Engineering of Recombinant *Escherichia coli* Towards Methanol Sensing Using *Methylobacterium extroquens* Two-component Systems

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Five genes (*mxhDM*, *mxhQE* and *mxhAB*) are responsible for the transcription of methanol oxidation genes in *Methylobacterium* strains. Among these, *MxhDM* and *MxhQE* constitute the two-component system (TCS) regulating methanol metabolism. In this study, we integrated the methanol-sensing domain of *MxhD* and *MxhQ* with the *EnvZ/OmpR* from *Escherichia coli*. The domain-swapping strategy resulted in chimeric histidine kinases (HK's) *MxhDZ* and *MxhQZ* AM1 containing recombinant *E. coli*. Real-time quantitative PCR was used to monitor *OmpC* expression mediated by the chimeric HK and response regulator (RR) *OmpR*. Further, an *ompC* promoter based fluorescent biosensor for sensing methanol was developed. GFP fluorescence was studied both qualitatively and quantitatively in response to environmental methanol. GFP measurement also confirmed *ompC* expression. Maximum fluorescence was observed at 0.05% methanol and 0.01% methanol using *MxhDZ* and *MxhQZ* AM1, respectively. Thus the chimeric HK containing *E. coli* were found to be highly sensitive to methanol, resulting in a rapid response making them an ideal sensor.

Keywords: Methanol, biosensor, chimeric two-component system, green fluorescent protein, *Escherichia coli*

Introduction

Methanol, one of the highly produced C1 compounds, also considered an alternative fuel. It is an additive and preservative in various products including processed foods, beverages, paints, coating products, and cosmetics. The versatility of methanol demands increased methanol production. Bioengineering of microbes could help in coping with methanol production cost-effectively. In addition, it is hard to sense methanol at lower concentrations, hence requiring a highly sensitive methanol sensing system. It could speed up the development of methanol producers. Two different methanol sensors grouped into physical and electrochemical [1] are used to

detect methanol. Although the above sensing systems are robust and reliable, the limited industrial usage roots from a lack of miniaturization [2]. It is a high time to develop a scalable system for high throughput sensing of methanol.

Bacteria ubiquitously sense environmental changes by a two-component system (TCS) [3, 4]. The TCS functions by coupling stimulus and response mechanisms using sensor kinase (SK) and response regulators (RR) [4, 5]. Most of the SKs are periplasmic histidine kinase (HK); composed of a sensor, a linker, and a transmitter domain, respectively. Binding of a signal molecule with the sensor domain autophosphorylate transmitter domain further, donating the phosphate to RR. The phosphate bounded RR activates the of a control protein, which leads the transcription. Thus, the sensor domain plays a pivotal role in sensing the compounds present in the environment. A chimeric TCS constructed domain

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swap could help in engineering recombinant bacteria to sense desired compounds [6–15].

Methylophiles are a group of bacteria that can use methanol as a sole source of carbon. It contains a well-established methanol sensing TCS. *Methylobacterium extorquens* are another group of methylophilic which can grow on multiple carbon sources like acetate, methanol (C1), ethanol, and ethylamine (C2), pyruvate (C3), or succinate (C4). Since methanol may play a major role as future alternative carbon sources *M. extorquens* has become a target for biotechnological applications [6]. It oxidizes methanol by enzymes methanol dehydrogenase and methylamine dehydrogenase [16]. Further methanol oxidation is regulated by *mxcQE*, *mxbDM*, *mxaB* [17–19] where *mxcQE* and *mxbDM* are TCS with SK (*mxcQ*, *mxbD*) and the RR (*mxcE*, *mxbM*).

In previous studies our group has engineered HK from *Paracoccus* sp. into *E. coli* sense methanol. Considering the growing importance of *Methylobacterium extorquens* as C1 compound producers in our present study, we engineered chimeric HKs by integrating methanol sensing domain *mxbD* from *M. extorquens* and *mxcQ* AM1 from *M. extorquens* AM1. The EnvZ-OmpR is an extensively studied TCS it regulates the expression of outer membrane porins, OmpF, and OmpC, as an osmolarity change response. The resulting chimeric HK can sense extracellular methanol further donates phosphate group to the RR OmpR. It induces the expression of the *ompC* was quantified by real-time quantitative PCR (qRT-PCR). OmpC promoter based fluorescent methanol sensor was developed. A Green fluorescent protein (*gfp*) gene was cloned under the control of *ompC* promoter (Fig. 1). The fluorescence was visualized and measured

Fig. 1. *E. coli* engineered to respond to exogenous methanol.

