Thesis for the Degree of Master of Science

Identification and Characterization of Cyclic-di-GMP-specific Phosphodiesterase in *Zymomonas mobilis*

by

Hye-Sook Heo

Department of Microbiology

The Graduate School

Pukyong National University

February 22, 2019

Identification and Characterization of Cyclic-di-GMP-specific Phosphodiesterase in *Zymomonas mobilis*

(*Zymomonas mobilis*에서 Cyclic-di-GMP 특이적 Phosphodiesterase 특성 및 역할 규명)

Advisor: Prof. Young-Jae Jeon

by

Hye-Sook Heo

A thesis submitted in partial fulfillment of the requirements

for the degree of

Master of Science

in Department of Microbiology, The Graduate School,

Pukyong National University

February 22, 2019

Identification and Characterization of Cyclic-di-GMP-specific Phosphodiesterase in *Zymomonas mobilis*

A dissertation

by

Hye-Sook Heo

Approved by:

(Chairman) Gun-Do Kim

(Member) Myung-Suk Lee

(Member) Young-Jae Jeon

February 22, 2019

CONTENTS

Identification and Characterization of Cyclic-di-GMP-specific Phosphodiesterase in *Zymomonas mobilis*

Hye-Sook Heo

Department of Microbiology, The Graduate School,
Pukyong National University

Abstract

Zymomonas mobilis has been known as a promising ethanologen used in fuel ethanol production process using lignocellulosic materials. The cell flocculation mutant strain *Z. mobilis* ZM401 with self-immobilization phenotype has been previously isolated via conventional chemical mutagenesis. This strain showed several important industrial robustness characteristics for ethanol production. With this reason, further studies on this flocculent strain with transcriptomic microarray and genome sequencing analysis have been carried out, and the results suggested that a single point mutation (A526V) in a putative phosphodiesterase(PDE) encoded by ZMO1055 EAL domain is one of the main causes for the cell flocculation characteristics. The PDE was predicted as one of major proteins involved in cell flocculation by controlling the intracellular levels of c-di-GMP, one of the bacterial second messengers. Therefore, this study investigated the expression levels of genes involved in the c-di-GMP synthesis/degradation, its enzymatic function and activities associated with the effect of the single point mutation on the ZMO1055 PDE via heterologous expression of this protein in *Escherichia coli*. The qRT-PCR analysis showed that five genes involved in c-di-GMP synthesis/degradation including ZMO1055 from ZM401 were 2-3 times up-regulated as compared to those from ZM4. The PDE enzyme assay indicated that total PDE activities of crude extracts

from ZM401 and purified ZMO1055 PDE of ZM401 showed 0.8- and 0.75-times lower hydrolytic activities than those of ZM4. These results suggest that the point mutation (A526V) in the ZMO1055 EAL domain of ZM401 influenced lowing the PDE activities in the flocculent strain ZM401 resulting in maintaining the higher intracellular level of c-di-GMP than that in the planktonic strain ZM4. In addition, the sequence and domain analysis of ZMO1055 confirmed that the point mutation (A526V) in α13 helix of ZMO1055 EAL domain is likely to be involved in PDE dimer formation. The dimerization of PDE plausibly induces the c-di-GMP binding site to be in less active form by expanding and stabilizing the helix loop of the DDFGTG(YSS) motif, the c-di-GMP binding site of the EAL domain. Consequently, this study indicated that ZMO1055 phosphodiesterase is a key enzyme involved in the regulation of c-di-GMP levels, and its enzyme function was potentially determined by induction of active form of the c-di-GMP binding site through dimerization of PDE.

1. Introduction

Over the past few decades, fossil fuels have been consumed excessively as the world's leading energy which are contributing to environmental pollution and significant greenhouse gas emissions. Fossil fuels will eventually run out in the near future[1]. Alternative sustainable and cleaner source of renewable energies including solar, wind power, biomass and geothermal energy are attracting public attention. Current transportation biofuels such as bioethanol and biodiesel which are extracted from sugar cane, corn and soybean can replace petroleum-based transportation fuels[2,3].

Biofuels are generally classified as the first, second and third generations depending on the feedstock used in fuel production. The first generation biofuels are sugar, starch and vegetable oils produced from agricultural crops and the second generation biofuels are non-edible lignocellulose and wastes produced from agricultural and food processing. The third generation biofuels are raw materials derived from photosynthetic microorganisms such as algae. Although the third generation biofuels are a promising source, but still in its early stages. Currently, the first generation biofuels have raised concerns about increase in arable land usage competition between energy and food or animal feeds production although it has been successfully commercialized. The second generation biofuels using non-edible feedstocks such as agricultural residues and waste cooking oils can

overcome this dilemma because there is no competition with food, and can provide various environmental benefits such as greenhouse gas neutralization[4-6].

Lignocellulosic materials for second generation biofuel production are required to destruct the structural polymers in plant such as cellulose, hemicellulose and lignin through the pretreatment followed by the enzyme hydrolysis to produce glucose[7]. During such a destruction step inevitably generating fermentative inhibitory compounds produced such as organic acids, furfurals, hydroxymethyl furfural and lignin derivatives. These inhibitory compounds can reduce the rate of growth and ethanol production for the microorganisms used in the fermentation steps such as *Zymomonas mobilis*, *Saccharomyces cerevisiae*[8,9]. In addition to such inhibitors, the ethanologenic microorganisms also exposure to various environment stresses such as high temperature, high osmotic pressure and ethanol toxicity. In this regard, it is necessary to develop robust microorganisms which can tolerate against such stressful environments to efficiently produce ethanol.

Z. mobilis is a Gram-negative bacterium and has been recently introduced to an industrial scale ethanol production using lignocellulosic materials such as corn stover due to its favorable physiological characteristics such as high bioethanol productivity, tolerance to high concentration of ethanol, high substrate-uptake rate[10,11] and efficiently modified pentose sugar utilization by metabolic engineering[8,12,13]. In previous studies, *Z. mobilis* ZM401, cell flocculation strain, was obtained by inducing chemical mutation in *Z. mobilis* ZM4[14,15]. Self-

cell immobilization characteristics of this strain has shown potential advantages such as increased tolerance to high substrate concentration, low exposure to inhibitory compounds and increased ethanol productivity due to prolonged cellular stability in batch system. It also has the advantage of easy end-product recovery and cell-recycling in the bioethanol production system[16,17]. Owing to such useful characteristics for ethanol production process, several previous studies have been attempted to understand genetic makeup of cell flocculation mutant strain ZM401 via genome and transcriptomic analyses. Both studies indicated that one of the mutations occurred in the EAL domain of the gene ZMO1055 which is involved in the regulation of the cellular secondary signaling molecule cyclic diguanylate(c-di-GMP)[15,18]. The ZMO1055 gene contains GGDEF and EAL domains. The GGDEF domain encodes diguanylate cyclase(DGC) which synthesize c-di-GMP from two guanosine triphosphate(GTP). The EAL domain encodes phosphodiesterase which converts c-di-GMP to linear 5′-phosphoguanylyl-(3′-5′)-guanosine(pGpG)[19].

In previous study, our group constructed the ZMO1055 phosphodiesterase deficient strains ZM4*ΔZMO1055*^{EAL}(ZAM1) and ZM401*ΔZMO1055*^{EAL*}(ZAM2). The recombinant strains showed higher c-di-GMP accumulation in cell and lower motility than wild type ZM4, and similar cell flocculation characteristics to ZM401[18].

