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Construction of methanol sensing *Escherichia coli* by the introduction of novel chimeric MxcQZ/OmpR two-component system from *Methylobacterium organophilum* XX

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Abstract–*Methylobacterium organophilum* XX is a type II facultative methylotroph that can grow on methanol. In *M. organophilum* XX, the MxcQ/MxcE two-component system (TCS) is involved in methanol metabolism. EnvZ/OmpR in *E. coli* TCS was exploited to develop a methanol biosensor by engaging the MxcQ/MxcE TCS system. The MxcQZ/OmpR methanol sensing chimeric TCS was constructed by integrating the sensing domain of *M. organophilum* MxcQ with the transmitter domain of *E. coli* EnvZ. The response regulator of the chimeric TCS system is OmpR, which regulates the expression of the *ompC* and *gfp*. The expression of *ompC* was monitored by real-time quantitative PCR analysis. The expression of *gfp* also confirmed the expression of the *ompC*. The maximum expression of *ompC* and *gfp* occurred with 0.05% of methanol, and the expression started to decline with further increases in methanol concentration. This system delivers rapid detection of methanol in the environment.

Keywords: Biosensors, Chimeric Two-component System, Fluorescence Sensors, Methanol, Synthetic Biology

INTRODUCTION

Methanol is a colorless liquid that occurs naturally in wood and volcanic gasses. It is also produced in larger quantities for versatile applications. Industrially, methanol is a toxic alcohol used as a solvent, pesticide, antifreeze agent, paints, preservatives, as a precursor for various compounds and alternative fuel. It is released into the environment from industries and causes adverse effects in the environment, depending on the concentration and duration of exposure. Some of these adverse effects in humans include severe abdominal, leg and back pain, loss of vision, sleep disorders and gastrointestinal problems. To avoid unintended methanol exposure and to monitor the methanol concentration, a highly sensitive methanol sensor is necessary.

Due to the importance of methanol sensing, various methanol sensors have been developed [1]. Methanol sensors can be classified as electrochemical and physical. Electrochemical sensors provide many advantages over other methanol sensors, such as simplicity in the structure and the fabrication process, but the narrow operating voltage range, slow response due to diffusion and degradation of the membrane limit the industrial applications [2]. Though physical sensors are reliable and robust, usage is limited because of the requirement of complex auxiliary driving devices such as pressure sensors and pumps to maintain the flow rate as well as the inability to miniaturize the sensors [2]. Thus, the demand for a simple and cost effective methanol sensor is high.

Bacteria use TCS as a primary apparatus to sense environmental change; it functions based on basic stimulus-response coupling mechanism [3,4]. TCS is composed of the sensor kinase (SK) and response regulators (RR) [4,5]. Usually, the SK is a periplasmic histidine kinase (HK), which contains a sensor domain, a linker domain, and a transmitter domain. Signal molecules bind to the sensor domain, leading to autophosphorylation in the transmitter domain and acting as phosphate donors for RR. The phosphorylated RR activates the control protein, leading to transcription. One of the best understood TCS is EnvZ/OmpR, which regulates the expression of outer membrane porin in response to osmolarity change. Chimeric TCS with novel properties can be constructed by domain swapping, in which the sensing and catalytic domains from different HKs are combined without affecting the functions of the proteins. The chimeric HK has proven to be an efficient tool for the construction of novel TCS that can sense environmental changes [6-12].

Methylobacterium organophillum XX is a facultative type II methylotroph that can utilize methanol as a sole source of carbon and energy. It was reported that the methanol metabolism of *M. organophillum* XX is tightly regulated by enzyme methanol dehydrogenase (MDH) and methylamine [13]. The expression of MDH is regulated by the methanol sensing HK, *mxcQ* of *M. organophillum* XX [14]. In the present work, MxcQZ chimeric HK was con-

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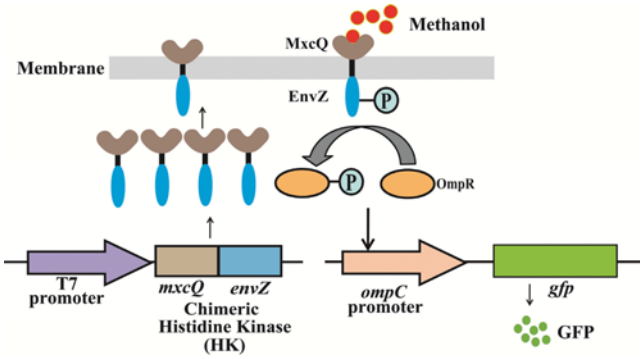

