1 Identification and Evaluation of a Panel of Strong Constitutive 2 Promoters in *Listeria monocytogenes* for Improving the Expression 3 of Foreign Antigens

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11
12 **Abstract:** Attenuated *Listeria monocytogenes* (*L. monocytogenes*) could be used as
13 a vaccine vector for immunotherapy of tumors or pathogens. However, the lack of
14 reliable promoters limits its ability to express foreign antigens. In this work, 21
15 promoters from *L. monocytogenes* were identified by RNA-seq analysis under two
16 conditions of pH 7.4 and pH 5.5. Based on the constructed fluorescence report system,
17 7 constitutive promoters showed higher strength than that of P_{help}, a previously
18 reported strong promoter. Further, the selected 5 constitutive promoters also showed
19 high activity in the production of UreB, a widely used antigen against *Helicobacter*
20 *pylori* (*H. pylori*). In particular, a well-characterized constitutive promoter P₁₈, which
21 performed best in both fluorescence intensity and UreB production, was proved to be
22 highly active in vitro and in vivo. In summary, we provide a useful promoter library
23 for *Listeria* species and offer a reference for constitutive promoter mining in other

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24 organisms.

25 **Key points**

- 26 ● 21 promoters from *L. monocytogenes* were identified by RNA-seq.
- 27 ● Fluorescent tracer of *L. monocytogenes* (P₁₈) was performed in vitro and in vivo.
- 28 ● A well-characterized constitutive promoter P₁₈ could improve the expression level
- 29 of a foreign antigen UreB in *L. monocytogenes*

30

31 **Key Words** : *L. monocytogenes*; Constitutive promoter; RNA-seq; Acid stress

32

33 **Introduction**

34 *L. monocytogenes* is a gram-positive bacterium that could stimulate innate response
 35 and cellular immune responses including CD4⁺ T cells and CD8⁺ T cells[1, 2]. Due to
 36 the endogenous pathway of antigen processing, attenuated *L. monocytogenes* such as
 37 *L. monocytogenes*Δ*actA/inlB* strain was widely applied as a vaccine vector in the
 38 research of microbial immunotherapy for controlling tumor and infectious
 39 diseases[3-6]. To date, in the construction of vaccine strain of attenuated *L.*
 40 *monocytogenes*, the promoter of *hly* gene (P_{*hly*}) was generally used to elicit the
 41 expression of foreign antigens[7, 8]. In the previous study, we found that P_{*hly*} was not
 42 strong enough to meet the expression levels of the foreign antigen and further provoke
 43 a specific immune response[9]. P_{help}, modified from P_{*hly*}, has higher promoter strength,
 44 but it is still to be improved[10]. Besides, the live vaccine based on *L. monocytogenes*
 45 vector is forced to bear the acidic environments like the macrophage phagosome and
 46 tumor microenvironment[11, 12]; the oral *L. monocytogenes*-vector vaccine also
 47 encounters stomach acid environment[13]. This requires the promoters to be stable
 48 enough to adapt to the acidic environments. Thus, one of the key limitations is the
 49 lack of a panel of well-characterized promoters to regulate the expression of foreign
 50 antigen in *L. monocytogenes*.

Constitutive promoters are widely used for fine-tuning the expression levels of key genes in metabolic engineering and synthetic biology[14, 15]. Usually, constitutive promoters are screened from the promoters of essential genes because of their constant transcript levels[16]. Hence, a method based on RNA-seq analysis could be applied to obtain native promoters according to different transcriptional strength. More recently, in the field of synthetic biology, this method has been developed to construct the native promoter libraries of various industrial microorganisms such as *Streptomyces albus*, *Streptococcus thermophilus*, and *Pseudomonas*[17-19]. A panel of strong constitutive promoters could be obtained by selecting the potential promoters based on RNA-seq analysis and evaluating their activities based on fluorescent reporter genes.

With the increasing interest in *L. monocytogenes* in microbiological immunotherapy of tumors and pathogens, the development of transcriptome sequencing technology offers an opportunity to mine reliable constitutive promoters. In this work, a panel of constitutive promoters was identified 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 the production of UreB, a widely used antigen against *H. pylori*. These constitutive promoters enriched the promoter library of *Listeria*, which should be of great value for metabolic engineering and synthetic biology in this genus.

2 Materials and methods

2.1 Strains, plasmids, and medium

All bacteria strains and plasmids used in this study were listed in Table S1. *Escherichia coli* DH5 α was grown on Luria-Bertani broth (LB) medium at 37 °C. It

was applied for plasmid construction and propagation. *L. monocytogenes* wild-type EGD-e and EGD-e $\Delta actA/inlB$ were cultured in brain heart infusion medium (BHI, Beijing Land bridge, China) at 37 °C.

2.2 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 in PBS (10 mM, pH 7.4), and adjusted to 1.0 at OD₆₀₀ 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, respectively) to 0.2 at OD₆₀₀ nm (3.6×10⁸ CFU/mL). The growth curve was assessed for 12 h at 37 °C with shaking. The bacterial solution was taken for determination at OD₆₀₀ nm and 1-h interval using a SpectraMax M2 microplate reader (Molecular Devices, USA). Three independent experiments were performed and the results were reported as average.

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

Based on the growths of wild-type EGD-e under different pH conditions, eight kinds of pre-acid stress treatments (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 for screening the optimal treatment. Due to that the bacteria was barely growing at pH 4.5 and 5.0, the initial inoculum at pH 4.5 and 5.0 was 1.0×10⁹ CFU/mL (OD₆₀₀ = 0.4), while that at pH 5.5 and 6.0 was 3.6×10⁸ CFU/mL (OD₆₀₀ = 0.2). The acid-treated cultures were collected by centrifugation at 5000 × g for 10 min at 4 °C, washed in PBS, and adjusted to 0.4 at OD₆₀₀ nm. The cultures were then plated onto BHI agar after appropriate dilutions for counting surviving bacteria. At the same time, 1 mL of cultures (OD₆₀₀=0.4) were harvested, washed once in PBS, resuspended in isometric BHI broth (pre-adjusted to pH 3.0, death acidity), and cultured at 37 °C for 20 min. After that, the cultures were collected, resuspended in isometric BHI broth (pH 7.4), and plated onto BHI agar. The acid

107 resistance of bacteria was characterized by survival rate, which was defined as the
108 ratio of survival number after acid lethal treatment to that after acid lethal treatment.
109 Survival rates are reported as the mean of three independent experiments, each
110 performed in duplicate.