Thus, in this study, we performed the analysis including transcription levels of the genes related c-di-GMP synthesis and/or degradation, and enzyme activities of ZMO1055 phosphodiesterase of ZM4 and ZM401 to clarify the characteristic differences of ZM4 and cell flocculation mutant strain ZM401. This helps to understand the mechanism of c-di-GMP regulation and to develop useful strains for bioethanol production.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains and plasmid used in this study are listed in Table 1 and 2. The strains of *Z. mobilis* were cultured in Rich Medium (RM) at 30 ℃ without shaking. The RM medium was composed of 20 g L^{-1} glucose, 10 g L^{-1} yeast extract, and 2 g L^{-1} KH_2PO_4, 1 g L^{-1} $(NH_4)_2SO_4$ and 1 g L^{-1} $MgSO_4$ with an addition of 1.5% agar for solid media[20]. This medium was separately autoclaved with carbon and other sources. When the strain was stored for a long term, bacterial cultures were suspended with 80% (v/v) glycerol and stored at -70℃ in cryogenic vials. For the growth of *E. coli* DH5α and BL21(DE3)pLysS, Luria-Bertani medium (LB)[21] was used. Its medium composition was as followed; 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl. Antibiotics were used at the following concentrations : ampicillin, 50 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol, 33 μg ml^{-1}.

Table 1. The list of strains used in this study

Bacterial strain	Genetic markers/phenotype	Reference
Escherichia coli strains		
DH5α	*hsd*R17(r - m +) *rec*A1 *end*A1	RBC
DH5α::pRBC::*ZMO1055*^{EAL}	*ZMO1055*^{EAL}, Amp^{R}	This study
DH5α::pRBC::*ZMO1055*^{EAL*}	*ZMO1055*^{EAL*}, Amp^{R}	This study
DH5α::pRBC::*ZMO1055*^{W}	*ZMO1055*^{W}, Amp^{R}	This study
DH5α::pRBC::*ZMO1055*^{W*}	*ZMO1055*^{W*}, Amp^{R}	This study
BL21(DE3)pLysS	*hsd*S$_B$($r_B^- m_B^-$)*galdcm*(DE3) pLysS (Cm^{R})	Novagen
BL21(DE3)pLysS::pET28a(+) ::*ZMO1055*^{EAL}	*ZMO1055*^{EAL}, Cm^{R}, Kan^{R}	This study
BL21(DE3)pLysS::pET28a(+) ::*ZMO1055*^{EAL*}	*ZMO1055*^{EAL*}, Cm^{R}, Kan^{R}	This study
BL21(DE3)pLysS::pET28a(+) ::*ZMO1055*^{W}	*ZMO1055*^{W}, Cm^{R}, Kan^{R}	This study
BL21(DE3)pLysS::pET28a(+) ::*ZMO1055*^{W*}	*ZMO1055*^{W*}, Cm^{R}, Kan^{R}	This study
Zymomonas mobilis strains		
ZM4	A wild-type strain, ATCC 31821	[20]
ZM401	A cell flocculation mutant strain of ZM4, ATCC 31822	[14]

EAL : only EAL domain sequence of ZMO1055 gene, *W* : whole sequence of ZMO1055 gene, * : a point mutation(A526V)

Table 2. The list of plasmids used in this study

Plasmid	Genetic markers/phenotype	Reference
pRBC	TA cloning vector, Amp^R	RBC
pRBC::*ZMO1055*^{EAL}	*ZMO1055*^{EAL}, Amp^R	This study
pRBC::*ZMO1055*^{EAL*}	*ZMO1055*^{EAL*}, Amp^R	This study
pRBC::*ZMO1055*^{W}	*ZMO1055*^{W}, Amp^R	This study
pRBC::*ZMO1055*^{W*}	*ZMO1055*^{W*},Amp^R	This study
pET28a(+)	Expression vector, Kan^R , His-tag	Novagen
pET28a(+)::*ZMO1055*^{EAL}	*ZMO1055*^{EAL}, Kan^R	This study
pET28a(+)::*ZMO1055*^{EAL*}	*ZMO1055*^{EAL*}, Kan^R	This study
pET28a(+)::*ZMO1055*^{W}	*ZMO1055*^{W}, Kan^R	This study
pET28a(+)::*ZMO1055*^{W*}	*ZMO1055*^{W*}, Kan^R	This study

EAL : only EAL domain sequence of ZMO1055 gene, *W* : whole sequence of ZMO1055 gene, * : a point mutation(A526V)

2.2 Isolation, amplification, and modification of DNA

To isolate genomic DNA and recombinant plasmids, HiGene™ Genomic DNA Prep Kit (Biofact, Korea) and Plasmid DNA Extraction Mini Kit (Favorgen, Taiwan) were used respectively. The purification of DNA fragments from PCR products and agarose gels was achieved by GEL/PCR purification Kit (Favorgen, Taiwan) according to the manufacturer's instructions. All PCR reactions were performed in 2720 PCR System (Thermo Scientific, USA) and using TaKaRa Ex Taq polymerase (Takara, Japan) for DNA amplification. The composition of PCR was set up with 0.25 µl Taq-polymerase (5 U/µl), 4 ul dNTP mixture (2.5mM each), 1µl/1µl forward/reverse primers (10 pmol), 5 ul buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM Dithiothreitol, 0.5% Tween 20, 0.5% NP-40, 50% glycerol), 1 µl Template(<500µg) and the rest was filled with Distilled water up to 50 µl. All PCR products and DNA fragments were separated on 1% agarose gels in TAE buffer (2 M Tris-acetate, 50 mM EDTA) and the size of DNA was compared with ExcelBand 1 kb Plus DNA Ladder (SMOBIO, Taiwan). PCRs were performed 30 cycles and the conditions were as follows; initial denaturation at 98℃ for 5 min, denaturation at 98℃ for 10 sec, annealing at 55℃ for 30 sec, extension at 72℃ for 1 min and final extension at 72℃ for 7 min. All gene constructs were confirmed by sequencing using the Sanger method[22].

2.3 RNA isolation and qRT-PCR

Total RNA was isolated from *Zymomonas mobilis* ZM4 and its mutant strain ZM401 using HiYield Total RNA Mini kit (RBC, Taiwan), according to manufacturer's instructions. The concentration of RNA was measured at 260 nm and 280 nm by a UV/VIS Spectrophotometer (Mecasys, Korea). The extracted RNA was treated with RQ1 RNase-Free DNase (Promega, USA) to remove DNA contamination. The cDNA was synthesized from 1000 ng of total RNA by Prime script RT Master mix (Takara, Japan). The synthesized cDNA was used as PCR templates. qRT-PCR was performed on a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems, USA) using AccuPower 2x GreenStar qPCR Master Mix (Bioneer, Korea). The composition of PCR was set up with 50 ng cDNA, 10 µl 2X GreenStar Master Mix, 1µl/1µl forward/reverse primers (10 pmol), and the rest was filled with deionized water up to 20 µl. The QuantStudio 6 Flex sequence detector (Applied Biosystems, USA) was programmed with the following PCR conditions; 40 cycles of denaturation at 95℃, 15 sec and amplification 60℃, 30 sec. All reactions were run in triplicate and calibrated with the wild type strain ZM4 (reference sample) and 16s rRNA sequence (endogenous control). The amount of mRNA obtained was calculated as the relative concentration using the value $2^{-\Delta\Delta Ct}$[23], and the results were compared. All primers used for qRT-PCR are shown in Table 3. The unpaired, two-tailed Student's *t*-test was used for statistical comparisons. A *p* value less than 0.05 was considered significant.

