Identification and evaluation of a panel of strong constitutive promoters in *Listeria monocytogenes* for improving the expression of foreign antigens

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Abstract

Attenuated *Listeria monocytogenes* could be a potential vaccine vector for the immunotherapy of tumors or pathogens. However, the lack of reliable promoters has limited its ability to express foreign antigens. In the present study, 21 promoters were identified from *Listeria monocytogenes* through RNA-seq analysis under two pH conditions of pH 7.4 and pH 5.5. Based on the constructed fluorescence report system, 7 constitutive promoters exhibited higher strength than P_{help} (1.8-fold to 5.4-fold), a previously reported strong promoter. Furthermore, the selected 5 constitutive promoters exhibited higher UreB production activity than P_{help} (1.1-fold to 8.3-fold). Notably, a well-characterized constitutive promoter P_{18} was found with the highest activity of fluorescence intensity and UreB production. In summary, the study provides a panel of strong constitutive promoters for *Listeria monocytogenes* and offers a theoretical basis for mining constitutive promoters in other organisms.

Key points

• *Twenty-one promoters were identified from L. monocytogenes through RNA-seq.*
• *Fluorescent tracer of L. monocytogenes (P_{18}) was performed in vitro and in vivo.*
• *A well-characterized constitutive promoter P_{18} could improve the expression level of a foreign antigen UreB in L. monocytogenes*

Keywords *L. monocytogenes* · Constitutive promoter · RNA-seq · Acid stress

Introduction

Listeria monocytogenes is a gram-positive bacterium that could stimulate innate response and cellular immune responses including CD4^{+} T cells and CD8^{+} T cells (Guleria and Pollard 2001; Mittrucker et al. 2000). The *L. monocytogenes* Δ*actA/inlB* strain, an attenuated *L. monocytogenes*, has been widely applied as a vaccine vector in microbial immunotherapy for controlling tumor and infectious diseases due to its endogenous pathway of antigen processing (Le et al. 2012; Paterson and Johnson 2004; Stark et al. 2009; Yoshimura et al. 2007). The promoter of *hly* gene (P_{hly}) has been used to elicit the expression of foreign antigens during the construction of vaccine strains of attenuated *L. monocytogenes* (Chen et al. 2012; Yang et al. 2014). In the previous study, P_{hly} was found inadequate to meet the expression levels of foreign antigens and further provoked a specific immune response (Ding et al. 2019). Although P_{help} modified from P_{hly} has higher promoter strength, it still needs further improvement (Riedel et al. 2007). In the previous studies, the live vaccine based on *L. monocytogenes* vector was forced to bear the acidic environments like the macrophage phagosome and tumor microenvironment (Beauregard et al. 1997; Gallagher et al. 2008). In another study, the oral *L. monocytogenes* vector vaccine was forced to encounter the stomach acid environment (Spears et al. 2011). However, this action requires the promoters to be stable enough to adapt to the acidic environments. Thus, one of the key limitations is the lack of a panel of well-characterized

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promoters to regulate the expression of foreign antigens in *L. monocytogenes*.

Constitutive promoters have been widely adopted for fine-tuning the expression levels of key genes in metabolic engineering and synthetic biology (Li et al. 2015; Sun et al. 2012). Usually, constitutive promoters are screened from the promoters of essential genes due to their constant transcript levels (Partow et al. 2010). In this regard, developing a method based on RNA-seq analysis could be helpful to obtain native promoters according to different transcriptional strengths. In recent years, this method has attracted much attention in the field of synthetic biology to construct the native promoter libraries of various industrial microorganisms, such as *Streptomyces albus*, *Streptococcus thermophilus*, and *Pseudomonas* (Jin et al. 2020; Kong et al. 2019; Luo et al. 2015). A panel of strong constitutive promoters could be obtained by selecting the potential promoters through RNA-seq analysis and fluorescent reporter genes.

With the emerging interest in L. monocytogenes, the development of transcriptome sequencing technology offers an opportunity to mine reliable constitutive promoters. The present study identified a panel of constitutive promoters based on the systematic analysis of transcriptome data of L. monocytogenes cultivated in different pH conditions. The green fluorescent protein (GFP) reporter was used for identifying the characterization of these promoters, and the activity of a well-characterized promoter was evaluated in vitro and in vivo. Based on our further needs, several promoters with different strengths were selected for experimentally evaluating UreB production, a widely used antigen against Helicobacter pylori. These constitutive promoters enriched the promoter library of L. monocytogenes, which might add value to metabolic engineering and synthetic biology in this genus.

Materials and methods

Strains, plasmids, cells, and medium

The bacterial strains and plasmids used in this study are listed in Supplemental Table S1. *Escherichia coli* DH5α was cultured on Luria-Bertani broth (LB) medium at 37 °C. It was applied for plasmid construction and propagation. *L. monocytogenes* wild-type EGD-e (ATCC BAA-679) and EGD-eΔ*actA/inlB* were cultured in brain heart infusion medium (BHI, Land bridge, Beijing, China) at 37 °C. Macrophage cell line Raw264.7 was purchased from Shanghai Cell Bank (Shanghai, China), and cultured in RPMI-1640 medium (Sigma, Saint Louis, USA).