Fig. 1. The Chimeric HK MxcQZ phosphorylates response regulator OmpR in the presence of methanol further initiates GFP expression by activating *ompC* promoter.

structed by integrating the sensor domain of *M. organophillum* MxcQ with the cytoplasmic catalytic domain of *E. coli* EnvZ [6,7]. The resulting chimeric HK can sense extracellular methanol and activate OmpR, which induces the expression of the *ompC* and *gfp* genes regulated by the *ompC* promoter [6] (Fig. 1). The expression profile of the *ompC* gene was measured by real-time quantitative PCR (qRT-PCR). The expression of the green fluorescent protein (GFP) was also monitored to assess the active response to methanol.

EXPERIMENTS AND METHODS

1. Bacterial Strains and Culture Conditions

The plasmids and bacterial strains used for recombinant DNA manipulation and the expression of the recombinant proteins are listed in 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 37 °C under vigorous shaking [15].

2. Molecular Modeling of Chimeric HK (MxcQZ)

Molecular modeling studies were performed to predict the fusion site and study the structure of chimeric HK MxcQZ. Modeling of the peptides is aimed at providing their function in terms of structure and interaction with the environment. The three-dimensional structure of EnvZ protein was solved by X-ray diffraction and is available in the Protein Data Bank (PDB ID 4CTI) [16]. The homology model of EnvZ was fused with the MxcQ peptide modeled using the graphical user interface program Easy Modeller 4.0 [17] with 2JIN as a template. The refined structure of the homology model was obtained by the 3Drefine Protein Structure Refinement Server [18].

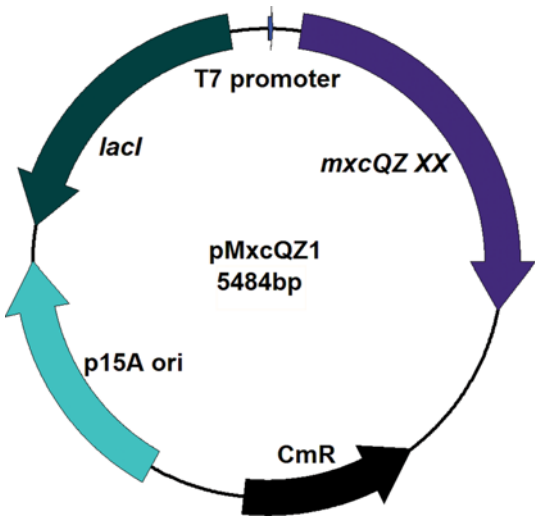

Fig. 2. Plasmid pMxcQZ1 constructed to sense methanol.

3. Construction of Plasmids for Chimeric MxcQZ TCS

The recombinant plasmid pMxcQZ1 contains the chimeric HK MxcQZ under the control of the T7 promoter induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). The periplasmic sensor domain of *mxcQ* (777 bp) (5′-**GAGCTC**ATGGGTCAGAGGCCGGAT-3′, 5′-**CTGCAG**TGAGCGAGCTTCACTCAA-3′) and the cytoplasmic catalytic domain of *envZ* (699 bp) (5′**CTGCAG**ATGGCGGCTGGTGTTAAGCAA3′, 5′**AAGCTT**TTACCCTTCTTTTGTCGTGCC 3′) were amplified from the chromosomal DNA of *M. organophillum* XX and *E. coli* XL1-blue, respectively [19], by polymerase chain reaction (PCR) with the MJ mini personal thermal cycler (BioRad Laboratories, USA) using the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Mannheim, Germany). The PCR products were cloned into the low copy number plasmid pACYCDuet-1 using *Sac*I and *Hin*dIII restriction sites to construct the pMxcQZ1 plasmid (Fig. 2).

