ACS SyntheticBiology

pubs.acs.org/synthbio

Research Article

Engineering of a Biosensor in Response to Malate in *Bacillus licheniformis*

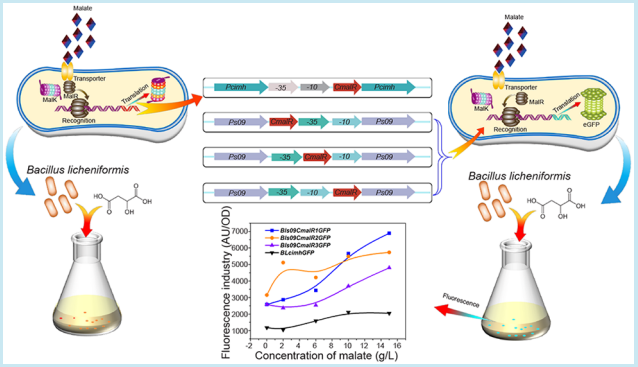
Yupeng Zhang, Youran Li,* Fengxu Xiao, Hanrong Wang, Liang Zhang, Zhongyang Ding, Sha Xu, Zhenghua Gu, and Guiyang Shi*

Cite This: *ACS Synth. Biol.* 2021, 10, 1775−1784

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Malate is an essential intermediate in the tricarboxylic acid (TCA) cycle; it also has valuable uses in medicine and food. The production of malate with a microbial synthesis method is still in its early stages. One of the key problems in metabolic engineering is that the dynamic and subtle changes in malate are difficult to detect. It remains critical to develop techniques with direct and precise detection of malate in microbial metabolism, which facilitates high-throughput screening of the engineered strains. In this study, a genetically encoded biosensor in response to malate was constructed in *B. licheniformis*. Key regulator MalR and the action site of the biosensor were first identified. Then, the output of the reporter gene expression was amplified by introducing a strong constitutive promoter and iteratively tuning the action sites. The engineered biosensor can respond to malate from 5 to 15 g/L; within this range, it shows a linear correlation between eGFP fluorescence and malate concentration. This biosensor enrich our toolbox of synthetic biology in pathway engineering for malate production in microorganisms.



KEYWORDS: *malate, biosensor, Bacillus licheniformis, malate regulator*

INTRODUCTION

Malate, also known as hydroxysuccinic acid, is a C4-dicarboxylic acid. It is widely used in food, cosmetics, pharmaceuticals, water treatment, fabric dying, and biodegradable polymers.[1,2] At present, L-malate is produced mainly in two ways: chemical synthesis and biochemical pathway.[3,4] The biochemical pathway for malate production has advantages in mild reaction conditions, low cost, and low contaminant emissions, which have attracted a lot of attention. At present, researches on the microbial production of malate have mainly focused on *E. coli* and *Aspergillus oryzae*.[1,5] Compared with the above strains, *B. licheniformis* possesses unique industrial characteristics, such as moderate growth rate, excellent stress tolerance, and highly antimicrobial activities.[6−8] Hence, the modification of malate metabolism in *B. licheniformis* has shown huge promise in a wide range of applications. With the development of synthetic biology and metabolic engineering, the modification of malate metabolism had attracted significant attention in recent years.[9] However, malate is an intermediate metabolite of the tricarboxylic acid cycle with highly dynamic metabolic flux. The change in intracellular malate content caused by metabolic pathway engineering is difficult to detect. Thus, novel high-throughput screening systems for malate metabolic should be developed to direct and detect subtle changes in malate to guarantee the modification of malate metabolism efficiently.

In nature, various transcription factors have evolved to sense the concentration of small molecules in the environment. Biosensors, encoded by the report gene and the response gene, have been used to detect the concentration of metabolites or metabolic processes.[10−13] Hence, these methods may overcome these limitations. Therefore, many regulators have been designed to detect metabolite production. For example, LysG has been used to detect L-lysine in *Corynebacterium glutamicum*.[14] TyrR has been used to detect L-Phe in *Escherichia coli*.[10] FdeR from *Herbaspirillum seropedicae* and QdoR from *Bacillus subtilis* were used in *E. coli* to perform *in vivo* detection of metabolites on a single cell level.[15] In addition, some operons have been designed to detect the metabolite. LacI has been mutated and constructed into a whole-cell lactulose biosensor.[16] As for malate, most biosensors are enzyme based and mainly used to detect malate in food or

Received: April 19, 2021
Published: July 2, 2021

https://doi.org/10.1021/acssynbio.1c00170
ACS Synth. Biol. 2021, 10, 1775−1784

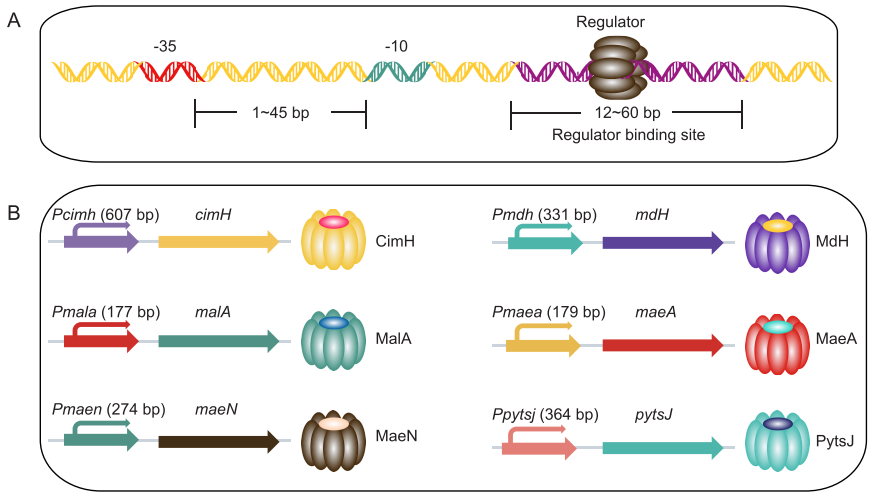


Figure 1. Schematic view of promoter selected to construct malate biosensor. (A) Basic structure of the promoter that was selected to construct the malate biosensor containing a −10 region, a −35 region and regulator binding site. (B) Location of promoters that we selected to construct the malate biosensor. All promoters were located at the upstream of target genes. Sequence numbers of the target gene: *cimH* (citrate/malate/H+ symporter), AGN38462.1; *maeA* (NAD-dependent malate dehydrogenase), AGN38281.1; *maeN* (Na+/malate symporter), AGN37731.1; *mdH* (malate dehydrogenase), AGN37485.1; *ytsJ* (NADP-dependent malic enzyme), AGN37496.1; *malA* (6-phospho-*α*-glucosidase), AGN35353.1.

