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Construction of malate-sensing *Escherichia coli* by introduction of a novel chimeric two-component system

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Abstract In an attempt to develop a high-throughput screening system for screening microorganisms which produce high amounts of malate, a MalKZ chimeric HK-based biosensor was constructed. Considering the sequence similarity among *Escherichia coli* (*E. coli*) MalK with *Bacillus subtilis* MalK and *E. coli* DcuS, the putative sensor domain of MalK was fused with the catalytic domain of EnvZ. The chimeric MalK/EnvZ TCS induced the *ompC* promoter through the cognate response regulator, OmpR, in response to extracellular malate. Real-time quantitative PCR and GFP fluorescence studies showed increased *ompC* gene expression and GFP fluorescence as malate concentration increased. By using this strategy, various chimeric TCS-based bacteria biosensors can be constructed, which may be used for the development of biochemical-producing recombinant microorganisms.

Keywords Malate · Chimeric two-component system · Green fluorescent protein · *Escherichia coli*

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Introduction

Malate is a member of the C_4-dicarboxylates, along with succinate and fumarate, and is an intermediate of the TCA cycle. It can also be formed from pyruvate as one of the anaplerotic reactions and functions biologically in the production of energy in both aerobic and anaerobic conditions [1]. Considering its core role in the generation of cellular energy, malate has been used in pharmaceuticals, cosmetics and the food industry [2–4]. Recently, biological production of chemicals and polymers from renewable sources has gained intensive interest, considering the limited nature of petroleum resources. The US Department of Energy has identified malate and other C_4-dicarboxylates as chemical building blocks of interest, which can be made in large quantities from renewable carbohydrates via biological or chemical processes.

Though it has been reported that malate can be produced by various microorganisms such as *Saccharomyces cerevisiae*, *Aspergillus flavus* and recombinant *Escherichia coli* (*E. coli*) [4], the development of more enhanced malate producers and processes is necessary to meet industrial requirements and compete with petroleum-based processes. To accomplish the development of efficient malate-producing recombinant strains, a massive number of metabolically engineered microorganisms must be constructed and tested. Then, the development of a novel high-throughput screening system for malate overproducing microorganisms is mandatory to achieve the final goal efficiently. Bacterial biosensors can be considered as one of the most promising candidates of high-throughput screening systems.

Two-component systems (TCS) are bacterial environmental stimulus-sensing apparatus. TCS consist of a membrane-bound histidine kinase (or sensor kinase, HK)

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and a cytoplasmic response regulator (RR) [5, 6]. Binding of an environmental signal ligand to the sensory domain of the HK induces autophosphorylation of the catalytic transmitter domain which serves as a phosphor donor for its cognate RR, activating the control protein and leading to transcriptional regulation. It was reported that *E. coli* senses environmental malate along with other C_4-dicarboxylates by the use of DcuS/DcuR TCS [7, 8]. The *dcuB* gene is strongly expressed under anaerobic conditions due to activation of DcuS/R, while the *dctA* gene is weakly expressed under aerobic conditions. However, DcuS/R-mediated expression characteristics were not found to be suitable for biosensors, especially under aerobic conditions. Therefore, the construction of a novel malate-sensing TCS is required.

The chimeric HK strategy has been proven to be a powerful tool for engineering TCS with novel properties. HK engineering has mainly employed the domain-swapping strategy, by which the sensory domain and catalytic domain from different HKs are combined. To construct a ribose-sensing TrZ1 chimeric HK, the sensory domain of Trg was integrated with the transmitter domain of EnvZ [9]. Similar to the example above, a light-sensing chimeric Cph8 was created by fusing the cyanobacterial light-sensing phytochrome Cph1 with EnvZ [10]. A TGF-beta–EnvZ chimera was also constructed by replacing the extracellular sensor domain of EnvZ with the TGF-beta receptor, by which it became able to sense an array of human TGF-beta ligands [11]. A similar strategy was employed for the MzrA–EnvZ two-hybrid system, which influences the EnvZ/OmpR regulon [12].

