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Expression characteristics of the *maeA* and *maeB* genes by extracellular malate and pyruvate in *Escherichia coli*

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Abstract−The malate-pyruvate conversion pathway is catalyzed by two malic enzyme isomers, MaeA and MaeB. qRT-PCR was carried out under malate and pyruvate supplemented conditions to understand the dynamics of *maeA* and *maeB* gene expression. *maeA* expression was elevated by malate, and *maeB* expression was inhibited by levels of both malate and pyruvate higher than 0.1 mM. Green fluorescent protein (GFP) reporter plasmids were also constructed by integration of the *maeA*/*maeB* promoter with the *gfp* gene. We showed that *maeA* driven GFP expression was positively and negatively correlated with extracellular malate and pyruvate induction. In contrast, no significant changes in *maeB* driven GFP expression were observed under both malate and pyruvate supplemented conditions.

Key words: Malate, Pyruvate, *maeA*, *maeB*, Green Fluorescent Protein, *Escherichia coli*

INTRODUCTION

Malate is a C_4-dicarboxylic acid that is a key intermediate in the tricarboxylic acid (TCA) cycle [1-5]. Malate has traditionally been used in pharmaceuticals and cosmetics [6,7], and recently has received much interest for use as a monomer in polymer industries [8]. The U.S. Department of Energy has designated malate as one of the top building block chemicals that could be made in large quantities from renewable carbohydrates [9]. Recent increases in oil prices and global warming have generated interest in the production of malate by recombinant bacteria from renewable sources [10,11].

For the overproduction of malate, redirection of carbon flux from the C_3 pathway to the C_4 pathway is required (Fig. 1). Several C_3-C_4 connecting enzymes have been overexpressed in previous approaches: phosphoenol-pyruvate carboxylase (Ppc) from *Escherichia coli* [12], phosphoenolpyruvate carboxy kinase (Pck) from *E. coli* [13] and *Actinobacillus succinogens* [14], and pyruvate carboxylase (Pyc) from *Rhizobium elti* [15]. Although malic enzyme was successfully employed for succinic acid overproduction [16,17], which is another important C_4 intermediate, malic enzyme has not been tested for malate overproduction.

Malic enzyme is a key enzyme in the malate pathway that also links the C_4-C_3 pathway as it catalyzes the reversible decarboxylation of malate to pyruvate and CO_2, using either NAD^+ or $NADP^+$ as the redox cofactor [18]. In *E. coli*, both the *maeA* (or *sfcA*) and *maeB* (or *ypfF*) genes encode the malic enzyme by using NAD^+ and $NADP^+$ as a coenzyme, respectively [16,19]. The kinetics of *maeA* and *maeB* were recently determined in detail [20]. The *maeA* gene is involved in gluconeogenesis by providing pyruvate and has been used in metabolic engineering [16], while *maeB* contributed to the generation of NADPH, which is needed for bacterial growth on two carbon compounds [21].

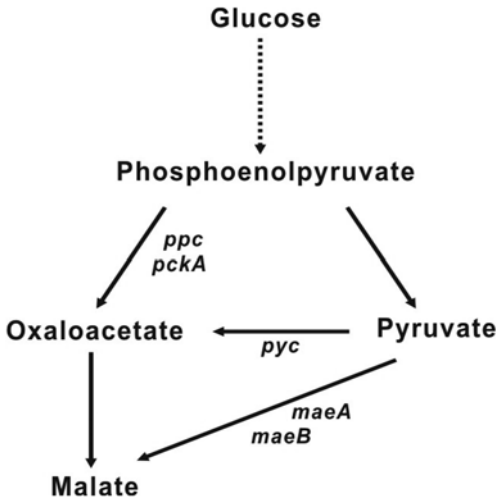

Fig. 1. Malic acid pathway in *E. coli*. Enzymes encoded by the genes shown are: *maeA*/*maeB*, malic enzyme; *pckA*, phosphoenolpyruvate carboxykinase; *ppc*, phosphoenolpyruvate carboxylase; *pyc*, pyruvate carboxylase.

