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

[†] Authors contributed equally

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*, that 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 tunable 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 (1,2) often providing health benefits in the form of anti-inflammatory, anti-pathogenic and immunomodulatory activities (3,4). 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 (5), mastitis(6), atopic dermatitis (7), bacterial vaginosis (8) and periodontitis(9). Apart from their health benefits, lactobacilli are also vital for numerous fermentation processes in the food industry, for example in the production of yogurt (10), cheese (11), sourdough bread (12), beer (13) and wine (14). Due to this ubiquity in our lives, there is considerable interest to genetically enhance and expand the capabilities of these bacteria for both healthcare applications(15). 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 (16), Human Immunodeficiency Virus (HIV) infection (17) and respiratory infections (18). They 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 (19). 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 diarrhea, pneumonia, viral infections (HIV, HPV, influenza, coronaviruses, etc.) and more (19,20). 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* (21,22).

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* (23,24) and *B. subtilis* (25,26) . 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 etc. Most of these have been developed in a few species that were found to be amenable to genetic modification, among which *Lactiplantibacillus plantarum* (27) is widely reported (28). 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 (29) , constitutive promoters with a wide range of expression strengths (30), a few inducible promoters that can be triggered by peptides (31) or sugars (32), signal peptides sequences enabling protein secretion (33) or surface display (34) and food-grade plasmid retention

systems based on resistance to external stressors (e.g. bacteriocins) (35,36) or auxotrophy complementation requiring genomic knockout of a metabolic gene and providing it in the plasmid (37,38). However, the available set of well-characterized genetic parts are still miniscule 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 drives protein expression at levels considerably higher than previously reported strong *L. plantarum* promoters and (ii) toxin-antitoxin systems as a third strategy for food-grade plasmid retention that does not require external stressors or genome manipulation. The novel promoter provides a hint for finding high-performance genetic parts in unrelated species, a space that has thus far been poorly explored. The toxin-antitoxin systems introduce a thus-far unexplored modality of plasmid retention in lactobacilli that enables the generation of temporary GMOs, 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.

MATERIALS AND METHODS

Strain, Media and Plasmids

L. 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) (39) and Prof. Geir Mathiesen (Addgene plasmid # 122030) (33) respectively. The plasmid pTlpA39-Wasabi was a kind gift from Prof. Mikhail Shapiro (Addgene plasmid # 86116) (40). The plasmid pUC-GFP-AT was a kind gift from Prof. Chris Barnes (Addgene plasmid # 133306) (41). 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 enyzme were purchased from New England BioLabs (NEB, Germany). PCR was performed using Q5 High Fidelity 2X 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 has been shown in Supplementary Figure S1. The promoter sequences used in this study are provided in

Supplementary Table S1 and the nucleotide sequences of the toxin-antitoxin modules have been highlighted in Supplementary 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 OD_{600} 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 $MgCl_2$ 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 were 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 lukewarm 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 (42) 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. 2000 ng of the purified PCR product was mixed with 2.5 µl of 10X 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 minutes and then at 70 °C for 10 minutes 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 10X 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 were transformed into *L. plantarum* WCFS1 electrocompetent cells.

Notably, since *L. plantarum* harbors 3 endogenous plasmids (43), 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 OD_{600} in 3 mL of antibiotic supplemented fresh MRS media and propagated at 30°C, 250 rpm. At $OD_{600} = 0.3$, the cultures were dispensed into Fisherbrand™ 0.2mL 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 (SppIp) with the sequence NH_2-

MAGNSSNFIHKIKQIFTHR-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 μL of 1X PBS and added to the clear bottom 96-well microtiter plate (Corning® 96 well clear bottom black plate, USA). The samples were then 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 for recording the mCherry fluorescent intensity were set to 19442 μm 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 = Fluorescence/OD_{600}.