Growths of *L. monocytogenes* under different pH conditions

The overnight-grown wild-type EGD-e strain was collected by centrifugation at 5000×g at 4 °C, washed with PBS (10 mM, pH 7.4), and then adjusted to 1.0 at OD_{600} nm. The cultures were then diluted 1:100 in fresh BHI broth (pre-adjusted to pH 4.5, 5.0, 5.5, 6.0, or 7.4 using hydrochloric acid, respectively) to obtain 0.2 at OD_{600} nm ($3.6×10^8$ CFU/mL, CFU: colony-forming units). The growth curve was assessed for 12 h at 37 °C with shaking. Afterward, the bacterial solution was taken for optical density determination at OD_{600} nm and 1-h interval using a SpectraMax M2 microplate reader (Molecular Devices, San Jose CA, USA). Three independent experiments were performed and the results were reported as average.

Acid resistance determination of pre-acid-treated *L. monocytogenes*

Based on the growth of wild-type EGD-e under different pH conditions, eight types of acid pretreatments (pH 4.5, 30 min; pH 4.5, 1 h; pH 5.0, 30 min; pH 5.0, 1 h; pH 5.5, 3 h; pH 5.5, 6 h; pH 6.0, 3 h; and pH 6.0, 6 h) were performed to explore acid resistance of EGD-e to screen the optimal treatment. Since the bacteria were barely growing at pH 4.5 and 5.0, the initial inoculum at these pH conditions was $1.0×10^9$ CFU/mL (OD_{600} = 0.4), while it decreased to $3.6×10^8$ CFU/mL (OD_{600} = 0.2) at pH 5.5 and 6.0. The acid-treated cells were collected by centrifugation at 5000×g for 10 min at 4 °C, washed with PBS, and adjusted to 0.4 at OD_{600} nm. After appropriate dilutions, the cultures were then plated onto BHI agar to count the surviving bacteria. Meanwhile, 1 mL of culture (OD_{600}=0.4) was harvested, washed once with PBS, resuspended in isometric BHI broth (pre-adjusted to pH 3.0, lethal acidity of *L. monocytogenes*), and cultured at 37 °C for 20 min. Afterward, the cultures were collected, resuspended in isometric BHI broth (pH 7.4), and plated onto BHI agar. The bacterial acid resistance was characterized by survival rate, defined as the ratio of survival number after lethal acid treatment to survival number after acid pretreatment. Survival rates were reported as the mean of three independent experiments, each performed in duplicate.

RNA-seq

The wild-type EGD-e strain was cultured in BHI medium under pH 7.4 and 5.5 for 3 h at 37 °C with shaking. The initial inoculum was $3.6×10^8$ CFU/mL. The cultures were collected by centrifugation at 5000×g at 4 °C, and washed with diethyl pyrocarbonate (DEPC) water. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA), and then, ribosomal RNA was removed by a Ribo-Zero Magnetic kit

(Epicentre Biotechnologies, Madison, USA). RNA quality was checked by NanoDrop (Thermo Fisher Scientific, Waltham, USA) followed by RNA degradation and contamination verification on 1% agarose gel. The cDNA libraries were prepared using the TruSeq RNA Library Preparation kit (Illumina, San Diego, USA) and sequenced on Illumina HiSeq 4000 platform (Illumina, San Diego, USA) by Sangon Biotechnology Co. Ltd (Shanghai, China). Clustering and sequencing were performed by Sangon (Shanghai, China) using spliced reads to determine the connectivity.

Raw FASTQ data were trimmed for sequencing adapters and low-quality bases using Trimmomatic v. 030 (Bolger et al. 2014) in double-end mode, retaining reads with a minimum Phred quality score of 20 and a minimum length of 35. The specific parameters used were "SE-phred33 ILLUMINACLIP: LEADING: 20 TRAILING: 20 MINLEN: 35." The adapter sequence used was AGATCGGAAGAGCACACGTCTGAAC (forward) and AGATCGGAAGAGCGTCGTGTAGGGA (reverse). FASTQC v. 0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was run on the trimmed and the raw FASTQ data to verify that trimming improved sequence quality. Bowtie2 v. 2.1.0 (Langmead and Salzberg 2012) was used to align the trimmed data against the *L. monocytogenes* EGD-e reference genome (GenBank accession AL591824.1) using −k 1 and −N 1 options (report 1 alignment per read and allow only 1 mismatch in a seed alignment during multiseed alignment, respectively). Raw read counts were generated using RSeQC (Wang et al. 2012) with the NCBI GFF gene model file for GenBank accession AL591824.1. FPKM (fragments per kilobase of gene model per million mapped reads), considering the effect of sequencing depth and gene length on reads counts, is a commonly used method for evaluating gene expression levels (Trapnell et al. 2010). FPKM was calculated using featureCounts (Liao et al. 2014). Furthermore, FPKM could reflect the promoter strength.

Construction of plasmids and strains

Firstly, the green fluorescent protein (GFP) gene from plasmid pUC57-GFP was amplified and the promoters of 25 highly expressed genes from wild-type EGD-e genome were cloned. Each cloned promoter and amplified *gfp* sequence was inserted into plasmid pERL3 using ClonExpress® MultiS One Step Cloning Kit (Vazyme, Nanjing, China) to create a series of pERL3 derivatives, pERL3-P_1-GFP to pERL3-P_{25}-GFP. The widely used promoters P_{hly} and P_{help} were applied as controls to generate pERL3-P_{hly}-GFP and pERL3-P_{help}-GFP using the same method. All primers used in this study are listed in Supplemental Table S2. The promoter sequences are listed in Supplemental Table S3. The constructed plasmids

with different promoters were proliferated in *E. coli* DH5α, identified by sequencing, and transformed into wild-type EGD-e using electroporation.