To see if malic enzyme is appropriate for the redirection of carbon flux from C_3 (pyruvate) to C_4 (malate), expression characteristics of malic enzyme on malate and pyruvate needed to be studied. To understand the expression behaviors of the *maeA* and *maeB* genes

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Table 1. Strains, plasmids and primers used in this study

Strain, plasmids and primers	Relevant genotype and/or property	Source or ref.
Strains		
Escherichia coli		
XL1-Blue	SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F' ($proAB^+$ $lacI^q$ lacZM15 Tn10 (tet^R))	Laboratory stock
Plasmids		
pUC19	Amp^R	New England Biolabs[a]
pMAGFP1	pUC19 (156 bp), *maeA'-gfp* transcriptional fusion vector, containing *maeA* promoter region, Amp^R	This work
pMBGFP1	pUC19 (288 bp), *maeB'-gfp* transcriptional fusion vector, containing *maeB* promoter region, Amp^R	This work

[a]New England Biolabs, Beverly, MA, U.S.A.

at the transcription level, real-time quantitative PCR (qRT-PCR) studies were carried out at varying concentrations of malate and pyruvate. Then, both the *maeA* and *maeB* promoter expression time profiles were studied using a green fluorescence protein (GFP) based monitoring system, in which GFP was expressed under the control of respective promoters.

MATERIALS AND METHODS

1. Bacterial Strains and Plasmids

E. coli XL1-Blue was used as a general cloning host. Construction of the plasmids and bacterial strains used in this study are listed in Table 1. All *E. coli* strains were grown in a M9 minimal salts medium (2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1% thiamine HCl and 0.4% glucose as a carbon source) supplemented with antibiotics (ampicillin, 100 mg/*l*) at 37 °C with vigorous shaking (250 rpm).

2. Construction of Plasmids

The genomic region containing 156 bp of *maeA* (tentatively 5'UTR <89 bases) promoter gene was amplified with oligonucleotides FSalI GGATCCGGTTAATCCTCCAGTGGTTGTC and RBamHI GTCGACCTTCTTCTTTGCCTGCTCATCCC and 288 bp of *maeB* promoter gene (tentatively 5'UTR<127 bases) was amplified with oligonucleotides FEcoRI GAATTCAGGAAATACTCCTTGAAA and RBamHI GGATCCTTGGTTGTTCCTTTCACG from *E. coli* genomic DNA. At the same time, *gfp* gene encoding GFP was amplified from plasmid pPROBE-NT' [22] genomic DNA with oligonucleotides FBamHI GGATCCATGAGTAAAGGAGAAGAACTTTTC and RKpnI GGTACCCCTTAGCTCCTGAAAATCTCG for *maeA* promoter and with oligonucleotides FBamHI GGATCCATGCAGTCTAAAGGAGAA and RSalI GTCGACTTATTAATGGTGATGGTG for *maeB* promoter. PCR was performed with the MJ mini Personal Thermal Cycler (BioRad Laboratories, USA) using an Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). Then, the *maeA* promoter was integrated with GFP by overlap PCR using oligonucleotides FSalI and RKpnI, and the *maeB* promoter was integrated with GFP by using PCR with oligonucleotides *maeB* FEcoRI and GFP RSalI. To construct pMAGFP1 and pMBGFP1plasmids (SI Fig. 1), the PCR products were then ligated into the pUC19 vector (NEB, USA) and were transformed into chemically competent *E. coli* cells [23]. The sequences of the resulting plasmids pMAGFP1 and pMBGFP1 were confirmed by primer extension sequencing using oligonucleotides *maeA* FSalI and GFP RKpnI and *maeB* FEcoRI and GFP RSal.

