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4 **Inducible expression system for the marine cyanobacterium**
5 ***Synechococcus* sp. strain NKBG 15041c**
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ABSTRACT

Cyanobacteria have been recognized as promising host organisms to produce biofuel materials including hydrogen and hydrocarbons due to high biomass productivity and capability of transformation. Metabolic engineering has recently been employed for further enhancement of bioenergy production, while excess fuel materials could give rise to cytotoxicity. Therefore genetic tools are required in order to precisely control the gene expression involved in the fuel production metabolism. Thus far, inducible-expression systems in cyanobacteria have been established mainly in freshwater strains. However there are a few reports with regard to marine strains, which can use vast of ocean for cultivation. In this study, we established a tetracycline-inducible gene expression system in the marine cyanobacterium *Synechococcus* sp. strain NKBG 15041c. Since tetracycline-inducible system is a non-native regulation, it is expected for inherent metabolisms not to be disrupted. The *de novo* designed promoter including tetracycline operator elements was inactivated by the repressor protein, and inducer addition successfully initiated target protein expression. This is the first report to demonstrate tetracycline-induction system with strict on/off switching in marine cyanobacteria, and it should be useful for future bioenergy production.

Key words

Marine cyanobacterium; Biofuel; Inducible expression; Anhydrotetracycline

1. Introduction

Photosynthetic production of biofuels is a promising solution for sustainable energy supply. Among a number of renewable energy sources, microalgal biomass has attracted much attention due to high growth rate and high production capability of a range of molecular species for biofuel, both of which are necessary for massive fuel production. In particular, cyanobacteria have advantageous characters of not only fast growth but also ease of genetic engineering, which could exploit further potential of microalgae by altering the inherent metabolic pathways [1, 2]. Cyanobacteria have been used for the production of valuable compounds including alkane [3], butanol [4, 5], acetone [6] and isoprene [7] through the expression of heterogeneous genes. Recently, hydrogen production was also attempted via metabolic engineering approach [8]. Nowadays, biofuel production is required not to use freshwater, food crops and arable lands in order to avoid competition with agriculture. To fulfill these requirements, marine strains have attracted more attention because they can use vast of ocean environments for their cultivation. Furthermore, marine and freshwater cyanobacteria can exhibit different preferences in useful component synthesis; e.g., with regard to carbohydrate fuel production, a freshwater cyanobacterium, *Synechococcus elongatus* PCC7942 synthesizes various alka(e)ne with the aid of acyl-ACP reductase and aldehyde deformylation oxygenase [9], while a marine *Synechococcus* sp. PCC7002 exclusively produces α -olefin through different pathway using olefin synthase [8]. Thus we should chose marine cyanobacteria as host strains depending on the target products, although biochemical production has been heavily studied in freshwater strains, and usage of marine strains is still in infancy.

In general high content of fuel materials exhibits cytotoxicity, and thus the gene expressions governing their biosynthesis pathways should be precisely regulated. More particularly in hydrogen

production, expression of active heterologous hydrogenase required a number of accessory proteins [8].

Excess and constant expression of such multiple genes itself may result in cell growth inhibition. Given these

facts, establishment of inducible expression systems is a key to achieve efficient fuel production in cyanobacteria. Thus far, such genetic tools have mainly been studied in *Synechocystis* sp. PCC6803 and

Synechococcus elongatus PCC7942, both of which are model freshwater cyanobacteria. As induction signals,

nitrate ion[10, 11], various metal ions (Cd^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Fe^{3+} , and Zn^{2+}) [12-18], IPTG [1, 19-21],

tetracycline-derivative [22], low CO_2 -level [23, 24] and light stimulation [25] were available. Among them,

non-native inducible systems including tetracycline- and IPTG-systems have been reported with the

advantages of high on/off ratio and no disruption to native pathways because heterogeneous strong promoters

were engineered and utilized [22]. Indeed, [NiFe] hydrogenase expression was demonstrated using

IPTG-system in the freshwater strain, *Synechococcus elongatus* [8]. In contrast, inducible expression systems

in marine cyanobacteria were rarely demonstrated. Only 4 studies were reported with the induction signals of

Fe^{3+} [26], low CO_2 -level [27] and light/dark conditions [28, 29]; all of these studies used native promoters

which might interfere in the inherent cyanobacterial systems, and were performed only in a model marine

cyanobacterium, *Synechococcus* sp. PCC 7002. In order to further emphasize the usefulness of marine

cyanobacteria for biofuel production, it is desired to demonstrate the availability of non-native inducible

systems in a variety of marine strains.