111

112 2.4 RNA-seq

113 The wild-type EGD-e strain was cultured in BHI medium under two conditions of
114 pH 7.4 and 5.5 at 37 °C for 3 h with shaking. The initial inoculum was 3.6×10^8
115 CFU/mL. Cultures were collected by centrifugation at $5000 \times g$ at 4 °C, and washed in
116 DEPC water. Total RNA was extracted using Trizol reagent (Invitrogen, USA) and
117 then ribosomal RNA was removed by a Ribo-Zero Magnetic kit (Epicentre
118 Biotechnologies, USA). RNA quality was checked by NanoDrop (ThermoFisher
119 Scientific, USA) followed by RNA degradation and contamination verification on 1%
120 agarose gel. The cDNA libraries were prepared using the TruSeq RNA Library
121 Preparation kit (Illumina, USA) and sequenced on Illumina HiSeq 4000 platform by
122 Sangon Biotechnology Co. Ltd., Shanghai, China. Clustering and sequencing were
123 performed by Sangon that employed spliced reads to determine connectivity. FPKM
124 (Fragments Per Kilobase of gene model per Million mapped reads) was calculated
125 using featureCounts. FPKM, which also considers the effect of sequencing depth and
126 gene length on reads counts, is a commonly used method for evaluating gene
127 expression levels[20]. Promoter strength could be reflected by FPKM.

128

129 2.5 Construction of plasmids and strains

130 The green fluorescent protein (GFP) gene was amplified from plasmid pUC57-GFP
131 and the promoters of 25 highly expressed genes were cloned from wild-type EGD-e
132 genome. Every cloned promoter and amplified *gfp* sequence were inserted into
133 plasmid pERL3 using ClonExpress® MultiS One Step Cloning Kit (Vazyme, Nanjing,
134 China) to create a series of pERL3 derivatives, pERL3-P₁-GFP to pERL3-P₂₅-GFP.

135 The widely used promoters P_{hly} and P_{help} , as controls, were applied to generate
136 pERL3- P_{hly} -GFP and pERL3- P_{help} -GFP using the same method. All primers used in
137 this study were listed in Table S2. The promoter sequences were listed in Table S3.
138 The constructed plasmids with different promoters were proliferated in *E. coli* DH5 α ,
139 identified by sequencing, and transformed into wild-type EGD-e using
140 electroporation.

141

142 2.6 Measurement of fluorescence intensity of GFP

143 The constructed strains were cultured for 12 h, 24 h, 36 h, and 48 h, respectively.
144 Cultures were collected by centrifugation, washed twice with 20mM Tris-HCl, and
145 suspended. After adjusting to the appropriate absorption at 600 nm (OD₆₀₀), the
146 fluorescence intensity of GFP (excitation at 485 nm and emission at 525 nm) was
147 measured in microtiter plates (Assay Plate, 96 wells, Black Polystyrene; Corning,
148 USA) using a SpectraMax M2 microplate reader (Molecular Devices, USA).

149

150 2.7 Fluorescent tracer of *L. monocytogenes* in macrophage RAW264.7

151 EGD-e and EGD-e $\Delta actA/inlB$ carrying the plasmid pERL3- P_{18} -GFP were chosen
152 for fluorescent tracer of *L. monocytogenes* in macrophage RAW264.7. Approximately
153 2×10^5 RAW264.7 cells were seeded on cover glass (Thermo Fisher Scientific, USA)
154 in a 12-well plate per well overnight. The cells were infected with bacteria at the
155 multiplicity of infection (MOI) of 100 for 2 h. After washing three times, gentamicin
156 was added for 30 min to eliminate the extracellular bacteria. Then cells were fixed
157 with 4% paraformaldehyde in PBS at room temperature for 30 min, and
158 permeabilized in 0.1% TritonX-100 in PBS for 5 min. Actin-stain 488 (Red,
159 Cytoskeleton Inc.) and DAPI (Blue, H-1200, Vector Lab.) were utilized to stain actin
160 and label the cell nucleus, respectively. Images were observed and captured on Leica
161 DM 2500 fluorescence microscope (Leica, Germany).

162

163 2.8 Fluorescent tracer of *L. monocytogenes* in vivo

164 In vivo imaging of EGD-e Δ actA/inlB carrying fluorescent reporting plasmid was
165 tested in traditional C57BL/6 mice. P₁₈, as the strongest promoter, was selected to
166 observe its activity in vivo. EGD-e carrying the plasmid pERL3-P₁₈-GFP were
167 cultured overnight, washed, and resuspended using PBS. Mice were inoculated with
168 EGD-e Δ actA/inlB (5×10^7 CFU/mouse) by intravenous injection. Three days
169 postinoculation, the mice were anesthetized with isoflurane and imaged in a Perkin
170 Elmer IVIS Lumina II system.

171

172 2.9 Measurement of Urease B subunit (UreB) production

173 The promoters with different strengths P₁₈, P₇, P₁₂, P₉, P₂₄, P_{help}, and P_{hly} were
174 chosen for evaluating their abilities to express foreign antigen UreB. The plasmids
175 pERL3-P₁₈/P₇/P₁₂/P₉/P₂₄/P_{help}/P_{hly}-UreB were constructed and transformed into
176 EGD-e Δ actA/inlB. The constructed strains were cultured overnight and the total
177 soluble proteins were extracted by ultrasonic crushing apparatus. The expression
178 levels of UreB under different promoters in EGD-e Δ actA/inlB were evaluated by
179 western blotting probed with a mouse anti-UreB polyclonal antibody. At the same
180 time, the purified UreB proteins with gradient dilution as standard were set for
181 quantitative analysis of UreB in EGD-e Δ actA/inlB by gray scan using ImageJ. In this
182 part, the quantitation of UreB was based on total protein normalization and the
183 proteinic concentration was assayed by BCA Kit (Solarbio, China).

184

185 2.10 Statistical analyses

186 All data were analyzed with the GraphPad Prism 5 software (GraphPad Software
187 Inc., La Jolla, CA, USA) and expressed as mean $\pm$ standard deviation (SD). Statistical
188 significance was tested using one-way ANOVA. P < .05 was considered as
189 statistically significant (*P < .05, **P < .01, ***P < .001, ****P < .0001; ns: not
190 significant).

3 Results

3.1 Growths of *L. monocytogenes* under different pH conditions

Acid stress could delay or stop the growth of *L. monocytogenes*. As shown in Fig. 1 a, compared with the normal condition (pH 7.4), the growth rate of wild-type EGD-e was inhibited by acid stress. The growth at pH 6.0 was slightly inhibited that its stationary phase was delayed by 4 hours and the bacterial concentration of stable period was about 0.68 at OD₆₀₀ nm, which was still close to that at pH 7.4 (0.71). The growth at pH 5.5 was moderately inhibited. The growths at pH 5.0 and 4.5 were severely inhibited, which were barely changed.

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 OD₆₀₀ 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.

212 3.2 The optimal acid stress treatments for *L. monocytogenes* RNA-seq 213 analysis.

214 Acid-inducing tolerance response under sublethal conditions could improve the
215 survival rate of *L. monocytogenes* under acid lethal conditions[21, 22]. In this study,
216 the survival rate was to characterize bacterial acid resistance. The bacteria with
217 stronger acid resistance have higher expression levels of acid stress-associating
218 proteins. As shown in Figure 1 b, the surviving bacteria after pre-acid treatments (pH
219 5.5, 3 h; pH 6.0 3 h) were 9.13×10^8 and 9.33×10^8 CFU/mL respectively, which were
220 close to that under the normal condition (1.0×10^9 CFU/mL). The lethality of bacteria
221 after these two acid stress pretreatments was extremely low. The surviving bacteria
222 were reduced to 4.70×10^8 and 4.43×10^8 CFU/mL respectively, when the acid
223 induction (pH 5.5 and 6.0) time was extended to 6 h. The surviving bacteria of the
224 other acid stress treatments (pH 4.5, 30 min; pH 4.5, 1 h; pH 5.0, 30 min; pH 5.0, 1 h)
225 were 4.9×10^7 , 2.5×10^6 , 3.33×10^8 , and 6.6×10^7 CFU/mL respectively, which indicated
226 that the strong-acid treatments have higher lethality even for a shorter time.