E. coli MalK was tested as a novel malate-sensing HK, considering its nucleotide sequence similarity with the *Bacillus subtilis* malate sensor MalK (renamed from YufL) and with the *E. coli* fumarate sensor DcuS. MalK/EnvZ (MalKZ) chimeric HK was constructed by fusing the sensory domain of MalK with the cytoplasmic catalytic domain of EnvZ (Fig. 1). The resulting MalKZ chimeric HK sensed extracellular malate to activate OmpR, inducing the *ompC* promoter. The expression profile of the *ompC* promoter was monitored by qRT-PCR and green fluorescent protein (GFP) under aerobic conditions to assess the dynamic response of the chimeric TCS in the presence of malate and other C_4-dicarboxylates.

Materials and methods

Bacterial strains and culture conditions

E. coli BL21-DE3 and XL1-Blue were used as host strains for recombinant DNA manipulation. The bacterial strains and plasmids used in this study are listed in SI Table 1. *E. coli* strains were grown aerobically in Luria–Bertani (LB) broth (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract and 5 g/L NaCl) and in M9 minimal salts medium (Sigma) supplemented with 4 g/L of glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 1 % thiamine HCl, at both 37 °C and 30 °C under vigorous shaking.

Construction of chimeric MalKZ TCS and GFP-based reporter system

For construction of the MalKZ chimeric HK, the truncated sensory domain of *malK* (540 bp) and the catalytic domain of *envZ* (687 bp) were amplified from *E. coli* XL1-Blue genomic DNA [13]. The PCR products were then cloned into the low copy number plasmid pACYCDuet-1 using the *Bam*HI and *Hin*dIII restriction sites to construct the pMalKZ1 plasmid (SI Fig. 1a). Expression of pMalKZ1 was placed under the control of the T7 promoter, induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG).

The genomic region containing 250 bp of the *ompC* promoter was amplified from *E. coli* XL1-Blue genomic DNA with the oligonucleotides OmpC_F_*Eco*RI and OmpC_R_*Bam*HI (SI Table 2). Oligonucleotides GFPm_F_*Bam*HI and GFPm_R_*SalI* (SI Table 2) were used to amplify the *gfp* gene from GFP-mut3.1 which was previously cloned in the pET expression system [14]. The *ompC* promoter was then integrated with the *gfp* gene by the overlap PCR method, after which it was cloned into pUC19 to construct pOGFP1 (SI Fig. 1b).

Monitoring of expression by real-time quantitative PCR

The transcriptional activities of the *ompC* gene in response to the presence of malate were measured by qRT-PCR. A single colony of an *E. coli* strain harboring pMalKZ1 was grown overnight in LB medium at 37 °C. It was then diluted 100-fold in fresh M9 medium (supplemented with 25 μg/mL chloramphenicol and 100 μg/mL ampicillin) and incubated at 37 °C in an orbital shaker at 250 rpm until the optical density at 600 nm (OD_{600}) reached 0.5. At that time, 10 μM IPTG was added to the culture and the cells were grown aerobically for an additional 4.5 h at 30 °C in the presence of varying concentrations of malate. After 4.5 h, the cells were harvested by centrifugation for total RNA preparation, which was carried out using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen), followed by treatment with DNase. Reverse transcription (RT) was performed with a cDNA synthesis kit (Applied Biosystems) using a random hexamer primer mix, according to the manufacturer's instructions. Specific primers were designed with OLIGO software (version 5.0; Molecular Biology Insights, Cascade, CO) for analysis of

the quantitative expression of the o*mpC* gene and 16sRNA (SI Table 2). Samples with the omission of the RT step were used as negative controls to check that the extracted RNA was not contaminated with DNA. qRT-PCR reactions were performed on a Mini-Opticon detection system using SYBR Green PCR Master mix, as recommended by the manufacturer. Each qRT-PCR experiment was performed in triplicate for biological samples, using separate cultures grown under identical aerobic conditions ($n = 3$) and calculations were performed automatically by the Mini-Opticon software using 16S rRNA as an internal control [15].

Monitoring of expression by fluorescence

A single colony of the *E. coli* BL21-DE3 strain harboring pMalKZ1 and pOGFP1 was grown overnight at 37 °C in LB medium. The overnight culture was diluted 100-fold in fresh M9 medium supplemented with 25 μg/mL chloramphenicol and 100 μg/mL ampicillin and incubated aerobically at 37 °C in an orbital shaker at 250 rpm until OD_{600} reached 0.5. The culture then was incubated at 30 °C after adding 10 μM of IPTG to induce the expression of the chimeric HK in aerobic conditions.