4. Monitoring of ompC Gene Expression by qRT-PCR

Transcription of the *ompC* gene in response to methanol was measured by qRT-PCR [7,20]. A single colony of *E. coli* BL21 (DE3) (pMxcQZ1) was grown overnight in LB medium at 37 °C. It was then diluted 100-fold in fresh M9 medium and incubated at 37 °C

Table 1. List of bacterial strains and plasmids used in this study

Strain/Plasmid	Relevant genotype and/or property	Source
***Escherichia coli* strains**		
BL21 (DE3)	*F⁻ ompT gal dcm lon hsdS$_B$(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	Novagen
XL1- Blue	*SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F′(proAB⁺ lacI^q lacZΔM15 Tn10 (tet^R))*	Stratagene
Plasmids		
pOGFP1	pUC19 containing the *ompC* promoter and *gfp* gene, Amp^R	[7]
pACYCDuet-1	Cm^R	Novagen
pMxcQZ1	pACYCDuet-1 containing the chimeric *mxcQ* - *envZ* gene, Cm^R	This work

in an orbital shaker at 250 rpm until the optical density at 600 nm (OD_{600}) reached 0.5. 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 methanol. After 4.5 h, cells were harvested by centrifugation for total RNA preparation using the RNeasy Mini kit (Qiagen) followed by DNase treatment. Reverse transcription was performed with a cDNA synthesis kit (Applied Biosystems, USA) 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, USA) for quantitative expression of the *ompC* gene and elsewhere, 16sRNA (5′-CTTCAAAGGTGAAACTCA-GGTTACTG-3′ and 5′-GTTGCCCTGGATCTGATATTCC-3′). The samples prepared without the RT step were used as negative controls to ensure that the extracted RNA was not contaminated with DNA. qRT-PCR reactions were performed on the Mini Opticon detection system using the 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 conditions (n=3), and calculations were performed automatically by the Mini-Opticon software using 16sRNA [21].

5. Monitoring of the gfp Expression by Fluorescence

A single colony of *E. coli* BL21 (DE3) (pMxcQZ1 and pOGFP1) was grown overnight at 37 °C in LB medium. The overnight cultures were diluted 100-fold in fresh M9 minimal media supplemented with appropriate antibiotics and incubated aerobically in an orbital shaker at 37 °C and 250 rpm until OD_{600} reached 0.5. 10 μM IPTG was added to the *E. coli* culture medium to induce the expression of the chimeric HK. *E. coli* BL21 (DE3) (pMxc-QZ1 and pOGFP1) was then cultured aerobically at 30 °C [7].

The strains were also screened for fluorescence with a 100 x objective on a reflected fluorescence microscope with a cooled, charge-coupled device camera (B&W SenSys, KAF1401). Emission intensity was recorded using MetaMorph image analysis software (Molecular device, Sunnyvale, CA, USA) with excitation and emission filter sets optimized for EGFP imaging [22]. Cell density and fluorescence were measured under various concentrations of methanol during 8 h of culture. Cell density was monitored by measuring the optical density at 600 nm with a spectrophotometer (Shimadzu, Japan). GFP fluorescence was measured using an RF-5301PC spectrofluorometer (Shimadzu, Japan). The excitation wavelength of the spectrofluorometer was set at 485/10 nm and the emission wavelength was set at 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 [6,7]. At a minimum, triplicate measurements were obtained for each sample.

RESULTS AND DISCUSSION

1. Construction and Expression of the Chimeric MxcQZ TCS

The bacterium *M. organophillum* XX contains MxcQ/MxcE TCS; it is involved in methanol metabolism. The chimeric TCS was constructed by integrating the sensing domain of MxcQ with the transmitter domain of EnvZ. The constructed MxcQZ HK senses methanol and induces the expression of the *ompC* gene by activating RR OmpR [20,23]. The resulting recombinant *E. coli* can induce the OmpC promoter in the presence of methanol. Overexpression of the chimera may lead to membrane destabilization; hence, a low copy number plasmid pACYCDuet-1 was used [7].

Molecular modeling, the analysis showed that the peptide MxcQ was fused at 256th methionine residue of EnvZ. Methionine is a sulfur-containing hydrophobic amino acid. The hydrophobic nature makes the amino acid to be buried inside the protein structure, which results in a stable chimeric HK with optimum methanol sensing properties. Also, unlike another sulfur-containing amino acid in methionine, the sulfur group is connected with the methyl group and does not involve in any side reactions. The colors blue and magenta indicate MxcQ and EnvZ (Fig. 3). Methionine is a