Table 3. Primers for each amplified gene by qRT-PCR

Primer	Sequence(5' → 3')	Target genes	Reference
16s_F	CGTGCTACAATGGCGGTGAC	ZMOr003 (16s ribosomal RNA)	This study
16s_R	GCGATTCCGCCTTCATGCTC		
ZMO1055_F	GGCACTTCTGACGTTTGGCA	ZMO1055 (GGDEF-EAL hybrid)	
ZMO1055_R	CGATCCGGTCATCCATCAACGA		
ZMO0401_F	ACCATTGCCGGAAACATAAG	ZMO0401 (GGDEF-EAL hybrid)	
ZMO0401_R	GGCAAACCGGAATGGTTAT		
ZMO1365_F	TATTAGGAAAGCCGGACGAT	ZMO1365 (GGDEF)	
ZMO1365_R	CGGAAAGAGATGGCTAATCC		
ZMO0919_F	AGCAGGGCAACACTCTTTCT	ZMO0919 (GGDEF)	
ZMO0919_R	TGCTGCTACCGACTATCCAG		
ZMO1487_F	AGCCAGACGTTATCCACTTGT	ZMO1487 (EAL)	
ZMO1487_R	CGTGAGATAACATGGCGAAT		
ZMO0614_F	CAACTCTGTTCGGTATTCAGG	ZMO0614 (FlgB)	
ZMO0614_R	ATCCCGCGCTTTATAGTTAG		
ZMO1084_F	AGGCGAAAATCGTATCAATC	ZMO1084 (BcsB)	
ZMO1084_R	GAAAGACCGCTTATTGGAAA		

2.4 Construction of ZMO1055^{W} and ZMO1055^{EAL} protein expression vectors

To construct the ZMO1055^{W} and ZMO1055^{EAL} protein expression vector, the genes coding for whole sequence and partial EAL domain sequence of ZMO1055 from ZM4 and ZM401 respectively were cloned for heterologous expression in *E.coli*. The PCR and sequencing primers used for recombinant vectors construction and genetic confirmation are the list in Table 4.

To construct the ZMO1055^{EAL} protein expression vector pET28a(+)::*ZMO1055^{EAL}*, the partial gene of EAL domain of ZMO1055 was amplified from the genomic DNAs of wild type ZM4 by PCR using primers, EAL_F(*BamH* I) and EAL_R(*Not* I). The 772 bp PCR products were cloned into a pRBC vector using RBC TA cloning kit (RBC, Taiwan) via an overnight ligation reaction at 4 ℃. The ligation mixture was transferred into *E. coli* DH5α competent cells (RBC, Taiwan). The objective recombinant plasmids were screened by blue-white selections and the putative recombinant strains *E. coli* DH5α (pRBC::*ZMO1055^{EAL}*) were further confirmed by PCR analysis using primer, M13F and M13R. The target gene in pRBC::*ZMO1055^{EAL}* was digested with *BamH* I and *Not* I and cloned into the *BamH* I and *Not* I sites of pET28a(+) vector (Novagen, USA) to generate both N-terminal and C-terminal hexa-histidine tagged protein. The ZMO1055 EAL domain protein expression vector pET28a(+)::*ZMO1055^{EAL*}* of the flocculant strain ZM401 was constructed with the same manner as described earlier.

To construct the ZMO1055^{W} protein expression vector pET28a(+)::*ZMO1055^{W}*, the whole sequence of ZMO1055 was amplified from the genomic DNAs of wild type ZM4 by PCR using primers, ZMO1055_F(*BamH* I) and ZMO155_R(*Xho* I). The 1758 bp PCR products were cloned into a pRBC vector using RBC TA cloning kit (RBC, Taiwan) via an overnight ligation reaction at 4 ℃. The ligation mixture was transferred into *E. coli* DH5α competent cells (RBC, Taiwan). The objective recombinant plasmids were screened by blue-white selections and the putative recombinant strains *E. coli* DH5α (pRBC::*ZMO1055^{W}*) were further confirmed by PCR analysis using primer, M13F and M13R. The target gene in pRBC::*ZMO1055^{W}* was digested with *BamH* I and *Xho* I and cloned into the *BamH* I and *Xho* I sites of pET28a(+) vector (Novagen, USA) to generate both N-terminal and C-terminal hexa-histidine tagged protein. The ZMO1055^{W*} protein expression vector pET28a(+)::*ZMO1055^{W*}* of the flocculant strain ZM401 was constructed in the same manner.

Plasmid DNA harboring the whole sequence and partial EAL domain of ZMO1055 were transformed into *E. coli* BL21(DE3)pLysS competent cells for protein expression. The objective recombinant strains were selectively screened by culturing in media containing kanamycin and chloramphenicol and further confirmed by PCR analysis using primer, T7 promoter and T7 terminator. The recombinant strains were also confirmed by sequencing using the Sanger method[23].

Table 4. Primers for each amplified gene by qRT-PCR

Primer	Sequence(5' → 3')	Reference
EAL_F(*Bam*HI)	ATAGGATCCACGACCTTCGACGTGCCATTG	This study
EAL_R(*Not*I)	ATTGCGGCCGCTTAATTCCTGTTGGACGCCATA	
ZMO1055_F(*Bam*HI)	AGAGCACGGATCCCCAGATCCTATATTAAAAAC	This study
ZMO1055_R(*Xho*I)	ATAACTCGAGAATTCCTGTTGGACGCCATA	
M13F	TTTCCCAGTCACGACGTTGTAA	RBC
M13R	TCACACAGGAAACAGCTATGAC	
T7 promoter	TAATACGACTCACTATAGGG	Novagen
T7 terminator	GCTAGTTATTGCTCAGCGG	

2.5 Protein sample preparation

The recombinant strains BL21(DE3)pLysS::pET28a(+)::*ZMO1055*^{W}, BL21(DE3)pLysS::pET28a(+)::*ZMO1055*^{W*}, BL21(DE3)pLysS::pET28a(+)::*ZMO1055*^{EAL}, BL21(DE3)pLysS::pET28a(+)::*ZMO1055*^{EAL*} were grown in LB medium supplemented with 50 μg ml^{-1} kanamycin and 33 μg ml^{-1} chloramphenicol at 37 ℃, 150 rpm to an optical density (OD_{660}) of ~1.0. Protein expression was induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside(IPTG) for overnight (~16 h) at 10 ℃, 150 rpm after which cells were harvested by centrifugation at 35,000 g for 20 min at 4 ℃

and washed twice with Lysis buffer A (60 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM 1,4-Dithiothreitol, 5% glycerol). The cell pellets were resuspended in Lysis buffer A and transferred to prefilled tubes containing beads. The suspension was homogenized in a Bead homogenizer (Benchmark Scientific, USA) for 1 min at 4,000 rpm and then incubated on ice for 1 min. This procedure was repeated 10 times. The soluble protein fractions were separated by centrifugation for 10min at 13,000 rpm, 4 ℃. The total protein fractions represent whole cell lysates before centrifugation. After centrifugation the insoluble protein pellet was additionally washed and resuspended in Lysis buffer A.

2.6 SDS PAGE and western blotting analysis

Samples for SDS-PAGE separation were prepared as follows: 80 µl of protein sample (whole cell lysate, soluble fraction), 20 µl of 5x SDS-PAGE loading buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 0.1% bromophenol blue, 40% glycerol, 100 mM 1,4-Dithiothreitol). Samples were heated for 5 min at 100 ℃. 20 µl of samples were applied to each lane of a 12% SDS-PAGE gel and run at 200 V for 80 min. The protein samples were transferred to a PVDF membrane (BIO-RAD, USA). Membranes were blocked with Blocking buffer (5% powder milk in 1X TBS buffer, 0.05% Tween 20) for 1 h at room temperature and washed in TBS buffer (65 mM Tris-base, pH 7.5, 150 mM NaCl) three times. The membranes were incubated with a mouse monoclonal antibody IgG_{2b} (Santa Cruz Biotechnology, USA) diluted

1:1,000 in Blocking buffer for 1hr at 37 ℃ and washed in TBS buffer three times. The membranes were then incubated with the secondary antibody, goat anti-mouse IgG horseradish peroxidase conjugate (BIO-RAD, USA) diluted 1:3,000 in Blocking buffer for 1hr at room temperature. After washing with TBS buffer, the immunoreactive bands were detected with a western ECL substrate kit (BIO-RAD, USA) according to the manufacturer's instructions and imaged by exposing to X-ray film.