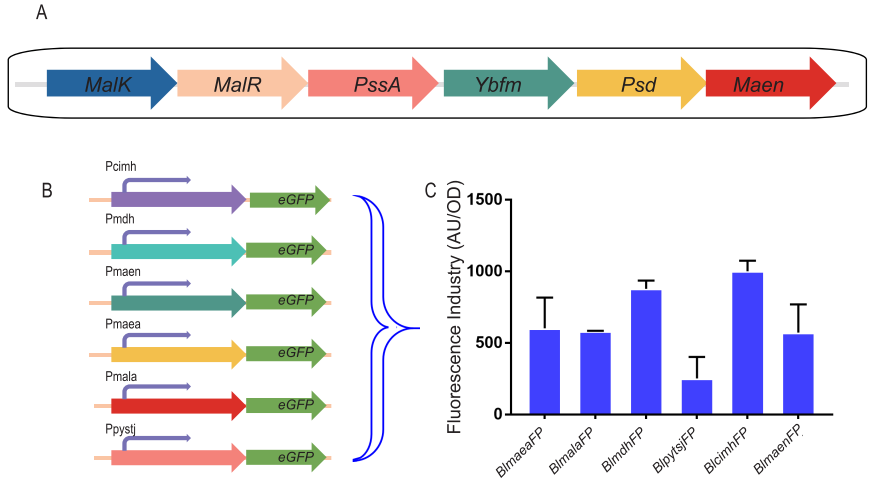


Figure 2. Schematic view of a genetically encoded malate biosensor. (A) Schematic of malate metabolic operon in *B. licheniformis*. MalK, AGN37726.1; MalR, AGN37727.1; PssA, AGN37728.1; Ybfm, AGN37729.1; Psd, AGN37730.1; Maen, AGN37731.1. (B) Schematic of biosensor. Promoters and *eGFP* are indicated by arrows. Arrows indicate the direction of transcription. (C) Specific fluorescence of *B. licheniformis* containing different biosensors was determined after 24 h in the presence of the indicated malate (15 g/L). The experiments were repeated three times. *B. licheniformis* containing different biosensors was determined after 24 h in the absence of the malate, which were regarded as a negative control. The value of fluorescence was the value of the experimental group minus the negative control group.

wine.[17] Malate-sensing *E. coli* used to be constructed by the introduction of a novel chimeric two-component system.[18] But, malate-sensing *Escherichia coli* is sensitive to extracellular malate, with a maximum output signal produced even at only 0.1 mM malate. Currently, a genetically encoded biosensor response to malate has not been reported in *Bacillus*.

Because of the great application potential, *B. licheniformis* is used not only for various applications in the field of fermentation but also as an excellent platform for exogenous gene expression.[19,20] However, there are few high-throughput screening tools for malate detection in *B. licheniformis*. These limited the development of the modification of malate metabolism. Therefore, the development of high-throughput screening tools for malate may help overcome the above limitations in *B. licheniformis*.

This study presents a biosensor encoded by genetics for malate, an intermediate metabolite of the tricarboxylic acid (TCA) cycle. It develops a whole-cell biosensor that enables the monitoring of malate. The results demonstrate that the specific fluorescence of *B. licheniformis* correlates with the concentration of malate. In addition, it reveals the working principle of the biosensor designed in this study. MalR is a regulator response for the malate usage in *B. licheniformis*. A special sequence TTAATTTAAACAAAAA has been recognized by MalR. The middle of the special sequence is flexible and does not influence the recognition of MalR with the sequence. According to the above results, the sensitivity for malate biosensor increased 2−3-fold. This study provides a high-throughput screening tool for screening malate production strains in *B. licheniformis*. It provides a basis for the further design of biosensors for malate production.

https://doi.org/10.1021/acssynbio.1c00170
ACS Synth. Biol. 2021, 10, 1775−1784

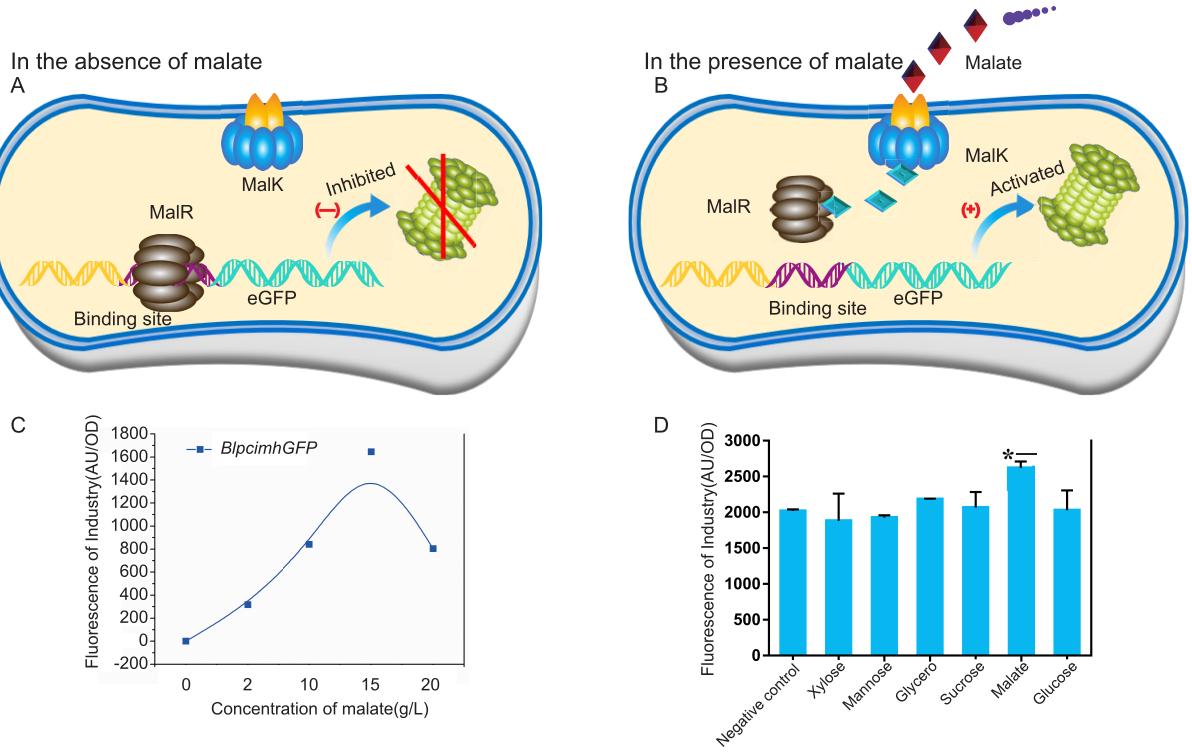


Figure 3. Schematic view and characterization of the malate biosensor. (A) Regulation of *Pcimh* by MalR in the absence of malate in *B. licheniformis*. MalR binds with the binding site, and the expression of *eGFP* was repressed. (B) Regulation of *Pcimh* by MalR at the presence of malate in *B. licheniformis*. MalR was released from the binding site, and the expression of *eGFP* was activated. (C) Fluorescence intensity was measured as a function of the malate concentration. The specific fluorescence of *BlcimhFP* was measured after 24 h of cultivation in the presence of various concentrations of malate. (D) Effector specificity of *BlcimhFP*. The specific fluorescence of *BlcimhFP* was determined after 24 h of cultivation in the presence of the indicated effectors (15 g/L). The error bars are shown as ± standard deviation ($n = 3$) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

RESULTS AND DISCUSSION

Screening for Malate Responsive Elements. In previous studies, active transport systems were mainly seen as a way to utilize carboxylic acids and were induced *via* two-component systems in *B. subtilis*.[21] The utilization of malate may also be induced *via* two-component systems in *B. licheniformis*. The genes involved in malate metabolism may be induced by malate. Promoters located in the upstream of genes involved in malate metabolism have the potential to be used to design the biosensor response to malate. On the basis of a previous study, the promoter used as a part of the biosensor contained a −10 region, a −35 region, and the binding site of the regulator.[22] Additionally, previous studies demonstrate that the length between the −10 and −35 region is flexible.[23] The length was about 1−45 bp (Figure 1A). As for the binding site of the regulator, many studies have demonstrated that the length of regulatory sites is also flexible.[24,26] On the basis of the above analysis, the length of the promoter should be more than 100 bp. Six potential promoters (*Pcimh*, *Pmaea*, *Pmaen*, *Pmdh*, *Pmala*, and *Pytsj*) were mined in the genome of *B. licheniformis* (Figure 1B). All the promoters we selected in this study were directly or indirectly correlated with malate metabolism (Table S3). In this study, the promoters located in the upstream of genes involved in malate metabolism were mined to construct a biosensor to respond to malate. Doan et al. proved that the *ywkA* expression of *B. subtilis* is induced in the presence of malate in the medium.[26] Since *B. subtilis* and *B. licheniformis* are similar, the feasibility of using promoters to construct the biosensor response to malate was high.