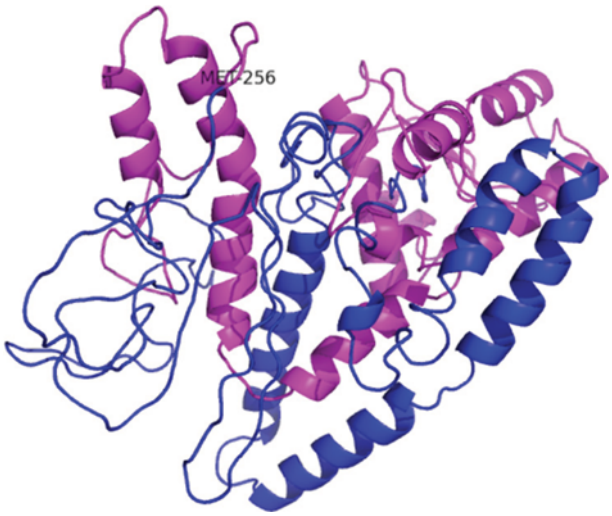

Fig. 3. Molecular modeling image of chimeric protein MxcQ (Blue) - EnvZ (Magenta).

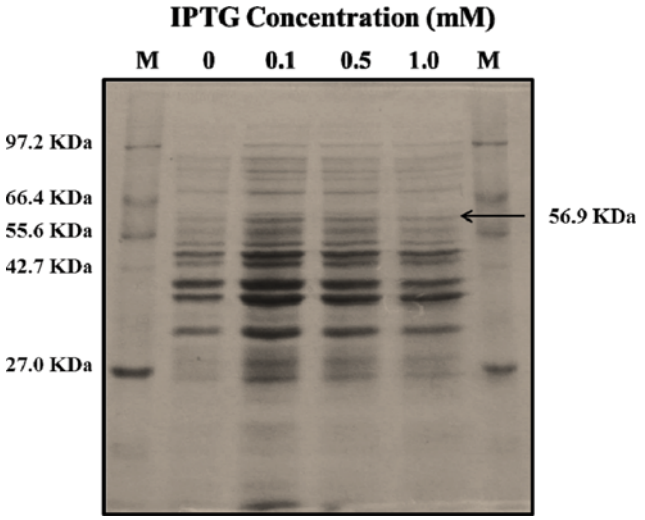

Fig. 4. SDS-PAGE expression analysis of MxcQ/EnvZ fusion protein extracts in the culture medium of pMxcQZ1 transformed BL21 (DE3) cells with non-induced recombinant plasmid as a control. M Molecular weight marker in kDa.

inside the protein core, which contributes to the stability of the chimeric HK MxcQZ. In methionine, sulfur is bonded with the methyl group and does not interfere with the function of the chimeric HK; hence, it can be considered an inert amino acid. The expression of the MxcQZ chimera was analyzed by SDS-PAGE with various concentrations of IPTG (0-1.0 mM) [24] (Fig. 4). The molecular weight of the chimeric protein (MxcQZ) is 56.9 KDa. The expression of the chimeric HK reached a maximum at 0.1 mM IPTG and decreased with further increases in IPTG concentration.

2. Monitoring ompC Gene Expression by qRT-PCR

The expression of the *ompC* gene was estimated by qRT-PCR in minimal media supplemented with varying concentrations of methanol (0.01-8%). The qRT-PCR result shows that the transcriptional level of *ompC* increased with increasing methanol concentration, and the maximum expression was observed with 0.05%. The expression level decreased as the methanol concentration increased further. This might be due to the interruption of cellular activity at higher methanol concentrations [25]. It was reported that methanol has antibacterial activity above 50 ppm (0.005%); however, in the present study, the expression of *ompC* was high even with 0.05% (500 ppm) of methanol, reflecting the methanol sensing ability of chimeric HK MxcQZ [26]. The correlation between methanol concentration and the relative transcriptional level was estimated to be 0.96939 for methanol concentration in the range of 0.001%-8.0% (Fig. 5). This suggests that *ompC* gene expression is specifically regulated by chimera MxcQZ in response to extracellular methanol. These results indicate that the chimera MxcQZ/