227 Further, after acid lethal treatment, the survival rates of pre-acid treatments (pH 5.5,
228 3 h; pH 5.5, 6 h) were 8.98% and 9.01%, which were the top 2 of all pre-acid
229 treatments. And there was no significant difference between 3 h and 6 h at pH 5.5. A
230 similar trend could be seen at pH 6.0 that the survival rates of pre-acid treatments (pH
231 6.0, 3 h; pH 6.0, 6 h) were both about 3.0%. Besides, the survival rates of pre-acid
232 treatments (pH 5.0, 1 h) could reach 6.8%, while those of the other pre-acid
233 treatments (pH 4.5, 30 min; pH 4.5, 1 h; and pH 5.0, 30 min) were 0.12%, 0.97%, and
234 1.22%. As a whole, the pre-acid treatment (pH 5.5, 3 h), which did well in both
235 bacterial activity and acid resistance, was selected for *L. monocytogenes* RNA-seq
236 analysis under acid stress.

237

238 3.3 Characterization of constitutive promoters via RNA-seq.

239 The transcriptional profiling of all 2952 genes in wild-type EGD-e genome was
 240 performed via RNA-seq. Firstly, RNA quality, sequencing saturation, and redundant
 241 sequence distribution frequency were analyzed for quality control of transcriptome
 242 data (Fig. S1, Supporting Information). For two conditions of pH 7.4 and 5.5, each
 243 sample was sorted from highly expressed to low expressed according to the FPKM
 244 value. Compared with the normal condition of pH 7.4, 333 genes were up-regulated
 245 and 339 genes were down-regulated under the acid stress condition of pH 5.5 (Fig. 2a).
 246 The top 2.0 % of highly expressed genes under each condition were selected for gene
 247 overlap analysis. Among the 59 selected genes, 34 genes were co-expressed under
 248 two conditions (Fig. 2b). Since there are 15 genes collectively distributed under 6
 249 different operons, 25 genes were chosen for cloning their promoters, which were
 250 listed in Table 1. And the promoter sequences were listed in Table S3.
 251

Number	Gene	Length	CDS product	FPKM	
				pH 5.5	pH 7.4
1	lmo1634	501	Aldehyde-alcohol dehydrogenase	13392.83	52369.24
2	lmo2653	108	Elongation factor Tu	41554.43	23758.48
3	lmo2637	447	Hypothetical protein	5134.74	48137.68
4	lmo2459	441	Glyceraldehyde-3-phosphate dehydrogenase	23215.88	18740.49
5	lmo0045	278	Single-strand binding protein	21808.48	17971.34
6	lmo1468	197	Hypothetical protein	17174.67	22337.2
7	lmo2654	192	Elongation factor G	24542.36	10582.53
8	lmo1439	300	Superoxide dismutase	27940.96	4269.49
9	lmo2556	168	Fructose-1,6-bisphosphate aldolase	8363.89	17402.44
10	lmo1541	156	Hypothetical protein	11821.15	11596.22
11	lmo1364	198	Cold-shock protein	10301.34	7921.58
12	lmo1003	306	Phosphotransferase system enzyme I	6824.64	10706.6
13	lmo2612	495	Preprotein translocase subunit	10711.67	6707.29
14	lmo2615	426	30S ribosomal protein S5	10020.13	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	10782.57	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

252 Table 1 Selected 25 promoter regions from wild-type EGD-e via RNA-seq.

253

254 To verify these putative constitutive promoters, the fluorescence intensities of GFP
 255 under different promoters in *L. monocytogenes* were measured for evaluating the
 256 promoter activity with constitutive promoters P_{help} and P_{hly} as references. According to
 257 the schematic diagram of plasmid construction (Fig. 2c), 21 fluorescent reporting
 258 plasmids with different promoters were successfully constructed and transformed into
 259 wild-type EGD-e. The strength of each promoter was similar at 12 h, 24 h, 36 h, and
 260 48 h under the condition of pH 7.4 (Fig. 2d), confirming that they are constitutive
 261 promoters. Seven promoters P_7 , P_8 , P_9 , P_{12} , P_{18} , P_{23} , and P_{24} showed high activities
 262 enhanced 1.8-fold to 5.4-fold compared with that of P_{help} in EGD-e. Under the
 263 condition of pH 5.5 (Fig. 2e), the fluorescence intensity of every well-characterized
 264 promoter has varying degrees of decline compared to that of pH 7.4. Nonetheless, the
 265 promoter P_{18} still showed a higher activity enhanced 5.3-fold compared to P_{help} .

266

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 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.

3.4 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 P₁₈ was selected for the tracer of bacteria in vitro and in vivo. The invasion of EGD-e and EGD-e $\Delta actA/inlB$ (pERL3-P₁₈-GFP) in macrophage RAW264.7 could be observed (Fig. 3a). Further, the fluorescence signal of EGD-e $\Delta actA/inlB$ (pERL3-P₁₈-GFP) was observed, and after dissection, could be assigned to livers (Fig. 3b), the major sites of listerial infections in mice. This indicates that the promoter P₁₈ also has high activity in vivo.

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.

3.5 Measurement of UreB production

Attenuated *L. monocytogenes*, as a live vector for expressing foreign antigens in microbial immunotherapy research, needs a strong promoter to improve the expression of foreign antigens urgently. For this purpose, the promoters P₁₈, P₇, P₁₂, P₉, and P₂₄ were selected to verify the expressing ability of the antigen UreB, a typical antigen of *H. pylori* with the promoters P_{help}, P_{hly}, and P_{None} (No promoter) as controls. As shown in Fig. 4a, the expression of UreB in EGD-eΔ*actA/inlB* under different promoters could be determined by anti-UreB polyclonal antibody using western blotting, which could be seen that the promoter P₁₈ still had the highest ability to express UreB. With the gray-scale analysis of purified UreB, we carried out a

quantitative analysis of UreB production under different promoters in EGD-e Δ actA/inlB (Fig. 4b). The promoters P₁₈, P₇, P₁₂, P₉, and P₂₄ showed high production of UreB enhanced 1.1-fold to 8.3-fold compared with promoter P_{help}. There was no doubt that the promoter P₁₈ still performed noticeably well in the expression of antigen UreB.

Fig. 4 Measurement of UreB production. **a.** Measurement of UreB production in EGD-e Δ 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 < 0.001; ****, P < 0.0001.

4 Discussion

In metabolic engineering and synthetic biology, constitutive promoters play a great role in fine-tuning gene expression. The promoter with a proper strength could not only maximize the target production but also maintain the biological activity of the host engineering bacteria, which means that a mature engineering bacteria system needs a constitutive promoter library to select. Since *Listeria* is not a common engineered bacterium, the lack of available constitutive promoters in *Listeria* urges us to enrich this toolbox for *Listeria* species.