2.7 Expression and purification of His6-target protein

The recombinant strains were grown in LB medium supplemented with 50 μg ml^{-1} kanamycin and 33 μg ml^{-1} chloramphenicol at 37 ℃, 150 rpm to an optical density (OD_{660}) of ~1.0. Protein expression was induced with 0.5 mM IPTG for overnight (~16 h) at 10 ℃, 150 rpm after which cells were harvested by centrifugation. For protein purification, Cell pellets were resuspended in Lysis buffer B (50mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM $MgCl_2$, 5 mM 1,4-Dithiothreitol) containing 1 mM phenylmethylsulphonyl fluoride (PMSF, in isopropanol). The suspension was prechilled on ice for 20 min and lysed by French Press cell (Thermo Scientific, USA) under 7,000 psi, three times. Cellular debris was removed by centrifugation at 35,000 g for 20 min at 4 ℃. The Ni-NTA columns (Qiagen, USA) were thoroughly washed and equilibrated with Binding buffer (50 mM Sodium phosphate, 500 mM NaCl, 10 mM Imidazole, pH 8.0) and then loaded with purified

lysate. The column was washed repeatedly with 10-bed volumes of Binding buffer until the OD_{280} of the flow through reached to below 0.01. The bound hexa-histidine tagged protein was eluted from the column with Elution buffer (50 mM Sodium phosphate, 500 mM NaCl, 150 mM Imidazole, pH 8.0). Fractions containing objective protein, as judged by SDS-PAGE analysis, were dialyzed against PBS buffer for overnight in 4 ℃. Proteins were stored at 4 ℃ in a solution of the following conditions; 50-300 mM NaCl, 25 mM Tris-Cl, 1 mM 1,4-Dithiothreitol, 1 mM PMSF and 1mM EDTA. For long-term storage of the protein, 20-50% glycerol was added and stored at -70 ℃.

2.8 Phosphodiesterase colorimetric assay

2.8.1 Total phosphodiesterase assay for crude extracts from *Z. mobilis*

Cells were harvested by centrifugation at 35,000 g for 20 min at 4 ℃ and washed twice with D.I water. The cell pellets were resuspended in D.I water and transferred to prefilled tubes containing beads. The suspension was homogenized in a Bead homogenizer(Benchmark Scientific, USA) for 1 min at 4,000 rpm and then incubated on ice for 1 min. This procedure was repeated 10 times. The soluble protein fractions were separated by centrifugation for 10 min at 13,000 rpm, 4 ℃. The soluble fractions from ZM4 and ZM401 were assayed for the enzyme activity of PDE against c-di-GMP using Cyclic Nucleotide Phosphodiesterase Assay kit (Enzo Life Sciences, USA). For PDE activity analysis on c-di-GMP, the soluble

fractions were loaded onto desalting resin to remove excess phosphates and nucleotides which interfere with the assay. These samples were injected with 0.5 mM cGMP substrate, 5`-nucleotidase, PDE solution (type I cyclic AMP phosphodiesterase) and incubated at 30 ℃ for 30 min in assay buffer (10 mM Tris-HCl, pH 7.4). The product was injected with BIOMOL green reagent (Enzo Life Sciences, USA) and allowed color to develop for 20 min. The enzyme activities were quantified using Microplate reader (Molecular Devices, USA) at OD_{620} nm. The value (amount of 5'-GMP released) was standardized to the amount of total protein, which was estimated using the Pierce 660 nm Protein Assay kit (Thermo Scientific, USA) with bovine serum albumin (BSA) in each sample.

2.8.2 Phosphodiesterase assay for ZMO1055^{EAL} and ZMO1055^{EAL*}

The purified proteins of ZMO1055^{EAL} and ZMO1055^{EAL*} from *E. coli* BL21(DE3)pLysS were assayed for the enzyme activity of PDE against c-di-AMP and c-di-GMP using Cyclic Nucleotide Phosphodiesterase Assay kit (Enzo Life Sciences, USA). For PDE activity analysis on c-di-AMP, the purified proteins were loaded onto desalting resin to remove excess phosphates and nucleotides which interfere with the assay. The samples were injected with 0.5 mM cAMP substrate, 5`-nucleotidase, PDE solution (type I cyclic AMP phosphodiesterase) and incubated at 30 ℃ for 30 min in assay buffer (10 mM Tris-HCl, pH 7.4). The product was injected with BIOMOL green reagent (Enzo Life Sciences, USA) and allowed color to develop

for 20 min. The enzyme activities were quantified using Microplate reader (Molecular Devices, USA) at OD_{620} nm. PDE activity analysis on c-di-GMP was carried out with the same manner except that c-di-GMP substrate was added instead of c-di-AMP substrate. The value (amount of 5'-AMP and 5'-GMP released) was standardized to the amount of total protein, which was estimated using the Pierce 660nm Protein Assay kit (Thermo Scientific, USA) with bovine serum albumin (BSA) in each sample.

The unpaired, two-tailed Student's *t*-test was used for statistical comparisons. A *p* value less than 0.05 was considered significant.

2.9 Bioinformatics analysis

The *Z. mobilis* genome database was searched for gene and protein sequences composed of GGDEF and EAL domain. Protein sequences were analyzed by the SMART[24] and Pfam[25] domain databases. RCSB PDB[26] was employed to identify homologous proteins in other bacteria. Multiple sequence alignments were constructed using Bioedit software and the Figures were generated using ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).

3. Results

3.1 Domain organization of predicted GGDEF and EAL domain in *Z. mobilis*

In previous studies, our group identified 5 genes involved in c-di-GMP synthesis/degradation in the genome of *Z. mobilis* ZM4 by gene function prediction and protein sequence analysis[18]. These genes include two GGDEF-EAL hybrid proteins, one EAL domain protein and two GGDEF domain proteins. Previously, it was confirmed that a point mutation was present in ZMO1055 of the cell flocculation mutant strain ZM401. This mutation, one of the point mutations present in ZM401, is located at the 526 amino acid position of ZMO1055 EAL domain and converts amino acid alanine to valine (A526V) by changing GCU codon to GUU[15]. By using the SMART algorithm, the predicted domains of these 5 genes were presented and listed in Table 5. The gene locus on the genome of ZM4 strain, ZMO0401, ZMO0919 and ZMO1365 contain transmembrane region (TM) or Reg_prop and YYY domain. The Reg_prop domain is part of the periplasmic sensor domain and the YYY domain is one of the bacterial signal transduction domains, however the exact function of these domains is not known[27]. These three genes are likely to be either membrane bound or exported membrane proteins. Only two genes contain EAL or dual GGDEF-EAL domain, i.e., ZMO1487 and ZMO1055, respectively. These proteins were predicted to be located within the inner membrane because of lacking obvious TM or signal domains.