The *M. extorquens* periplasmic sensor kinase domain of MxbD/MxcQ AM1 and the cytoplasmic catalytic domain of EnvZ was fused to form the MxbDZ/MxcQZ AM1 chimeric protein, which phosphorylates the response regulator OmpR of the TCS while senses methanol. This activates the *ompC* promoter, resulting in GFP expression in *E. coli*.

by microscopy and spectrofluorometer, respectively. The expression of GFP is considered as an active response of chimeric TCS (MxbDZ and MxcQZ AM1) towards methanol.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains for recombinant DNA manipulation and the expression of recombinant proteins were *E. coli* XL1-Blue (XB) and BL21 (DE3) respectively. The bacterial strains and plasmids used in this study are listed in Table 1. The strains were grown under constant shaking (250 rpm) in Luria-Bertani (LB) broth (10 g/l

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

Strain/Plasmid	Relevant genotype and/or property	Source
<i>Escherichia coli</i> strains		
BL21 (DE3)	F^- <i>ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [<i>lacI lacUV5-T7 gene 1 ind1 sam7 nin5</i>])</i>	Novagen
XL1-Blue	<i>SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F'(proAB⁺ lac^R lacZΔM15 Tn10 (tet^R))</i>	Stratagene
Plasmids		
pUC19	Amp ^R	NEB ^a
pOGFP1	pUC19 containing the <i>ompC</i> promoter and <i>gfp</i> gene, Amp ^R	[16]
pACYCDuet-1	Cm ^R	Novagen
pMxbDZ	pACYCDuet-1 containing the chimeric <i>mxcD-envZ</i> gene, Cm ^R	This work
pMxcQZ AM1	pACYCDuet-1 containing the chimeric <i>mxcQ-envZ</i> gene, Cm ^R	This work

^aNew England Biolabs, U.S.A.

bacto-tryptone, 5 g/l bacto-yeast extract and 5 g/l NaCl) and in M9 minimal salts medium (Sigma-Aldrich, USA) supplemented with 4 g/l of glucose, 2 mM MgSO₄, 0.1 mM CaCl₂ and 1% thiamine HCl at 37°C.

Molecular modeling of chimeric protein MxbDZ

Molecular modeling was done to predict the optimal fusion site for chimeric sensor kinase (MxbDZ and MxcQZ AM1). Further the interaction between the methanol sensing and transmitter domain of the peptides was structurally investigated by molecular modeling. X-ray diffraction solved the three-dimensional structure of the EnvZ protein is available in the Protein Data Bank (PDB ID 4CTI) [20]. The homology model of EnvZ was fused with the peptide structure (MxbD and MxcQ AM1) modeled with 2JIN as a template by a graphical user interface program Easy Modeller 4.0 [21]. 3Drefine Protein Structure Refinement Server was used to refine the homology model [22].

Chimeric SK construction

Based on the computational studies, the chimeric SK (MxbDZ and MxcQZ AM1) was constructed. The periplasmic sensor domain of *mxhD* (912 bp), *mxhQ* AM1 (777 bp) and the cytoplasmic catalytic domain of *envZ* (684 bp) were amplified from the chromosomal DNA of *M. extroquens* and *E. coli* by polymerase chain reaction (PCR), respectively. PCR was performed with the MJ mini Personal Thermal Cycler (Bio-Rad, USA) using the Expand High Fidelity PCR system (Roche Molecular Biochemicals, Germany). The primers used in this study are listed in Table 2. The PCR products were cloned into the low copy number plasmid pACYCDuet-1 using *SacI* and *HindIII* restriction sites to construct the plasmids

pMxbDZ1 and pMxcQZ AM1. The expression of chimeric SK was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) regulated by the T7 promoter.

ompC expression profile

The dynamics of the chimeric TCS MxbDZ/OmpR and MxcQZ AM1/OmpR, towards *ompC* expression in response to varying concentrations of methanol (0–8%) were measured by qRT-PCR. To perform qRT-PCR a single colony of *E. coli* BL21 (DE3) harboring pMxbDZ1 and pMxcQZ1 AM1 were cultured overnight in LB medium at 37°C was then diluted 100-fold in fresh M9 medium and incubated at 37°C with vigorous shaking (250 rpm) until an optical density of 0.5 at 600 nm (OD600) reached. IPTG was added to the culture, and the cells were grown cultured at 30°C for 4.5 h in the presence of methanol. The 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, USA) for quantitative expression of the *ompC* gene and 16sRNA (Supplementary Table 1). Samples for which the RT step was omitted were used as negative controls to ensure that the extracted RNA was not contaminated with DNA. The 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 using separate cultures grown under identical conditions (n = 3) and was calculated automatically by the Mini-Opticon software using 16s RNA as an internal control [23].

gfp expression profile

Recombinant *E. coli* harboring (pMxbDZ1, pMxcQZ1 AM1 and pOGFP1) were grown overnight and the cultures were diluted to 100-fold in fresh M9 medium supplemented with appropriate antibiotics and incubated in an orbital shaker (250 rpm) at 37°C until OD600 reached 0.5. Then 0.5 mM IPTG was added to the culture medium and incubated with varying concentrations of methanol (0–8%) at 30°C to induce the expression of the

Table 2. Oligonucleotide primers used in this study.