Design and Construction of Malate Biosensor. To construct a genetically encoded biosensor that responds to malate, promoters related to the metabolism of malate can be part of the biosensor. In a previous study, a transcriptional regulator belonging to the MarR family binding to malE promoter DNA was designated as MalR (malate response regulator) and regulated malate metabolism.[27,28] Moreover, it was also demonstrated that the malate metabolism was regulated by malK (malate sensor histidine kinase)/MalR in *B. subtilis*.[26] Fortunately, the same regulator (MalK, AGN37726.1; MalR, AGN37727.1) was present in *B. licheniformis*, as is shown in Figure 2A. The amino acid sequence homology showed that the similarity of malate regulators between *B. subtilis* and *B. licheniformis* was 79.15%. In addition to its amino acid sequence homology, a regulator of *B. licheniformis* shares a strong tertiary structure similarity with the regulator of *B. subtilis* (Figure S1). It was suggested in this system that the regulator MalR binds to specific DNA sites in the absence of malate, causing the expression level downstream binding sites repressed.[25] When MalK encoding the malate sensor histidine kinase receives the extracellular malate signal, a communication between MalK and MalR would occur *via* a phosphoryl group transfer.[26,27] Then, the repression was relieved and a measurable output in response to malate could be observed.

This native regulatory system in *B. licheniformis* was used to create a malate biosensor capable of converting the malate

https://doi.org/10.1021/acssynbio.1c00170
ACS Synth. Biol. 2021, 10, 1775−1784

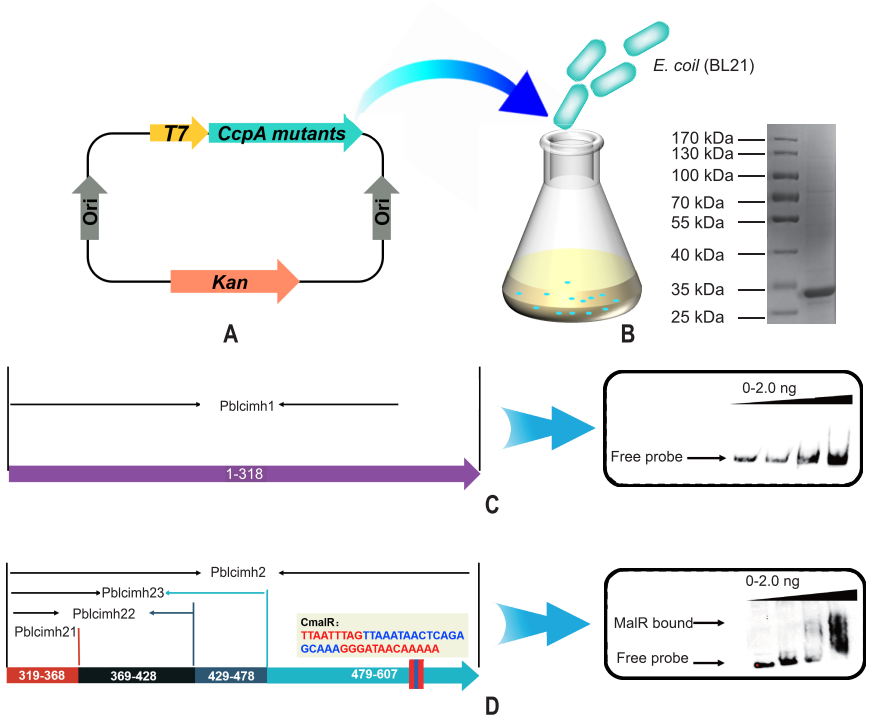


Figure 4. Heterologous expression of MalR and validation of the binding region of MalR for *Pblcimh*. (A) Plasmid that was used for the heterologous expression of MalR. (B) Purification of MalR, verified by SDS-PAGE. The size of MalR was 27.4 kDa. (C) EMSA of MalR binding to *Pblcimh1* (1−318 bp). (D) EMSA of MalR binding to *Pblcimh2* (319−607 bp). The sequences highlighted with red were regarded as the conserved region. The sequences highlighted with blue were regarded as the flexible region.

concentration into an optical fluorescence output. Therefore, the promoters in *B. licheniformis* were related to the metabolism of malate, which could be used to design a biosensor responding to malate. A single plasmid was constructed containing both the promoter and *eGFP*. The direction of the promoter transcription was the same as that shown in the genome (Figure 2B).

The expression of *eGFP* was expected to be regulated by malate alone in *B. licheniformis*. The plasmid used in this study was a high-copy plasmid. If MalR is the functional, the expression of *eGFP* will be repressed in the absence of malate, and the presence of malate will lend to the expression of *eGFP*. Therefore, the fluorescence intensity of *B. licheniformis*, which contains different biosensors, was measured using a microplate reader at the location of the malate. As is shown in Figure 2C, fluorescence values were determined as 600 ± 152 (*BlmaeaFP*), 582 ± 1 (*BlmalaFP*), 878 ± 41 (*BlmdhFP*), 252 ± 106 (*BlpytsjFP*), 1000 ± 53 (*BlcimhFP*), 571 ± 139 (*BlmaenFP*), and 63 ± 23 (*BlpHY*). All promoters located at the upstream of the target gene, which were involved in malate metabolism. In the presence of malate, the expression of the target gene was activated by MalR. Similarly, MalR is disengaged from the binding site in the presence of malate, and the change of fluorescence was detected in the malate biosensor. *B. licheniformis* containing different biosensors had different fluorescence values for identical malate content, where *BLcimhFP* had a larger response value. Fluorescence output, which was converted by the concentration of malate, was more readily detectable by microplate readers. Therefore, *BLcimhFP* was chosen to construct a high-performance biosensor for malate in this study. The value of the biosensor constructed was 1000 ± 53 in the presence of malate.

Characterization of the Malate Biosensor. The resulting model is shown in Figure 3A. In the absence of malate, MalR binding with *Pcimh* and no change in fluorescence were detected. In contrast, the fluorescence measured in the presence of malate exhibited high fluctuations. Next, the performance of the transcriptional regulator MalR in the constructed malate biosensor in the host was examined. *B. licheniformis* cells transformed with the *pPblcimhGFP* construct were grown in TB medium supplemented with different concentrations of malate. The responses of *BlcimhFP* cells to various concentrations of malate were measured (Figure 3C). The transformant cells clearly responded to induction by malate. As shown in Figure 3C, the fluorescence intensity of *BlcimhFP* increases with the increase in malate concentration (0−15 g/L), and the value of fluorescence intensity changes of eGFP was about 0−1400. In addition, the presence of various carbon sources in the growth procedure might lend itself to the changes of fluorescence intensity. To avoid carbon source influence, the responses of *BlcimhFP* cells to five effectors (xylose, mannose, glycero, sucrose, and glucose) were measured. As expected, *BlcimhFP* showed a significant response to induction by malate ($P < 0.05$) (Figure 3D). Moreover, there is a significant positive correlation between the value of fluorescence and malate content.