Table 5. Predicted domain organizations of proteins involved in the turnover of c-di-GMP in *Z. mobilis* ZM4

Gene locus	Domain organization	Similarity		
		% seq identity	Gene locus	Reference
ZMO0401		37	PA1727	*Pseudomonas aeruginosa* [28]
ZMO1055		39	PA4601	*Pseudomonas aeruginosa* [29]
ZMO1487		35	KPN_01598	*Klebsiella pneumoniae subsp. Pneumoniae* [30]
ZMO0919		45	CCNA_02546	*Caulobacter vibrioides* [31]
ZMO1365		39	CCNA_02546	*Caulobacter vibrioides* [31]

The light blue represents the transmembrane helix region

3.2 Transcription analysis of genes involved in c-di-GMP in *Z. mobilis*

3.2.1 Gene expression related to c-di-GMP turnover

Previously, our group confirmed that the flocculation mutant strain ZM401 showed a higher intracellular c-di-GMP concentration than that of its parent strain ZM4[18]. ZM401 was expected to up-regulate genes involved in synthesis and degradation of c-di-GMP. To determine the transcription levels of these genes in ZM4 and ZM401, qRT-PCR analysis was performed. The results were confirmed that measurements are significant by using two-tailed Student's *t*-test ($p<0.05$) analysis on the means from three independent experiments and are detailed in Figure 1. The four genes including the GGDEF and GGDEF-EAL hybrid domains were up-regulated 2 to 3 times higher and the ZMO1487 with only EAL domain was significantly up-regulated 14 times higher in the ZM401 as compared to those in the wild type ZM4. These results suggest that the various mutations found in the genome of ZM401[37] and the resulting changes in cell flocculation mutant strain ZM401 promote transcription of these genes.

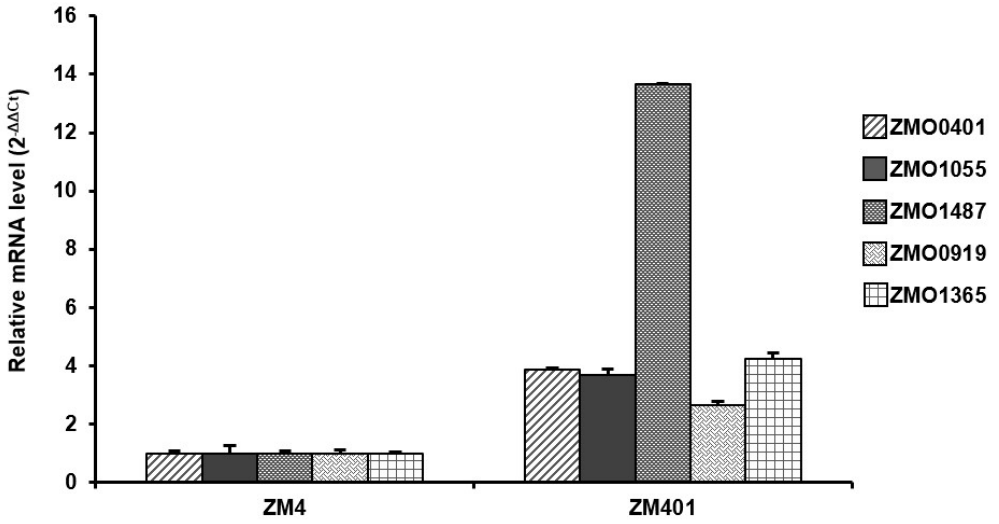


Figure 1. The expression levels of genes composed with GGDEF and EAL domain related to c-di-GMP synthesis and degradation in ZM4 and ZM401. The expression levels of the selected genes relative to 16s rRNA gene ZMOr003 were compared, and the values are presented as -fold differences in the expression of cell flocculation mutant strain ZM401 relative to wild type ZM4. Bars indicated mean ± SD (n=3, $p < 0.05$: unpaired, two-tailed Student's *t*-test).

3.2.2 Gene expression related to motility and cellulose synthesis

The c-di-GMP regulates diverse cellular activities including motility, adherence, biofilm formation, virulence and cell cycle progression depending on its intracellular levels[19]. The cell flocculation mutant strain ZM401 was expected to have a difference in the expression of genes involved in these cell processes as compared with its parent strain ZM4. To determine the transcription levels of these genes such as ZMO0614 and ZMO1084 in ZM4 and ZM401, qRT-PCR analysis was performed. ZMO0614 encode the flagellar basal body rod protein FlgB which is involved in motility and ZMO1084 encode the cellulose synthase subunit B. The results showed significant by using two-tailed Student's *t*-test ($p<0.05$) analysis on the mean value from three independent experiments and are detailed in Figure 2. The gene expression level of ZMO0614 was similar in the wild type and cell flocculation mutant strain ZM401. ZMO1084 was up-regulated 3.8 times higher in ZM401 than that in the wild type strain ZM4.

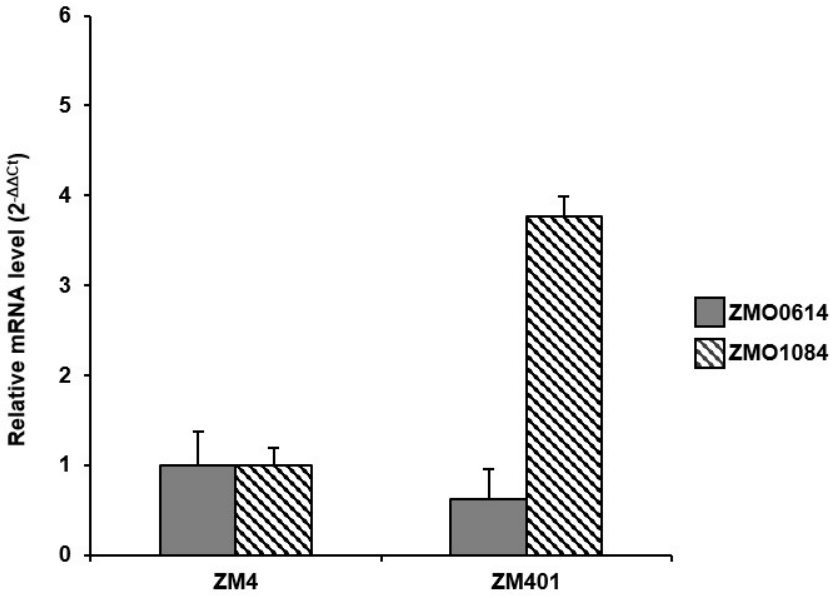


Figure 2. The expression levels of genes related motility and cellulose synthesis regulated by c-di-GMP levels in ZM4 and ZM401. The expression levels of the selected genes relative to 16s rRNA ZMOr003 were compared, and the values are presented as -fold difference in the expression of cell flocculation mutant strain ZM401 relative to wild type ZM4. Bars indicated mean ± SD (n=3, $p < 0.05$: unpaired, two-tailed Student's *t*-test).

3.3 Total PDEs activities of crude extracts from *Z. mobilis*

The cell flocculation mutant strain ZM401 showed higher intracellular c-di-GMP levels than that of parent strain ZM4 even though up-regulation of the genes containing EAL domain involved in degradation of c-di-GMP as described above. To determine the activities of phosphodiesterases (PDEs) from EAL domains in both strains, total PDE enzyme assays using soluble fraction of crude extracts from ZM4 and ZM401 were carried out. The results are shown in Figure 3. The activities of total PDE from ZM401 was 0.8-times lower than that of wild type strain. This result indicates that the higher intracellular c-di-GMP level from the flocculation strain ZM401 was due to the lower activities of total PDEs rather than the up-regulation of genes involved in c-di-GMP turnover.