Considering that *L. monocytogenes*, as a vaccine vector, has to encounter the acid environment in the host, the promoter is required to maintain certain activity under

322 acid stress conditions. Different acidity and processing time could result in different
323 acid resistance of *L. monocytogenes*. In this work, the treatment of pH 5.5 for 3 h was
324 selected as the acid stress condition of RNA-seq from all eight kinds of acid stress
325 treatment. The bacteria under this treatment had the strongest acid resistance so that
326 the down-regulated genes under acid stress could be filtered out as much as possible.

327 Based on RNA-seq and co-expression analysis under two conditions of pH 7.4 and
328 pH 5.5, the characterizations of 21 promoters were identified by GFP reporter. Under
329 the normal condition of pH 7.4, the activities of 7 promoters P₇, P₈, P₉, P₁₂, P₁₈, P₂₃,
330 and P₂₄ were higher than P_{help}, a previously reported strong promoter. And under acid
331 stress of pH 5.5, the fluorescence intensity of all promoters was decreased compared
332 with that of pH 7.4, but there were still 2 promoters P₁₂ and P₁₈, whose fluorescence
333 intensity was higher than that of P_{help}. As far as we know, this is the first
334 well-characterized constitutive promoter library in *L. monocytogenes*.

335 Further, the well-characterized promoter P₁₈ was evaluated in vitro and in vivo via
336 *L. monocytogenes* strains carrying fluorescent reporter plasmid. Interestingly, the
337 bacterial invasion in macrophage RAW264.7 and the bacterial enrichment in the liver
338 of mice could be observed, which confirmed the feasibility of constitutive promoter
339 P₁₈ in the application. Also, the selected constitutive promoters P₁₈, P₇, P₁₂, P₉, and P₂₄
340 with different strength had high activities in the production of the specific antigen
341 UreB. Due to the dependence of antigen dose and antibody response, the higher
342 antigen dose means the stronger immune response of specific antibodies[23]. So it
343 was worth noting that the performance of constitutive promoter P₁₈ was still far ahead
344 in antigen production in *L. monocytogenes*.

345 In conclusion, we identified 21 candidate promoters from *L. monocytogenes* by
346 RNA-seq under two conditions of pH 7.4 and pH 5.5. Based on the constructed
347 fluorescent reporter system, 7 constitutive promoters showed great strength than that
348 of P_{help}, a previously reported strong promoter. To demonstrate their utility, 5
349 well-characterized constitutive promoters were used to successfully activate a foreign
350 UreB biosynthetic pathway. In particular, the promoter activity in practical

351 applications and the unprecedented antigen production of P₁₈ were verified by the
352 tracer of fluorescent reporter strains and UreB production. It could greatly enhance
353 the effectiveness of live vector vaccines. Moreover, this study not only provides a
354 useful toolkit for *Listeria* species but also offers a reference for constitutive promoter
355 mining in other organisms.

356

357 Authors' contributions

358 Conceived and designed experiments: JF Ma, and Q Liu. Performed the experiments:
359 JF Ma, and QY Ji. Analyzed the data: JF Ma, SY Wang, and JX Qiu. Wrote and
360 revised the paper: JF Ma, and SY Wang.

361

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369

370 Declarations

371 Ethical approval

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

374 Conflict of interest

375 The authors declare that they have no conflict of interest.

376

377 Supplementary Information

Fig. S1 Descriptive statistics on RNA-seq. (A) Agarose gel electrophoresis of RNA samples from the conditions at pH 5.5 (1) and 7.4 (2). (B) Sequencing saturation analysis of RNA-seq. (C) Redundant sequence distribution frequency analysis of RNA-seq.

Table S1 Strains and plasmids in this study.

Strains/plasmids	Description	Sources
<i>E. coli</i> DH5α	Host for cloning	Purchased from Vazyme
EGD-e	<i>L. monocytogenes</i> wild-type	A gift from Qin Luo
EGD-eΔactA/inlB	<i>L. monocytogenes</i> deleting gene <i>actA</i> and <i>inlB</i>	Our lab
Plasmids		
pUC57-GFP	pUC57 derived, carrying a <i>gfp</i> gene, appropriate for <i>L. monocytogenes</i> after codon optimization	Our lab
pERL3	<i>L. monocytogenes</i> shuttle vector, Em ^r	Our lab
pERL3-P ₁ -GFP	pERL3 derived, carrying P ₁ promoter-GFP cassette	This study
pERL3-P ₂ -GFP	pERL3 derived, carrying P ₂ promoter-GFP cassette	This study
pERL3-P ₃ -GFP	pERL3 derived, carrying P ₃ promoter-GFP cassette	This study
pERL3-P ₄ -GFP	pERL3 derived, carrying P ₄ promoter-GFP cassette	This study
pERL3-P ₅ -GFP	pERL3 derived, carrying P ₅ promoter-GFP cassette	This study
pERL3-P ₆ -GFP	pERL3 derived, carrying P ₆ promoter-GFP cassette	This study
pERL3-P ₇ -GFP	pERL3 derived, carrying P ₇ promoter-GFP cassette	This study
pERL3-P ₈ -GFP	pERL3 derived, carrying P ₈ promoter-GFP cassette	This study
pERL3-P ₉ -GFP	pERL3 derived, carrying P ₉ promoter-GFP cassette	This study

pERL3-P ₁₀ -GFP	pERL3 derived, carrying P ₁₀ promoter-GFP cassette	This study
pERL3-P ₁₁ -GFP	pERL3 derived, carrying P ₁₁ promoter-GFP cassette	This study
pERL3-P ₁₂ -GFP	pERL3 derived, carrying P ₁₂ promoter-GFP cassette	This study
pERL3-P ₁₃ -GFP	pERL3 derived, carrying P ₁₃ promoter-GFP cassette	This study
pERL3-P ₁₄ -GFP	pERL3 derived, carrying P ₁₄ promoter-GFP cassette	This study
pERL3-P ₁₅ -GFP	pERL3 derived, carrying P ₁₅ promoter-GFP cassette	This study
pERL3-P ₁₆ -GFP	pERL3 derived, carrying P ₁₆ promoter-GFP cassette	This study
pERL3-P ₁₇ -GFP	pERL3 derived, carrying P ₁₇ promoter-GFP cassette	This study
pERL3-P ₁₈ -GFP	pERL3 derived, carrying P ₁₈ promoter-GFP cassette	This study
pERL3-P ₁₉ -GFP	pERL3 derived, carrying P ₁₉ promoter-GFP cassette	This study
pERL3-P ₂₀ -GFP	pERL3 derived, carrying P ₂₀ promoter-GFP cassette	This study
pERL3-P ₂₁ -GFP	pERL3 derived, carrying P ₂₁ promoter-GFP cassette	This study
pERL3-P ₂₂ -GFP	pERL3 derived, carrying P ₂₂ promoter-GFP cassette	This study
pERL3-P ₂₃ -GFP	pERL3 derived, carrying P ₂₃ promoter-GFP cassette	This study
pERL3-P ₂₄ -GFP	pERL3 derived, carrying P ₂₄ promoter-GFP cassette	This study
pERL3-P ₂₅ -GFP	pERL3 derived, carrying P ₂₅ promoter-GFP cassette	This study
pERL3-P _{hly} -GFP	pERL3 derived, carrying P _{hly} promoter-GFP cassette	This study
pERL3-P _{help} -GFP	pERL3 derived, carrying P _{help} promoter-GFP cassette	This study
pERL3-P ₁₈ -UreB	pERL3 derived, carrying P ₁₈ promoter-UreB cassette	This study
pERL3-P ₇ -UreB	pERL3 derived, carrying P ₇ promoter-UreB cassette	This study
pERL3-P ₁₂ -UreB	pERL3 derived, carrying P ₁₂ promoter-UreB cassette	This study
pERL3-P ₉ -UreB	pERL3 derived, carrying P ₉ promoter-UreB cassette	This study
pERL3-P ₂₄ -UreB	pERL3 derived, carrying P ₂₄ promoter-UreB cassette	This study
pERL3-P _{hly} -UreB	pERL3 derived, carrying P _{hly} promoter-UreB cassette	This study
pERL3-P _{help} -UreB	pERL3 derived, carrying P _{help} promoter-UreB cassette	This study