Name	Sequence
MxbD_F_SacI	5'-GAGCTCATGAGATCTTAAGAGTT-3'
MxbD_R_PstI	5'-CTGCAGCCGCTGGAGCGGTCCAC-3'
MxcQ AM1_F_SacI	5'-GAGCTCATGGCTCAATCAGTGCGC-3'
MxcQ AM1_R_PstI	5'-CTGCAGATCGGATAATGCACCTGC-3'
EnvZ_F_PstI	5'-CTGCAGATCGGATAATGCACCTGC-3'
EnvZ_R_HindIII	5'-AAGCTTTTACCCTTCTTTTGTCTGCC-3'
OmpC_RT_Fwd	5'-CTTCAAAGGTGAACTCAGTTACTG-3'
OmpC_RT_Rev	5'-GTTGCCCTGGATCTGATATTCC-3'

chimeric SK reported [13, 14, 18]. The strains were visualized for fluorescence with 100x objective on a reflected fluorescence microscope with a cooled, charge-coupled device camera (B&W SenSys, KAF1401). Emission intensity was recorded using Meta-Morph image analysis software (Molecular Device, USA) with excitation and emission filter sets optimized for EGFP imaging [13, 14, 24]. Fluorescence were also quantitatively measured for the strains harboring chimeric SK at regular time intervals for 8 h. 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), which was set at an excitation and the emission wavelength of 485/10 and 515/10 nm, respectively. The specific fluorescence intensity (SFI) was described as the raw fluorescence intensity expressed in relative fluorescence units divided by the optical density at 600 nm measured at regular time intervals [7, 13, 14, 24]. The measurements were performed at least in triplicate for each sample.

Results and Discussion

M. extroquens strains are methylotrophs, it is gaining more attention to produce value-added products from the single carbon (C1) compounds. The C1 compounds are metabolized methanol dehydrogenase and alcohol oxidase. The transcription of above genes was activated by five genes, it includes two TCS *mxhDM*, *mxhQE*. TCS were exploited for the development of a fluorescence-

based methanol sensor.

Construction and expression of the chimeric SK MxbDZ & MxcQZ AM1

To engineer the methanol sensing TCS in *E. coli* a chimeric HK was constructed by fusing the methanol sensing domain *mxhD* and *mxhQ* AM1 from *M. extroquens* strains with the transmitter domain of *E. coli* *envZ*. As overexpression of membrane protein may cause destabilization of cell membrane. A low copy number plasmid pACYCDuet-1 was used in the construction of chimeric SK. Both the chimeric SK could methanol, sequentially activates the RR *ompR* and induces *ompC* expression. The ideal site for the construction of chimeric SK was evaluated by molecular modelling using the 3Drefine Protein Structure Refinement Server (Fig. 2). The colors magenta and blue indicate MxbD and EnvZ (Fig. 2A) subsequently, MxcQ AM1 and EnvZ are represented in blue and white respectively (Fig. 2B). The structural analysis revealed that more stable and active chimeric HK can be constructed by fusing the MxbD and MxcQ AM1 at the 305th and 254th methionine residue of EnvZ. Since methionine is considered as a hydrophobic amino acid, it tends to be buried inside the protein core results in a stable fused protein structure. The methyl group in methionine is connected to sulfur; hence the fusion does not intervene in the activity of chimeric SK [13, 14]. Based on the modelling studies a recombinant plasmid was constructed (Figs. 3A and B). The overexpression of chimeric SK (MxbDZ and MxcQZ AM1) was carried out in varying concentration of IPTG and was

Fig. 2. Molecular modelling, image of chimeric SK (A) MxbD (Magenta) - EnvZ (Blue); (B) MxcQ AM1(Blue) - EnvZ (White).

Fig. 3. Construction of recombinant plasmid with chimeric SK (A) pMxbDZ1, (B) pMxcQZ AM1; Recombinant protein expression analysis by SDS-PAGE (C) MxbDZ, (D) MxcQZ AM1; M. Molecular weight marker in KDa.

analysed by SDS-PAGE [25] (Figs. 3C and D). Both chimeric SK exhibited maximum expression at 0.5 mM IPTG, with further increase the expression declined.

