcatcat
cagaccaagccttaatacatcagattcaacctcgattaagtcagtggttttaaatgcacctttggagaattgttcattctcctcgacgaatt
ttttcatgacgtaattattagaaagtgttctcaacggttcttgcataagaatcataatctcttttgataatacaacaactgtatcttctacat
ctttactgttacaataagtgttccagcatcctcattaactgtttcatataactacgtaaatgttggcgaaattgaataagctactgct
tccattcttttcccccttattatttctataaaacaattgtacattatattgtacaattaagcaaagattaatattctaacttcttataatt
aaaagtgcctatcacttttagtttttaggataaaaaggtgacgtcacttttgagtgtggttacctcaaatcctgcttt

Supplementary Table S2. Nucleotide Sequences of the toxin/antitoxin modules tested in this study.

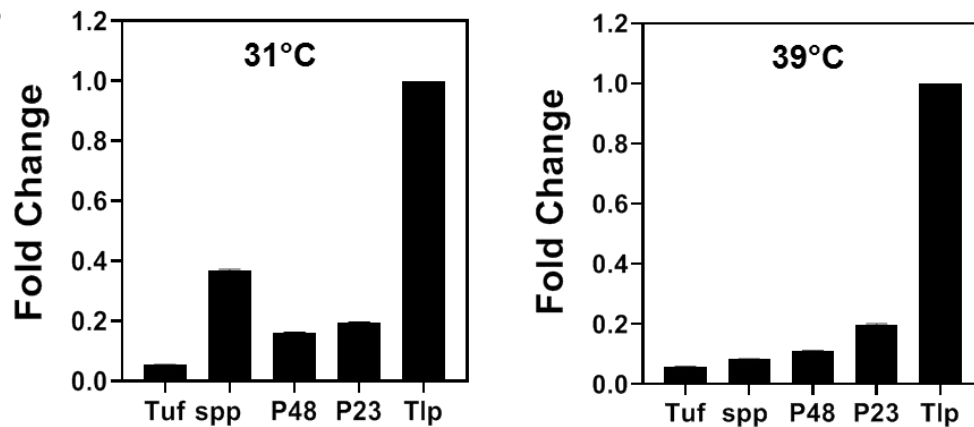


Supplementary Figure S2. (A) Flow Cytometry analysis of P_R , P_L , and P_{tIpA} driven mCherry expression in *L. plantarum* WCFS1 after 18 h incubation at 37°C. (B) Fluorescence Spectroscopy analysis of the P_R , P_L , and P_{tIpA} driven mCherry expression after 18 h incubation in the Thermocycler setup at thermal gradients ranging from 31°C to 41°C (See Materials and Methods section “Microplate reader Setup for Thermal Gradient Analysis”). (C) Fold Change of P_{tIpA} driven mCherry expression in comparison to P_R and P_L promoters at 37°C (D) RFU Plot of mCherry production by plasmids pTIpA and pTIpA39 after 18 h incubation at thermal gradients ranging from 31°C to 41°C. The pTIpA39 plasmid encodes for additional P_{48} promoter-driven codon-optimized TIpA39 repressor. The data in B, C, and D, corresponds to three independently conducted biological replicates with column heights representing means and whiskers representing SD.