3. qRT-PCR Analysis of Gene Expression

E. coli cells harboring pMAGFP1 and pMBGFP1 were harvested after a 4.5 h culture by centrifugation for RNA isolation. Total cellular RNA was extracted by using the RNEasy Mini kit (Qiagen, USA) according to the manufacturer's instructions, followed by DNase treatment. Specific primers were designed using Primer Express software (Applied Biosystems, USA) for *maeA* and *maeB* genes. For *maeA*, Fwd Primer is CGTGCTGTTGCAGTTTGAAGA and Rev Primer is AAATTTCATTGCGATAGCGGTTA. For *maeB*, CATGTTCGGCATCACCAGAA and CGTATGCCGGACAGCTCTTT is used as Fwd and Rev primer. Primer specificity and efficiency were checked by PCR amplification on *E. coli* XL1-Blue genomic DNA. For the RT reaction, about 200 ng of RNA was incubated with a mix using random hexamer primers (50 ng for 20 μl of final volume, Applied Biosystems, USA). RNA was reverse transcribed for 2 h at 37 °C followed by incubation at 85 °C for 5 min. RT reaction product (0.1 μl) was used for further studies. Samples for which the RT step was omitted were used as negative controls to check that the extracted RNA was not contaminated with DNA. qRT-PCR reactions were performed on the Mini Opticon detection system by using SYBR Green PCR Master mix as recommended by the manufacturer (Applied Biosystems, USA). The amplification program consisted of one cycle of 95 °C with a 60 s hold, followed by 40 cycles of 95 °C with a 15 s hold, then to a specified annealing temperature with a 20 s hold, followed by 60 °C with a 40 s hold. The generation of specific PCR products was confirmed by melting curve analysis (one cycle at 95 °C with 0 s hold, 65 °C with 10 s hold, and 95 °C with 0 s hold) and gel electrophoresis. Each qRT-PCR experiment was performed on three replicates for the biological sample using separate cultures grown under identical conditions (n=3). 16S RNA was used as a reference gene as it is known to be a stable housekeeping gene [24].

4. Evaluation of the Malic Enzyme Expression System

E. coli strains harboring pMAGFP1 and pMBGFP1 were grown independently at 37 °C. The overnight culture was diluted to 100-fold in fresh M9 medium supplemented with 100 μg/ml of ampicillin and incubated at 37 °C in an orbital shaker at 250 rpm until the OD_{600} reached 0.5. Then, the bacterial cells were cultured with

various concentrations of L-malic acid disodium salt to induce the expression system for about 16 h. Another set of *E. coli* strains were induced by various concentrations of sodium pyruvate. During the incubation period, cell growth was measured at an optical density of 600 nm using a UV-visible spectrophotometer (Shimadzu, Japan), and the GFP fluorescence was measured with an RF-5301PC spectrofluorimeter (Shimadzu, Japan). The excitation wavelength of the spectrofluorimeter was set at 490/10 nm, and the emission wavelength was set at 510/10 nm. At least three measurements were obtained for each sample.

Malate and pyruvate induced cells were harvested after 4.5 h by centrifugation at 3,500 ×g for 5 min at 4 °C for analysis of *maeA* and *maeB* promoter driven expression of GFP in M9 medium. The cells were washed with phosphate buffered saline (PBS) solution and were resuspended in PBS solution supplemented with 0.3% agarose. Then, cells were screened for fluorescence with a 100x objective on a reflected fluorescence microscope (Olympus, Japan) with a cooled charge-coupled device camera (B&W SenSys, KAF1401). Emission intensity was recorded by using MetaMorph image analysis software (Molecular Devices, Silicon Valley, CA, USA) with excitation and emission filter sets optimized for EGFP imaging.

RESULTS AND DISCUSSION

1. qRT-PCR Expression Studies

The qRT-PCR was carried out to study the expression profile of both *maeA* and *maeB* genes under varying concentrations of malate and pyruvate in M9 medium. The qRT-PCR results suggested that expression of *maeA* gene was induced by extracellular malate while it was repressed with high concentration of pyruvate (Fig. 2(a)). When extracellular malate concentration increased from 0.1 to 50 mM, the expression level of *maeA* generally increased about sevenfold. The correlation coefficient between malate concentration and the relative transcriptional level was estimated to be 0.995 in the