Synechococcus sp. strain NKBG 15041c was isolated as a fast-growing marine cyanobacterium from

coastal seawater at Okinawa in Japan [30]. It is not a naturally competent strain, while gene transfer technique

was already established [31]. Several biotechnological applications of this strain have been demonstrated by genetic engineering approach [32, 33], and the potential of this strain as host cells for producing eicosapentnoic acid [33] and alka(e)ne (unpublished data) has been shown. Furthermore, we have recently reported the draft genome sequences of the strain [34]. Given these achievements, we recognized this strain as a promising biofuel producer, and undertook development of the tetracycline-inducible expression system in this strain. A strong and tetracycline-inducible promoter was designed, and conjugated with the tetracycline-repressor (TetR) expression vector. Induction efficiency was confirmed by monitoring the reporter gene expression. To our best knowledge, this is the first report to demonstrate the tetracycline-inducible expression system in marine cyanobacteria.

2. Materials and methods

2.1. Materials

Oligonucleotides for PCR were prepared by Operon Biotechnology (Tokyo, Japan). DNA polymerases and all restriction endonucleases were from TaKaRa Bio Inc. (Shiga, Japan) or New England Biolabs (MA, USA). All other reagents were commercially available analytical reagents and were purchased as laboratory grade. Deionized and distilled water was used in all procedures.

2.2. Bacterial strains and culture conditions

Escherichia coli DH5 α , TOP10 (Life Technologies, CA, USA) and JM109 (TaKaRa Bio Inc., Shiga, Japan) were used as hosts for gene cloning. Cells were cultured in LB medium containing 50 μ g/ml streptomycin or 50 μ g/ml ampicillin at 37°C. *Synechococcus* sp. strain NKBG 15041c was isolated from

Japanese coastal seawater [30]. *Synechococcus* sp. strain NKBG 15041c was grown in marine BG11 medium (ATCC catalogue, medium no. 617, supplemented with 3% of NaCl). Cultures were maintained at 26°C under continuous white light at 50 $\mu\text{mol}/\text{m}^2\text{s}$ with continuous agitation (120 rpm). Transformants were cultured under the same conditions containing 75 $\mu\text{g}/\text{ml}$ streptomycin. Cell growth was monitored by cell counting.

2.3. Construction of plasmids

The DNA fragment including *trc* promoter [35, 36] conjugated with tetracycline operator derived from *E. coli* (termed P_{trc}^{tetO} , Fig. 1), and *green fluorescent protein* (*gfp*) gene with optimized codon usage for *Synechococcus* sp. PCC 7002 were synthesized and cloned (pIDT-tetOGFP, Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Additionally, the tetracycline-repressor gene under the control of the phycocyanin promoter derived from *Synechococcus* sp. strain NKBG 15041c (P_{cpc}) was also created (pIDT-tetR). The fragment including P_{trc}^{tetO} and *gfp* gene was obtained by cleaving pIDT-tetOGFP with *EcoRI* and *SphI*, and ligated into the *EcoRI*- and *SphI*-digested pUC19 (pUC-tetOGFP). Then, the fragment including P_{cpc} and *tetR* gene was obtained by cleaving pIDT-tetR with *SphI* and *HindIII*, and ligated into the *SphI*- and *HindIII*-digested pUC-tetOGFP (pUC-tetORGFP). Finally, the fragment including *gfp* gene under P_{trc}^{tetO} and *tetR* gene under P_{cpc} was obtained by cleaving pUC-tetORGFP with *EcoRI* and *ApaLI*, and ligated into the *EcoRI*- and *ApaLI*-digested pKT230 (Km^r Sm^r), broad host-range vector for gram negative bacteria. The constructed plasmid was referred to as pKT-tetORGFP.