384

385 Table S2 The primers used in this study.

Gene/Promoter	Primer (5'→3')
<i>gfp</i> -F	ATGGTTTCTAAAGGTGAAGA
<i>gfp</i> -R	TGGCTGCAGGTCGACGGATCCTTAATGATGATGATGATGAT
P ₁ -F	CCCCGGAATTCCCGGGGATCCTTTTTACTTGTGTTTATCTT
P ₁ -R	ACCTTTAGAAACCATTTTTCCAGAACCTCCTAATAT
P ₂ -F	CCCCGGAATTCCCGGGGATCCTTGATTTTTTTTCGCAACATC
P ₂ -R	ACCTTTAGAAACCATTTTAAAATATCCTCCTCGAT
P ₃ -F	CCCCGGAATTCCCGGGGATCCATGCTTCCTCAACTCCTTGT
P ₃ -R	ACCTTTAGAAACCATGTTTTGCTCCACCTTTTGT
P ₄ -F	CCCCGGAATTCCCGGGGATCCAAGACGTATTTTGTCTCACG
P ₄ -R	ACCTTTAGAAACCATTTTAAATTGCTCCTCATCTT
P ₅ -F	CCCCGGAATTCCCGGGGATCCGCTTTGAAAAATGAGAATCC
P ₅ -R	ACCTTTAGAAACCATCTACTCTACACCTCCTTGTG
P ₆ -F	CCCCGGAATTCCCGGGGATCCCATTTCAAAGTTTATACGA
P ₆ -R	ACCTTTAGAAACCATCTCTCTTTCCCTCCCTC
P ₇ -F	CCCCGGAATTCCCGGGGATCCTTAACTAAATTCAAGTAAC
P ₇ -R	ACCTTTAGAAACCATGGGATAAATTCCTCCTTC
P ₈ -F	CCCCGGAATTCCCGGGGATCCTCTTTTTTCAACACCTCG
P ₈ -R	ACCTTTAGAAACCATTAATAAATTCCTCCTTGTATT
P ₉ -F	CCCCGGAATTCCCGGGGATCCAGCGTTTACACCAGAAAATT
P ₉ -R	ACCTTTAGAAACCATAATACAATTCCTCCCTCAG
P ₁₀ -F	CCCCGGAATTCCCGGGGATCCAATCAACAATTGGTTGACAT
P ₁₀ -R	ACCTTTAGAAACCATACTTGACCTCCTCATATAC
P ₁₁ -F	CCCCGGAATTCCCGGGGATCCTCATAATTATCTAGTAATTC
P ₁₁ -R	ACCTTTAGAAACCATGTTTCATGTTCCCTCCTC
P ₁₂ -F	CCCCGGAATTCCCGGGGATCCTTTTGAACCTTCCTTAAATT
P ₁₂ -R	ACCTTTAGAAACCATAATTTACATTCTCCTTTGTA
P ₁₃ -F	CCCCGGAATTCCCGGGGATCCGCTTAAAATTATTATGCAAT

P ₁₃ -R	ACCTTTAGAAACCATTAGATCACCTCAGTTTTTCC
P ₁₄ -F	CCCCGGAATTCCCGGGGATCCTAATGTTAGTTCCTAAACGT
P ₁₄ -R	ACCTTTAGAAACCATTGCTTCACCACCAATTTCT
P ₁₅ -F	CCCCGGAATTCCCGGGGATCCGGAGTGAGCTGTGCTGAATC
P ₁₅ -R	ACCTTTAGAAACCATATGTTTGCTACCTCCTTCTT
P ₁₆ -F	CCCCGGAATTCCCGGGGATCCTTTTAGTTATTACTTTAGTT
P ₁₆ -R	ACCTTTAGAAACCATTATAATTCTCTCCTTTGTT
P ₁₇ -F	CCCCGGAATTCCCGGGGATCCTTCATTGGAAAGGAGGACT
P ₁₇ -R	ACCTTTAGAAACCATTATGCGAGCACCTCCTCTAC
P ₁₈ -F	CCCCGGAATTCCCGGGGATCCAAAAATCTTGCAACTATACA
P ₁₈ -R	ACCTTTAGAAACCATGAGACATACCTCCTTAAGTC
P ₁₉ -F	CCCCGGAATTCCCGGGGATCCGAGTTGTTGTTTTTAGAAGG
P ₁₉ -R	ACCTTTAGAAACCATTGATAACCTCCCTTTTTTTT
P ₂₀ -F	CCCCGGAATTCCCGGGGATCCATCTGACACCTCCATTTGAT
P ₂₀ -R	ACCTTTAGAAACCATGTTTTTCTCTCCTTCCAACA
P ₂₁ -F	CCCCGGAATTCCCGGGGATCCTAATGTTAGTTCCTAAACGT
P ₂₁ -R	ACCTTTAGAAACCATTGCTTCACCACCAATTTCT
P ₂₂ -F	CCCCGGAATTCCCGGGGATCCTATAAAAACCCTGTTATAAG
P ₂₂ -R	ACCTTTAGAAACCATATTTACAAAACCTCCAAAAA
P ₂₃ -F	CCCCGGAATTCCCGGGGATCCAAAAAATCACTCCACTCATT
P ₂₃ -R	ACCTTTAGAAACCATTTCGAATTCCTCCTATAAAA
P ₂₄ -F	CCCCGGAATTCCCGGGGATCCATGATTGATATCCAAAAAAT
P ₂₄ -R	ACCTTTAGAAACCATAATCCAAAATACTCCTTTGT
P ₂₅ -F	CCCCGGAATTCCCGGGGATCCAATTTAACTTGACCAGCCCC
P ₂₅ -R	ACCTTTAGAAACCATTTTTCCACCTCCGTCCAACA
P _{hly} -F	CCCCGGAATTCCCGGGGATCCCGACGATAAAGGGACAGCAG
P _{hly} -R	ACCTTTAGAAACCATCATGGGTTTCACTCTCCTTC
<i>ureB</i> -F	ATGAAAAAGATTAGCAGAAA