Cell density and fluorescence were measured under various concentrations of malate during 8 h of culturing. Other metabolites such as fumarate, succinate and aspartate were also tested in addition to malate. Cell density was monitored by measuring OD_{600} with a spectrophotometer (Shimadzu, Japan), and GFP fluorescence was measured using an RF-5301PC spectrofluorimeter (Shimadzu, Japan). The excitation wavelength of the spectrofluorimeter was set to 485/10 nm, with an emission wavelength of 515/10 nm. The specific fluorescence intensity (SFI) was defined as the raw fluorescence intensity, expressed in relative fluorescence units, divided by the optical density at 600 nm measured at each time point. At a minimum, triplicate measurements were obtained for each sample.

Principal component analysis (PCA)

PCA is a useful statistical technique for the evaluation of the effects of multiple factors [16]. The input to the analysis was a 4×3 matrix, wherein each row corresponds to the four C_4-dicarboxylic acids, and each column corresponds to the different induction concentrations (0.1, 1.0 and 10) used in this study. The output of the PCA was a 4×3 matrix. In this form, each C_4-dicarboxylic acid was represented as a point in three dimensions, where each axis was a principal component. Each principal component was a linear combination of the three different concentrations of metabolites used. The contributions of each concentration were PC1 and PC2, where each metabolite corresponded to activation of the chimeric MalKZ/OmpR TCS. PC1 and PC2 together captured 99.9 % of the variance of the original dataset.

Results

Monitoring of the *dcuB* gene expression

First, DcuS/R TCS, which is a known as C_4-dicarboxylate-sensing TCS, was tested as a candidate aerobic malate-sensing apparatus. To investigate the dynamic characteristics of the DcuS/R TCS in response to malate, the expression induced by the *dcuB* promoter was monitored under various malate concentrations using an *E. coli* strain harboring pDBGFP1. Plasmid pDBGFP1 contains the *gfp* gene downstream of the *ducB* promoter [17]. A significant increase of fluorescence was not observed in the control strain, harboring pUC19. When 0.1 mM of malate was added to the culture media, elevated fluorescence was obtained under aerobic conditions (Fig. 2). Increasing malate concentrations, however, did not provide a further increase of fluorescence. Theseresults suggested that DcuS/R TCS is too sensitive to extracellular malate, with a maximum output signal produced even at only 0.1 mM of malate. To be used as a biosensor, a gradual increase of the output signal in response to the malate concentration is required. As *E. coli* DcuS/R TCS demonstrated poor gene expression characteristics, it is not suitable to be applied as an aerobic biosensor. Therefore, the construction of a novel TCS with the desired characteristics was subsequently conducted in this study.

Sequence alignment

The MalK/MalR TCS was previously reported to control the uptake and metabolism of malate in *B. subitilis* [18]. It can be supposed that *E. coli* MalK may also have the function of a malate-sensing HK, although MalR has not been found in *E. coli*. Nucleotide sequence alignment was performed among *E. coli malK*, *E. coli dcuS* and *B. subitilis malK* genes (SI Fig. 2). The *E. coli malK* gene showed 38.87 % sequence identity with the *B. subitilis malK* gene, and 42.69 % sequence identity with the *E. coli dcuS* gene. This result suggested that *E. coli* MalK may have key conserved malate-sensing residues and the function of a malate-sensing HK in addition to its primary known function, which is for ATP hydrolyzation involved in maltose transport [19, 20].

Construction and expression of the chimeric MalK/EnvZ TCS

The MalKZ chimeric HK was constructed by integration of the putative sensor domain of MalK and the cytoplasmic

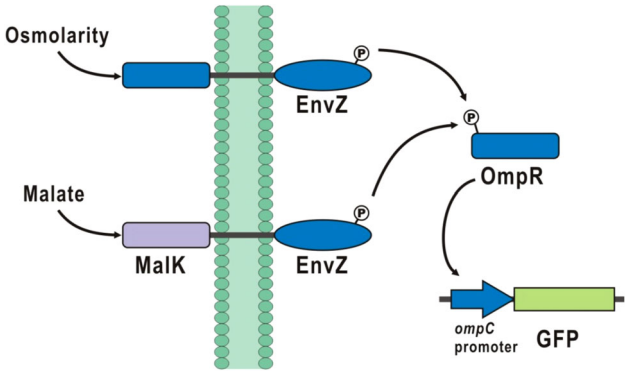

Fig. 1 *E. coli* engineered to respond to exogenous malate. Maltose receptor MalK and the histidine kinase domain of EnvZ/OmpR from *Escherichia coli* were fused to form a chimeric protein which phosphorylates the response regulator OmpR of the TCS. This activates the synthetic genetic circuit of MalKZ/OmpR TCS, resulting in malate sensing