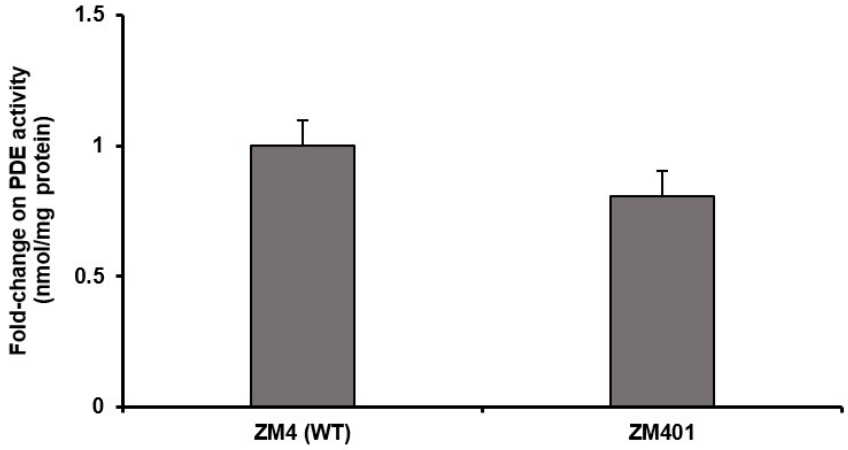


Figure 3. The enzyme activities of total PDEs against substrates. c-di-GMP/PDE activity for soluble fractions of crude extracts from *Z. mobilis* ZM4 and ZM401 were measured in Phosphodiesterase colorimetric assay. The values are presented as -fold difference in the PDEs activities of cell flocculation mutant strain ZM401 relative to wild type ZM4. Bars indicated mean. (n=2, $p < 0.05$: unpaired, two-tailed Student's *t*-test).

3.4 Heterologous expression of ZMO1055^{W} and ZMO1055^{EAL} in *E. coli*

3.4.1 Construction of ZMO1055^{W} protein expression vector

To determine the enzyme activities of ZMO1055 hybrid protein and the effect of single point mutation(A526V) in ZMO1055 EAL domain of ZM401, the entire sequence of ZMO1055 (ZMO1055^{W}) containing both GGDEF and EAL domains from ZM4 was subcloned into each pET28a(+) vector. This plasmid was separately transformed into *E. coli* BL21(DE3)pLysS. The expression of ZMO1055^{W} with N-terminal His6-tags was induced by adding IPTG. The western blotting analysis was used to confirm whether the constructed proteins were expressed, and the result is shown in Figure 4. The whole protein expression vector of ZMO1055^{W*} of ZM401 was constructed and expressed in the same manner. The predicted size of the protein ZMO1055^{W} and ZMO1055^{W*} is approximately 64.5 kDa. Both ZMO1055^{W} of ZM4 and ZMO1055^{W*} of ZM401 were expressed in whole cell lysates but not expressed in soluble fractions. Therefore, the proteins could not be further purified.

3.4.2 Construction of ZMO1055^{EAL} protein expression vector

To determine the enzyme activities of ZMO1055 EAL domain protein and the effect of single point mutation in ZMO1055 EAL domain of ZM401, the sequence of only ZMO1055 EAL domain (ZMO1055^{EAL}) from ZM4 was subcloned into each pET28a(+) vector as the same manner described in the earlier section. The western blotting analysis was used to confirm whether the constructed proteins were expressed, and the result is shown in Figure 4. The protein expression vector of ZMO1055^{EAL*} from the flocculating strain ZM401 was constructed and expressed in the same manner. The predicted sizes of the protein ZMO1055^{EAL} and ZMO1055^{EAL*} is approximately 29.6 kDa. Both ZMO1055^{EAL} of ZM4 and ZMO1055^{EAL*} of ZM401 were expressed in whole cell lysates and soluble fractions. To perform the enzyme assay, the ZMO1055^{EAL} and ZMO1055^{EAL*} were isolated and purified from soluble fraction of cell lysates.

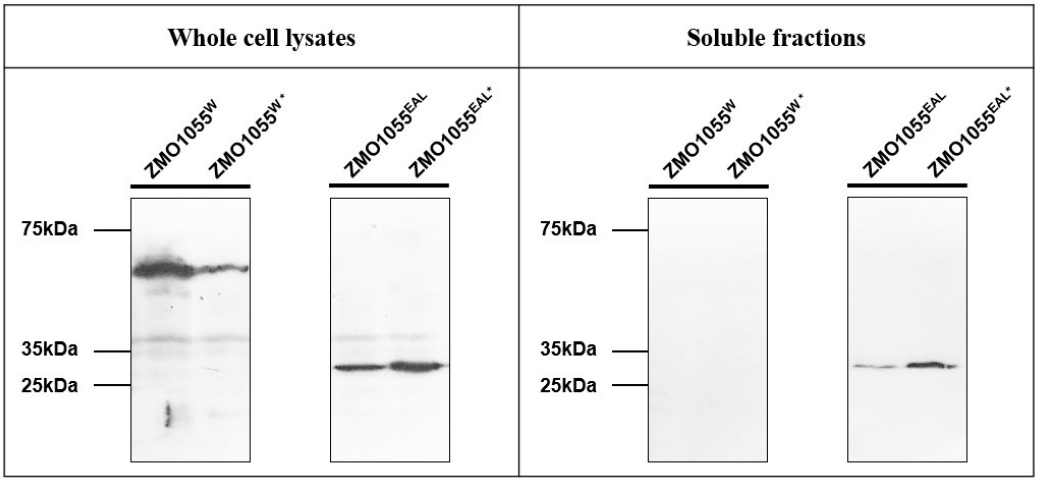


Figure 4. The expression of poly his-tagged ZMO1055^{W} and ZMO1055^{EAL} proteins from ZM4 and ZM401. The expression of ZMO1055^{W} and ZMO1055^{EAL} proteins from ZM4 and ZM401 was measured by western blotting analysis on whole cell extracts and soluble fractions; ZM1055^{W} is a protein of the entire ZMO1055 sequence; ZMO1055^{EAL} is a protein of only ZMO1055 EAL domain sequence; The superscript * after the protein name indicates the single point mutation in the ZMO1055 EAL domain of cell flocculation mutant strain ZM401.

3.5 PDE activity of purified ZMO1055^{EAL} and ZMO1055^{EAL*}

3.5.1 Enzyme activity of ZMO1055^{EAL} against c-di-AMP and c-di-GMP

To identify the substrate specificity of ZMO1055 EAL domain protein, a putative phosphodiesterase, the His6-ZMO1055^{EAL} was expressed in *E. coli* BL21(DE3)pLysS and purified. The purified protein was tested for the PDE activity through colorimetric assay, and the results are shown in Figure 5. ZMO1055 EAL wild type domain protein of ZM4 showed 1.38 times higher hydrolytic activity against c-di-GMP than that against c-di-AMP. The results indicate that ZMO1055 EAL domain protein is a cyclic nucleotide phosphodiesterase and prefer c-di-GMP as a substrate rather than c-di-AMP.