Fluorescence Microscopy Analysis

Bacterial cultures were grown overnight in 5 mL of MRS media (supplemented with 10 μg/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 SppIp 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 (15700 × g, 5 min, 4 ◦C), washed twice with Dulbecco's 1X PBS (Phosphate Buffer Saline) and finally resuspended in 1 mL of 1X PBS. 10 μL of the suspensions were 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 100X 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 were 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 were 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 were harvested by centrifugation at 13000 rpm (15700 × g). The supernatant was discarded and the pellet was resuspended in 1 mL of sterile Dulbecco's 1X PBS. The samples were then serially diluted by a 10^4 Dilution Factor (DF) and 5,000 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 done using the Luminex GuavaSoft 4.0 software for EasyCyte.

Toxin/Antitoxin Module based Plasmid Construction

Similar to previous reports in *E. coli* (41), the effect of Txe/Axe (toxin/antitoxin) module from *E. faecium* (44) was tested in *L. plantarum* WCFS1 to test its capability for antibiotic-free plasmid retention. TA Finder version 2.0 tool (45) was used to select further type-II TA (Toxin/Antitoxin) systems present in *Lactobacillus* genomes. *L. acidophilus*, *L. crispatus*, *L. casei*, *L. reuteri*, and *L. plantarum* WCFS1 genomes were retrieved from NCBI Genome. TA systems harbored 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, 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 (41). 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, 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 1X PBS. The resuspended bacterial solution 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_λ/Em_λ = 300 nm/600 nm) and Cy3 channel (Ex_λ/Em_λ = 554 nm/568 nm) to visualize the cell population producing mCherry fluorescence.

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 microtiter 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 sequence 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 (46). 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 (47).

For the multiple sequence alignment, protein sequences of the σ70 subunits from *L. plantarum*, *E. coli* and *S. typhimurium* RNA polymerases were first retrieved from Uniprot (48). Sequences were aligned using the tool MUSCLE (49). Jalview was used to visualize and edit the multiple sequence alignment (50).
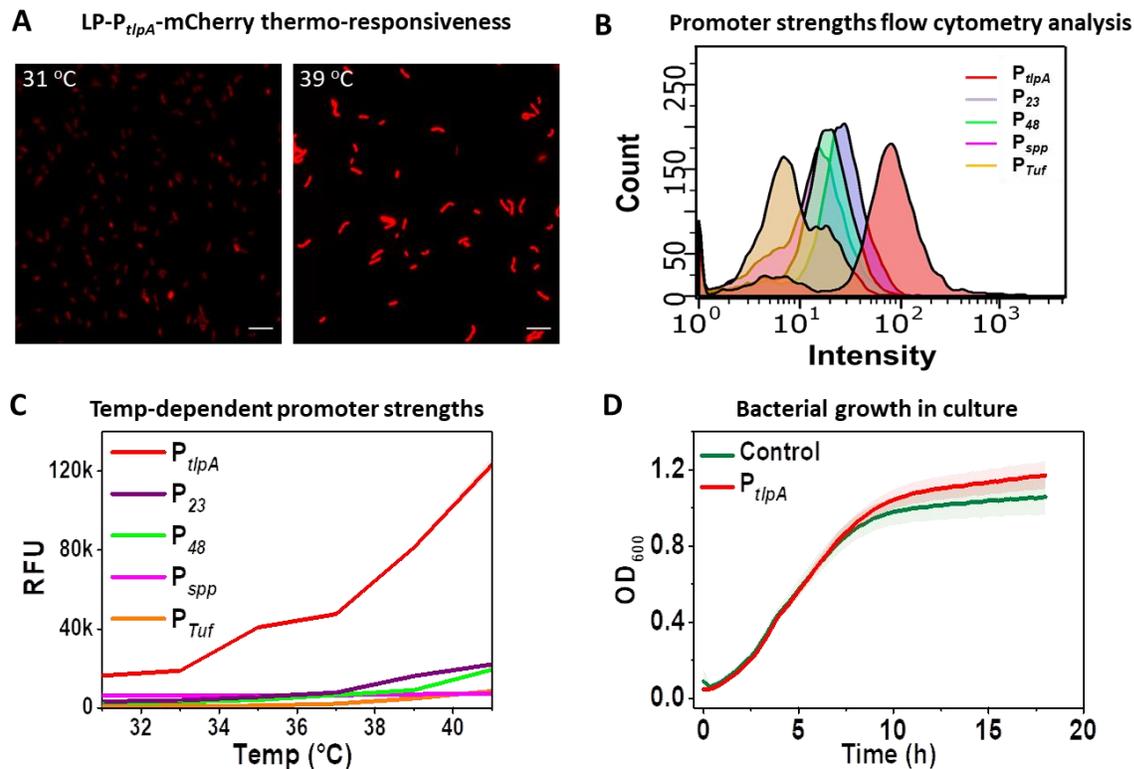
SnapGene was used to identify DNA sequences similar to P_{tlpA} within the genome of *L. plantarum* WCFS1 using the feature "Find Similar DNA Sequences". The search allowed a mismatch or gap/insertion every 4 bases. BPROM, an online tool for predicting bacterial promoters, was used to identify the -35 and -10 boxes within this promoter (51). BlastP was used to identify the protein encoded by the gene driven by this promoter. Promoter alignment was performed using MUSCLE (49).