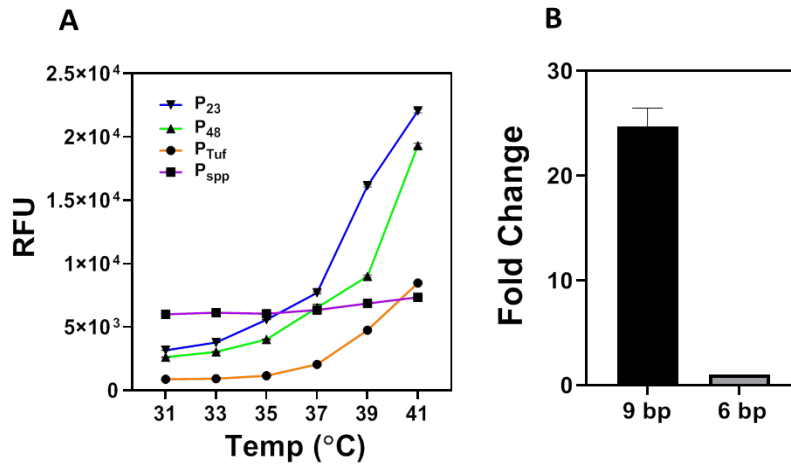
A



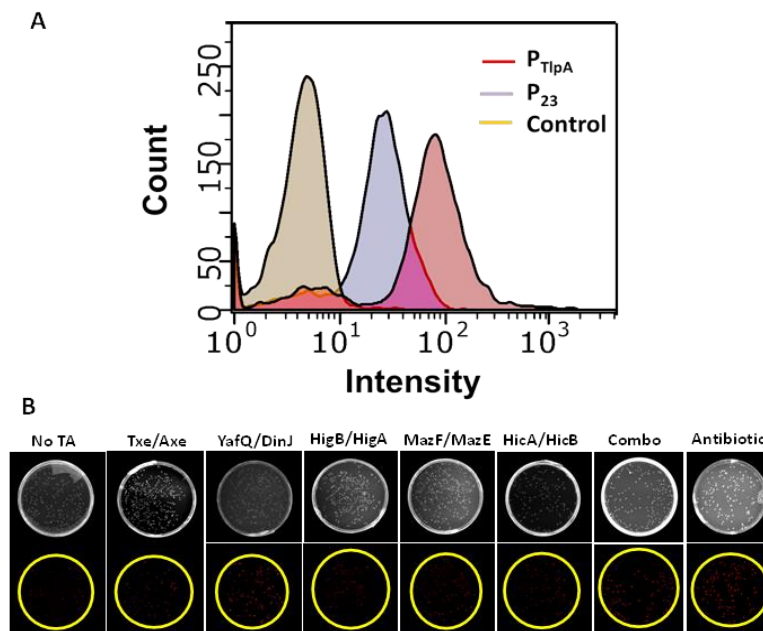
B



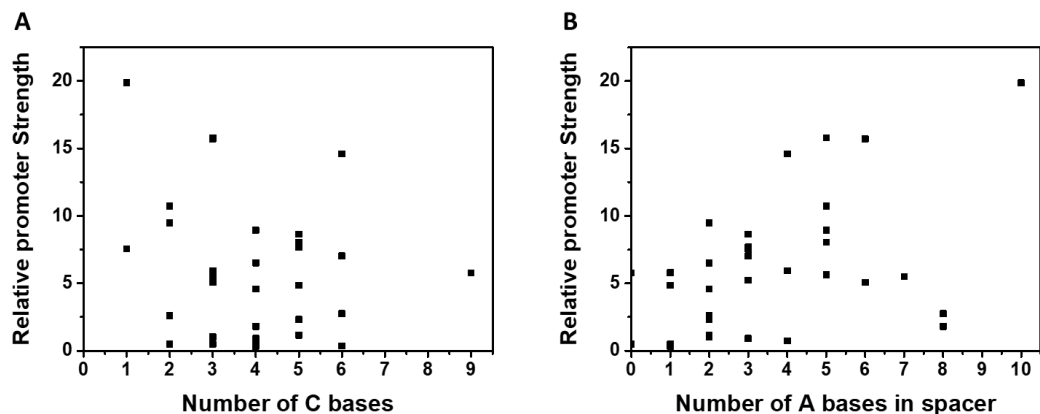
Supplementary Figure S3. (A) Fluorescence microscopy images of P_{tlpA} , P_{23} , P_{48} , P_{spp} , P_{tuf} driven mCherry expression and Empty Vector constructs in *L. plantarum* WCFS1 cultivated at 37°C for 18 h (Scale = 10μm). (B) Fold Change of the P_{tuf} , P_{23} , P_{48} , P_{spp} driven mCherry expression normalized to the expression level of the P_{tlpA} promoter at 31°C and 39°C, respectively. The data corresponds to three independently conducted biological replicates with whiskers as SD.