Action Site of Malate Biosensor. To get better insight into the biosensor response, further research is required. Many studies have determined that the binding sites of many regulators were inverted repeats.[29,30] However, clear repeat sequences in the *Pblcimh* promoter sequence were not identified. The heterologous expression of MalR used *pET28a* plasmid in *E. coli* BL21 to identify the binding site of MalR (Figure 4A,B). The result is shown as sodium

https://doi.org/10.1021/acssynbio.1c00170
ACS Synth. Biol. 2021, 10, 1775−1784

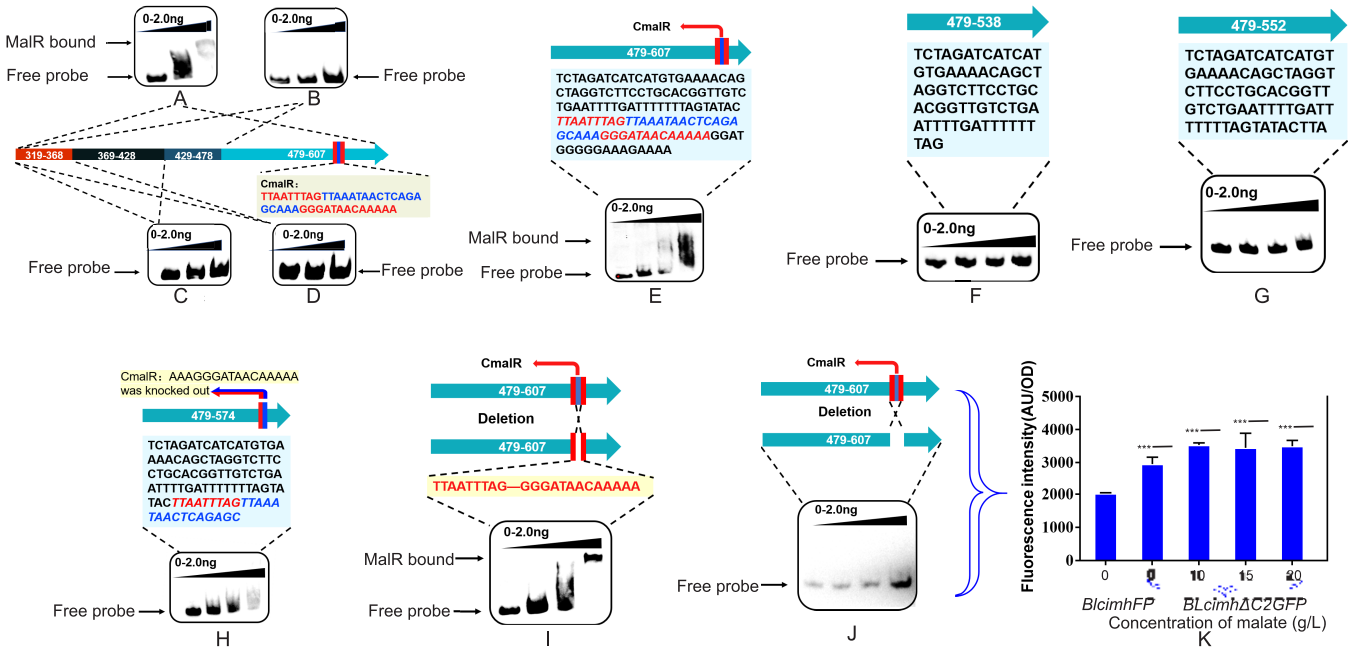


Figure 5. Screening of the binding site of MalR. (A–J) EMSA verification of MalR binding to the putative *CmalR*. The sequences highlighted with red and blue indicate the putative *CmalR*. The sequences highlighted with red were regarded as the conserved region. The sequences highlighted with blue were regarded as the flexible region. (K) Specific fluorescence of *B. licheniformis* cells containing *pPblcimhΔC2GFP*. The error bars are shown as ± standard deviation (n = 3) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) in Figure 4B. The size of the purified MalR was 27.4 kDa, consistent with the size of the expected value. Then, the *in vitro* binding of fragments of the 0–318 bp upstream and the 319–607 bp downstream of MalR was tested by electrophoretic mobility shift assay (EMSA) (Figure 4C,D). As the concentration of MalR increased, a shifted binding was not detected in the upstream (0–318 bp) of *Pblcimh*. In contrast, a shifted binding was detected when the probe was incubated downstream (329–607 bp) with increasing amounts of MalR (Figure 4D). Therefore, it can be inferred that the binding site of MalR is located downstream (329–607 bp) of *Pblcimh*. To confirm the specificity binding site of MalR, the downstream fragment was divided into four portions (*Pblcimh21* (319–368 bp), *Pblcimh22* (319–428 bp), *Pblcimh23* (319–478 bp), and *Pblcimh2* (319–607 bp)) (Figure 4D). These four portions were labeled with biotin and incubated with increasing amounts of MalR. The results indicate the prominent binding of MalR to *Pblcimh2*, whereas *Pblcimh21*, *Pblcimh22*, and *Pblcimh23* exhibited no binding (Figure 5A–D). According to these results, it can be inferred that the binding site of MalR is located at the fragment of 479–607 bp. To validate the above results, the sequence of 479–607 bp was labeled with biotin and incubated with increasing amounts of MalR. As expected, a significant band shift was detected (Figure 5E).

The sequence of 479–607 bp of *Pblcimh* was analyzed according to the above results. It was found that a nested structure was present in the fragment, as is shown in Figure 5E. The nested structure is made up of repeating segments of (A/T) (A/T)CAAA. It was annotated in red. Meanwhile, the same structure was shown in the middle. It was annotated in blue (Figure 5E). The sites of the nested structure are referred to as *CmalR*. To validate the above results, the fragments in front of *CmalR* were labeled with biotin and incubated with MalR. As is shown in Figure 5F,G, shifted binds were not detected in both experimental sets. The weak trailing shifted band was detected when the probe contained portions of *CmalR* (Figure 5H). According to the above results, a complete sequence of *CmalR* appears to be essential for the recognition of MalR. Some studies have demonstrated that regulator binding sites are flexible, and the key domain was the conversed domain located at the two ends of the sequence.[24,30,31] To verify whether *CmalR* was flexible, the middle area and the whole *CmalR* were removed. Then, the sequences were labeled with biotin and incubated with MalR. As shown in Figure 5I, the shifted band was detected when sequences of the middle area were deleted. As expected, the results showed that the deletion of *cmalR* eliminated the binding of MalR to the probe (Figure 5J). Prior studies have noted that recognition sites are pivotal for binding the regulator to a DNA sequence. Insertions or deletions of recognition sites will affect the binding of the regulator to a DNA sequence and further affect metabolism.[22,32]

In vitro experiments demonstrate that the action site of the malate biosensor is a nested structure called *CmalR*. Next, *in vivo* experiments were performed to prove that the nested structure regulates metabolic processes. The transformant cells containing *pPblcimhΔC2GFP*, whose *CmalR* was knocked out, were cultured with different malate contents (0, 10, 15, and 20 g/L), and fluorescence was detected. As expected, compared with that of *BLcimhFP*, the fluorescence of *BLcimhΔC2GFP* increased significantly, and the fluorescence of *BLcimhΔC2GFP* was about 1.5 times that of *BLcimhFP* (Figure 5K). Moreover, a positive relationship between the fluorescence and malate content was not detected.