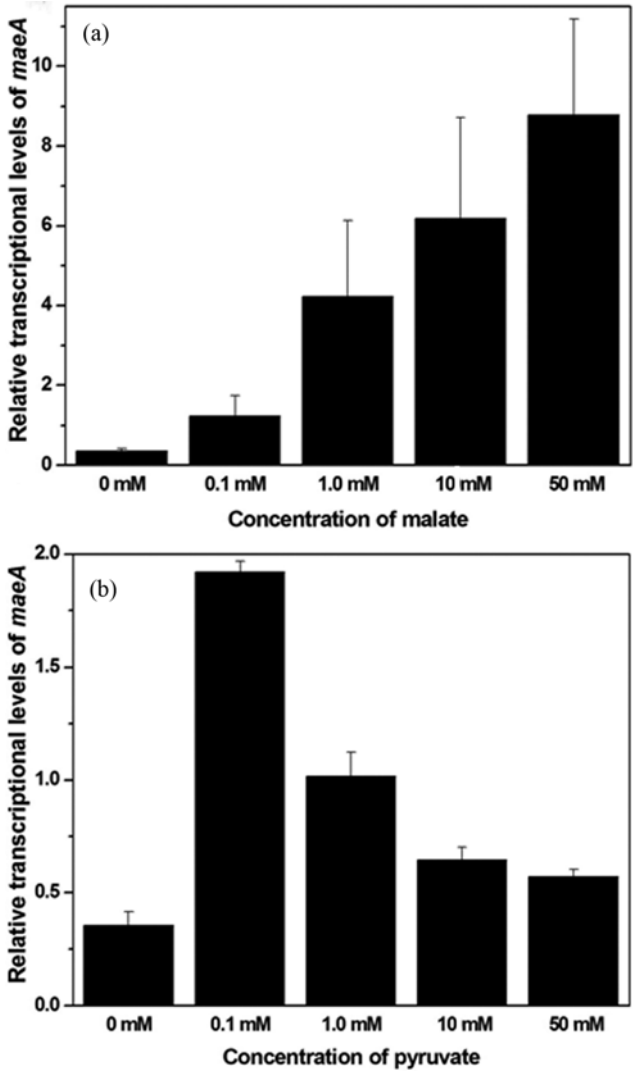

Fig. 2. Relative transcriptional levels of *maeA* in M9 medium at varying concentrations of malate (a) and pyruvate (b) were determined. The error bars indicate one standard deviation from the mean. The data are aggregate results from malate and pyruvate treatments from replicate experiments (n=3).

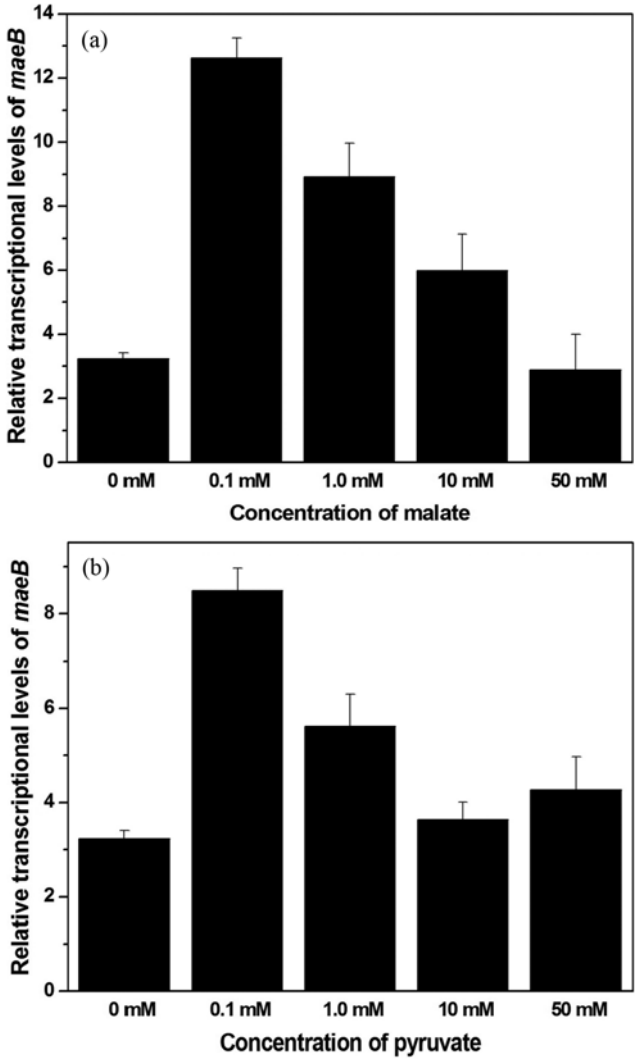

Fig. 3. Relative transcriptional levels of *maeB* in M9 medium at varying concentration of malate (a) and pyruvate (b) were determined. The error bars indicate one standard deviation from the mean. The data are aggregate results from malate and pyruvate treatments from replicate experiments (n=3).