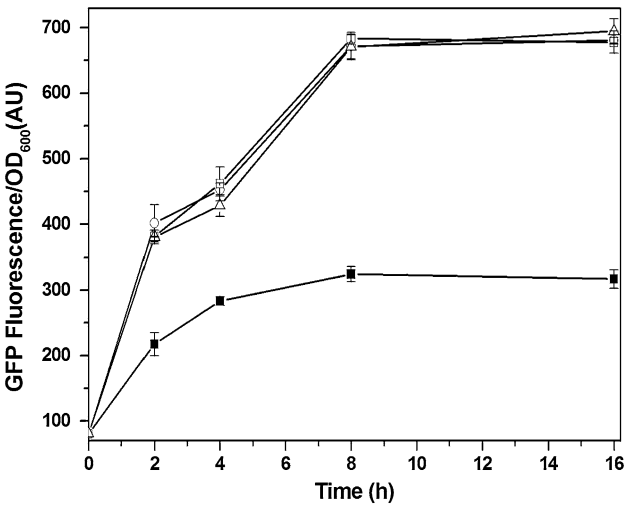

Fig. 2 Time course of GFP fluorescence of the *E. coli* XL1-Blue strain harboring pDBGFP1 after induction with varying concentrations of malate in M9 medium. Control (*closed square*); 0.1 mM (*open circle*); 1.0 mM (*open square*); 10 mM (*open triangle*). The data represent the aggregate results from replicate experiments ($n = 3$)

catalytic domain of EnvZ. If MalKZ chimeric HK was expressed more highly in the cellular membrane, a stronger output signal could be obtained in response to extracellular malate. However, it also may cause the cellular membrane to become unstable, which could lead to a reduction of cellular activity or cell lysis. Therefore, MalKZ should be expressed at low levels, which was achieved by cloning the *malKZ* gene into the very low copy number vector, pACYCDuet-1. The expression of MalKZ was analyzed by SDS-PAGE, and the results showed that MalKZ, with the

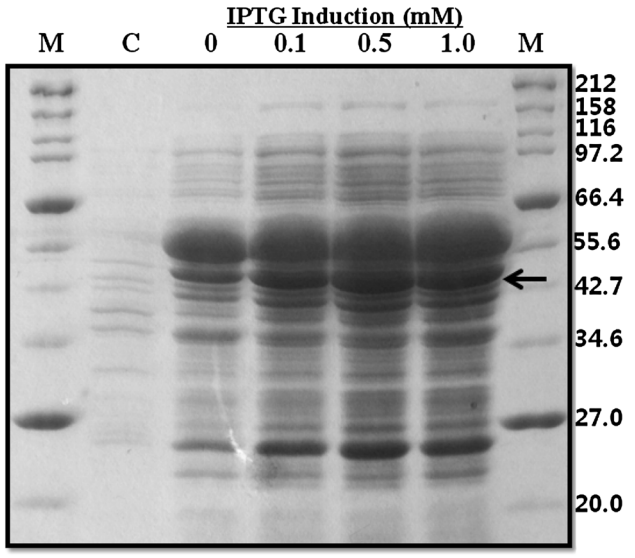

Fig. 3 SDS-PAGE expression analysis of MalK–EnvZ fusion protein extracts in the culture medium of pMalKZ1-transformed *E. coli* BL21 (DE3) cells, with non-induced recombinant plasmid and vector plasmid as controls. *M* Molecular weight marker in kDa

size of 46.6 kDa, was successfully expressed by the addition of various concentrations of IPTG (Fig. 3).

A pOGFP1 reporter plasmid was constructed (SI Fig. 1b) to monitor the output signal of the MalKZ chimeric HK in response to extracellular malate. In pOGFP1, GFP-mut3.1 was expressed under the control of the *ompC* promoter, which was induced by the activated OmpR. GFP-mut3.1 is a rapid-folding type of engineered GFP, and has been used in various studies due to its fast protein expression and folding [21–23]. By introduction of the pMalKZ1 and pOGFP1 plasmids into *E. coli*, a bacterial malate biosensor was constructed, which sensed extracellular malate and expressed fluorescent protein as an output signal.