Measurement of fluorescence intensity of GFP

The constructed strains were cultured in BHI medium at pH 7.4 or 5.5 at 37 °C for 12 h, 24 h, 36 h, and 48 h, respectively. The cultures were collected by centrifugation, washed twice with 20 mM Tris-HCl, and resuspended. After adjusting to appropriate absorption at 600 nm (OD_{600}), the fluorescence intensity of GFP (excitation at 485 nm and emission at 525 nm) was measured in microtiter plates (Assay Plate, 96 wells, Black Polystyrene; Corning, New York, USA) using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA).

Fluorescent tracer of *L. monocytogenes* in macrophage RAW264.7

EGD-e and EGD-eΔ*actA/inlB* carrying the plasmid pERL3-P_{18}-GFP were chosen for fluorescent tracer of *L. monocytogenes* in macrophage RAW264.7. Approximately $2×10^5$ RAW264.7 cells were seeded on cover glass (Thermo Fisher Scientific, Waltham, USA) in a 12-well plate per well overnight. The cells were infected with bacteria at the multiplicity of infection (MOI) of 100 for 2 h. After washing thrice, gentamicin was added for 30 min to eliminate the extracellular bacteria. Then, the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, and permeabilized in 0.1% TritonX-100 in PBS for 5 min. Actin-stain 488 (red, Cytoskeleton Inc., Yeasen, Shanghai, China) and DAPI (4′,6-diamidino-2-phenylindole, blue, H-1200, Vector Lab., Yeasen, Shanghai, China) were utilized to stain actin and label the cell nucleus, respectively. The images were captured and observed on a Leica DM 2500 fluorescence microscope (Leica, Solms, Germany).

Fluorescent tracer of *L. monocytogenes* in vivo

In vivo imaging of EGD-eΔ*actA/inlB* carrying fluorescent reporting plasmid was tested in traditional C57BL/6 mice. P_{18}, as the strongest promoter, was selected to observe its activity in vivo. EGD-eΔ*actA/inlB* carrying the plasmid pERL3-P_{18}-GFP was cultured in BHI medium at 37 °C overnight, washed, and resuspended using PBS. Mice were inoculated with EGD-eΔ*actA/inlB* ($5×10^7$ CFU/mouse) by intravenous injection. Three days post-inoculation, the mice were anesthetized with isoflurane and imaged in a PerkinElmer IVIS Lumina II system (PerkinElmer, Waltham, USA).

Measurement of urease B subunit (UreB) production

The promoters with different strengths of P_{18}, P_7, P_{12}, P_9, P_{24}, P_{help}, and P_{hly} were selected for determining their abilities to express foreign antigen UreB. The plasmids pERL3-P_{18}/P_7/P_{12}/P_9/P_{24}/P_{help}/P_{hly}-UreB were constructed and transformed into EGD-e$\Delta actA/inlB$. The constructed strains were cultured in BHI medium at 37 °C overnight, and the total soluble proteins were extracted by an ultrasonic crushing apparatus. The expression levels of UreB under different promoters in EGD-e$\Delta actA/inlB$ were evaluated by western blotting probed with a mouse anti-UreB polyclonal antibody. Meanwhile, the purified UreB proteins with gradient dilution as standards were set for quantitative analysis of UreB in EGD-e$\Delta actA/inlB$ by gray scan using ImageJ. In this regard, the quantitation of UreB was based on total protein normalization and the proteinic concentration was assayed by BCA Kit (Solarbio, Beijing, China).

Statistical analyses

All data were analyzed with the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA) and expressed as mean ± standard deviation (SD). Statistical significance was analyzed using one-way ANOVA. $P < .05$ was considered statistically significant (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$; ns: not significant).

Results

Growths of *L. monocytogenes* under different pH conditions

Acid stress could defer or terminate the growth of *L. monocytogenes*. As depicted in Fig. 1a, the growth rate of wild-type EGD-e was inhibited by acid stress compared with the normal condition (pH 7.4). At pH 6.0, the growth was slightly inhibited, which delayed its stationary phase by 4 h, and the bacterial concentration of the stable period was about 0.68 at OD_{600} nm, which was still close to the growth at pH 7.4 (0.71). The growth at pH 5.5 was moderately inhibited, while the growth at pH 5.0 or 4.5 was severely inhibited.