<i>ureB-R</i>	TGGCTGCAGGTCGACGGATCC CTAGAAAATGCTAAAGAGTT
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386

387 Table S3 The sequences of selected promoters.

Promoter Number (Gene)	Sequence (5'→3')
P ₁ (lmo1634)	TTTTTACTTGTTGTTTATCTTACATAAGAAAATCAC GAAAGTCAAGAAAATAAACTACTTTTATACTAA AAAGGTTTCAAAACGATAATTAATAACAAGTA AGAAAAAGAACTACCCATCTAAAATATATTTCA CTTCAGAAAAAGCATGCTATTCTCTCTATTATAAT GAACACTAACTTCCTTAAAAACCCTATAAAATAT ATGTTTTCAAATGATAATTCGCTTAAATCCCAATG ATAAAAACACTCATATAAACTATGCCTAATTA ATAATACAACCTTTACAAAAGGACTTTTCAACCAT CTTTATCAATTTGTGAAGTTTTTTCACGTGAAACA CTGGACAACTTTTATTGATGGATTATACTAACG GTGTAATCAAATAAAACAAAAGAATACGGCAAA GCAACAAGCTAAATTTTTTTTGGATTATCAGGGAC GTCAAAAGTCTACTATGGACGTTTGTAGTCCCG AACAAATATTAGGAGGTTCTGGAAA
P ₂ (lmo2653)	TTGATTTTTTTCGCAACATCAAGTATAACTTTAGT TAGAAGTATTACTTAGTTTAAATTTAAGCTAAGT AAAAAATAATTATCGAATTATCGAGGAGGATATT TTAAA
P ₃ (lmo2637)	ATGCTTCCTCAACTCCTTGTAAGTTTATTCACAA AAATAAAATGAGTCCAAAATTCCCCACAGTATTA TTATAACCTATCATAGTTTAAACAGAAACCTCCTA CTAATGTCTAGATAGTAAAGAACAATTCTAATCT AAATTTGAAAAATAGGACTTTGTGCAAATGTTAC ATAATTAAGCAGGCTAAAAGTCCTTGTTTAACAT

	AGGATGTTAGACATTTTTGTTTGCTATTTTGCTAG AAAAGGTTGGTTTAAGCCATCTTTTGTATATCAA TAGTCATTATTTTGAAAATTAGATGAATTATGTCT TTAGAATTACAGTAGACAAAACCGGGCGCTGAT GGTATCATTTGTATTGTAAAAGTGATATCGCTTTC GCAGACTATCGGTTTGTGTTACGAAATCACAA ACAAAGTTAATCAATAAAACAAAAGGTGGGAGC AAAAC
P ₄ (lmo2459)	AAGACGTATTTTGTCTCACGAGCGGTATGTGGG ACAAAATACGTCTTTTAAAACAAAAAATCAC TAAGAGCATATTTTCATTTTTTAGAGAGGTGGAAA ACAAC TAGTGTAGCCTATTTAGAAGTTTGCAGTT TAAGAATTAATTGTAAATTTTCAGGTGAATATTTCA TAAATGTCCCACCTAACACTTGAAGATTTGTGCC AAGACTGTTAGAATAGGGAACGTAGGGTAGTTA AAGAACATAAAAAAAGATTCACCAGCGTAAGCA GAAAGAGAAGGGGTAAAAATCACATTGAGAGA CACTTTGTTAACAAGCTGAAGTAGCTAGTTTAAT AGTCTTGAAAGTTGTAAAAGCCTCTATCTCTACT TTTTTTAGTTCTAACGGGACGTCAGATGACTAAG TGGGTCATTAAGCGTCCAAAGATGAGGAGCAAT TAAAA
P ₅ (lmo0045)	GCTTTGAAAAATGAGAATCCCCTTTCAAATGC GACTTTCGCTAAAAGTATTGTTGGCGATTTTAAG ATGTGCTATAGTGTTTTCAAACCAGAATTTTCGA GAAATATCTTGCTGGTTAGTTTGCCTATGATATA ATATTTTATTGTGAGTAATTATAAATTATTCCTTGC TCGCTCTTGATTTTAAGGCTTGCTAAAGGCAGTC TGGATTTACAGGGATATGGTATGGTTTCATGCTATA

	TCAGAGCCGCTTAAGACCACAAGGAGGTGTAG AGTAG
P ₆ (lmo1468)	CATTTCAAAGTTTATACGAAAGTTAACGATTTC ATGTTGACGATGCCCCGATGTTTCGTATATAATTTT TAATGAGTGAATGCTTATCTGCATTACTTCAGAAT AATTCATAATTTATTTTCCGTTATGTCTTATTCTGG AACTTAAATGAAGGATTAAAGTGAGTGCATGTA AGTGCCGGAGGGAGGGAAAGAGAG
P ₇ (lmo2654)	TTAACTAAATTTCAAGTAACACCTTGACATCTAA ACCTCAAGGTGTTACTATATTCAAGGTGCCTCTA CTATAAACAGAGGTCTGAAAATAAACAGCGTT GTAGGTCGAACTTACAGCCATTTTATTTTACCGA AAAAATGAACCACCTGGATGTGTGGAACACTA AATAAGGAAGGAGGAATTTATCCC
P ₈ (lmo1439)	TCTTTTTTCAACACCTCGCCTAATGGTTTAACTT TTGAGTTTCAGGGAAAAAGATACATGTATGTGC AAAAGAGCGTACTTTTCCACCTAACTTAATCAT AGCTTGAAATGGTCGCAAAATCAATGTTGAAAC ATCAATGAGCAGAAAAATTGCAAGCATTTCGGG AGCATGGTAGGCTAAATGGTGTAAGAAGAACT GTTTTTAAGGTTGATAGTAGTTCTATTGAAATAG GACATGAACTTTTGCCTTATACGTCATTCTTTT CACGTAAAAACAATACAAGGAGGAATTTTAA
P ₉ (lmo2556)	AGCGTTTACACCAGAAAATTTTCTAGTTTTTATG GTAAATTCATCACAAATCTGTTATCATATGTTTGT TAGGGGAAAATAGCTGCCACTATTTTTCACGGCG GAAATTCAAATCTTAGGGGAAATCAAAAATGGT TTTCCTAAATTCCTGAGGGAGGAAATTGTATT
P ₁₀ (lmo1541)	AATCAACAATTGGTTGACATAGGCTACTTGCTTA