Improvement of the Malate Biosensor Using Artificial Promoter. An increase was observed in the fluorescence intensity per cell in *BLcimhFP* cultures exposed to malate. A major incentive for developing biosensors is to create a device with the best possible sensitivity.[33,34] However, on the basis of the above results, the utilization of the nature promoter was a

https://doi.org/10.1021/acssynbio.1c00170
ACS Synth. Biol. 2021, 10, 1775–1784

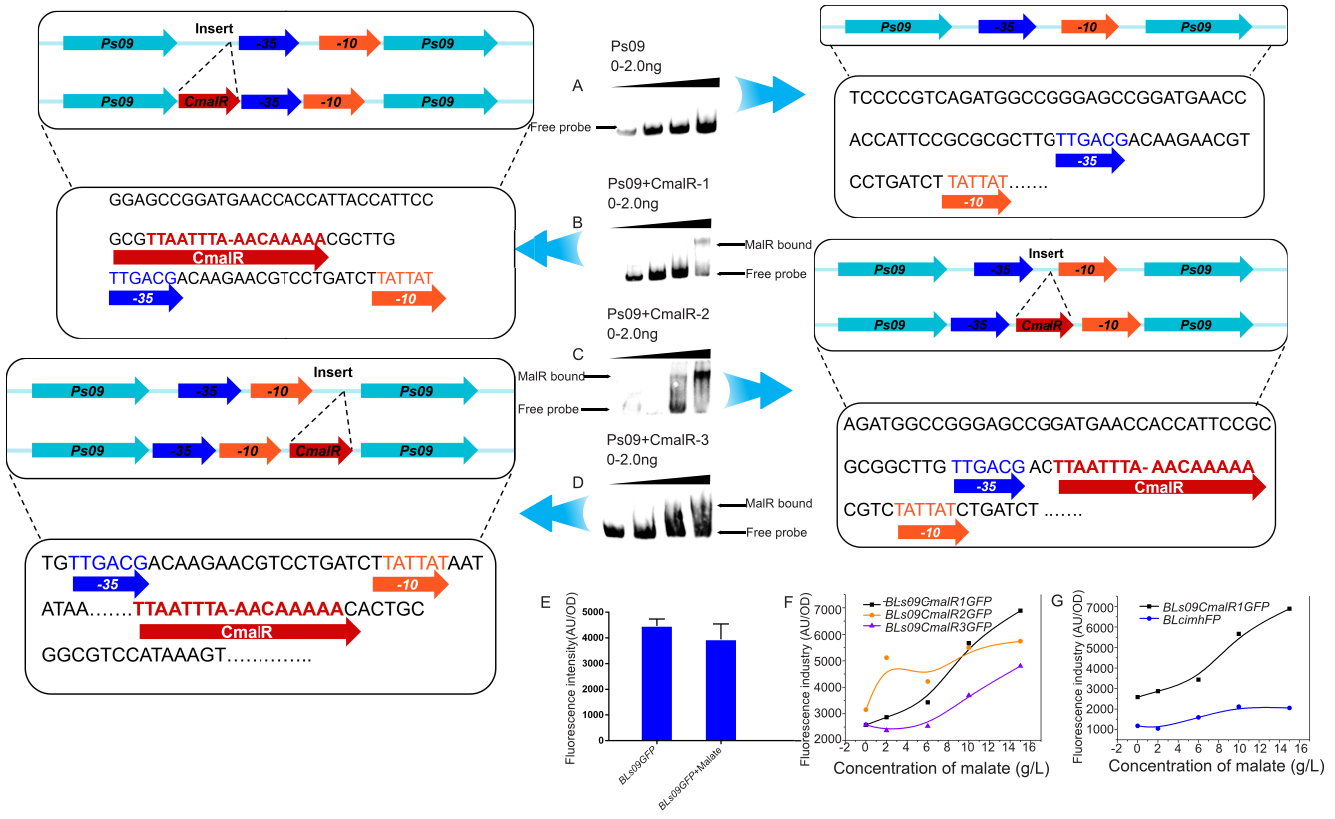


Figure 6. Transformation of *Pshuttle 09*. (A) EMSA verification of MalR binding to *Pshuttle 09*. (B–D) EMSA verification of MalR binding to *Pshuttle 09* with CmalR was inserted into different locations. (E) Specific fluorescence of *B. licheniformis* cells containing *ps09GFP*. (F) Specific fluorescence of *B. licheniformis* cells containing *ps09CmalR1GFP*, p*s09CmalR2GFP*, and p*s09CmalR3GFP* as a function of the malate concentration. (G) Comparison of the specific fluorescence of *BlcimhFP* and *BLs09CmalR1GFP*.

limitation in the construction of a highly sensitive biosensor. The malate biosensor constructed in this study involved the interaction of the regulator with the promoter as well as the regulation of the expression of *eGFP* in the presence of malate. The promoter remodeling strategy is effective in constructing sensitive sensors. In previous studies, promoter remodeling and engineering an allosteric transcription factor have been used to respond to new effectors.[22,35,36] Some studies have noted that efficient promoters were necessary to construct a sensitive biosensor.[22,37] Promoter *Pshuttle 09* has been shown to be an efficient promoter and has an 8-fold intensity of P43 in *Bacillus*.[38]

To create a malate biosensor with efficient detection ability, the sensitivity of the plasmid-based *pPblcimhGFP* construct was further improved. The promoter response to malate was amplified by coupling the *CmalR* with *Pshuttle 09*. The recombinant biosensors were named *ps09CmalR1GFP*, p*s09CmalR2GFP*, and *ps09CmalR3GFP* (Figure 6). To demonstrate that the recombinant biosensors were effective, *in vitro* and *in vivo* experiments were performed. First, EMSA was performed and analyzed as described (Figure 6A–D). As expected, the results did change significantly. Shifted bands were not detected when *Pshuttle* 09 was incubated with MalR (Figure 6A). In contrast, shifted bands were detected when *CmalR* was inserted into different sites of *Pshuttle 09* (Figure 6B–D).

In the view of the above *in vitro* results, reconstructed-promoters were recognized by MalR. But, the effectiveness of the reconstructed malate biosensor needed to be validated *in vivo*. Before performing *in vivo* validated studies, *Pshuttle 09* needed to demonstrate that it was not a response to malate. Transformants containing *ps09GFP* were cultured in TB medium with malate. As shown in Figure 6E, *Pshuttle 09* did not respond to malate. Then, the recombinant biosensors were cultured in TB medium supplemented with different concentrations of malate. Results are shown in Figure 6F. Compared with *BLs09CmalR1GFP* and *BLs09CmalR3GFP*, a strong correlation was observed between the fluorescence and concentration of malate in *BLs09CmalR2GFP*. Additionally, the change in fluorescence caused by malate was easier to detect than *pPblcimhGFP*.

Transcriptional amplifiers of genetically encoded biosensors can enhance transcriptional signals with a large output dynamic range.[39] In this study, a new malate biosensor platform was used to improve the malate biosensor response to malate. The *CmalR*-based signal amplifier increased the dynamic range of malate detection (1000–2000 to 2500–6500). Due to the recognition of *CmalR*, this special site can be applied to construct a variety of biosensors. Above this range, *CmalR* was recognized and regulated by MalR. Therefore, the insertion or deletion of *CmalR* will change the metabolic process regulated by malate.

MATERIALS AND METHODS

Bacterial Strains and Reagents. The plasmids and strains used in this study are presented in Table S1. The *E. coli* strain JM109 was used for the cloning of shuttle plasmids, and the strain *B. licheniformis* CICIM B1391 was used for the

https://doi.org/10.1021/acssynbio.1c00170
ACS Synth. Biol. 2021, 10, 1775–1784

construction of biosensors. Luria−Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) was used for cultivation. BR medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 0.5 M D-sorbitol, 0.5 M mannitol, and 10% glycose) was used for growth recovery after transformation. The medium (12 g/L tryptone, 24 g/L yeast extract, 12.54 g/L K_2HPO_4, and 2.31 g/L KH_2PO_4) was used for bacterial cultivation. Ampicillin (100 μg/mL) and tetracycline (20 μg/mL) may be added when necessary. High-fidelity polymerase used for polymerase chain reaction (PCR) experiments, and a one-step cloning kit was bought from Vazyme (Vazyme biotech, Nanjing, China). All restriction enzymes and T4 DNA ligase were purchased from Takara (Takara Bio, Dalian, China).

Plasmid and Strain Construction. All recombinant plasmids constructed were confirmed by Sanger sequencing and restriction analysis.