As a control vector lacking *tetR* gene under P_{cpc} , pKT-tetOGFP was constructed by ligating the fragment including P_{trc}^{tetO} and *gfp* gene into the *EcoRI*- and *HindIII*-digested pKT230 (termed pKT-tetOGFP).

-35 -10
 P_{trc} 5'-TATTCTGAAATGAGCTG**TTGACA**ATTAATCATCCGGCTCG**TATAAT**
 P_{trc}^{tetO} 5'-TCCCTATCAGTGATAGA**TTGACATCCCTATCAGTGATAGA****TATAAT**
 +1
 P_{trc} GTGTGGAATTGTGAGCGGATAACAATTTACACACAGGAAACAG-3'
 P_{trc}^{tetO} GTGTGGAATTGTGAGCGGATAACAATTTACACACAGGAAACAG-3'

Fig. 1 Nucleotide sequences of the original P_{trc} and tetracycline-inducible P_{trc}^{tetO}. Letters with underlines indicate the tet operator (tetO) elements. Black boxes indicate the putative -10 and -35 promoter consensus sequences. Gray boxes indicate the Shine-Dalgarno sequence in the ribosome binding site. The transcription start sites are indicated with + 1.

2.4. Transformation of cyanobacteria with electroporation

The transformation procedure used in this study was based on the previously report [37] with slight modification. Electroporation was performed with Gene Pulser Xcell Electroporation Systems (Bio-Rad Laboratories, CA, USA), at a capacitance of 25 μ F, resistance of 200 Ω , electric field strength of 7.5 kV/cm, time constant of 3ms, and cuvettes with 0.4 cm of interelectrode distance. Exponential phase of NKBG 15041c was harvested and washed with EP buffer (10 mM TES, 1 mM $MgCl_2$, 272 mM sucrose, pH 7.5). The cells were re-suspended in the EP buffer at 10^{10} cells/ml, and 40 μ l of the cell suspension was mixed with 400 ng of each plasmid. The mixture was subjected to single pulse electroporation, immediately transferred to 5 ml of marine BG11 medium and incubated for 24 h. Then, the cells were harvested and re-suspended in the 1 ml of marine BG11 medium, and transferred on marine BG11 plates (0.8 % agar) containing 75 μ g/ml

streptomycin.

2.5. cDNA synthesis and reverse-transcription PCR

The cyanobacterial cells at the logarithmic phase (1×10^9 cells) were re-suspended in 1 ml of TRIZOL reagent (Life Technologies) and heated for 20 min at 65°C. Total RNA was extracted from the samples using RNA purification kit (Life Technologies, CA, USA), and treated with RNase-free DNaseI to eliminate contaminating chromosomal DNA, according to the manufacturer's instructions. For cDNA synthesis, 1 µg of RNA sample was used to generate cDNA using PrimeScript® II 1st strand cDNA Synthesis Kit (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Amplification of *tetR* was performed with the specific primers (5'-TGAGAATGCGCTCTATGCCTTGAG-3' and 5'-CGTTCTTCTTTGGCCACCTGATGT-3'). The ribonuclease P gene (*rnpB*) of NKBG15041c was amplified as a housekeeping gene [38], with the specific primers (5'-CCGGTTGGCAAATTATC-3' and 5'-AATGCCCATGGAATACCT-3').

2.6. Fluorescent microscopy

Synechococcus sp. strain NKBG 15041c transformants were incubated until the logarithmic phase. Anhydrotetracycline (ATc, final concentration of 0-10 µg/ml) were added, and the cultures were further incubated for 24 h. The resulting cells were observed using a fluorescent microscope, BX51 (Olympus Co., Tokyo, Japan) and an NIBA filter set.