The optimal acid stress treatments for *L. monocytogenes* RNA-seq analysis

Acid-inducing tolerance response at sublethal conditions could improve the survival rate of *L. monocytogenes* at lethal acid conditions (Cheng et al. 2015; Stack et al. 2007). In this study, the survival rate was to characterize bacterial acid resistance. As depicted in Fig. 1b, the surviving bacteria after acid pretreatments (pH 5.5, 3 h; pH 6.0 3 h) were 9.13 ±0.02×10^8 and 9.33±0.01×10^8 CFU/mL, respectively, which were close to that under the normal condition (1.0×10^9 CFU/mL). The lethality of bacteria after these two acid stress pretreatments was extremely low. When the acid induction (pH 5.5 and 6.0) time was extended to 6 h, the surviving bacteria were reduced to 4.70±0.01×10^8 and 4.43±0.02×10^8 CFU/mL,
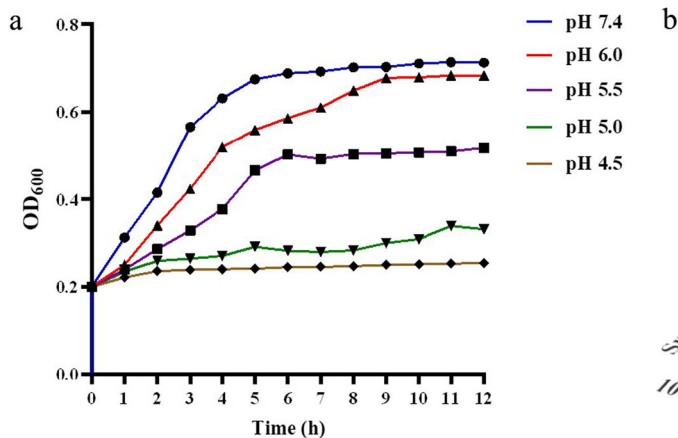

Fig. 1 The screening of acid stress treatments for *L. monocytogenes* RNA-seq analysis. **a** The growth characteristics of *L. monocytogenes* at different pH conditions. The initial inoculation amount of *L. monocytogenes* was 3.6×10^8 CFU/mL. **b** Acid resistance determination of *L. monocytogenes* with different acid stress treatments. The viable bacteria number under eight kinds of different acid stress (pH 4.5, 30 min;
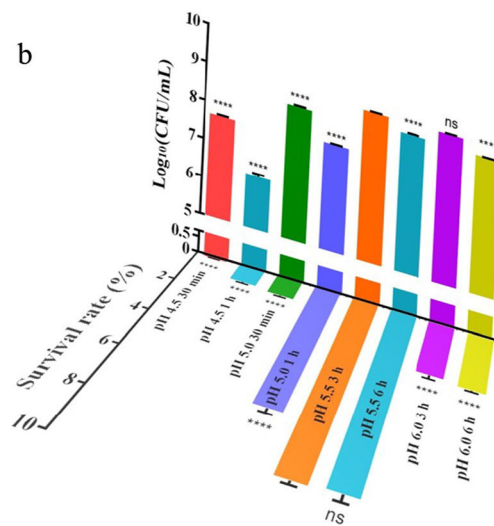
pH 4.5, 1 h; pH 5.0, 30 min; pH 5.0, 1 h; pH 5.5, 3 h; pH 5.5, 6 h; pH 6.0, 3 h; and pH 6.0, 6 h) and survival rate after further acid lethal treatment (pH 3.0, 20min) were measured. Bacteria were adjusted to OD_{600} nm of 0.4 for counting. The error bars indicate the standard deviations from three independent replicates. Statistical significance was compared to the group of pH 5.5, 3 h: ns, no significant, ****, $P < 0.0001$

respectively. The surviving bacteria of the other acid pretreatments (pH 4.5, 30 min; pH 4.5, 1 h; pH 5.0, 30 min; pH 5.0, 1 h) were $4.90\pm0.03\times10^7$, $2.5\pm0.06\times10^6$, $3.33\pm0.02\times10^8$, and $6.60\pm0.01\times10^7$ CFU/mL, respectively, which indicated that the strong-acid treatments have higher lethality even for a shorter time.

Furthermore, the survival rates of acid pretreatments (pH 5.5, 3 h; pH 5.5, 6 h) were $8.98\pm0.12\%$ and $9.01\pm0.24\%$ after lethal acid treatment, indicating the top 2 of all acid pretreatments. Moreover, there was no significant difference between 3 h and 6 h at pH 5.5. A similar trend was observed at pH 6.0, in which the survival rates of acid pretreatments (pH 6.0, 3 h; pH6.0, 6 h) were $3.0\pm0.16\%$ and $3.0\pm0.06\%$. Besides, the survival rates of acid pretreatment (pH 5.0, 1 h) could reach $6.8\pm0.18\%$, while the other acid pretreatments (pH 4.5, 30 min; pH 4.5, 1 h; and pH 5.0, 30 min) were $0.12\pm0.01\%$, $0.97\pm0.05\%$, and $1.22\pm0.06\%$. Overall, the acid pretreatment pH 5.5, 3 h, which did well in both bacterial activity and acid resistance, was selected for *L. monocytogenes* RNA-seq analysis under acid stress.

Characterization of constitutive promoters by RNA-seq

The transcriptional profiling of all 2952 genes in the wild-type EGD-e genome was performed by RNA-seq. Firstly, RNA quality, sequencing saturation, and redundant sequence distribution frequency were analyzed for quality control of transcriptome data (Fig. S1, Supporting Information). For two conditions of pH 7.4 and 5.5, each sample was sorted from highly expressed to low expressed according to the FPKM value. Compared with the normal condition of pH 7.4, 333 genes were up-regulated and 339 genes were down-regulated under the acid stress condition of pH 5.5 (Fig. 2a). The top 2.0% of highly expressed genes under each condition were selected for gene overlap analysis. Of the 59 selected genes, 34 genes were co-expressed under two conditions (Fig. 2b). Since 15 genes were collectively distributed under 6 different operons, 25 genes were selected for cloning their promoters, as listed in Table 1. Additionally, the promoter sequences are listed in Supplemental Table S3.