RESULTS

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

In pursuit of a heat-inducible promoter for therapeutic applications, we encoded mCherry as a fluorescent reporter protein downstream of the Salmonella-derived P_{tlpA} promoter along with its thermo-responsive TlpA repressor (40,52). While the repressor did not repress gene expression (Supplementary Figure S2D), 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 ^{o}C to 39 ^{o}C) (Figure 1A). In contrast, the commonly used heat-inducible pR and pL promoters from *E. coli* lambda phage, were found to perform very weakly, driving low levels of mCherry expression (Supplementary Figure S2A, Supplementary S2B). Fluorescence spectroscopy revealed that the strength of the P_{tlpA} promoter at 37 ^{o}C was 26 and 39-fold higher than the pR and pL promoter respectively (Supplementary Figure S2C). Most remarkably, fluorescence microscopy and flow cytometry analysis also revealed that mCherry expression levels driven by the P_{tlpA} promoter significantly exceeded the levels driven by some of the strongest promoters previously reported in *L. plantarum* - P_{23} (53), P_{48} (30), P_{spp} (54) and P_{Tuf} (55) (Figure 1B, Supplementary S3A). At 31 ^{o}C, mCherry expression levels were at least 2-fold higher than these other promoters, while this increased to 5-fold at 39 ^{o}C (Figure 1C, Supplementary Figure S3B). All constitutive promoters (P_{23}, P_{48}, P_{Tuf}) were mildly thermo-responsive, while the inducible promoter (P_{spp}) was not (Supplementary Figure S4A). Notably, in all cases, the spacer length between the ribosome binding site (RBS, 5'-AGGAGA-3') and the start codon strongly influenced