2.7. Western blotting

The cyanobacterial cells at the logarithmic phase (1×10^9 cells) were collected by centrifugation,

re-suspended in 30 μ l of 1% (w/v) sodium dodecyl sulfate (SDS) in aqueous solution and boiled for 10 min. After centrifugation, supernatant was collected, and SDS sample buffer was added (final concentration of 62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 5% sucrose, and 0.002% bromophenol blue). Denatured proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% (w/v) gel, and transferred to a polyvinylidene difluoride membrane. GFP was then detected using alkaline phosphatase (ALP)-labeled anti-GFP antibody (Rockland Immunochemicals Inc., Gilbertsville, PA, USA, 1/5,000 dilution from stock in PBS containing 0.05% Tween 20). BCIP/NBT-Blue (Sigma, St. Louis, Mo. USA) was used as the ALP substrate for visualization. In order to compare signal intensity from Western blotting data, the images were analyzed with Image J program. The images were converted into 8-bit black-and-white images and inverted. Then, regions of interest including each specific band were selected and gray scales were measured. Signal intensity was indicated as a product of the area and gray scale.

3. Results and discussion

3.1. *De novo* design of the tetracycline-inducible promoter

We aimed to develop a tetracycline-inducible protein expression system in *Synechococcus* sp. strain NKBG 15041c to prevent the toxic effects of biofuel production in cyanobacterial cells. The tetracycline-inducible expression system is based on the tetracycline resistance operon of *E. coli* transposon Tn10 [39]. In the present study, the tetracycline analog, anhydrotetracycline (ATc) was used as an inducer due

to its high affinity to tetracycline repressor (TetR) and low antibiotic effect. The inducer molecule can pass through the phospholipid bilayer by simple diffusion without transporter proteins [40], bind to the tetracycline repressor with high affinity ($Ka = 2.8 \times 10^9 \text{ M}^{-1}$) [41], collapse the interaction between the TetR protein and the tetO element in promoters, and induce gene expression [39]. This simple induction system has been successfully adapted for various organisms [42-46].

We designed a novel promoter, P_{trc}^{tetO} , capable of repression with TetR, and strong induction with tetracycline derivatives in cyanobacteria. P_{trc}^{tetO} was constructed based on the P_{trc} , which was demonstrated to show high-level expression in cyanobacteria [21]. Two tetO elements were inserted at the adjacent sequences of -10, and -35 regions, respectively (Fig. 1).

3.2. Growth of *Synechococcus* sp. strain NKBG15041c in the presence of ATc

To test whether the inducer molecule exhibits negative impacts on cell growth, cell density of *Synechococcus* sp. strain NKBG wild type was monitored over time in the presence or absence of ATc. Addition of ATc with the final concentrations of 0.1, 1 and 10 $\mu\text{g/ml}$ caused no growth inhibition, indicating that ATc with the concentration analyzed here is available as a gene expression inducer (Fig. 2). In the previous study with *E. coli*, cytotoxic effect appeared when ATc concentration in the culture exceeded 2 $\mu\text{g/ml}$ [47]. On the other hand, 10 $\mu\text{g/ml}$ ATc was available for freshwater cyanobacterium *Synechocystis* sp. PCC6803 [22], suggesting high tolerance of cyanobacteria against ATc.

Fig. 2 Cell growth of *Synechococcus* sp. strain NKBG15041c wild type with and without anhydrotetracycline (ATc). ATc was added at 96 h (arrow). Filled circles, open circles, filled triangles and open triangles are cultures with 0, 0.1, 1, and 10 $\mu\text{g/ml}$ ATc.

3.3. Expression of tetracycline repressor

In the plasmids used in this study, *tetR* gene locates at the downstream of an endogeneous P_{cpc} promoter. P_{cpc} is capable of showing substance expression in *Synechococcus* sp. strain NKBG 15041c (un-published data). Expression of *tetR* repressor was examined with RT-PCR (Fig. 3). Specific amplification was shown in the transformants, while wild type showed no signal, confirming the expression of *tetR* gene in the transformants.