The fluorescence intensities of GFP under different promoters in *L. monocytogenes* were measured to evaluate the promoter activities with constitutive promoters P_{help} and P_{hly} as references. According to the schematic diagram of plasmid construction (Fig. 2c), 21 fluorescent reporting plasmids with different promoters were successfully constructed and transformed into wild-type EGD-e. The strength of each promoter was similar at 12 h, 24 h, 36 h, and 48 h under the condition of pH 7.4 (Fig. 2d), confirming them to be the constitutive promoters. Seven promoters (P_7, P_8, P_9, P_{12}, P_{18}, P_{23}, and P_{24}) exhibited high activities from 1.8-fold to 5.4-fold than P_{help} in EGD-e. Compared with pH 7.4, the fluorescence intensity of

each well-characterized promoter had varying degrees of decline under pH 5.5 (Fig. 2e). Nonetheless, promoter P_{18} still showed higher activity of 5.3-fold than P_{help}.

Fluorescent tracer of *L. monocytogenes* in vitro and in vivo

To assess the application potential of the well-characterized promoter, *L. monocytogenes* carrying fluorescent report plasmid with the strongest constitutive promoter P_{18} was selected for the tracer of bacteria in vitro and in vivo. Figure 3 a depicts the invasion of EGD-e and EGD-e$\Delta actA/inlB$ (pERL3-P_{18}-GFP) in macrophage RAW264.7. Furthermore, the fluorescence signal of EGD-e$\Delta actA/inlB$ (pERL3-P_{18}-GFP) was observed, which could be assigned to livers (Fig. 3b), the major sites of listerial infections in mice (Radoshevich and Cossart 2018) after dissection. This result confirmed the high activity of promoter P_{18} also in vivo.

Measurement of UreB production

Attenuated *L. monocytogenes* could improve the expression of foreign antigens by a strong promoter. For this purpose, promoters P_{18}, P_7, P_{12}, P_9, and P_{24} were selected to verify the expressing ability of the antigen UreB, a typical antigen of *H. pylori* with promoters P_{help}, P_{hly}, and P_{None} (no promoter) as controls. As depicted in Fig. 4a, the expression of UreB in EGD-e$\Delta actA/inlB$ under different promoters could be determined by anti-UreB polyclonal antibody using western blotting. This result indicated that promoter P_{18} still had the highest ability to express UreB. With the gray-scale analysis of purified UreB, a quantitative analysis of UreB production was performed under different promoters in EGD-e$\Delta actA/inlB$ (Fig. 4b). Compared with promoter P_{help}, promoters P_{18}, P_7, P_{12}, P_9, and P_{24} showed high UreB production from 1.1-fold to 8.3-fold. This result indicated that promoter P_{18} still performed noticeably well in the expression of the UreB antigen.

Discussion

Attenuated *L. monocytogenes*, as a vaccine vector, exerts excellent application prospects in microbial immunotherapy. However, the low expression level of foreign antigen could not induce a strong specific immune response in the previous study (Ding et al. 2019). In another study, a strong promoter was used to improve the expression levels of foreign antigens to enhance the efficacy of the modified vaccinia virus Ankara (Becker et al. 2014). Therefore, mining the strong promoters from *L. monocytogenes* might be the key to enhance the efficacy of vaccines based on an attenuated *L. monocytogenes* vector.

Table 1 The information of 25 promoters from wild-type EGD-e selected by RNA-seq

Number	Gene	Length	CDS product	FPKM	
				pH 5.5	pH 7.4
1	lmo1634	501	Aldehyde-alcohol dehydrogenase	13,392.83	52,369.24
2	lmo2653	108	Elongation factor Tu	41,554.43	23,758.48
3	lmo2637	447	Hypothetical protein	5134.74	48,137.68
4	lmo2459	441	Glyceraldehyde-3-phosphate dehydrogenase	23,215.88	18,740.49
5	lmo0045	278	Single-strand binding protein	21,808.48	17,971.34
6	lmo1468	197	Hypothetical protein	17,174.67	22,337.2
7	lmo2654	192	Elongation factor G	24,542.36	10,582.53
8	lmo1439	300	Superoxide dismutase	27,940.96	4269.49
9	lmo2556	168	Fructose-1,6-bisphosphate aldolase	8363.89	17,402.44
10	lmo1541	156	Hypothetical protein	11,821.15	11,596.22
11	lmo1364	198	Cold-shock protein	10,301.34	7921.58
12	lmo1003	306	Phosphotransferase system enzyme I	6824.64	10,706.6
13	lmo2612	495	Preprotein translocase subunit	10,711.67	6707.29
14	lmo2615	426	30S ribosomal protein S5	10,020.13	5383.06
15	lmo2610	386	Translation initiation factor IF-1	9943.54	5379.53
16	lmo2455	135	Enolase	6747.16	7920.58
17	lmo2625	648	50S ribosomal protein L16	10,782.57	3878.09
18	lmo0248	126	50S ribosomal protein L11	6298.52	7050.71
19	lmo1847	842	Metal ABC transporter	8066.96	4621.05
20	lmo1787	207	50S ribosomal protein L19	6125.42	6160.1
21	lmo2621	426	50S ribosomal protein L24	8505.77	3758.96
22	lmo2016	288	Cold-shock protein	4096.5	8136.36
23	lmo0210	293	L-lactate dehydrogenase	4121.04	7400.75
24	lmo2411	1223	Hypothetical protein	6852.28	4470.22
25	lmo0250	247	50S ribosomal protein L10	4045.77	5270.73

The constitutive promoters play significant roles in fine-tuning the gene expression in metabolic engineering and synthetic biology. The promoter with a proper strength could maximize the target production and also maintain the biological activity of the host engineering bacteria, suggesting that a mature engineering bacteria system needs a constitutive promoter library (Gilman and Love 2016). Since *L. monocytogenes* is not an ordinary engineered bacterium, the lack of availability of constitutive promoters in *L. monocytogenes* urges to enrich this toolbox.