Monitoring of *ompC* gene expression, as controlled by the chimeric MalKZ/OmpR TCS using qRT-PCR

If extracellular malate was sensed by the MalKZ chimeric HK, it activated OmpR, leading to induction of the *ompC* promoter. The expression of the *ompC* gene in pMalKZ1-harboring *E. coli* was monitored by qRT-PCR in minimal media supplemented with varying concentrations of malate. The results of qRT-PCR showed that the transcriptional level of the *ompC* gene gradually increased as the extracellular malate concentration increased (Fig. 4). This result suggested that the chimeric MalKZ/OmpR TCS generated an output signal which is strong enough to induce the expression of the *ompC* gene. The correlation between the malate concentration and the relative

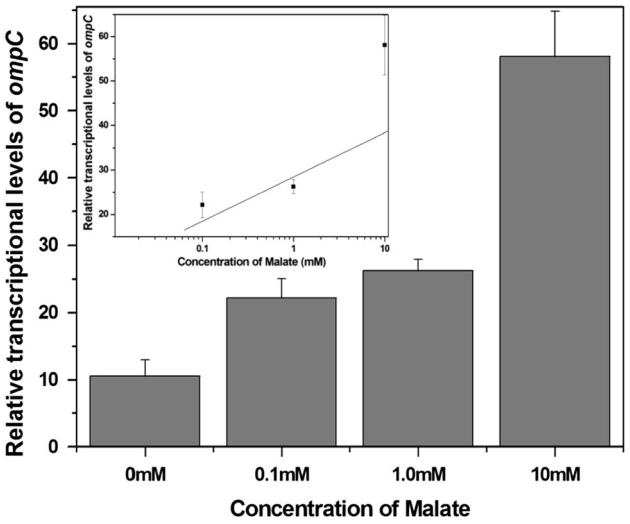

Fig. 4 Comparative study of transcriptional levels of the *ompC* gene in M9 medium after a 4.5 h exposure to malate. After exposure, the C_t value was normalized using the 16sRNA C_t value as an internal control. The *error bars* indicate one standard deviation from the mean. The linear correlation between the relative transcriptional levels of the *ompC* gene induced by the chimera in M9 medium at varying concentration of malate is shown in the *box*. The data represent the aggregate results from replicated experiments ($n = 3$)

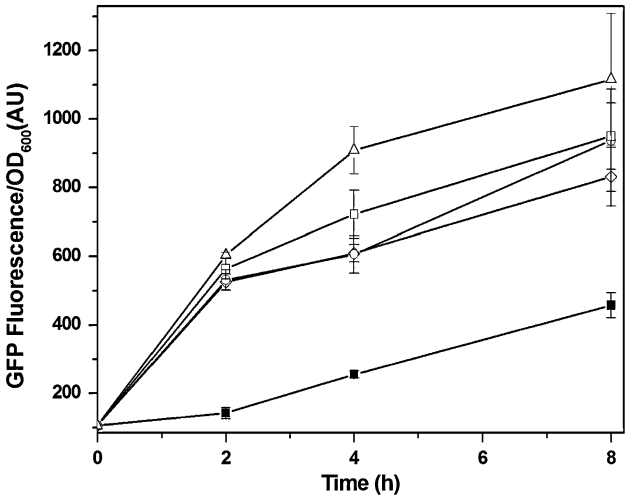

Fig. 5 Time course of GFP fluorescence of an *E. coli* strain harboring pMalKZ1 and pOGFP1 after induction with varying concentrations of malate in M9 medium. Control (*closed square*); 0.05 mM (*open diamond*); 0.1 mM (*open circle*); 1.0 mM (*open square*); 10 mM (*open triangle*). The data represent the aggregate results from replicated experiments ($n = 3$)

transcriptional level was estimated to be 9.947 for malate concentrations in the range of 0.1–10 mM (Fig. 4). The expression level of the *ompC* gene was found to be quite proportional to the extracellular malate concentration. Based on these results, it can be deduced that *ompC* gene expression is precisely regulated by chimeric MalKZ in response to extracellular malate concentration under aerobic conditions.