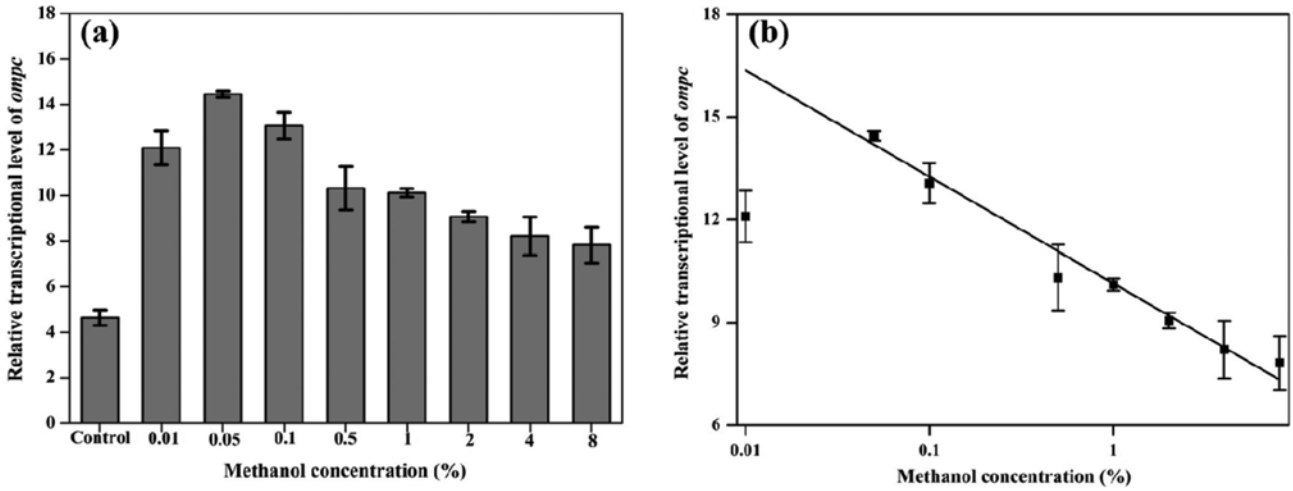

Fig. 5. Monitoring *ompC* gene expression by qRT-PCR. (a) Comparative study of transcriptional levels of the *ompC* gene in the M9 medium after a 4.5 h exposure to methanol. After exposure, the Ct value was normalized using the 16sRNA Ct value as an internal control. The error bars indicate one standard deviation from the mean. *E. coli* harboring pMxcQZ1 and pOGFP1 grown in the absence of methanol was included as the control and (b) The linear correlation between the relative transcriptional levels of the *ompC* gene of the chimera in the M9 medium at varying concentrations of methanol. The data are the aggregate results from replicate experiments (n=3).

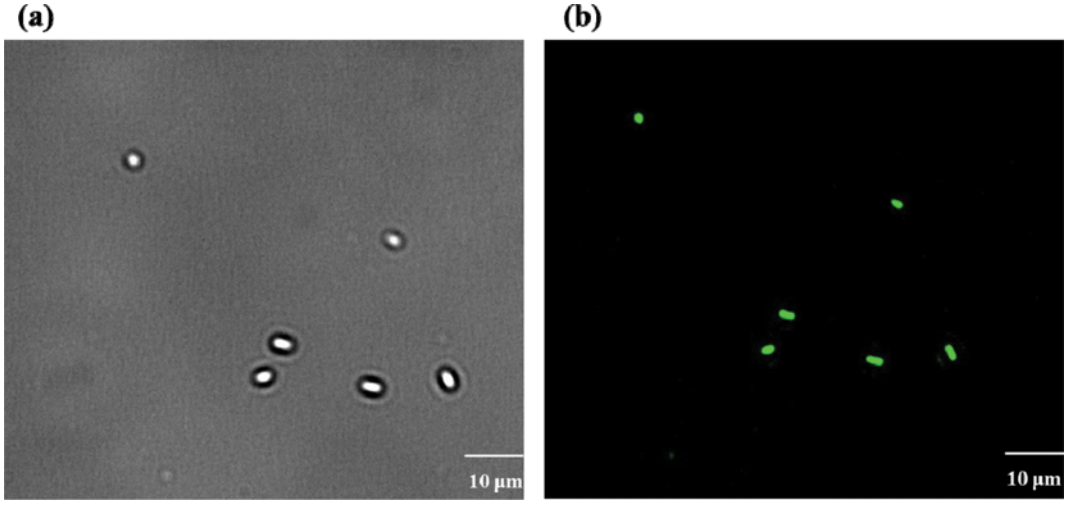

Fig. 6. The image of *E. coli* (pMxcQZ1 and pOGFP1) (a) Differential interference contrast (DIC) microscopy image of the recombinant cells carrying GFP and (b) The same cells showing the fluorescence in the reflected fluorescence microscope.

OmpR TCS produced a signal strong enough for *ompC* gene expression with extracellular methanol.