The effect of acidic environments on the activities of some promoters of engineering bacteria results in the excitation of the pH-induced promoters, such as base-responsive promoter P-atp2 from *Corynebacterium glutamicum* (Barriuso-Iglesias et al. 2013) or acid-responsive promoter P-asr from *E. coli* (Seputiene et al. 2003). When *L. monocytogenes*, as a vaccine vector, encounters the acid environment in the host, the promoter will maintain certain activity under acid stress conditions. Different acidity and processing time could develop different acid resistance in *L. monocytogenes* (Cheng et al. 2015). In this study, the treatment of pH 5.5 for 3 h was selected as the acid stress condition of RNA-seq from all eight

acid stress treatments. Under this treatment, *L. monocytogenes* with the strongest acid resistance could filter out the down-regulated genes as much as possible, thereby preventing the inactivity or low activity of screened promoters in the acid environment.

Recently, RNA-seq analysis has been widely adopted to construct the promoter library for engineered microorganisms, such as *Pseudomonas mendocina*, *Streptomyces albus*, and *Saccharomyces cerevisiae* (Gao et al. 2020; Luo et al. 2015; Zhao et al. 2019). Based on the RNA-seq and co-expression analysis under two conditions of pH 7.4 and pH 5.5, the characteristics of 21 promoters were identified by GFP reporter. Under the normal condition of pH 7.4, the activities of 7 promoters P_7, P_8, P_9, P_{12}, P_{18}, P_{23}, and P_{24} were higher than of P_{help}. Additionally, under the acid stress of pH 5.5, the fluorescence intensity of all promoters showed a decreasing trend compared with pH 7.4. However, 2 promoters P_{12} and P_{18} exhibited higher fluorescence intensity than P_{help}. To the best of our knowledge, this is the first well-characterized genome constitutive promoter study in *L. monocytogenes*, which provides a genetic tool for *L. monocytogenes*.
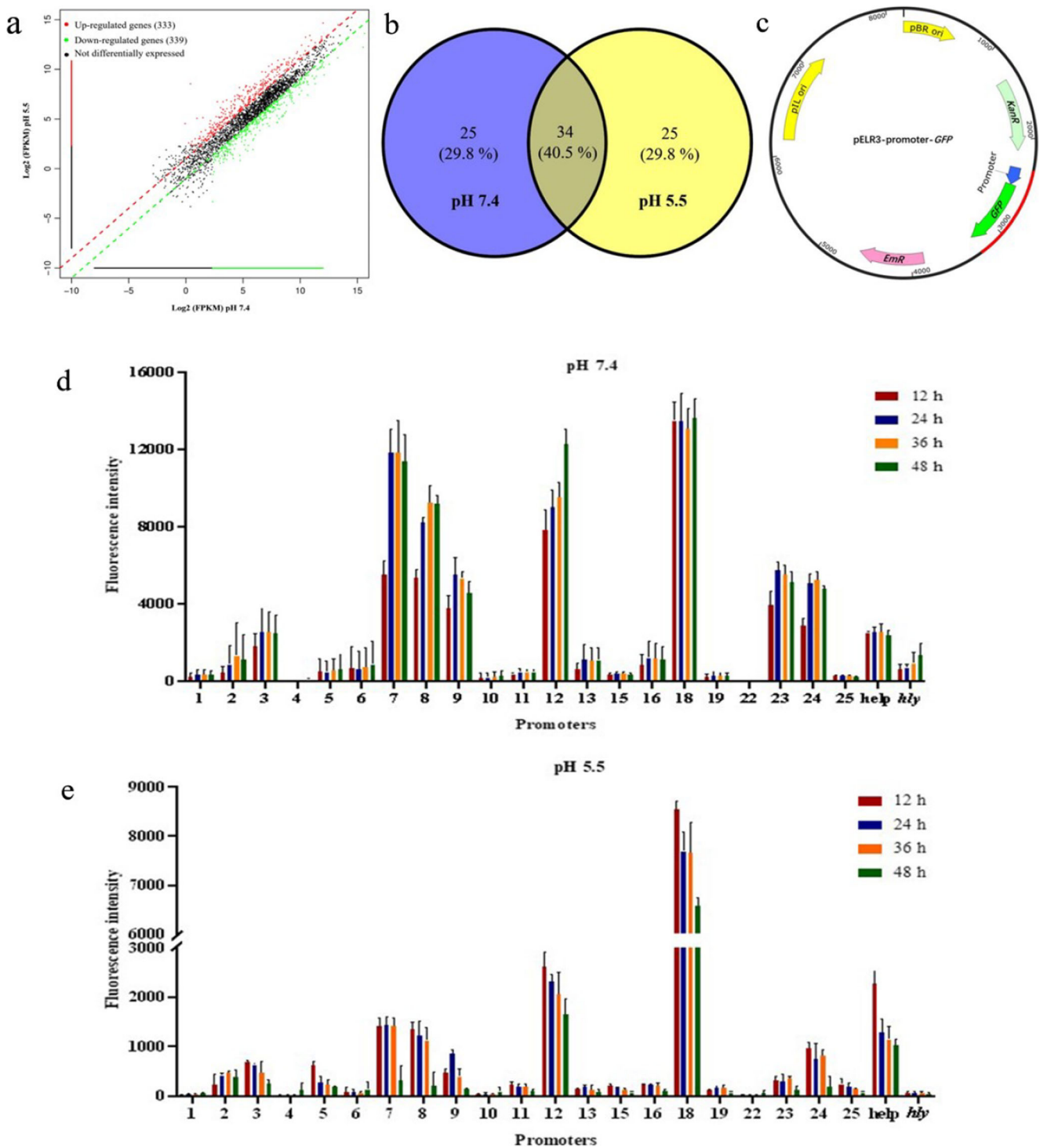
Fig. 2 Characterization of constitutive promoters via RNA-seq. **a** Scatter-plot of gene expression level at pH 5.5 and 7.4 via RNA-seq. The red spots indicate significantly up-regulated genes; the green spots indicate significantly down-regulated genes; black spots indicate nonsignificant genes. **b** Venn diagram of the number of the top 2.0% of the most highly expressed genes under two conditions by RNA-seq. **c** Schematic diagram of the promoter-GFP cassette for promoter screening. The sequence of the promoter-GFP cassette is highlighted in red color. Fluorescence intensity of GFP by different constitutive promoters in wild-type EGD-e at pH 7.4 (**d**) and 5.5 (**e**), respectively. The error bars indicate the standard deviations from three independent replicates