expression levels. Previous reports involving the P_{tlpA} promoter in *E. coli* have done so with a spacer length of 6 bp (40). However, in accordance with previous *L. plantarum* reports (29), high level mCherry expression was observed with a spacer length of 9 bp, which dropped significantly when reduced to 6 bp (25-fold lower for P_{tlpA}) (Supplementary Figure S4B). 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 surprisingly did not metabolically overburden the cell (Figure 1D).
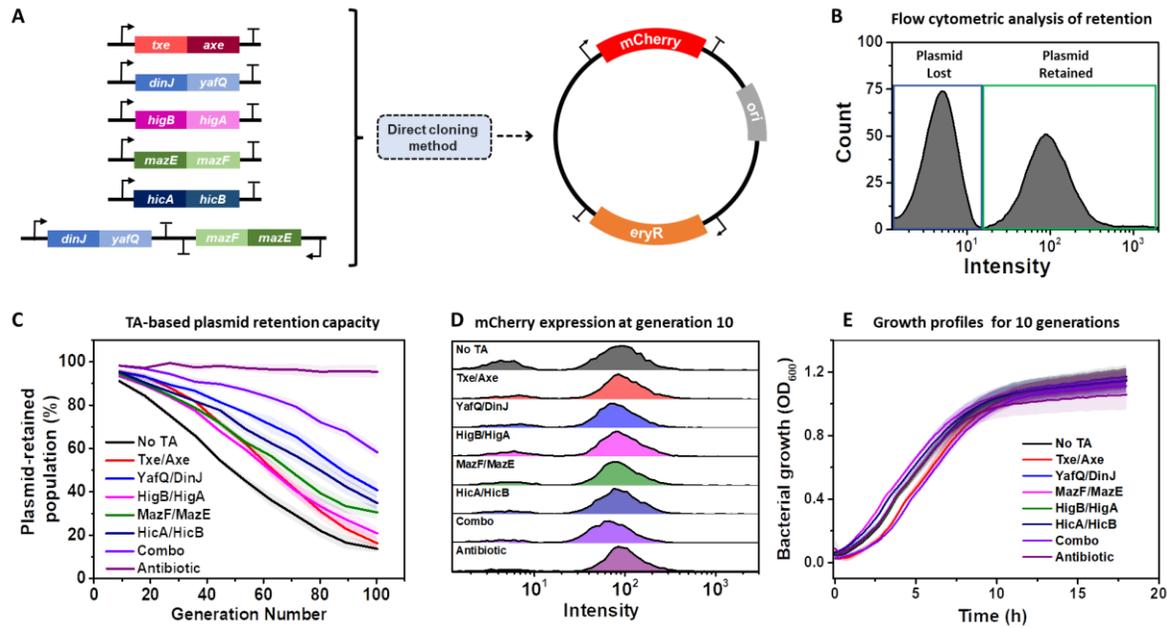

Figure 1. (A) Fluorescence microscopy of P_{tlpA} driven mCherry expression in *L. plantarum* WCFS1 cultivated at 31°C and 39°C for 18 h. Scale bar = 10 μm (B) Flow Cytometry analysis of P_{tlpA}, P_{23}, P_{48}, P_{spp} and P_{Tuf} driven mCherry expression in *L. plantarum* WCFS1 after 18 h incubation at 37°C. (C) Fluorescence Spectroscopy analysis of the P_{tlpA}, 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. (D) Growth rate (OD_{600})

measurement of *L. plantarum* WCFS1 strains containing a control plasmid and P$_{tlpA}$-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.

Toxin/Antitoxin based plasmid retention and transient GMOs

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 several natural TA systems have been studied (56) and some were investigated for plasmid retention in *E. coli* with promising results (41,57,58), their use in lactobacilli has not yet been explored. We first investigated the type II TA system, Txe/Axe, derived from *Enterococcus faecium* that was shown to ensure long-term plasmid retention in *E. coli (41)*.In this system, Txe is an endoribonuclease and Axe is its corresponding inhibitory protein. This module was added to the plasmid encoding P$_{tlpA}$-driven mCherry expression (Figure 2A) 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 (Supplementary 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 2B). 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 (Supplementary Figure S5A). In the absence of a TA system (P_{tlpA} 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 2C). 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. In order to identify potentially better performing TA systems, the bioinformatics tool, TA finder, was used and 4 other type II TA systems were selected – YafQ/DinJ from *L. casei* (59), HigB/HigA and MazF/MazE from *L. plantarum* WCFS1, and HicA/HicB from *L. acidophilus*. All these TA candidates involved an endoribonuclease toxin that targeted the RNA pool of the metabolically active and rapidly dividing microbial host. Similar to the Txe/Axe module, these TA modules were added to the P_{tlpA}_mCherry plasmid. Plasmid retention analysis revealed that the HigB/HigA and MazF/MazE sytems performed similarly to Txe/Axe 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 2C).