The constructions of p*GFP*, pPbl*mdhGFP*, pPbl*cimhGFP*, pPbl*maenGFP*, pPbl*malaGFP*, pPbl*maeaGFP*, pPbl*pytsjGFP*, *GFP*-F, and *GFP*-R were used as primers to acquire fragments of e*GFP*. Then, the fragment of e*GFP* was double digested by *Xho*I and *Eco*RI, followed by ligation with a previously double-digested vector. Other primers used in the study are presented in Table S2. Pbl*mdh*, Pbl*cimh*, Pbl*mala*, Pbl*maea*, Pbl*maen*, and Pbl*pytsj* were amplified from the genomic acid of *B. licheniformis* (CICIM B1391) using target-specific primers (P*mdh*-F and P*mdh*-R for Pbl*mdh*; P*cimh*-F and P*cimh*-R for Pbl*cimh*; P*maen*-F and P*maen*-R for Pbl*maen*; P*mala*-F and P*mala*-R for Pbl*mala;* P*maeA*-F and P*maeA*-R for Pbl*maea*). The fragments of Pbl*mdh*, Pbl*cimh*, Pbl*mala*, Pbl*maea*, and Pbl*pytsj* were double digested by *Hin*dIII and *Xho*I, followed by ligation with the previously double-digested p*GFP*. Pbl*maen* was inserted into p*GFP* using a one-step cloning kit (Vazyme biotech, Nanjing, China). All the plasmids constructed were transformed into *B. licheniformis* according to a method by Li et al.[40]

pET*malR*, *malR*-F, and *malR*-R were used as a primers to amplify *malR* from the genomic acid of *B. licheniformis*. Then, the fragment was double digested by *Bam*HI and *Xho*I followed by ligation with pET28a. The plasmid was transformed into a BL21 *E. coli* strain.

TPbl*cimh*, TPbl*cimh*1, TPbl*cimh*2, TPbl*cimh*21, TPbl*cimh*22, TPbl*cimh*23, TPbl*cimh*ΔC1, TPbl*cimh*ΔC2, and pPbl*cimh*ΔC2*GFP* were constructed. To identify the MalR recognition sites, *Pcimh* was divided into five parts (*Pcimh*1, *Pcimh*2, *Pcimh*21, *Pcimh*22, and *Pcimh*23) before undergoing ligation with pMD19T. TPbl*cimh*ΔC1 and TPbl*cimh*ΔC2 were constructed on the basis of TPbl*cimh*. Four fragments were amplified using target-specific primers (P*cimh*-F and P*cimh*ΔC1-R1 for *Pcimh*Δ1-1; P*cimh*ΔC1-F2 and P*cimh*ΔC1-R2 for *Pcimh*Δ1-2; P*cimh*-F and P*cimh*ΔC2-R1 for *Pcimh*Δ2-1; P*cimh*ΔC1-F2 and P*cimh*ΔC1-R2 for *Pcimh*Δ2-2); overlap-extension PCR was performed to delete sequence1 (ttaaataactcagagcaaa) and sequence2 (ttaatttagttaaataactcagagcaaagggataacaaaaa). Then, *cimh*Δ1 and *cimh*Δ2 were followed by ligation with pMD19-T-simple. The fragment *Pcimh*Δ2 was amplified using the primers P*cimh*-F and P*cimh*-R with TPbl*cimh*ΔC2 as a template. Then, the fragment was double digested by *Hin*dIII and *Xho*I, followed by ligation with p*GFP*. TPbl*cimh*, TPbl*cimh*1, TPbl*cimh*2, TPbl*cimh*21, TPbl*cimh*22, TPbl*cimh*23, TPbl*cimh*ΔC1, and TPbl*cimh*ΔC2 were transformed into *E. coli* JM109. pPbl*cimh*ΔC2*GFP* was transformed into *B. licheniformis*.

P*s09GFP*, P*s09CmalR*1*GFP*, P*s09CmalR*2*GFP*, and P*s09CmalR*3*GFP* were designed to replace the native *cimh* promoter. P*s09GFP* was constructed according to p*GFP*. The fragment of *Pshuttle09* was amplified using primers P*s09*-F and P*s09*-R and then was digested by *Hin*dIII and *Xho*I, followed by ligation with p*GFP*. P*s09CmalR*1*GFP*, P*s09CmalR*2*GFP*, and P*s09CmalR*3*GFP* were constructed according to P*s09GFP*. The P*s09CmalR*1*GFP* plasmid was constructed with the recognition site of MalR (*CmalR*) inserted upstream of the −35 region. The P*s09CmalR*2*GFP* plasmid was constructed with the recognition site of MalR (*CmalR*) inserted downstream of the −35 region and upstream of the −10 region. The P*s09CmalR*1*GFP* plasmid was constructed with the recognition site of MalR (*CmalR*) inserted upstream of the −10 region. The plasmids described above were transformed into *B. licheniformis*.

Culture Conditions and Malate Supplementation. Single *B. licheniformis* colonies harboring a biosensor plasmid were individually inoculated into LB medium containing tetracycline and cultured at 37 °C, 250 rpm overnight. Then, the cultures were inoculated at OD_{600} = 0.15 into fresh 30 mL of TB medium supplemented with tetracycline at a final concentration of 20 μg/mL in a 250 mL conical flask at 37 °C, 250 rpm. When the bacteria were cultured in OD_{600} = 3 (2, 6, 10, 15, and 20 g/L), it was added to the 250 mL conical flask. Fluorescence intensity was monitored after 24 h.

Fluorescence Determination. *B. licheniformis* cells harboring a biosensor plasmid were harvested by centrifugation, and the supernatant was removed. Then, the cells were washed twice with buffer (PB) and diluted to OD_{600} = 0.5−1.0. Two hundred microliters of each sample was then transferred to a 96-well plate. Fluorescence was measured using a microplate reader (Tecan, Männedorf, Switzerland) at 485/535 nm using black-walled 96-well polystyrene plates. OD_{600} was also measured using a microplate reader.

Protein Overexpression and Purification. The heterologous expression of MalR in *E. coli BL21* was undertaken using the pET28a vector, which contains six his tags. Recombinant plasmids containing *pETmalR* were transformed into *E. coli BL21* (DE3) competent cells according to Furst et al.'s method.[41] First, BL21pETMalR was activated on plants. A single colony was inoculated into 15 mL of LB medium and cultured overnight. The next day, 30 mL cultures were seeded with the 900 μL culture and incubated for 2 h at 37 °C. When the OD_{600} of the cultures reached 0.6−0.8, corresponding inducers (IPTG) were added.[42] After 12 h of incubation, cells were harvested by centrifugation and the supernatant removed. To remove any impurities on the surface of the cells, the cells were washed twice with buffer. The cells were then resuspended in buffer (PBS) and broken by sonication on ice and centrifuged at 8000 rpm for 10 min. His-tagged proteins were purified using Mag-Beads His-Tag Protein Purification (Sangon Biotech, Shanghai, China) and checked using SDS-PAGE.[43]

Electrophoretic Mobility Shift Assay. EMSA was performed using a Chemiluminescent biotin-labeled nucleic acid detection kit (Beyotime Biotechnology, Shanghai, China). The EMSA experiment used competing DNA. DNA probes were amplified using high-fidelity DNA polymerase (2 × Phanta Mix), and primers were labeled with biotin. Biotin-labeled probes were purified by agarose gel electrophoresis. First, MalR was mixed with a binding buffer and let to stand for 10 min at room temperature. Then, probes were added to the

https://doi.org/10.1021/acssynbio.1c00170
ACS Synth. Biol. 2021, 10, 1775−1784

above mixture. All biotin probes used in the study were incubated with various concentrations of MalR (0−2.0 ng) for 20 min at room temperature in a binding buffer. After the reaction was completed, the samples were separated by electrophoresis using 4% acrylamide gels in 0.5 × tris-borate EDTA (TBE) buffer. The samples were electroblotted from the acrylamide gels onto a nylon membrane (Beyotime, FFN15) and immobilized by UV cross-linking. The subsequent procedures were performed according to the manufacturer's instructions with cautionary notes.[44,45]

CONCLUSION

In this study, *BlcimhFP* biosensors for metabolites of the tricarboxylic acid cycle malate were created and improved. Malate has shown potential in areas such as food processing, manufacturing, and water treatment.[46] The regulator MalR was mined from the genome of *B. licheniformis*. The initial *BlcimhFP* biosensor showed a weak response to malate. The dynamic range of fluorescence was small. The weak fluorescence changes made it difficult to determine whether changes were caused by a change in malate content. After demonstrating the binding site of MalR, it was combined with an effective promoter (*Pshuttle 09*) to amplify fluorescence signals. Malate could be detected using a fluorescence signal that depends on malate concentration. This malate biosensor can be applied to identify novel enzymes and pathways involved in malate production, improve known malate synthases, or optimize malate production, thereby helping to develop microbial cell factories for malate production in *B. licheniformis*.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00170.