range of 0.1-50 mM malate concentration. This result indicated that *maeA* gene expression is quite precisely regulated by malate concentration. When the same strain was exposed to extracellular pyruvate, different expression profiles of *maeA* gene were observed. On addition of 0.1 mM of pyruvate to media, *maeA* gene expression increased about five-fold (Fig. 2(b)). Then, the expression level of *maeA* decreased approximately to 3.4-fold while extracellular pyruvate concentration increased from 0.1 to 50 mM. This result suggests that *maeA* gene expression is inhibited by higher pyruvate concentration. When pyruvate concentration increased from 0.1 to 1.0 mM, expression level decreased rapidly, and the gradient became less steep as pyruvate concentration increased.

The *maeB* gene expression study was also carried out under various malate and pyruvate concentrations. Similar gene expression profiles were obtained under both malate and pyruvate induced conditions (Fig. 3). When malate and pyruvate concentration increased from 0 to 0.1 mM, *maeB* gene expression increased approximately four- and 2.6-fold, respectively. Above 0.1 mM malate concentration, the expression level of the *maeB* gene decreased gradually as malate concentration increased (Fig. 3(a)). At 50 mM malate induction, the expression level of the *maeB* gene was as low as that obtained under 0 mM malate condition. While pyruvate concentration was increased from 0.1 to 1.0 mM, the expression level of the *maeB* gene decreased significantly (Fig. 3(b)). Then, it decreased slightly beyond 1.0 mM of pyruvate. In the range of 0.1-50 mM, the correlation between both malate and pyruvate concentration and the relative transcription level were found to linearly decreased. This result clearly showed the negative correlation of *maeB* expression by high concentrations of both malate and pyruvate.

2. GFP Reporter Plasmid Expression Studies

The *maeA* and *maeB* expression reporter plasmids were constructed to monitor malic enzyme expression profile using GFP. The *maeA* and *maeB* promoters were integrated with *gfp* reporter gene on the pUC19 plasmid to construct pMAGFP1 and pMBGFP1, respectively. GFP is expressed in response to the extracellular malate and pyruvate. The response of the pMAGFP1 harboring strain to malate was examined by fluorescence microscopy in minimal medium. Malate-exposed strains appeared green, indicating the expression of GFP was under the control of the *maeA* promoter (SI Fig. 2).

To understand dynamic characteristics of malic enzyme expression, the *maeA* and *maeB* promoter driven GFP expression was monitored at regular time intervals after the optimum value of 1.0 mM of malate and pyruvate induction. Without malate and pyruvate addition, a gradual increase in relative fluorescence was observed (Fig. 4(a)). As expected from the qRT-PCR results, pyruvate induction provides a meager change of relative fluorescence profile, while increased fluorescence was observed only with malate induction. After 4 h of malate induction, relative GFP fluorescence (GFP fluorescence/OD) began to increase and reached about 550 after 16 h (Fig. 4(a)). In *maeB* driven GFP expression studies no significant changes were observed (Fig. 4(b)), although a gradual increase was seen in the GFP production at 1.0 mM of both malate and pyruvate induction.

Malate from renewable resources is widely used as an acidulant and taste enhancer in the beverage and food industry. Furthermore, as a chemical, demand for malate is rising as it is used as a monomer in the polymer industry [8]. Though *E. coli* and *S. cerevisiae* serve as excellent platforms for malate production [25-27], more enhancement of yield and productivity is required for industrialization.