Figure 2. (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) 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. (E) Growth rate (OD$_{600}$) of strains with the TA modules, no TA and antibiotic retention over 10 generations at 37°C. In (C) and (E), the solid lines represent mean values and the lighter bands represents SD calculated from three independent biological replicates. Combo = MazF/MazE + YafQ/DinJ

Fundamental studies have shown that different TA systems can cumulatively offer better plasmid retention capabilities (60), based on which we combined the best-performing TA system endogenous to *L. plantarum* WCFS1 (MazF/MazE) with the best-performing non-endogenous system (YafQ/DinJ). Interestingly, we did observe

better plasmid retention capabilities with this combination with a 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 harboring TA modules, mCherry expression levels (Figure 2D) and bacterial growth rates (Figure 2E) were found to be minimally impacted compared to "No TA" or antibiotic-retention conditions, suggesting the that the toxins did not drastically impede the regular functioning of the cells. The greatest drop in protein expression was observed in the strain harboring the TA combo, although even in this case, the P_{tlpA} driven mCherry intensity was at least 4-fold higher than that of the next strongest promoter, P_{23} (as seen in Figure 1B). Notably, in the case of the TA systems, loss of plasmid only reverts the bacteria to its natural genetically unmodified probiotic status, thereby enabling the construction of transient GMOs.

DISCUSSIONS

Due to the beneficial presence of lactobacilli in our bodies, food and environment, there is considerable interest to genetically engineer high level heterologous protein production in them for healthcare applications. Among various strains, *L. plantarum* is one of the most extensively investigated and the quest for strong promoters has typically involved either screening the genome of the host strain (30,61) or adapting those driving high-level protein expression in phylogenetically close lactic acid bacteria (62) (Figure 3A). Very few studies have tested promoters from phylogenetically distant species like *P. megaterium* (P_{xylA}) or *E. coli* (P_{T7} from

lambda phage) (32), although expression levels were found to be low in these cases. In this study, we surprisingly stumbled upon the P$_{tlpA}$ promoter from the phylogenetically distant gram-negative *Salmonella typhimurium* capable of driving protein expression levels higher than previously reported strong promoters in *L. plantarum* WCFS1. In Salmonella, P$_{tlpA}$ is a promoter of the σ70 sigma factors, which are involved in regulating the expression of housekeeping genes in most prokaryotes. Todt et al., (63) used a genome wide analysis approach to identify σ70 based promoter consensus sequences in *L. plantarum* WCFS1 and predicted 568 promoter regions in close proximity to the transcription start sites (<40 nt). Their results combined with similar analysis from other organisms (64, 65) suggest that the σ70 promoter-binding motifs are conserved across different genera and divergent promoters recruiting σ70 to drive transcription in these bacterial species might be possible. Multiple Sequence Alignment (MSA) among the major RNA polymerase σ70 proteins (RpoD) of *E. coli*, S. typhimurium, and *L. plantarum* strains (Figure 3B) 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.,(66) showed that the *L. plantarum* RpoD seems to promiscuously promote transcription from a wide variety of heterologous promoters. They expressed sigma factors from seven different organisms in *E. coli* and tested their capability to drive the expression of GFP encoded downstream of a library of genome fragments from a variety of sources. They found that only the RpoD from Lactobacillus plantarum (Lpl) was capable of initiating transcription from all sources of DNA they tested. 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.