Supplementary Figure S4. (A) RFU Plot of P_{23} , P_{48} , and P_{tuf} driven mCherry expression showing enhanced fluorescence expression in contrast to P_{spp} within the thermal gradients ranging from 31°C to 41°C. The data corresponds to three independently conducted biological replicates with whiskers as SD. (B) Fold Change of the P_{tlpA} promoter-driven mCherry expression with 9 bp spacer (between the RBS and the start codon) in comparison to the P_{tlpA} -6 bp spacer construct.



Supplementary Figure S5. (A) Flow cytometry plots showing that strains with P_{23} -driven mCherry expression produce relatively low intensities, with part of the population overlapping with the signal gained from bacteria that do not express any fluorescent proteins (control). In comparison, the signal from P_{tlpA} is clearly demarcated from that of control. (B) Agar-plate-based analysis of plasmid retention provided by the different TA systems. The total number of colonies can be determined from the brightfield images, and the red colonies visible in the fluorescent images represent the plasmid-retaining bacteria.



Supplementary Figure S6. Plots representing relative promoter strengths of 34 promoters in Table S3 vs (A) number of C bases in the -35 to -10 region and (B) number of A bases in the spacer between the -35 and -10 region (non-bold region in Table S3).

Promoter	Sequence	Relative strength
P ₂₀	-35 TTGACAACCTGTGGGCGGTTTGATTGTT -10	0.33
P ₁₇	-35 TTGACACTGATCCCGGCTGGTGGTAAATT -10	0.35
P ₁₃	-35 TTGACAGCGTGGGTTGGTGCTGGTAATTT -10	0.48
P ₄₁	-35 -TGACAGGGCTGTGATGGTGTGGTATTGT -10	0.50
P ₄₃	-35 TTGACAGGATAAAGGTCGCCTGGTATGGT -10	0.74
P ₄₀	-35 TCAACATACATGGATCG---TGGTATGTT -10	0.91
P ₄₄	-35 TTGACACTTGAGGGTTTGATGGTAATCT -10	1.02
P ₁₀	-35 TTGACATTGTTAATGGCCCCTGATATATT -10	1.15
P ₃₄	-35 TTGACAGACAAACATAAGGATGATATGCT -10	1.80
P ₉	-35 TTGACAGGGCTGAGCTGACCTGGTATGGT -10	2.33
P ₄₂	-35 TTGACA GAGTTGTCTGGTATTGATATTGT -10	2.60
P ₅	-35 TTGACACACAAAACCAGACATGGTATTAT -10	2.75
P ₃₃	-35 TTGACATGTGACCCGTTTTATGGTATTAT -10	4.58
P ₂₁	-35 TTGACATTCGGGGCATCTCGTGGTATAAT -10	4.84
P ₂₆	-35 TGGACAAGTGATAAACGGGTGATATGAT -10	5.06
P ₂₂	-35 TTGACAGATTAGGGCGGTCATGGTAAAAT -10	5.23
P ₁₈	-35 TTGACAGGGAGGCTTCGTTGTGATAAGAT -10	5.79
P ₄₇	-35 TTGACAGACGCGGGAGAATATGGTAAAGT -10	5.65
P ₁₆	-35 TTGACATCCCCCTCTCTTCTGGTATAAT -10	5.76
P ₃	-35 TTGACAAAGAAGCCGGGTTTTGGTATAAT -10	5.93
P ₂₉	-35 TTGACAGTTCTGGCTGGATATGGTAAACT -10	6.51
P ₆	-35 TTGACAGGCAGCCATCTCTATGGTAAAAT -10	7.04
P ₃₈	-35 TTGACAGAATGTTTTGTAGTGGTATAAT -10	7.56
P ₃₀	-35 TTGACAGCCGAGGTACCATGTGGTATAAT -10	7.69
P ₃₁	-35 TTGACAAAAGTCCAGGGTATGATATACT -10	8.04
P ₈	-35 TTGACAAATCCGGTGTCTATGGTATTCT -10	8.64