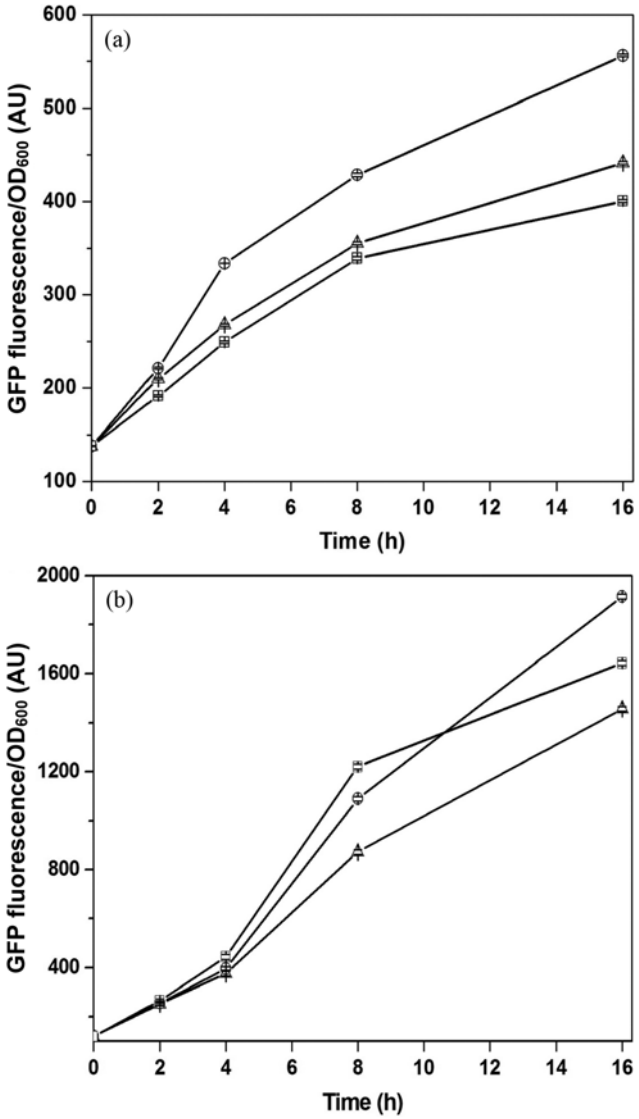

Fig. 4. Time course of relative GFP fluorescence for *E. coli* strain harboring pMAGFP1 (*maeA*) (a) and pMBGFP1 (*maeB*) (b) in M9 medium after induction with 1.0 mM concentration of malate and pyruvate: control (open triangle); malate induced (open circle); pyruvate induced (open square). The data are the aggregate results from malate and pyruvate treatments from replicate experiments (n=3).

Although the conversion of pyruvate to malate is thermodynamically favored [28,29], the expression and activity of malic enzyme are optimized for conversion of malate to pyruvate in enzyme level [16,20]. The kinetic parameters of malic enzymes also suggest that malate conversion to pyruvate is a more spontaneous metabolic reaction. The Km value of MaeA is 0.66 mM for malate and 2.59 mM for pyruvate [20]. The Km value of MaeB for malate (3.41 mM) is also lower than that for pyruvate (6.21 mM) [20]. To redirect pyruvate to malate, which is the opposite of native carbon flux, modification of the expression profile as well as the enzyme kinetics of malic enzyme is required.

To understand the dynamic characteristics of malic enzyme medi-

ated pyruvate-malate linkage, the expressions of *maeA* and *maeB* gene on extracellular malate and pyruvate were studied by using qRT-PCR and GFP reporter protein. The expression results showed that *maeA* gene expression is continuously activated by extracellular malate. When pyruvate was added, the maximum *maeA* gene expression was obtained at 0.1 mM pyruvate, and it decreased at higher pyruvate concentration. But the *maeB* gene showed different expression profiles. The highest *maeB* gene expression was obtained with 0.1 mM of malate or pyruvate, and it was reduced with higher concentration of malate and pyruvate.

In the present work, both *maeA* and *maeB* induced expression was decreased by a higher concentration of pyruvate. It has been already reported that the regulation of both the malic enzymes was correlated with acetyl-CoA and acetyl-P [20]. Acetyl-P inhibits the *maeA* expression and Acetyl-CoA inhibits *maeB* expression, and they are the direct products of pyruvate [30]. Thus, an increase in pyruvate concentration accompanied by an increase in Acetyl-coA and Acetyl-P production leads to negative correlation of *maeB* and *maeA* gene expression. To the best of our knowledge, this study provides the first example of *maeA* and *maeB* gene expression by malate and pyruvate induction in the aerobic minimal medium.