	<p>TGCTATCATTTTACTGTTATGTTTGTAGCGCACCA AGCTACAACCGCACAGAGCTGGGTCAAGTATTT ACTTCGTGTAAATCCCCTGTGATGGCGAGTCTTA GTATATGAGGAGGTGCAAGT</p>
P ₁₁ (lmo1364)	<p>TCATAATTATCTAGTAATTTCAAATTTTTTCACA TATATAATTCAAATTGATTTGCTTTTCCTAAAATA CCGTGTTATACTAATGTAAGATTATTTTTGTGGGT GAAAGATACGATTGTGAACAACCTTCCATCTCGT GCCGTTAAGCAAGAATAGTAAATAATTAGTGTGC ATAACACACGAGGAGGAACATGAAC</p>
P ₁₂ (lmo1003)	<p>TTTTGAACCTTCCTTAAATTTTGCCTAAAAAAC TATTAATCAACTATAAGCCATTAAAAAGTGCT ATTTAATTCAAACAGAAACAATTTAGTGATAAA TTAAAAAAACGCTCTATTTTTTCTATTTAAAAA GAGCGAAATAAGGTGCTCTACAAAAAACATTC TAAAAAATTTTAATCAAATAGTTGTAACAATAG GTAAACATGGTACTCTTTTAAATTGAGGGATAG TCTTTTGGCTGAAATTCTTTCGGCTTAAAAGGT TACAACAAATTTCTTTACAAAGGAGAATGTAAAT T</p>
P ₁₃ (lmo2612)	<p>GCTTAAAATTATTATGCAATTGATAGAATTTGTTA AATAGGAGGAGGTGCTTGACATGAACTACATG AACTTAAGCCTTCAGAAGGTTCTCGTAAAGAAC GTAATCGTGTTGGTCGTGGAACAGGCTCTGGTA ACGGCAAACTTCAGGACGCGGTCATAAAGGA CAAAAAGCTCGTTCTGGTGGTGGCGTACGTTTA GGTTTTGAAGGTGGACAACCTCCACTTTTCCGT CGTATTCCAAAACGTGGATTCACAAATATCAACC GTAAAGAATTTGCTATCGTGAACCTTAGATGTTTT</p>

	AAACCGCTTTGAAGATGGTACAGAAGTAACACC AGAACTTTTAGTTGAAACTGGAATTATTCGTAAT GAAAAATCCGGAATCAAGATTTTATCTAATGGAA ATATCGAGAAAAA ACTTACTGTGAAAGCGAACA AATTCTCTGCAGCTGCGAAAGAAGCAATTGAAG CAGCTGGCGGAAAAA ACTGAGGTGATCTA
P ₁₄ (lmo2615)	TAATGTTAGTTCCTAAACGTGTAAAATACCGTCG TGAATTCCGCGGAAACATGCGTGGACGCGCGAA AGGCGGA ACTGAAGTTGCATTGGTGAATATGG TCTTCAAGCAGTTGAAGCTTCTTGGATTACAAA CCGTCAAATCGAAGCAGCTCGTATCGCAATGAC TCGTTACATGAAACGTGGCGGTAAAGTTTGGATT AAAATTTTCCCTCATAAATCTTACACTTCTAAAC CAATCGGGGTTCGGATGGGTAAAGGTAAAGGTG CTCCGGAAGGTTGGGTAAAGCCAGTCAAACGTG GCAAAATTATGTTTGAAATCGCAGGTGTTCTCTGA AGATGTAGCGCGTGAAGCATTACGTCTAGCAGC ACACAAACTGCCGGTCAAACTAAGATCGTTAA ACGTGAAGAAATTGGTGGTGAAGCAA
P ₁₅ (lmo2610)	GGAGTGAGCTGTGCTGAATCACTGTCAAATCA ATGCGTGCAAATTAGATTAGTTTGTAGTATAATG GATAATTGGCAGTGGAATTAAAGGCATCTAAGC AAATCATGATGAACGAGAGAACCGTTTGAAGAT CATCCGAATGACGTATATGTTCTACCTTGATCTGT TCAGCCGCAATGCTTTT GCGAGTGACAGTTCTTC TGGTTTTTACGGATTTCACTTTTACAGGTTGTCT GAAAAGCTGGCGAAATGTGTTGCCTTTTAATGA AGTG CATCCATTTTGCATGTTTACGTTTTTTTC ATGTAAGAGAAATAGTTACACTGAAAGGATTAG

	GCCTGCATCAGGAACAAATCCAAGAATACAAGA AGGAGGTAGCAAACAT
P ₁₆ (lmo2455)	TTTtagttattacttttagtttttttgcaaacagag CTACAATAAATACGAATTTAAAAACGGATGGGTC TTTGAATTTGAAGTGAATATTATGTTCAATTTCAAT CAATTAATCCCAACAAAGGAGAGAATTATAA
P ₁₇ (lmo2625)	TTCATTTGGAAAGGAGGACTAACGAATGCCAAA ATTAAGCTTACTTAAACAAGATGGAACAAACGC TGGCGAAATTACTTTAAACGACACTGTTTTCGGT ATCGAACCAAATGAAAAAGTTGTTGTTGATGTG ATTTTGAGCCAACGTGCATCCCTACGTCAAGGG ACTCACAAAGTAAAAAATCGTTCAGAAGTACGT GGTGGCGGACGTAAACCATGGCGTCAAAAAGGT ACAGGTCGTGCCCCGTCAAGGTTCAATCCGTTCC CCGCAATGGCGTGGCGGTGGTGTCTGTTTCGGC CCAACACCTCGTTCATATGCTTACAAATTACCTA AGAAAGTTCGTCTGTTTAGCGATTAAATCAATTCT TTCTTCTAAAGTAAATGAAGAAAAATTAGTTGTA CTTGAAGGTTTGACTTTCGATGCACCTAAAACA AAAGAATTTGCGGCTTTTCTTAAAAATATCTCTG TAGATACTAAGGCACTAATCGTAGTTGCTGGTGA AAGTGAAAATGTAGAATTATCTGCACGCAACTTA CAAGGCATTACAGTTATTCCGGCTGAAAGTATCT CAGTACTAGAAGTTGCTAAACATGATAAATTAAT TATCACTAAAGCAGCTGTGAAAAAGTAGAGGA GGTGCTCGCATA
P ₁₈ (lmo0248)	AAAAATCTTGCAACTATACATTTAAAATGGTATC ATATATAAGTACGTTTTTGACGTATGTTTTAACGT GGGAGGGGAAATATCAGCCCCAGTCAACCACAT