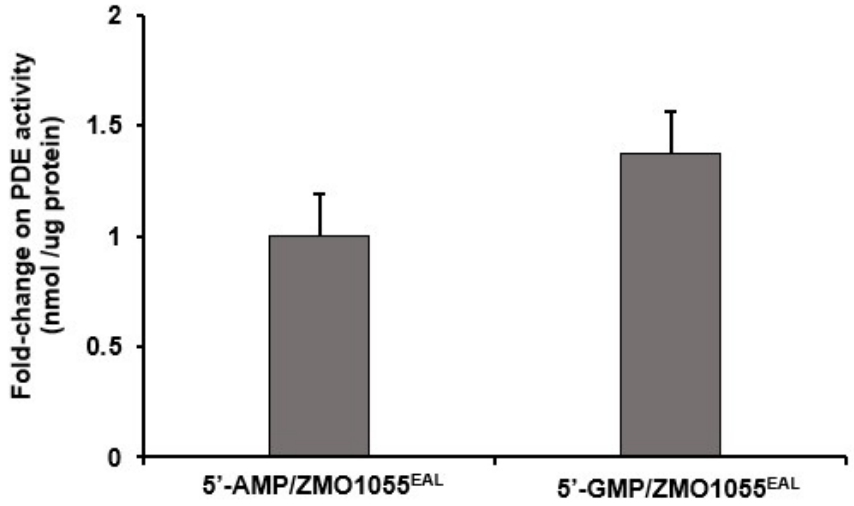
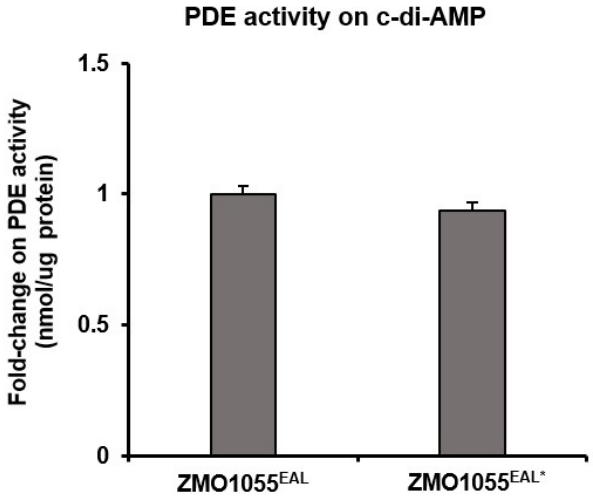


Figure 5. The enzyme activity of ZMO1055^{EAL} on substrates c-di-AMP and c-di-GMP. c-di-AMP/ZMO1055^{EAL} and c-di-GMP/ZMO1055^{EAL} activities were measured in phosphodiesterase colorimetric assay. The values are presented as -fold difference in the PDE activity of c-di-GMP/ZMO1055^{EAL} relative to c-di-AMP/ZMO1055^{EAL}. Bars indicated mean. (n=3, $p < 0.05$: unpaired, two-tailed Student's t-test).

3.5.2 Enzyme activity of ZMO1055^{EAL} and ZMO1055^{EAL*}

The PDE activity of ZMO1055^{EAL*} of the cell flocculation mutant strain ZM401 were compared with that of parent strain ZM4 to determine whether the single point mutation(A526V) in ZMO1055 EAL domain of ZM401 affects the enzyme function and activities against substrates. The results are shown in Figure 6. ZMO1055 PDE of ZM401(ZMO1055^{EAL*}, the mutant type) was 0.94- and 0.75-times lower activities on c-di-AMP and c-di-GMP respectively than those of ZM4 (ZMO1055^{EAL}, the wild type). These results suggest that the single point mutation in ZMO1055 EAL domain of ZM401 is associated with decreased in PDE activity.



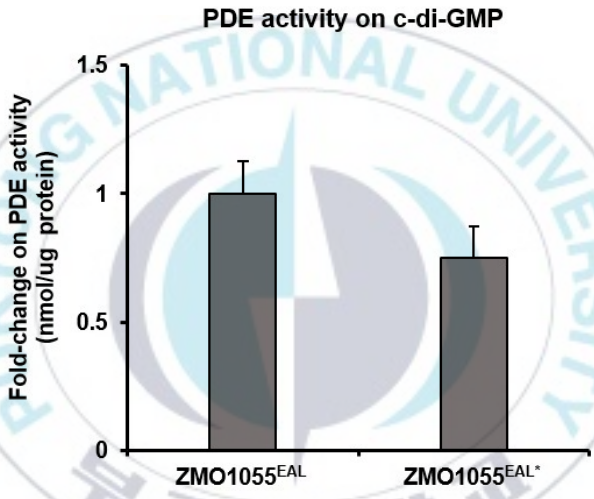


Figure 6. The enzyme activity of ZMO1055^{EAL} and ZMO1055^{EAL*} on substrates c-di-AMP and c-di-GMP. The PDE activities of ZMO1055^{EAL}(the wild type) and ZMO1055^{EAL*} (the mutant type) were measured in phosphodiesterase colorimetric assay. The values are presented as -fold difference in the PDE activity of ZMO1055^{EAL*} relative to ZMO1055^{EAL}. Bars indicated mean. (n=3, $p < 0.05$: unpaired, two-tailed Student's t-test). The superscript * after the protein name indicates the single point mutation in the ZMO1055 EAL domain of cell flocculation mutant strain ZM401.

3.6 Sequence and domain analysis of ZMO1055

To identify the structural characteristics of ZMO1055, homology search for amino acid sequence of ZMO1055 was performed using blast software at RCSB PDB server. ZMO1055 has 39% sequence similarity with MorA(locus tag : PA4601) of *P. aeruginosa* at the amino acid level. Although structural characteristics of ZMO1055 have not yet been elucidated, the structure of MorA showed the highest homology with ZMO1055 has been identified[29]. Therefore, multiple alignment between ZMO1055 and other proteins composed of GGDEF and EAL domain, including MorA, was performed using Bioedit software. Based on the secondary structure data of *P. aeruginosa* MorA obtained from the RCSB PDB database, the secondary structure depiction on those proteins was performed using ESPrinpt 3.0 and SWISS-MODEL, and the results are shown in Figure 7 and Figure 8.

Previous studies confirmed that both DDFGTG(YSS)motif and PDE-α6 helix in MorA of *P. aeruginosa* are involved in EAL domain dimerization, also those helixes influence active site formation of EAL domain[29]. The bioinformatic analysis showed that ZMO1055 EAL domain conserved EAL and DDFGTG(YSS) motifs, and its α13 helix was similar homology with the PDE-α6 helix of MorA of *P. aeruginosa*(Fig. 7). In addition to, the point mutation in ZMO1055 EAL domain of cell flocculating strain ZM401 was present in the α13 helix(the residue A526) and putatively associated with EAL domain dimerization(Fig. 8A). These results

suggest that DDFGTG(YSS) motif and α13 helix in ZMO1055 EAL domain are involved in dimer formation and activity of EAL domain, and the single point mutation in ZMO1055^{EAL*} of cell flocculation mutant strain ZM401 is associated with ZMO1055 PDE enzymatic function.