3. Monitoring *gfp* Gene Expression by Fluorescence Analysis

The response of chimeric TCS MxcQZ/OmpR to extracellular methanol concentration was evaluated via the expression of *gfp*, regulated by the *ompC* promoter [6]. *E. coli* was cultured with 0.01%-8% of methanol, and fluorescence was observed with a fluorescence microscope. The image clearly showed the ability of the constructed bacterial biosensor to detect environmental methanol and emit fluorescence as an output signal. The *E. coli* BL21 (DE3) strain harboring pMxcQZ1 and pOGFP1 was cultured with varying methanol concentrations and fluorescence was measured. The control strain, harboring pACYCDuet-1 and pUC19, yielded a negligible amount of fluorescence (Fig. 6). Though the IPTG inducer was not added, a low level of fluorescence was observed, and this might be the basal level of expression for the *ompC* promoter. Fluorescence was observed starting from 0.01% of methanol; maximum expression was found with 0.05% of methanol followed by a gradual decrease with further increases in methanol. This observation further confirmed the qRT-PCR results. The fluorescence of recombinant *E. coli* harboring pMxcQZ1 and pOGFP1 increased with time (Fig. 7). At the end of 8 h, maximum fluorescence was obtained only with 0.05% (500 ppm) of methanol. These results suggest that the MxcQZ/OmpR chimeric TCS is highly sensitive to methanol and is capable of detecting as little as 0.01% of methanol. The GFP signal was reduced above 0.1% methanol concentration, as higher methanol concentration affects the growth of microbes. Based on previous reports, 0.17% of methanol disrupts bacterial growth [16]; hence, the growth was reduced by the methanol sensing characteristics of the MxcQZ chimera. According to the Food and Drug Administration, the allowable limit of methanol in the environment is 200 ppm (0.2). The MxcQ/EnvZ TCS can sense methanol from 0.01%; thus, it can be used to detect methanol in food and drinks, as methanol is a commonly used preservative.

CONCLUSIONS

Considering the increasing demand for methanol sensors, a two-component system (TCS) based biosensor can be considered as an option. The advantages of TCS include a simple structure, low constructional cost, and high sensitivity. The TCS based sensor can be easily adopted because of its finely controlled gene expression. This chimeric TCS strategy imbues *E. coli* with a methanol sensing property and facilitates the use of recombinant *E. coli* with chimeric TCS MxcQZ/OmpR to sense methanol even at 0.05%. The low detection levels may help to ensure a rapid response to small amounts of adventitiously formed methanol or to methanol generated initially during growth with methylotrophic substrates. The biosensor developed in this study exploits the basic stimulus mechanism of bacteria, making the process cheap and reliable.

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REFERENCES

1. H. Zhao, J. Shen, J. Zhang, H. Wang, D. P. Wilkinson and C. E. Gu, *J. Power. Sources*, **159**, 626 (2006).
2. T. Okada, N. Nakamura, M. Yuasa and I. Sckine, *J. Electrochem. Soc.*, **144**, 2744 (1997).
3. M. T. Laub and M. Goulian, *Annu. Rev. Genet.*, **41**, 121 (2007).
4. A. M. Stock, V. L. Robinson and P. N. Goudreau, *Annu. Rev. Biochem.*, **69**, 183 (2000).
5. A. Y. Mitrophanov and E. A. Groisman, *Gen. Dev.*, **22**, 2601 (2008).
6. I. Ganesh, S. Ravikumar, S. H. Lee, S. J. Park and S. H. Hong, *J. Biotechnol.*, **168**, 560 (2013).
7. I. Ganesh, S. Ravikumar, I. K. Yoo and S. H. Hong, *Bioprocess. Biosyst. Eng.*, **38**, 797 (2014).

