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RESEARCH ARTICLE

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

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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

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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-Desnoulez 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 (Halbmayr 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 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 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 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 (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 $2–3 \times 10^2$ 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 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™ 0.2 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 (SppIp) 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 μL 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_λ/Em_λ = 587 nm/625 nm) were measured. The *z*-position and gain settings for recording the mCherry fluorescent intensity were set to 19,442 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 (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 μL 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 × *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_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. 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 σ70 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_{xylA}) 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_{tlpA}) 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_{tlpA} 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_{tlpA} 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 pTlpA39 plasmid, with the P_{tlpA} promoter driving expression of mCherry and the codon optimized TlpA repressor being expressed constitutively by the P_{48} promoter (Rud et al., 2006). However, the pTlpA39 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_{tlpA} 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

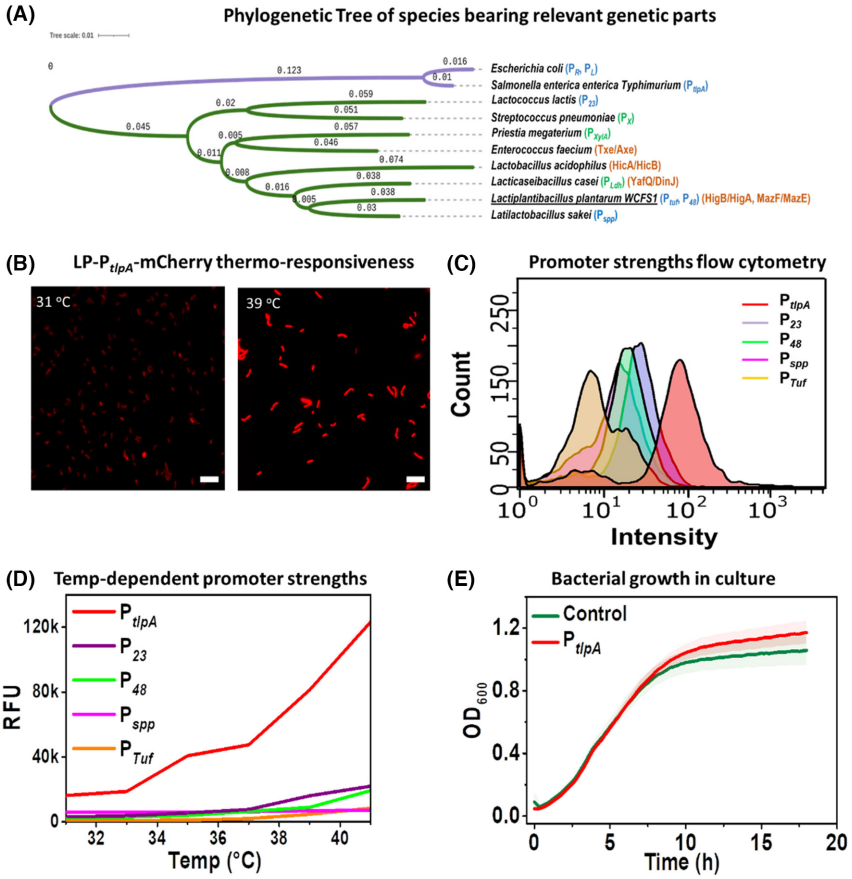

FIGURE 1 (A) Phylogenetic tree highlighting the distances between species from which various genetic parts have been tested in *Lactiplantibacillus plantarum*. Purple clade corresponds to Gram-negative bacteria. Green clade corresponds to Gram-positive bacteria. Promoters tested in this study are labelled 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_{tlpA} 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_{tlpA}, 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_{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. (E) 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.

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 overburden 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 σ70 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 σ70 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