	CACGGACTTAAGGAGGTATGTCTC
P ₁₉ (lmo1847)	GAGTTGTTGTTTTTAGAAGGTTTAATGCAATATA GTTTTTTACAAAAAGCCCTTATTACTTCTGTGAC GGTTGGTATTGTTTCAGGTGTTATTGGTAGTTTTA TTATTTTACGAGGTATGTGCTTATGGGGGATGC GATTTCTCATGCAGTGCTTCCAGGAGTGGCGATT TCTTATATGATGGGGATGAACTTCTTTATTGGTGC AGCTACATTCGGCATCGCGGCGGCACTTGGAAT CGGTTTTGTTAATCAAAAAAGTCGGATAAAAAA TGATACAGCGATTGGAATTGTTTTIAGTGCATTT TTTGCACTTGGAATTATTTTAATATCCTTTGCGAA AAGTAGTACGGATTTGTATCATATTTTATTGGAA ATGTGCTTGCGGTGCGGAGTTCGGATATGTGGAT GACAATTATTATTGCCATTATCGTGATTTTCATTAG TAGCCCTATTTTACAAAGAGTTTCTAGTTAGTTC GTTTGATCCAGTGATGGCAGAAGCATATGGTCTC AATGTGAAATTCTTGCATTACTTCTTGATGTIACT TTTAACACTTGTAACGGTTTCCGCTTTGCAAACG GTTGGAATTATTTTAGTTGTGGCGATGTTAATTAC GCCAGCTGCAACAGCTTATCTGCTTACGAATAAA TTATCCAAAATGATTGTTCTTGCTTCTACTTTTGG AGCAGTGAGTGCGATTATCGGACTTACTTTAGT TACATTTTCAACTTAGCATCTGGTGCGGCTATGG TTTTAGTTGCGACAATTATTTCTTTATTGCCTTT TTATTCGCACCGAAACAAGGCTTGCTATTTTCTA AAAAAAGGGAGGTATCGA
P ₂₀ (lmo1787)	ATCTGACACCTCCATTTGATATACTAAAGGTAAC ATTAAACCATACTCCAAGGTCAAGCTTTTTTATC AAAGAAAAAGTAAGAAAAAACTTTACAGCGA

	AACTAGTTTATGGTAACCTAAATAAGGTGACTGG AGAGACCAGTCTGATAACGATATCCGCTTCATT ATTAATATGAATGAATGTTTGTGGAAGGAGAGA AAAAC
P ₂₁ (lmo2621)	TAATGTTAGTTCCTAAACGTGTAAAATACCGTCG TGAATTCCGCGGAAACATGCGTGGACGCGCGAA AGGCGGAAGTGAAGTTGCATTTGGTGAATATGG TCTTCAAGCAGTTGAAGCTTCTTGGATTACAAA CCGTCAAATCGAAGCAGCTCGTATCGCAATGAC TCGTTACATGAAACGTGGCGGTAAAGTTTGGATT AAAATTTTCCCTCATAAATCTTACACTTCTAAAC CAATCGGGGTTCGGATGGGTAAAGGTAAAGGTG CTCCGGAAGGTTGGGTAAAGCCAGTCAAACGTG GCAAATATGTTTGAAATCGCAGGTGTTCTGA AGATGTAGCGCGTGAAGCATTACGTCTAGCAGC ACACAACTGCCGGTCAAACTAAGATCGTTAA ACGTGAAGAAATTGGTGGTGAAGCAA
P ₂₂ (lmo2016)	TATAAAAACCCTGTTATAAGAATTAGCTTATAGC AGGGTTTTTCAGATAGTAACTGAATAATCTATT TTGTAACCGTACAGCTTTTTTCACTTGATTTATTTG CTCGTTGGCGTTAATATATAAGTGTTGATAGTAGC TAATTTACTTATTATCAAGGATATTGATGTGAAGT GAGTATCTTTGCTAATATGTGAATTTTGTAATTGA AAAAATGATTAGCTTGTTGTTAACATCTTTTTCG TTAGTAAAAGAAACATATTAAGAAAATTTTGGGA GGTTTGTGAAAT
P ₂₃ (lmo0210)	AAAAAATCACTCCACTCATTCAATTTTGGTACAT CTACATTATATCTACCCTTTAGAAAAATAGCAAA ACAAAGGAATAGCCTTATTTTGTGTTTGTAAAGTT

	<p>TATAATTCTCAATAAAAAGGTAAACAGAAAATAGC GGTACTCGCTAATGTGGACGCTTCCAAACTGTA CTACAGCGTTTTACAAATTCATTATAACAACCTT TTTTAAATTTAGGACGTTCAACGCTTCACAAATG AAAAATACATGGTATACTGTACTAGTAAATAGAT TTTTTATAGGAGGAATTCGAA</p>
P ₂₄ (lmo2411)	<p>ATGATTGATATCCAAAAAATTCGGGCGGATTTTC CAATTTTAGCTCAAGAAATAAATGAAAAACCGC TAGCTTATTTAGATAATGCTGCCACTTCACAAAA GCCGAAACAAGTTATTGAAGCTTTAACACATTAC TATGAGTTTGATAATGCGAACGTTACCGTGCGT TGCATACGCTTGCGGCAAGAGCGACGGATGCTT ATGAATCAGCTCGAGGTAAAGTAGCCAAGTTTAT TCATGCGCGTGAAGTAGCAGAAATTATTTTCACA AGAGGCACTACTTCAGCGATTAATTTAGTTGTAG ATAGTTACGCGGAAGCAAATATTGAAGCTGGCG ATGAGATAGTTATTTCTTATTTAGAGCATCATTCT AATTTGATTCCATGGCAACAACCTAGCTAAACGC AAAGGCGCGGTTTTTAAATATATCGAACTAGAA GAAGATGGCACGATTTTCAGTGGAACAAGCGAA AAAAACGATTGGCGAGAAAACGAAAATCGTTG CGCTAGCACATGTTTCTAATGTTCTAGGGACAAT CACGCCAATTAAAGAAATCGCAGCAATTGCTCA TCAATTTGGAGCAGTCATTCTCGTTGACGGTGCG CAAGCTGTGCCTCATATGGAAGTCGATGTAGTTG ATTTAGATGCTGACTTTTATGCTTTTTTCAGGGCAT AAAATGATGGCTCCTACTGGCATTGGCGCTTTGT ATGGCAAACGTGAATTGCTTGATGCGATGGAAC CTACCGAATTTGGCGGAGAAATGATTGATTTTGT</p>

	<p>TGAATTATACGATTTCGACTTGGAAAGAACTACCT TGGAAATTTGAAGCCGGAACACCGATTATTGGC GGAGCAATTGCGCTAGGTGCGGCGATTGATTAC TTGGCAGAAGTCGGACTCGAAAACATTCACGCA CATGAACAAGCATTAGCCAGCTACGCGATGGAA GAAATGAGCAAAATCGAAGGCATTACCATTAC GGGCCGAAAGATGCAAGTAAACGTTGTGGTTTA GTGACTTTTAATTTAGAAGGCGCACACCCACAT GATATTGCGACTATTTTGGACGAAGATGGGGTGG CGATTGAGCTGGACATCACTGTGCACAACCGT TGATGAAATGGCTGGACGTTTCTTCCACAGCTC GCGCAAGCTTTTATATTTATAATAACAAAAGAAGA AATTGATGCGCTTATAGATGGCCTCAAGTTAACA AAGGAGTAT TTTGGATT</p>
P ₂₅ (lmo0250)	<p>AATTAACTTGACCAGCCCCCTTGTCTTTTGATAA GATAGGCTAGTTGAAACAATCAAATATCCTTACC GTAGACAGTTGGCGCGTTTATCGCTTAAATTTGC CACCCGAGGGATTCTTTTGAAGTGTGCGTCCG CGTGCATTTTACAAAAACCTCTGACGTCTACGGT GTCAGAGGTTTTTTGTTGCTGATCAAACGGAAC TTGTGTCTGTTTAATTAGATTAAATTGTTGGACGG AGGTGGAAAA</p>

388

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454