***ompC* expression profile**

The expression of *ompC* mediated by MxbDZ/OmpR and MxcQZ AM1/OmpR was quantified by qRT-PCR (Figs. 4A–D). The recombinant *E. coli* were grown in minimal media supplemented with varying concentrations of methanol. In case of MxbDZ and MxcQZ AM1, maximum expression of *ompC* was at 2% and 0.01% of methanol respectively. It started to decline with further increase in methanol concentration. This might be due to the interruption of cellular activity and protein expression by methanol [26, 27]. The correlation between methanol concentration and the relative transcriptional level was estimated to be 0.94592 (Figs. 4C

and D). The expression of *ompC* can be considered as dynamic response of chimeric TCS towards extracellular methanol.

***gfp* expression profile**

Expression of *ompC* was observed as a response to extracellular methanol. Based on the results we developed a fluorescent based methanol biosensor. To realize this, the *gfp* was cloned downstream of the *ompC* promoter, which is activated by OmpR. The *E. coli* BL21 (DE3) strain harboring Chimeric SK (MxbDZ and MxcQZ AM1) and pOGFP1 was cultured with various concentrations of methanol. Time profiles showed that fluorescence increased as culture time increased. The control strain, harboring pACYCDuet-1 and pUC19, yielded a negligible amount of fluorescence (Figs. 5A–C). Though the IPTG inducer was not added, a low level of

Fig. 4. Monitoring *ompC* expression by qRT-PCR; Comparative study of transcriptional levels of the *ompC* gene in the M9 medium after a 4.5 h exposure to methanol. *E. coli* harboring (A) pMxbDZ1 and (C) pMxcQZ1 AM1 grown in the absence of methanol was included as the control; The linear correlation between the relative transcriptional levels of the *ompC* gene of the (B) pMxbDZ1 and (D) pMxcQZ1 AM1 in the M9 medium at varying concentrations of methanol. The data are the aggregate results from replicate experiments ($n = 3$).

fluorescence was observed, this might be due to the basal level of *ompC* promoter expression. Both chimeric TCS sensed as low as 0.01% methanol. After 8 h maximum fluorescence was observed at 0.05% and 0.01% with chimeric TCS MxbDZ and MxcQZ AM1 respectively. According to food and drug administration, the allowable limit of methanol into the environment is 200 ppm (0.02%). Further increase in methanol resulted in a gradual decline in fluorescence. The GFP expression pattern of MxcQZ AM1 corroborates with the qRT-PCR results. In contrast with MxbDZ a shift in maximum fluorescence observed. According to qRT-PCR results maximum *ompC* expression was at 2% methanol. This may be due to the over-amplification of the GFP signal with the use of a high copy number-based reporter system (pOGFP1) [7]. This system could be employed to detect methanol in food and drinks as it is a commonly

used preservative. Fluorescence was visualized by microscopy (Supplementary Fig. 1). The image clearly showed the ability of the constructed bacterial biosensor to detect environmental methanol and emit fluorescence as an output signal.

Methanol is used as an initial compound for various products including pharmaceutical products, LPG, gasoline, adhesives, automobiles, shopping bags, PET bottles, and mobile phones. The global market for methanol is expected to continue to rise because of its versatile applications. According to the methanol institute, over 90 methanol plants worldwide have a combined production capacity of 100 million metric tons per year. Considering the increasing demand for methanol, biotechnological strategies like metabolic engineering were employed to create cost-effective methanol producers. We strongly emphasize that with

Fig. 5. Time course of GFP fluorescence for an *E. coli* strain harboring pMxbDZ1 (A), pMxcQZ1 AM1 (C) and pOGFP1 with varying concentrations of methanol in M9 medium. Control (closed square); 0.001% (Open square); 0.01% (closed upper triangle); 0.05% (open upper triangle); 0.1% (closed diamond); 0.5% (open diamond); 1.0% (closed circle); 2.0% (open circle); 4.0% (closed inverted triangle); 8.0% (open inverted triangle). The data are the aggregate results from replicate experiments ($n=3$); GFP fluorescence value of *E. coli* strain harboring pMxbDZ1 and pOGFP1 after induction with and without varying concentrations of methanol in M9 medium after 8 hours of incubation (B) pMxbDZ1, (D) pMxcQZ1 AM1.

high-throughput methanol sensors, accelerated methanol production could be achieved. TCS based methanol sensors can sense and monitor desired compounds from the environment. In this study, the TCS from one of the ecologically relevant methylotrophs was engineered in *E. coli* by domain strategy. The resulting chimeric HK was able to produce an output signal in the form of fluorescence depending on environmental methanol concentrations.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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