P ₂₅	-35 TTGACATTAAGGCACATCATTGATATGGT -10	8.94
P ₃₅	-35 TTGACATGCAGGGAGTTTGTGGTATAAT -10	9.49
P ₁	-35 TTGACAGGGATATTAGAGTATGGTATGCT -10	10.74
P ₄	-35 TTGACACGCGCAGCAAGGCATGATATAAT -10	14.60
P ₁₁	-35 TTGACAGAATGGACATACTATGATATATT -10	15.70
P ₄₈	-35 TTGACATGGAACGAGGAATGTGATAATCT -10	15.79
P ₂₃	-35 ATGACAAAAAGAGAAAATTTTGATAAAAT -10	19.86
P _{Tuf}	-35 TTTACAAATCAGATTAGGCTATATATAAT -10	5.49

Supplementary Table S3: Promoter sequences included in Figure S7 and their relative strengths. Sequences and strengths derived from reference “Rud, I., et al., *A synthetic promoter library for constitutive gene expression in Lactobacillus plantarum*. Microbiology, 2006. **152**(4): p. 1011-1019.” The relative strengths of P₂₃ and P_{Tuf} from this study were estimated based on the expression levels we determined in relation to P₄₈.

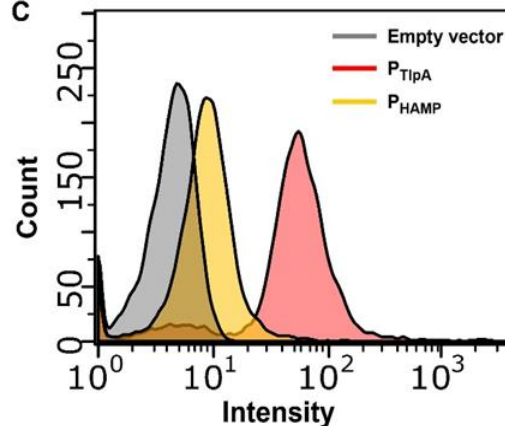
A

Hit	Upstream a gene	Position	Sequence	Sequence length	Identity Matrix
Hit 1	Yes	257,256..257,284	GTTTATGTTGGTTATTTACGTAATAAAAT	29	82.76
Hit 2	No	1,292,083..1,292,110	ATTTTAAACAACATAACAAAATGAAC	28	69.23
Hit 3	No	1,541,627..1,541,653	GTTATTTTATGTTTGTGTGAAATTT	27	77.78
Hit 4	No	2,772,508..2,772,536	ATACTTAAACACAACAAAAACACTTAAC	29	32.82
Hit 5	No	3,129,614..3,129,645	ATATGGTCAGTCAATGAAATCAACAAATAAAC	32	60.00
Hit 6	No	3,209,426..3,209,455	ATAGTAACCAACAGCACACCACAAGTAAAC	30	71.43

B

P_{HAMP} GTTTA--TGTTGG-TTATTTACGTAATAAAAT
P_{TlpA} -TTTATTTGTTGTTTGTGTTATAATAT
**** * * * * *

C



Supplementary Figure S7. (A) Results obtained after screening the entire genome of *L. plantarum* WCFS1 for a P_{tIpA}-like sequence. (B) Sequence alignment of P_{HAMP} and P_{tIpA} promoters. (C) Flow Cytometry analysis of P_{tIpA} and P_{HAMP} driven mCherry expression in *L. plantarum* WCFS1 after 18 h incubation at 37°C.