Furthermore, the well-characterized promoter P_{18} was evaluated in vitro and in vivo using *L. monocytogenes* strains with the fluorescent reporter plasmid. Interestingly, the bacterial invasion in macrophage RAW264.7 and the bacterial enrichment in the liver of mice were observed, which confirmed the potential application of constitutive promoter P_{18}.
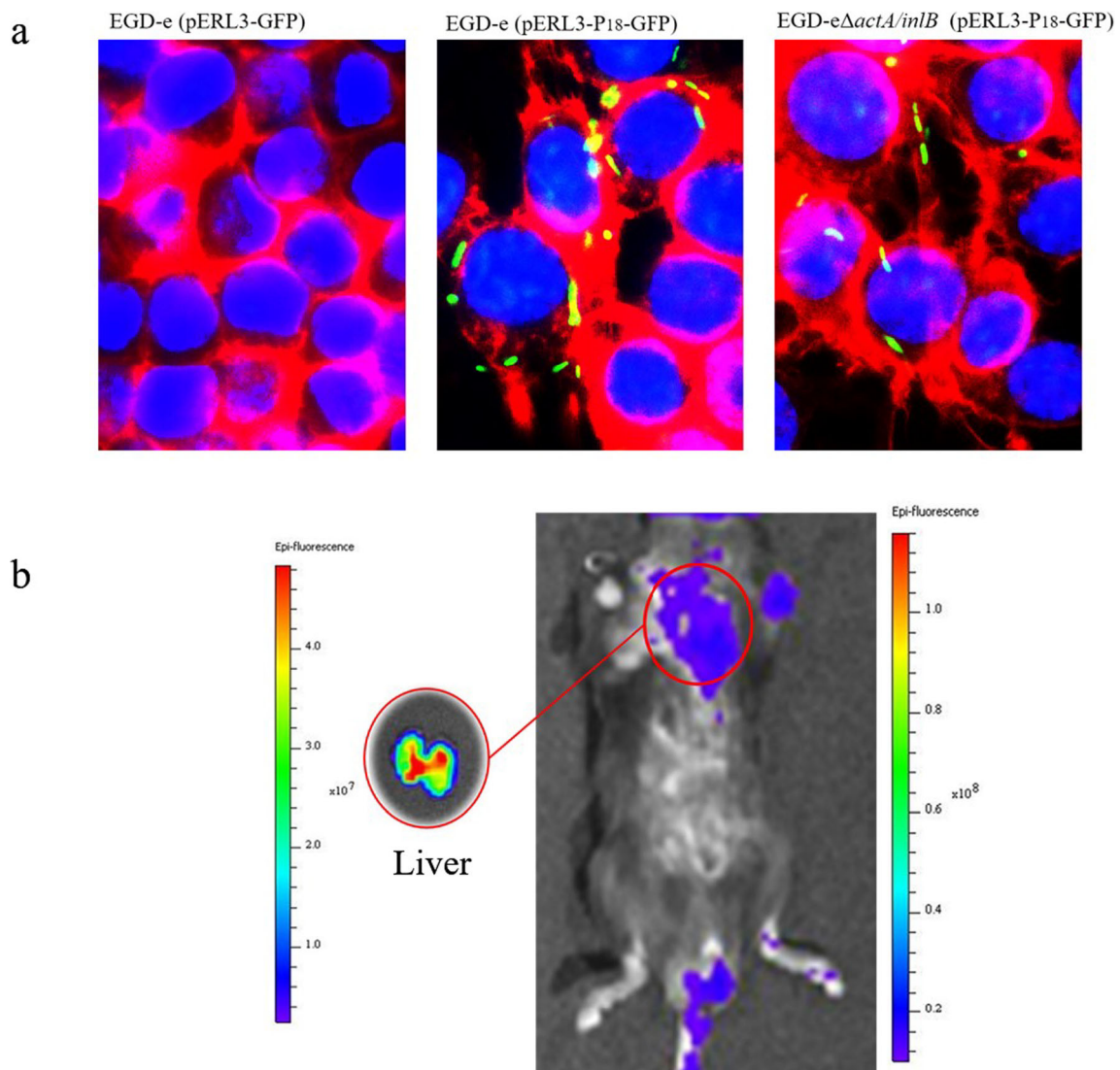
Fig. 3 Fluorescent tracer of *L. monocytogenes* in vitro and in vivo. **a** Fluorescent tracer of *L. monocytogenes* in macrophage RAW264.7. **b** Fluorescent tracer of *L. monocytogenes* in vivo