Fig. 3 Reverse transcription PCR for *Synechococcus* sp. strain NKBG15041c. Amplification was performed using *tetR*-specific primers and cDNA prepared from pKT-tetORGFP transformant (Lane 1). As control experiments, *tetR*-amplification from the cDNA sample prepared without reverse transcriptase (Lane 2), housekeeping ribonuclease gene-amplification from the transformants (Lane 3), and *tetR*-amplification from wild type cells (Lane 4) were attempted. Lane M represents DNA ladder.

3.4. ATc-induced GFP expression

GFP expression was observed with fluorescent microscopy (Fig. 4). Wild type cells showed no fluorescence. pKT-tetOGFP transformant cells emitted significant green fluorescence without ATc addition. This is reasonable because pKT-tetOGFP does not express TetR repressor, thus GFP expression should not be repressed. In contrast, pKT-tetORGFP transformant showed little fluorescence, and ATc addition enhanced its emission, implying the success in inducible expression. It should be noted that pKT-tetORGFP transformant culture with Atc addition did not show significant delay of growth as compared to that without Atc, suggesting that Atc addition and subsequent protein expression should not have negative impact on transformant cell growth.

Fig. 4 Differential interference contrast (DIC) and fluorescent microscopy for *Synechococcus* sp. strain NKBG15041c. pKT-tetORGFP transformant was observed with (10 μ g/ml) or without ATc (scale bar = 5 μ m).

For further confirmation, whole proteins extracted from wild type and each transformant were subjected to Western blotting with anti-GFP antibody (Fig. 5). Without ATc, no signal was detectable in pKT-tetORGFP transformant, suggesting the complete repression by TetR repressor. Strict repression confirmed in this study is one of the most important properties of the inducible system which will be applied for future biosynthesis of toxic chemicals. When Atc was added, GFP signal increased with increasing ATc concentration, suggesting the induction of GFP expression by ATc. These results indicate the establishment of tetracycline-inducible expression system in the marine cyanotacterium with strict on/off switching. The inducer molecule Atc with the concentration of 0.1 μ g/ml was sufficient to initiate target gene expression, while the maximum expression required Atc at 10 μ g/ml in the analyzed conditions. Similar expression variations depending on inducer concentration were demonstrated in the previous study using *Synechocystis*

1 sp. strain PCC6803 [22]. In contrast, less than 1 $\mu\text{g/ml}$ of ATc was often employed in other bacteria [43-46].

2 Thus more ATc tend to be needed in cyanobacteria than other bacteria. This could be due to the photolability

3 of ATc under the continuous light irradiation [22]. When the GFP expression levels were compared between

4 transformants harboring pKT-tetORGFP and pKT-tetOGFP which lacks *tetR* gene, pKT-tetORGFP

5 transformant showed approximately 4 times lower expression than pKT-tetOGFP transformant. Since these

6 plasmids share identical promoter sequence ($P_{\text{trc}}^{\text{tetO}}$), the difference in the expression level should be attributed

7 to TetR. Repression of TetR might not be fully removed even in the presence of 10 $\mu\text{g/ml}$ ATc.

8 Fig. 5 Western blotting to detect GFP from *Synechococcus* sp. strain NKBG15041c wild type (Lane 1),

9 pKT-tetOGFP transformant (Lane 2), and pKT-tetORGFP transformant with different concentration of ATc (0,

10 0.1, 1, and 10 $\mu\text{g/ml}$ in Lane 3-6, respectively). From band area and grayscale on the Western blotting

11 membrane (a), signal intensity of each band was measured (b).

Conclusion

We demonstrated the tetracycline derivative-inducible gene expression in the marine cyanobacterium *Synechococcus* sp. strain NKBG 15041c. It was demonstrated that ATc ranging from 0.1 to 10 µg/ml in cultures did not show any inhibition effects on the cell growth. *tetR* gene expression was confirmed by RT-PCT. Finally, increase in GFP expression stimulated by ATc addition was confirmed with both fluorescent microscopy and Western blotting. Since strict on/off regulation was demonstrated, we believe that the genetic tool developed in this study is useful for metabolic engineering of marine cyanobacteria with the aim of bioenergy production.

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