Figure of homology analysis of malate regulator between *B. licheniformis* and *B. subtilis* and tables of strains and plasmids used, primers used, and target genes mined (PDF)

AUTHOR INFORMATION

Corresponding Authors

Youran Li − *Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China;* orcid.org/0000-0003-3396-4132; Email: liyouran@jiangnan.edu.cn

Guiyang Shi − *Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China;* orcid.org/0000-0001-5147-0238; Email: gyshi@jiangnan.edu.cn

Authors

Yupeng Zhang − *Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China*

Fengxu Xiao − *Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China*

Hanrong Wang − *Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China*

Liang Zhang − *Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China*

Zhongyang Ding − *Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China*

Sha Xu − *Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China*

Zhenghua Gu − *Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China; Jiangsu Provincial Research Center for Bioactive Product Processing*

https://doi.org/10.1021/acssynbio.1c00170
ACS Synth. Biol. 2021, 10, 1775−1784

Technology, Jiangnan University, Wuxi, Jiangsu Province 214122, People's Republic of China

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.1c00170

Author Contributions

Y.Z., Y.L., and G.S. designed the study, carried out the experiments, analyzed data, and wrote the paper. F.X. and H.W. carried out the experiments. L.Z. and Z.D. analyzed data. Z.G. and S.X. designed the study.

Funding

This work was supported by National Key Research & Development Program of China (2020YFA0907700, 2018YFA0900300, and 2018YFA0900504), the National Natural Foundation of China (31401674), the National First-Class Discipline Program of Light Industry Technology and Engineering (LITE2018-22), and the Top-notch Academic Programs Project of Jiangsu Higher Education Institutions.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Dong, X., Chen, X., Qian, Y., Wang, Y., Wang, L., Qiao, W., and Liu, L. (2017) Metabolic engineering of *Escherichia coli* W3110 to produce L-malate. *Biotechnol. Bioeng. 114*, 656−664.

(2) Zou, X., Cheng, C., Feng, J., Song, X., Lin, M., and Yang, S.-T. (2019) Biosynthesis of polymalic acid in fermentation: advances and prospects for industrial application. *Crit. Rev. Biotechnol. 39*, 408−421.

(3) Goldberg, I., Rokem, J. S., and Pines, O. (2006) Organic acids: old metabolites, new themes. *J. Chem. Technol. Biotechnol. 81*, 1601−1611.

(4) Iyyappan, J., Baskar, G., Gnansounou, E., Pandey, A., Raaman, J. K., Bharathiraja, B., and Praveenkumar, R. (2019) Recent advances in microbial production of malic acid from renewable byproducts. *Rev. Environ. Sci. Bio/Technol. 18*, 579−595.

(5) Zhu, F., San, K. Y., and Bennett, G. N. (2020) Metabolic engineering of *Escherichia coli* for malate production with a temperature sensitive malate dehydrogenase. *Biochem. Eng. J. 164*, 107762.

(6) Wang, J., Yuan, H., Wei, X., Chen, J., and Chen, S. (2016) Enhancement of poly−glutamic acid production by alkaline pH stress treatment in *Bacillus licheniformis* WX-02. *J. Chem. Technol. Biotechnol. 91*, 2399−2403.

(7) Wei, X., Ji, Z., and Chen, S. (2010) Isolation of halotolerant *Bacillus licheniformis* WX-02 and regulatory effects of sodium chloride on yield and molecular sizes of poly-gamma-glutamic acid. *Appl. Biochem. Biotechnol. 160*, 1332−1340.

(8) Yuan, H., Xu, Y., Chen, Y., Zhan, Y., Wei, X., Li, L., Wang, D., He, P., Li, S., and Chen, S. (2019) Metabolomics analysis reveals global acetoin stress response of *Bacillus licheniformis*. *Metabolomics 15* (3), 25.

(9) Sun, W., Jiang, B., Zhang, Y., Guo, J., Zhao, D., Pu, Z., and Bao, Y. (2020) Enabling the biosynthesis of malic acid in *Lactococcus lactis* by establishing the reductive TCA pathway and promoter engineering. *Biochem. Eng. J. 161*, 107645.

(10) Rogers, J. K., Taylor, N. D., and Church, G. M. (2016) Biosensor-based engineering of biosynthetic pathways. *Curr. Opin. Biotechnol. 42*, 84−91.

(11) Liu, Y., Zhuang, Y., Ding, D., Xu, Y., Sun, J., and Zhang, D. (2017) Biosensor-based evolution and elucidation of a biosynthetic pathway in *Escherichia coli*. *ACS Synth. Biol. 6*, 837−848.

(12) Kim, S. K., Kim, S. H., Subhadra, B., Woo, S.-G., Rha, E., Kim, S.-W., Kim, H., Lee, D.-H., and Lee, S.-G. (2018) A genetically encoded biosensor for monitoring isoprene production in engineered *Escherichia coli*. *ACS Synth. Biol. 7*, 2379−2390.

(13) Zappi, D., Coronado, E., Soljan, V., Basile, G., Varani, G., Turemis, M., and Giardi, M. T. (2021) A microbial sensor platform based on bacterial bioluminescence (luxAB) and green fluorescent protein (gfp) reporters for in situ monitoring of toxicity of wastewater nitrification process dynamics. *Talanta 221*, 121438−121438.

(14) Binder, S., Schendzielorz, G., Staebler, N., Krumbach, K., Hoffmann, K., Bott, M., and Eggeling, L. (2012) A high-throughput approach to identify genomic variants of bacterial metabolite producers at the single-cell level. *Genome. Biol. 13*, R40.

(15) Siedler, S., Stahlhut, S. G., Malla, S., Maury, J., and Neves, A. R. (2014) Novel biosensors based on flavonoid-responsive transcriptional regulators introduced into *Escherichia coli*. *Metab. Eng. 21*, 2−8.

(16) Wu, J., Jiang, P., Chen, W., Xiong, D., Huang, L., Jia, J., Chen, Y., Jin, J.-M., and Tang, S.-Y. (2017) Design and application of a lactulose biosensor. *Sci. Rep. 7*, 45994.

(17) Guzman Vazquez de Prada, A., Ruiz-Barrio, M. A., Reviejo-Garcia, J., and Pingarron, J. M. (2011) Enzyme-based biosensors for analysis of glucose, fructose and malic acid in wines. *Alimentaria*, 70−74.

(18) Ganesh, I., Ravikumar, S., Yoo, I.-k., and Hong, S. H. (2015) Construction of malate-sensing *Escherichia coli* by introduction of a novel chimeric two-component system. *Bioprocess Biosyst. Eng. 38*, 797−804.

(19) Zhan, Y., Zhou, M., Wang, H., Chen, L., Li, Z., Cai, D., Wen, Z., Ma, X., and Chen, S. (2020) Efficient synthesis of 2-phenylethanol from L-phenylalanine by engineered *Bacillus licheniformis* using molasses as carbon source. *Appl. Microbiol. Biotechnol. 104*, 7507−7520.