Compared with the other fluorescence reporter *L. monocytogenes* strains in the previous studies (Fortineau et al. 2000; Freitag and Jacobs 1999), the fluorescence reporter *L. monocytogenes* strain based on P_{18} exhibited better functionality in vivo. During vaccine development based on microbial carrier, selecting an appropriate promoter is the key to optimize the expressions of foreign antigens (Chapman and Rybicki 2019; Galvin et al. 2000; Stojanov et al. 2020). In this study, the selected constitutive promoters P_{18}, P_7, P_{12}, P_9, and P_{24} with different strengths exhibited high activities during the production of the specific antigen UreB. Due to the dependency of antigen dose and antibody response, a higher antigen dose imposes a stronger immune response of specific antibodies (Li et al. 2020). Therefore, it is worth noting that the performance of constitutive promoter P_{18} is still far ahead in antigen production in *L. monocytogenes*. Compared with the previous studies that used P_{hly} as the promoter (Chen et al. 2014; Gunn et al. 2001; Singh et al. 2005), this study is significant for improving the efficiency of vaccines based on a *L. monocytogenes* vector.

In conclusion, 21 promoters were identified from *L. monocytogenes* by RNA-seq analysis under two conditions of pH 7.4 and pH 5.5. Based on the constructed fluorescent reporter system, 7 constitutive promoters showed higher activity than P_{help}. Additionally, 5 well-characterized constitutive promoters were used to activate a foreign UreB biosynthetic pathway successfully. In particular, the activity of a promoter in practical applications and the unprecedented antigen production of P_{18} were verified by the tracer of fluorescent reporter strains and UreB production, which could significantly enhance the effectiveness of live vector vaccines. This study provides a useful toolkit for *L. monocytogenes* and a
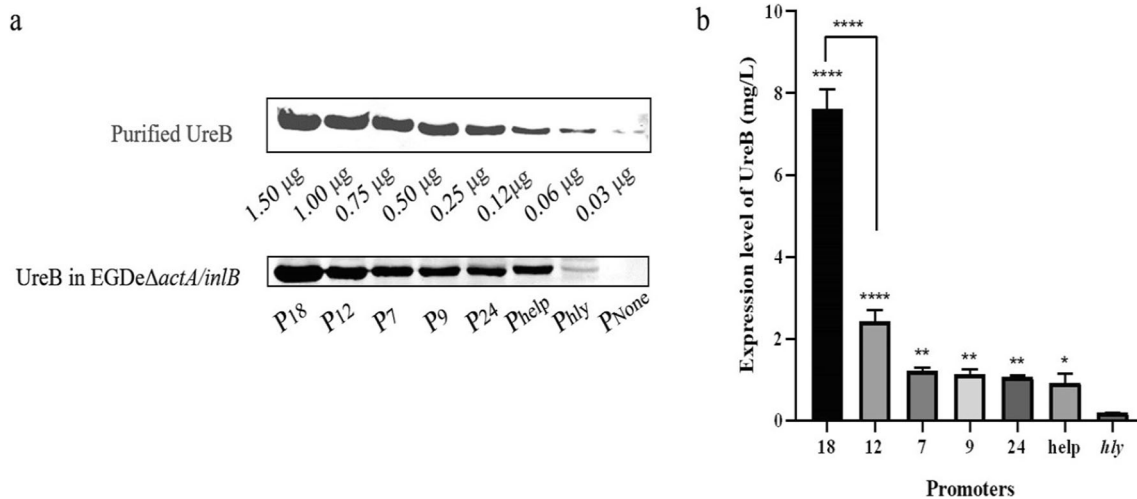
Fig. 4 Measurement of UreB production. **a** Measurement of UreB production in EGD-e$\Delta actA/inlB$ by western blotting. **b** Quantitative determination of UreB production based on the gray scan. The error bars indicate the standard deviations from three independent replicates. Statistical significance was compared to the group of P$_{hly}$: ns, no significant; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$

theoretical basis for mining constitutive promoters in other organisms.

Data availability Raw data of RNA-seq are deposited in the National Center for Biotechnology Information (NCBI) database under the SRA Study accession number PRJNA703423. The other data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Ethics approval This research does not contain any studies with human participants performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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