CONCLUSION

Based on our results, we concluded that maximum malic enzyme (*maeA*) expression could be achieved with higher malate concentration. Thus the *maeA* gene was used as a tool in the production of malate and succinate through metabolic engineering strategies [16,17]. These results can be applied for the development of enhanced malate producing bacterial systems.

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SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

REFERENCES

1. Y. Peleg, J. S. Rokem and I. Goldberg, *FEMS Microbiol. Lett.*, **67**, 233 (1990).
2. E. Battat, Y. Peleg, A. Bercovitz, J. S. Rokem and I. Goldberg, *Biotechnol. Bioeng.*, **37**, 1108 (1991).
3. O. Pines, S. Even-Ram, N. Elnathan, E. Battat, O. Aharonov, D. Gibson and I. Goldberg, *Appl. Microbiol. Biotechnol*., **46**, 393 (1996).
4. M. Kawagoe, K. Hyakumura, S. I. Suye, K. Miki and K. Naoe, *J. Ferment. Bioeng*., **84**, 333 (1997).
5. O. Taing and K. Taing, *Eur. Food Res. Technol.*, **224**, 343 (2007).
6. M. Rosenberg, H. Miková and L. Krištofíková, *Lett. Appl. Microbiol*., **29**, 221 (1999).
7. E. Bressler, O. Pines, I. Goldberg and S. Braun, *Biotechnol. Progr*., **18**, 445 (2002).
8. G. Li, D. Yao and M. Zong, *Eur. Polym. J*., **44**, 1123 (2008).
9. T .Werpy and G. Petersen, *Top value added chemicals from biomass*, U.S. Department of Energy, Washington, DC (2004).
10. I. Goldberg, J. S. Rokem and O. Pines, *J. Chem. Technol. Biotechnol.*, **81**, 1601 (2006).
11. C. Roa Engel, A. Straathof, T. Zijlmans, W. van Gulik and L. van der Wielen, *Appl. Microbiol. Biotechnol*., **78**, 379 (2008).
12. C. S. Millard, Y. P. Chao, J. C. Liao and M. I. Donnelly, *Appl. Environ. Microbiol*., **62**, 1808 (1996).
13. Y. D. Kwon, S. Y. Lee and P. Kim, *J. Microbiol. Biotechnol*., **16**, 1448 (2006).
14. P. Kim, M. Laivenieks, C. Vieille and J. G. Zeikus, *Appl. Environ. Microbiol*., **70**, 1238 (2004).
15. R. R. Gokarn, M. A. Eiteman and E. Altman, *Appl. Environ. Microbiol*., **66**, 1844 (2000).
16. L. Stols and M. I. Donnelly, *Appl. Environ. Microbiol*., **63**, 2695 (1997).
17. S. H. Hong and S. Y. Lee, *Biotechnol. Bioeng.*, **74**, 89 (2001).
18. G. G. Chang and L. Tong, *Biochem*., **42**, 12721 (2003).
19. T. Murai, M. Tokushige, J. Nagai and H. Katsuki, *Biochem. Biophys. Res. Commun.*, **43**, 875 (1971).
20. F. P. Bologna, C. S. Andreo and M. F. Drincovich, *J. Bacteriol*., **189**, 5937 (2007).
21. B.Wang, P. Wang, E. Zheng, X. Chen, H. Zhao, P. Song, R. Su, X. Li and G. Zhu, *J. Microbiol*., **49**, 797 (2011).
22. W. G. Miller, J. H. J. Leveau and S. E. Lindow, *Mol. Plant Microbe In.*, **13**, 1243 (2000).
23. J. Sambrook and D. W. Russell, *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, **1** (2001).
24. H. Eleaume and S. Jabbouri, *J. Microbiol. Methods*., **59**, 363 (2004).
25. S. Y. Moon, S. H. Hong, T. Y. Kim and S. Y. Lee, *Biochem. Eng. J*., **40**, 312 (2008).
26. R. M. Zelle, E. de Hulster, W. A. van Winden, P. de Waard, C. Dijkema, A. A. Winkler, J. M. A. Geertman, J. P. van Dijken, J. T. Pronk and A. J. A. van Maris, *Appl. Environ. Microbiol*., **74**, 2766 (2008).
27. X. Zhang, X. Wang, K. T. Shanmugam and L. O. Ingram, *Appl. Environ. Microbiol*., **77**, 427 (2011).
28. R. K. Thauer, K. Jungermann and K. Decker, *Bact. Rev*., **41**, 100 (1977).
29. R. N. Goldberg, Y. B. Tewari, D. Bell and K. Fazio, *J. Phys. Chem. Ref. Data*, **22**, 515 (1993).
30. W. R. McCleary and J. B. Stock, *J. Biol. Chem*., **269**, 31567 (1994).