(20) Silano, V., Baviera, J. M. B., Bolognesi, C., Cocconcelli, P. S., Crebelli, R., Gott, D. M., Grob, K., Lambre, C., Lampi, E., and Mengelers, M. (2020) Safety evaluation of the food enzyme phospholipase C from the genetically modified *Bacillus licheniformis* strain NZYM-VR. *EFSA J. 18* (7), e06184.

(21) Charbonnier, T., Le Coq, D., McGovern, S., Calabre, M., Delumeau, O., Aymerich, S., and Jules, M. (2017) Molecular and physiological logics of the pyruvate-induced response of a novel transporter in *Bacillus subtilis*. *mBio 8* (5), 18.

(22) Xu, X., Li, X., Liu, Y., Zhu, Y., Li, J., Du, G., Chen, J., Ledesma-Amaro, R., and Liu, L. (2020) Pyruvate-responsive genetic circuits for dynamic control of central metabolism. *Nat. Chem. Biol. 16*, 1261−1268.

(23) Wang, Y., Wang, H., Wei, L., Li, S., Liu, L., and Wang, X. (2020) Synthetic promoter design in *Escherichia coli* based on a deep generative network. *Nucleic Acids Res. 48*, 6403−6412.

(24) Zhang, L., Liu, Y., Yang, Y., Jiang, W., and Gu, Y. (2018) A novel dual-cre motif enables two-way autoregulation of CcpA in *Clostridium acetobutylicum*. *Appl. Environ. Microbiol. 84* (8), 1.

(25) Wu, R., Gu, M., Wilton, R., Babnigg, G., Kim, Y., Pokkuluri, P. R., Szurmant, H., Joachimiak, A., and Schiffer, M. (2013) Insight into the sporulation phosphorelay: Crystal structure of the sensor domain of *Bacillus subtilis* histidine kinase, KinD. *Protein Sci. 22*, 564−576.

(26) Doan, T., Servant, P., Tojo, S., Yamaguchi, H., Lerondel, G., Yoshida, K., Fujita, Y., and Aymerich, S. (2003) The *Bacillus subtilis* ywkA gene encodes a malic enzyme and its transcription is activated by the YufL/YufM two-component system in response to malate. *Microbiology 149*, 2331−2343.

(27) Krause, J. P., Polen, T., Youn, J. W., Emer, D., Eikmanns, B. J., and Wendisch, V. F. (2012) Regulation of the malic enzyme gene malE by the transcriptional regulator MalR in *Corynebacterium glutamicum*. *J. Biotechnol. 159*, 204−215.

(28) Wilkinson, S. P., and Grove, A. (2006) Ligand-responsive transcriptional regulation by members of the MarR family of winged helix proteins. *Curr. Issues. Mol. Biol. 8* (1), 51−62.

(29) Xiao, F., Li, Y., Zhang, Y., Wang, H., Zhang, L., Ding, Z., Gu, Z., Xu, S., and Shi, G. (2020) Construction of a novel sugar alcohol-inducible expression system in *Bacillus licheniformis*. *Appl. Microbiol. Biotechnol. 104*, 5409−5425.

(30) Yang, Y., Zhang, L., Huang, H., Yang, C., Yang, S., Gu, Y., and Jiang, W. (2017) A flexible binding site architecture provides new

insights into CcpA global regulation in gram-positive bacteria. *mBio 8* (1), 1.
(31) Yamamoto, H., Murata, M., and Sekiguchi, J. (2000) The CitST two-component system regulates the expression of the Mg-citrate transporter in *Bacillus subtilis*. *Mol. Microbiol. 37*, 898−912.
(32) Langa, S., Peiroten, A., Arques, J. L., and Landete, J. M. (2021) Catabolite responsive elements as a strategy for the control of heterologous gene expression in *Lactobacilli*. *Appl. Microbiol. Biotechnol. 105* (1), 225−233.
(33) Gimenez-Gomez, P., Gutierrez-Capitan, M., Capdevila, F., Puig-Pujol, A., Fernandez-Sanchez, C., and Jimenez-Jorquera, C. (2017) Robust L-malate bienzymatic biosensor to enable the on-site monitoring of malolactic fermentation of red wines. *Anal. Chim. Acta 954*, 105−113.
(34) Perrotta, P. R., Arevalo, F. J., Vettorazzi, N. R., Zon, M. A., and Fernandez, H. (2012) Development of a very sensitive electrochemical magneto immunosensor for the direct determination of ochratoxin A in red wine. *Sens. Actuators, B 162* (1), 327−333.
(35) Taylor, N. D., Garruss, A. S., Moretti, R., Chan, S., Arbing, M. A., Cascio, D., Rogers, J. K., Isaacs, F. J., Kosuri, S., Baker, D., et al. (2016) Engineering an allosteric transcription factor to respond to new ligands. *Nat. Methods 13* (2), 177−183.
(36) Ogasawara, H., Ishida, Y., Yamada, K., Yamamoto, K., and Ishihama, A. (2007) PdhR (pyruvate dehydrogenase complex regulator) controls the respiratory electron transport system in *Escherichia coli*. *J. Bacteriol. 189*, 5534−5541.
(37) Meyer, A. J., Segall-Shapiro, T. H., Glassey, E., Zhang, J., and Voigt, C. A. (2019) *Escherichia coli* "Marionette" strains with 12 highly optimized small-molecule sensors. *Nat. Chem. Biol. 15* (2), 196−204.
(38) Liu, Y., Shi, C., Li, D., Chen, X., Li, J., Zhang, Y., Yuan, H., Li, Y., and Lu, F. (2019) Engineering a highly efficient expression system to produce BcaPRO protease in *Bacillus subtilis* by an optimized promoter and signal peptide. *Int. J. Biol. Macromol. 138*, 903−911.
(39) Wang, B., Barahona, M., and Buck, M. (2014) Engineering modular and tunable genetic amplifiers for scaling transcriptional signals in cascaded gene networks. *Nucleic Acids Res. 42*, 9484−9492.
(40) Li, Y., Jin, K., Zhang, L., Ding, Z., Gu, Z., and Shi, G. (2018) Development of an inducible secretory expression system in *Bacillus licheniformis* based on an engineered xylose operon. *J. Agric. Food Chem. 66*, 9456−9464.
(41) Furst, A. L., Hoepker, A. C., and Francis, M. B. (2017) Quantifying hormone disruptors with an engineered bacterial biosensor. *ACS Cent. Sci. 3*, 110−116.
(42) Gao, C., Hou, J., Xu, P., Guo, L., Chen, X., Hu, G., Ye, C., Edwards, H., Chen, J., Chen, W., and Liu, L. (2019) Programmable biomolecular switches for rewiring flux in *Escherichia coli*. *Nat. Commun. 10* (1), 3751.
(43) Ray, L., Valentic, T. R., Miyazawa, T., Withall, D. M., Song, L., Milligan, J. C., Osada, H., Takahashi, S., Tsai, S.-C., and Challis, G. L. (2016) A crotonyl-CoA reductase-carboxylase independent pathway for assembly of unusual alkylmalonyl-CoA polyketide synthase extender units. *Nat. Commun. 7* (1), 13609.
(44) Hellman, L. M., and Fried, M. G. (2007) Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nat. Protoc. 2*, 1849−1861.
(45) Xiao, F., Li, Y., Zhang, Y., Wang, H., Zhang, L., Ding, Z., Gu, Z., Xu, S., and Shi, G. (2021) A new CcpA binding site plays a bidirectional role in carbon catabolism in *Bacillus licheniformis*. *iScience. 24* (5), 102400.
(46) West, T. P. (2017) Microbial production of malic acid from biofuel-related coproducts and biomass. *Fermentation. 3* (2), 14.

https://doi.org/10.1021/acssynbio.1c00170
ACS Synth. Biol. 2021, 10, 1775−1784