Figure 3. (A) Phylogenetic tree highlighting the distances between species from which various genetic parts have been tested in *L. plantarum*. Purple clade corresponds to Gram-negative bacteria. Green clade corresponds to Gram-positive bacteria. Promoters that have been tested in this study are labelled in blue. Promoters that have been tested by other authors in *L. plantarum* are labelled in green. Orange labels correspond to the TA systems tested in this study. (B) Homology analysis of σ70 RpoD genes from *L. plantarum*, *E. coli*, and *S. typhirmurium*. Height and brightness of the yellow bars indicate the extent to which individual residues are conserved across all 3 bacteria. (C) P*tlpA* promoter sequence with -35, spacer and -10 regions labelled

The P$_{tlpA}$ promoter sequence (Figure 3C) harbors the sigma70 consensus sequence at the -10 region (5′-TATAAT-3′) but not at the -35 region (5′-TTGACA-3′) (63). 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 (30,53,54,55). 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 (67,68). However, on analysis of 34 constitutive promoter sequences from the synthetic promoter library reported by Rud et al., (30) and those tested in this study (Supplementary Table S3), no correlation could be derived between promoter strengths and number of C bases within the -35 to -10 region or the A bases in the spacer (Supplementary Figure S6). If methylation could be influencing promoter strengths, it would be necessary to identify the methyltransferase recognition sequences in *L. plantarum* to derive meaningful correlations. These unique features of the P$_{tlpA}$ promoter sequence provide interesting clues for understanding factors affecting promoter strengths in *L. plantarum*. Accordingly, we searched for DNA sequences similar to P$_{tlpA}$ within the genome of *L. plantarum* WCFS1. Out of 6 hits (Supplementary Figure S8A), 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..257181), with an percent identity score of 82.76 compared to P$_{tlpA}$. This sequence (G**TTTATG**TTGGTTATTTACGTAA**TAAAAT**) 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 (Supplementary Figure S8B). Notably, P$_{HAMP}$ also contains an A and C base in the spacer. When the full promoter sequence (Supplementary Table S1) was cloned upstream of mCherry, only weak expression was observed (Supplementary Figure S8C), suggesting that one or more of these mismatches compared to P$_{tlpA}$ are essential for driving high-level gene expression. To gain deeper insights into P$_{tlpA}$'s unprecedented strength, further studies analyzing mutant libraries of the promoter and/or measuring DNA methylation patterns are required.

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. While genomic integration ensures stable retention of heterologous genes, plasmids provide far greater versatility and ease of engineering. Most commonly, retention is ensured by providing an antibiotic resistance gene in the plasmid and culturing the bacteria in the presence of the antibiotic. A food-grade variant of this strategy involves encoding bacteriocin resistance in the plasmid and culturing the bacteria in the presence of the bacteriocin (35,36). The main antibiotic-free retention strategy that does not require external selection pressure, involves generating auxotrophic strains by knocking out an essential metabolic gene and including it in the plasmid (37,38). However, the necessity to develop auxotrophic strains through gene knockout limits the widespread applicability of this strategy. For the first time, we have explored a third strategy involving Toxin-Antitoxin systems for plasmid retention in lactobacilli that require no genome manipulation and can be easily applied to different strains

without the need for external selection pressure conditions. Such TA systems have been tried in only a few bacterial species and have shown considerable retention capabilities in *E. coli.* Our results indicate that both homologous and heterologous TA systems help to slow down plasmid loss over multiple generations, although retention over 100 generations was between only 10 – 30%. Most interestingly, the combination of two TA systems in a single plasmid led to a significant improvement in retention, up to 60% over 100 generations. While this improved cumulative effect of TA systems has been observed in nature (60), it has not been previously shown with an engineered plasmid, especially with a combination of heterologous and homologous TA systems.