Monitoring of the *gfp* gene expression induced by the chimeric MalKZ/OmpR TCS

From the qRT-PCR results, it was shown that *ompC* gene expression was precisely controlled via the chimeric MalKZ/OmpR TCS in response to extracellular malate. The fluorescence-based malate biosensor system was then constructed. For construction of the reporter plasmid pOGFP1, the *gfp* gene was cloned downstream of the *ompC* promoter, allowing GFP to be expressed under the control of the *ompC* promoter (SI Fig. 1b). *E. coli* BL21 (DE3) harboring pMalKZ1 and pOGFP1 was cultured aerobically with various concentrations of malate (0.05–10 mM) and the fluorescence was measured. The control strain, harboring pACYCDuet-1 and pUC19 instead of pMalKZ1 and pOGFP1, exhibited a negligible amount of fluorescence (Fig. 5). The time profiles of fluorescence showed that fluorescence increased as the culturing time increased (Fig. 5). Although 0.05, 0.1, 1.0 and 10 mM concentrations of malate produced similar levels of fluorescence in the 2 h culture, higher concentrations of malate provided a more rapid increase of fluorescence. At the end of 8 h, the fluorescence increased as the malate concentration increased, with the highest fluorescence obtained with 10 mM of malate.

Analysis of TCS chimera specificity

To evaluate the specificity of the constructed MalKZ/OmpR TCS-based biosensor, the recombinant strain was cultured with other metabolites such as fumarate, succinate and aspartate, which have similar molecular structures with malate. The four metabolites, at concentrations of 0.1, 1.0 and 10 mM, were supplemented into the culture media and fluorescence was measured. At all test concentrations, malate provided the highest fluorescence, while the lowest fluorescence was obtained with aspartate (Fig. 6a). This result suggested that the newly constructed chimeric TCS can specifically sense malate, along with other metabolites with similar molecular structures.

PCA was applied to the fluorescence data obtained with malate, fumarate, succinate and aspartate to analyze their individual effects on the activity of chimeric TCS. PCA has been used in multivariate data analysis by visualizing the diversity and clustering of patterns [16]. PCA analysis was conducted here to estimate the output signal exhibition patterns in response to different C_4-dicarboxylates. The