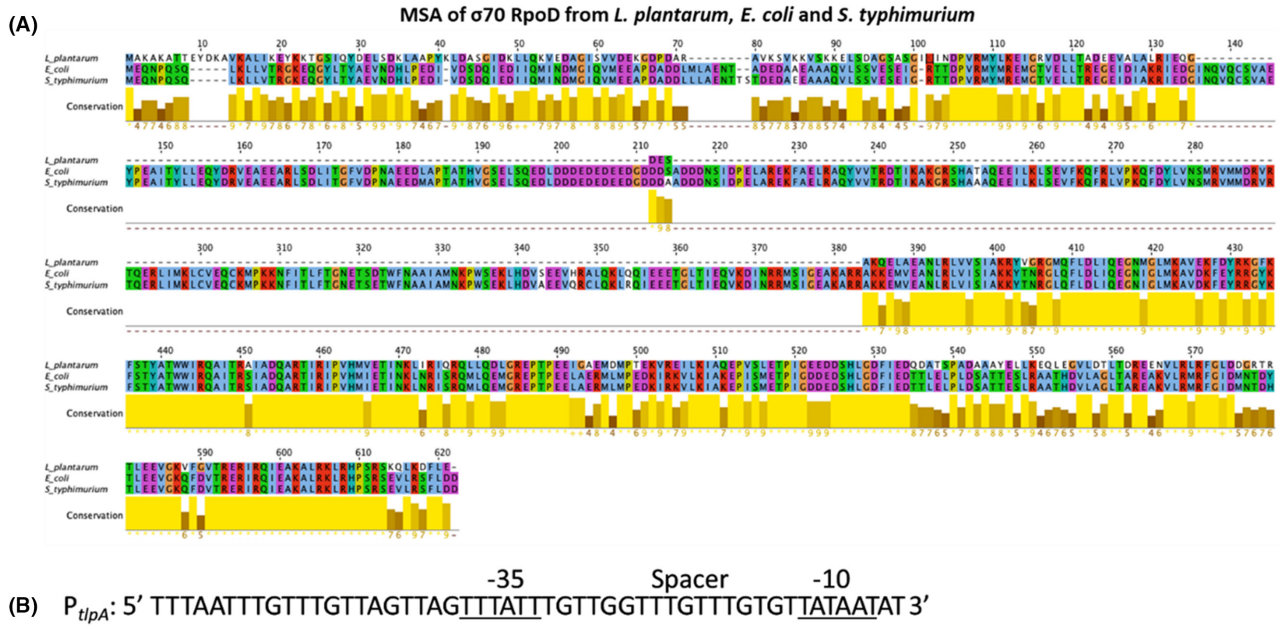

FIGURE 2 (A) Homology analysis of σ70 RpoD genes from *Lactiplantibacillus plantarum*, *Escherichia coli* and *Salmonella typhimurium*. Height and brightness of the yellow bars indicate the extent to which individual residues are conserved across all 3 bacteria. (B) P_{tlpA} promoter sequence with −35, 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 (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 (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}

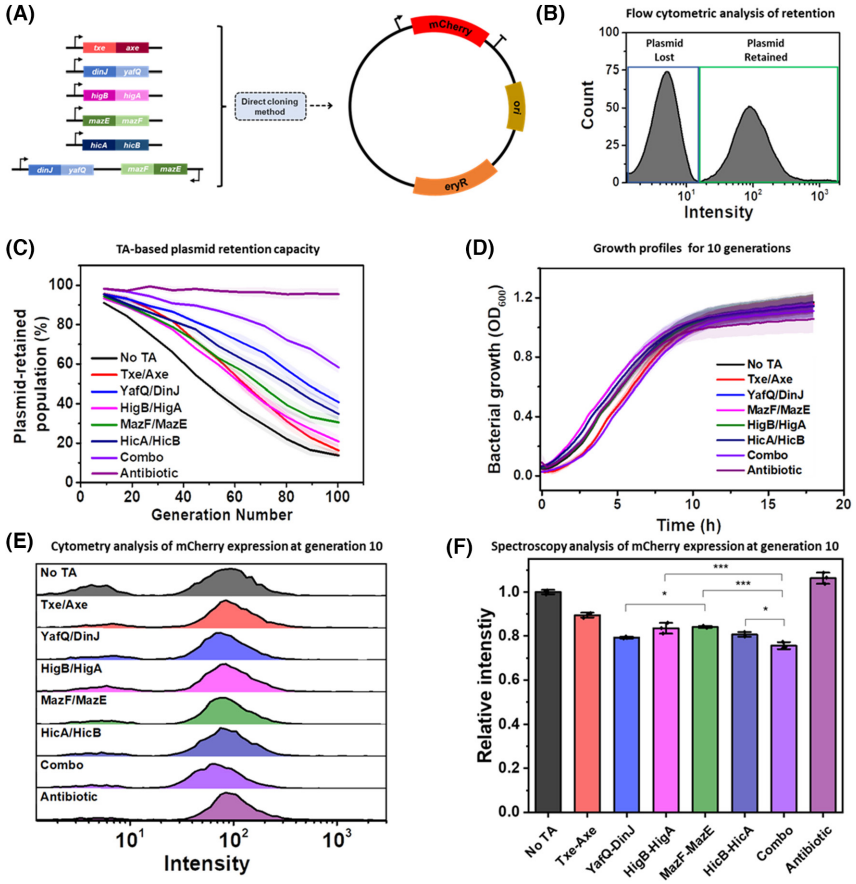

FIGURE 3 (A) Schematic representation of cloning the different TA genetic modules into the P_{tlpA}-mCherry plasmid. (B) Sample flow Cytometry histogram plot of the P_{tlpA}-mCherry plasmid containing strain without any TA module or selection pressure after 50 generations of serial passaging in the absence 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 (OD_{600}) 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 represents 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 ~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 (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 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.

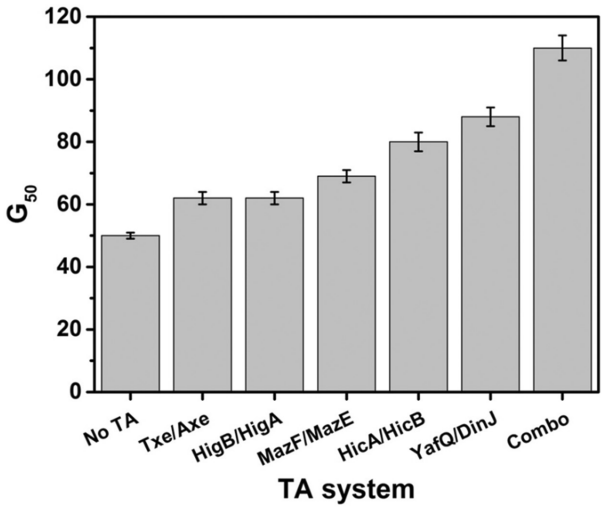

FIGURE 4 G_{50} values of the different TA systems tested in *Lactiplantibacillus plantarum*. Combo = MazF/MazE + YafQ/DinJ.

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).

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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.

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SUPPORTING INFORMATION

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