It is important to note that a single generation corresponds to a bacterial duplication, so 10 generations = 2^{10} or ~10^3 bacteria and 100 generations = 2^{100} or ~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 (18-20) or external growth restrictions (69). 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, since loss of the plasmid only reverts the bacteria to their non-GMO probiotic status, such transient GMOs might even be desirable for such applications. Accordingly, by varying the TA system used, the GM-lifetime of these organisms could be tuned. Based on this concept, we introduce a new metric, G_{50}, for characterizing such transient GMOs. 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 gens for the No TA condition up to 110 gens (extrapolated) for the combo system. Further exploration of additional TA systems in 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 (41). 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.
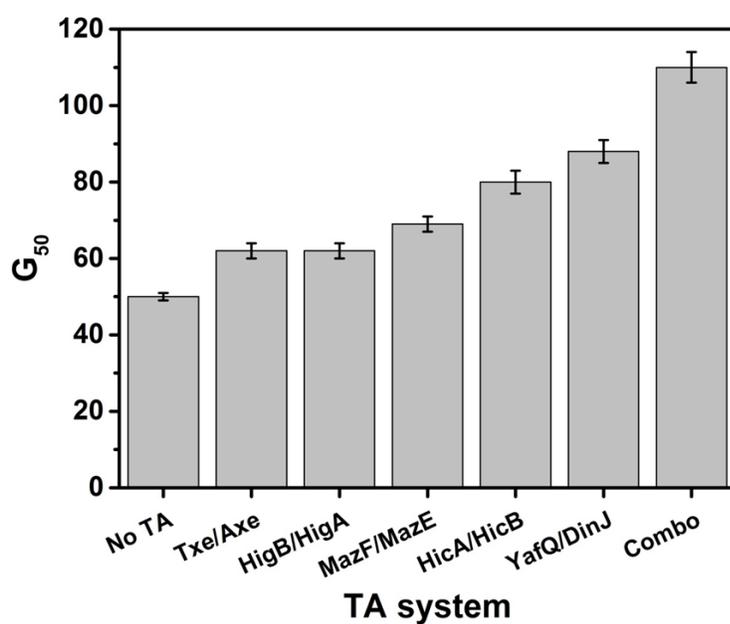
Figure 4. G_{50} values of the different TA systems tested in *L. plantarum.* Combo = MazF/MazE + YafQ/DinJ

DATA AVAILABILITY

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

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

CONFLICT OF INTEREST

The authors declare no conflicts.

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FIGURE LEGENDS

Figure 1. (A) Fluorescence microscopy of P_{tlpA} driven mCherry expression in *L. plantarum* WCFS1 cultivated at 31°C and 39°C for 18 h. Scale bar = 10 μm (B) Flow Cytometry analysis of P_{tlpA}, P_{23}, P_{48}, P_{spp} and P_{Tuf} driven mCherry expression in *L. plantarum* WCFS1 after 18 h incubation at 37°C. (C) Fluorescence Spectroscopy analysis of the P_{tlpA}, 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. (D) Growth rate (OD_{600}) measurement of *L. plantarum* WCFS1 strains containing a control plasmid and P_{tlpA}-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.

Figure 2. (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) 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. (E) Growth rate (OD$_{600}$) of strains with the TA modules, no TA and antibiotic retention over 10 generations at 37°C. In (C) and (E), the solid lines represent mean values and the lighter bands represents SD calculated from three independent biological replicates. Combo = MazF/MazE + YafQ/DinJ

Figure 3. (A) Phylogenetic tree highlighting the distances between species from which various genetic parts have been tested in *L. plantarum*. Purple clade corresponds to Gram-negative bacteria. Green clade corresponds to Gram-positive bacteria. Promoters that have been tested in this study are labelled in blue. Promoters that have been tested by other authors in *L. plantarum* are labelled in green. Orange labels correspond to the TA systems tested in this study. (B) Homology analysis of σ70 RpoD genes from *L. plantarum*, *E. coli*, and *S. typhirmurium*. Height and brightness of the yellow bars indicate the extent to which individual residues are conserved across all 3 bacteria. (C) P$_{tlpA}$ promoter sequence with -35, spacer and -10 regions labelled

Figure 4. G$_{50}$ values of the different TA systems tested in *L. plantarum.* Combo = MazF/MazE + YafQ/DinJ