Supporting Information

Expression characteristics of the *maeA* and *maeB* genes by extracellular malate and pyruvate in *Escherichia coli*

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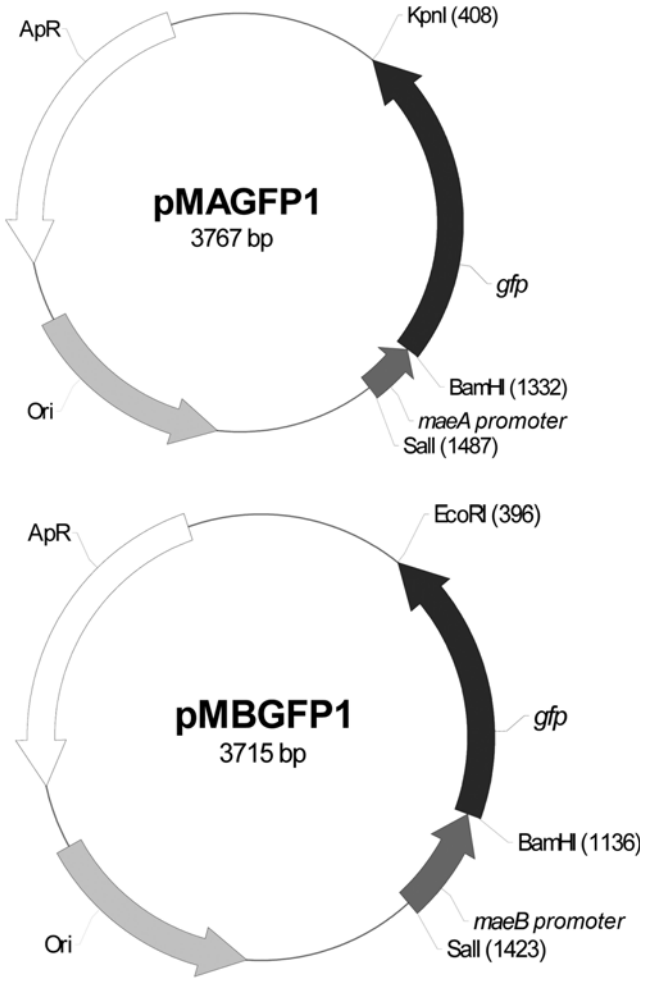

Fig. 1. Plasmid maps of pMAGFP1 and pMBGFP1.

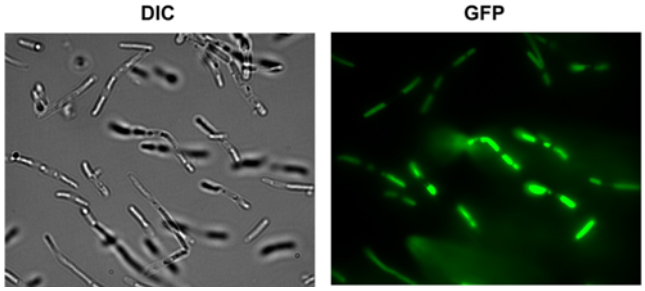

Fig. 2. *E. coli* system effectively responds to malate. The localization and expression of GFP were analyzed by reflected fluorescence microscopy. *E. coli* carrying pMAGFP1 senses malate (presence of 10 mM malate). Magnification 100x.