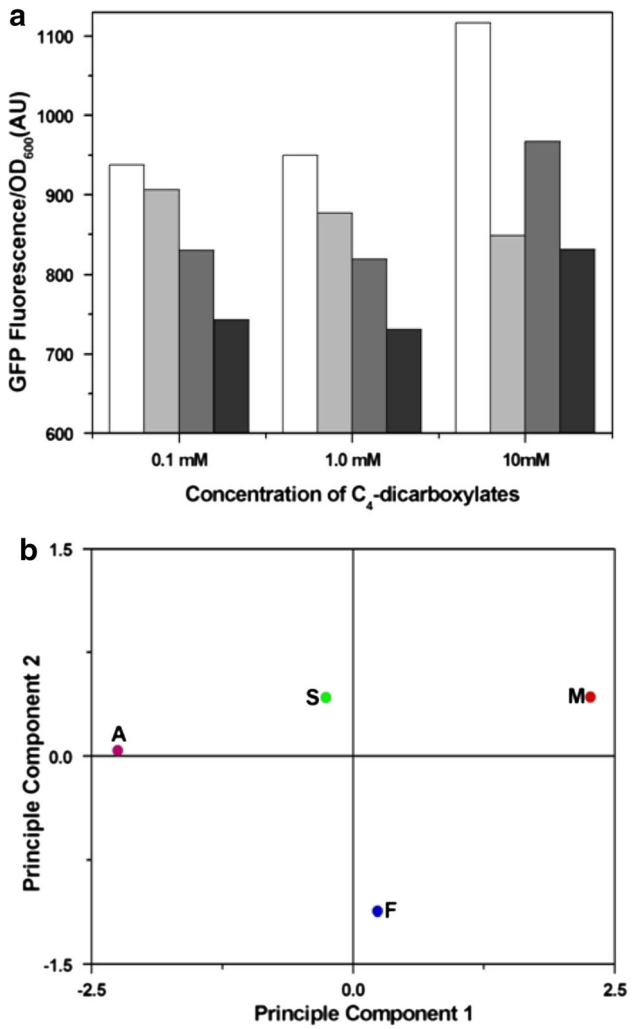

Fig. 6 **a** Comparative study of GFP fluorescence of an *E. coli* strain harboring pMalKZ1 and pOGFP1 after induction with varying concentrations of C_4-dicarboxylates in M9 medium after 8 h. The data represent the aggregate results from replicated experiments ($n = 3$). Malate (*white*), fumarate (*light gray*), succinate (*gray*) and aspartate (*dark gray*). **b** PCA was used to visualize the diversity in the sensor activity patterns produced by induction with 0.1, 1.0 and 10 mM concentrations of malate (M), fumarate (F), succinate (S) and aspartate (A) in cells harboring pMalKZ1 and pOGFP1. Each point corresponds to the intensity of the green fluorescent protein generated by the effectors used in a chimeric two-dimensional principal component space. The points are diverse in the two-dimensional principal component space with the time course data produced

fluorescence obtained at varying concentrations (0.1, 1.0 and 10) of the four different C_4-dicarboxylates (malate, fumarate, succinate and aspartate) was used as input data. The results showed that the fluorescence expression profile was strongly correlated with malate, followed by succinate and fumarate, while aspartate alone was located away from the other metabolites. These results suggested that the newly designed bacterial biosensor system has a unique expression pattern with respect to malate (Fig. 6b).

Discussion

Two-component systems are one of the common environmental sensors of free-living bacteria, and are used for the fine control of gene expression in response to frequently changing environmental conditions. In *E. coli*, DcuS/DcuR TCS senses malate under anaerobic conditions. However, the DcuS/DcuR TCS-mediated expression profile was not good enough to be used as an aerobic biosensor (Fig. 2). Therefore, construction of a novel malate-sensing TCS, which demonstrates a good gene expression profile under aerobic conditions, is required for application as a malate sensor. The putative sensor domain of MalK was integrated with the catalytic domain of EnvZ, considering the results of sequence alignment. Though MalK is an ATP-hydrolyzing subunit of the maltose network system, it has a similar nucleotide sequence with *B. subtilis* MalK and *E. coli* DcuS (SI Fig. 2). The constructed MalKZ/OmpR TCS successfully sensed environmental malate, and a good correlation between the output gene expression and malate concentration was achieved. This result implies that each TCS has unique signal profiles, and TCS can be engineered to have better characteristics including sensitivity and specificity by employing chimeric TCS strategy. Based on these results, it can be deduced that the chimeric TCS strategy is quite effective for the construction of novel TCS with the desired properties [24–27].

By employing the EnvZ/OmpR TCS as a basic apparatus, a stable output signal profile was obtained. EnvZ/OmpR TCS is one of the most studied TCS in *E. coli* and controls the expression of the outer membrane porins OmpF and OmpC in response to changes in osmolarity [28, 29]. Considering its well-characterized features, it has been applied in the construction of chimeric TCS by fusion of EnvZ with other HKs [30–34].

Though the qRT-PCR result showed that MalKZ-/OmpR-mediated output signal gradually increased as the malate concentration increased, a high level of fluorescence was observed even at low malate concentrations (Fig. 5). This may be because of overamplification of the output signal from use of the pUC19-based high copy number reporter plasmid (pOGFP1). It seems that using a low copy number plasmid for MalKZ TCS was not enough to control the fluorescence signal to low levels, and more modifications such as cloning the *ompC-gfp* reporter system in a medium or low copy number plasmid could be made. Though novel MalKZ/OmpR TCS showed malate-correlated expression profile, its specificity was not good enough to be directly used as a malate biosensor. Further engineering of the sensory domain of MalKZ is required to enhance the specificity.

These days, considering the limited nature of the petroleum-based industry, the biomass-based industry or

biorefineries attract extreme interest. For the commercialization of biomass-based products, the optimization of intracellular metabolism and the screening of efficient producers are necessary. Bacterial biosensors based on TCS are the potential candidates for high-throughput screening systems for the assessment of biochemical-producing organisms. Especially, various TCS can be constructed to sense the desired biochemical by employing a TCS chimera strategy, which was effectively employed in this study. Therefore, various chimeric TCS-based bacteria biosensors can be constructed, which may be used for the development of engineered microorganisms for producing biochemicals.

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