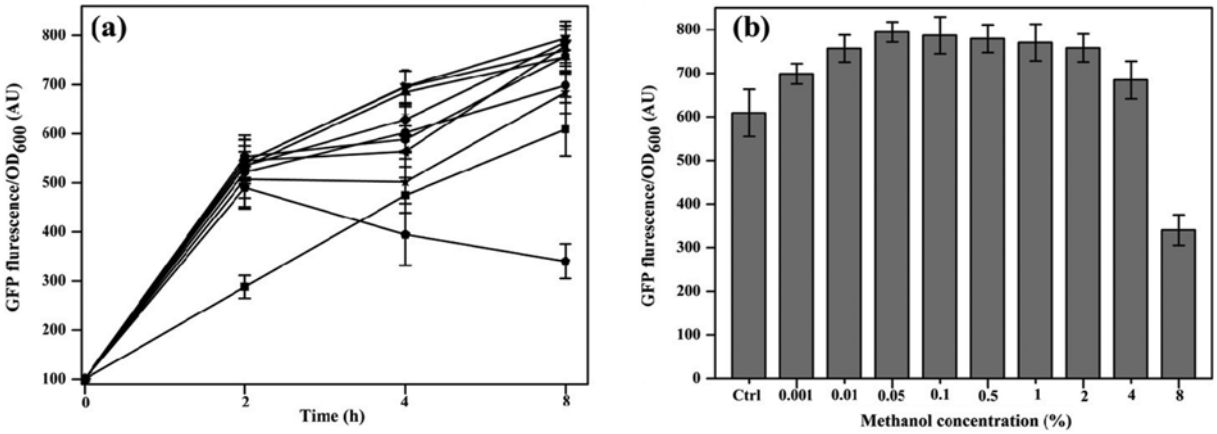

Fig. 7. Monitoring *gfp* gene expression by fluorescence analysis. (a) Time course of GFP fluorescence for an *E. coli* strain harboring pMxcQZ1 and pOGFP1 with varying concentrations of methanol in M9 medium. *E. coli* harboring pMxcQZ1 and pOGFP1 grown in the absence of methanol was included as the control. Control (square); 0.001% (circle); 0.01% (upper triangle); 0.05% (lower triangle); 0.1% (diamond); 0.5% (left triangle); 1.0% (right triangle); 2.0% (hexagon); 4.0% (star); 8.0% (pentagon). The data are the aggregate results from replicate experiments (n=3) and (b) GFP fluorescence value of *E. coli* strain harboring pMxcQZ1 and pOGFP1 after induction with varying concentrations of methanol in M9 medium at the 8th hour of incubation.

8. R. Utsumi, R. Brissette, A. Rampersaud, S. Forst, K. Oosawa and M. Inouye, *Science*, **245**, 1246 (1989).
9. A. Siryaporn and M. Goulian, *Mol. Microbiol.*, **70**, 494 (2008).
10. T. May and Okabe, *J. Bacteriol.*, **190**, 7479 (2008).
11. V. V. Rogov, N. Y. Rogova, F. Bernhard, A. Koglin, F. Löhr and V. Dötsch, *J. Mol. Biol.*, **364**, 68 (2006).
12. S. S. Rizk, M. J. Cuneo and H. W. Hellinga, *Science*, **15**, 1745 (2006).
13. L. Chistoserdova, S. W. Chen, A. Lapidus and M. E. Lidstrom, *J. Bacteriol.*, **185**, 2980 (2003).
14. H. H. Xu, J. J. Janka, M. Viebahn and R. S. Hanson, *Microbiol.*, **141**, 2543 (1995).
15. J. Sambrook and D. W. Russell, *Molecular cloning - A laboratory manual*, New York, Cold Spring Harbor (2001).
16. A. Baslé, G. Rummel, P. Storici, J. P. Rosenbusch and T. Schirmer, *J. Mol. Biol.*, **362**, 933 (2006).
17. D. Bhattacharya and J. Cheng, *Proteins*, **81**, 119 (2013).
18. N. Eswar, B. Webb, M. A. Marti-Renom, M. S. Madhusudhan, D. Eramian, My. Shen, U. Pieper and A. Sali, *Current Protocols in Protein Science*, Wiley (2007).
19. F. R. Blattner, G. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, V. J. Collado, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau and Y. Shao, *Science*, **277**, 1453 (1997).
20. S. A. Forst and D. L. Roberts, *Res. Microbiol.*, **145**, 363 (1994).
21. H. Eleaume and S. Jabbouri, *J. Microbiol. Meth.*, **59**, 363 (2004).
22. M. Maruthamuthu, I. Ganesh, S. Ravikumar and S. H. Hong, *Biotechnol. Lett.*, **37**, 659 (2015).
23. H. Aiba and T. Mizuno, *FEBS Lett.*, **261**, 19 (1990).
24. U. K. Laemmli, *Nature*, **227**, 680 (1970).
25. D. Bourque, B. Ouellette, G. André and D. Groleau, *Appl. Microbiol. Biotechnol.*, **37**, 7 (1992).
26. E. L. Okoye, B. O. Ulba, P. C. Uhobo, A. N. Oli and M. N. Ikegbunam, *J. Scientific Research and Reports*, **3**, 255 (2013).