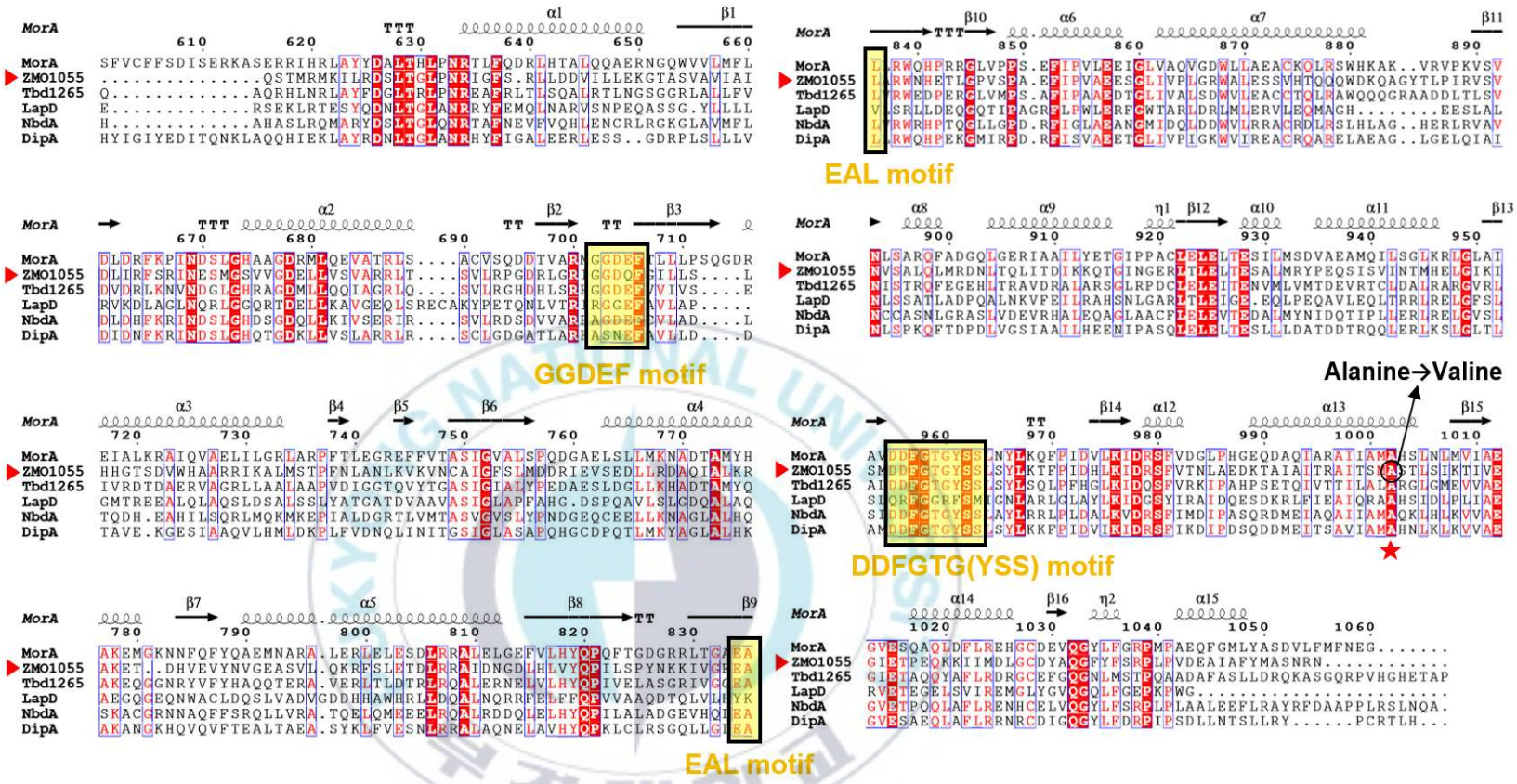


Figure 7. Sequence alignments of GGDEF and EAL domain of ZMO1055. Structure-based amino acid sequence alignment of ZMO1055 and homologous domains from other genes containing GGDEF-EAL domain. The numbering of the residues and secondary structure elements are based on the MorA sequence(locus tag : PA4601) of *P. aeruginosa* referred to RCSB PDB database. This model is based on ZMO1055-template sequence alignment of 39% sequence identity. Strictly conserved residues are highlighted in red. The catalytic motifs of the PDE domain ("EAL" motif and "DDFGTG" motif) are highlighted in yellow boxes. Red star indicates the position of a single point mutation present in ZMO1055 EAL domain of ZM401. Accession numbers are as follows: MorA, WP_073670889.1; Tbd1265, WP_011311777.1; LapD, WP_011331847.1; NBDA, WP_048305406.1 and DipA, NP_253704.4

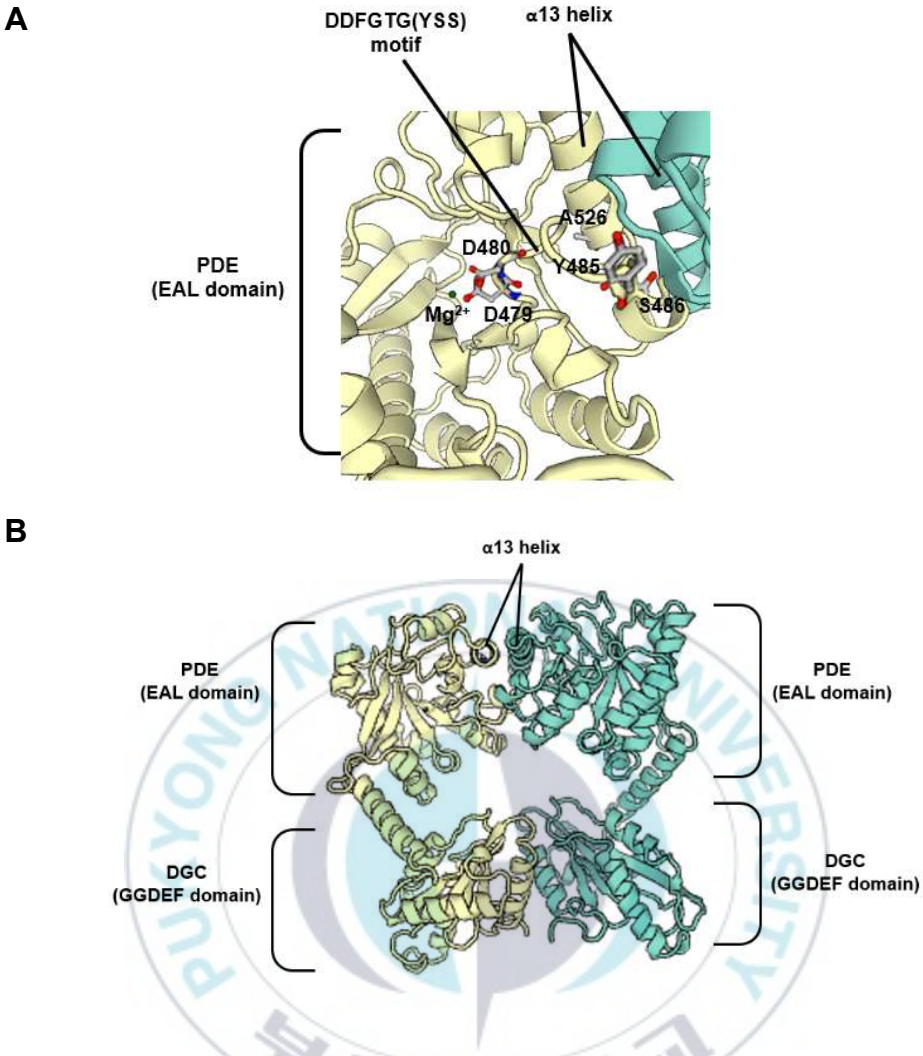


Figure 8. Putative crystal structure of the dimeric form of ZMO1055 GGDEF-EAL domain. The structure model of ZMO1055 of *Z. mobilis* ZM4 is based on the structure of *P. aeruginosa* MorA protein with 39% sequence similarity to the ZMO1055 amino acid sequence and is shown using Protein Model Portal and SWISS-MODEL. (A) The dimeric interface is formed through contact of certain parts of monomer A(yellow) and B(green); DDFGTG(YSS) motif Y485/S486 and α13 helix. The two residues D479 and D480, which are part of the DDFGTG(YSS) motif, acts as an active site that coordinates Mg^{2+} ion. (B) The structure represents a dimeric form of the GGDEF-EAL hybrid domain composed of 2 monomers[29].

4. Discussion

The cell flocculation is one of the useful properties of fermentation microorganisms in the ethanol fermentation industry. The stable cell flocs can be easily separated from the fermentation broth at the end of fermentation and re-used in continuous fermentation. High density cells obtained by flocculation also shorten the fermentation time and provide resistance to high-ethanol and other environmental stress[32]. These advantages, which are useful for the fermentation process, have led to many studies for the development of industrial strains with cell flocculation properties. In previous literature, c-di-GMP is one of the factors associated with cell flocculation and regulates various cellular activities including motility, biofilm formation, toxicity and cell cycle progression depending on its intracellular levels[19]. The c-di-GMP, a second messenger used in signal transduction, is synthesized from two GTP molecules by a diguanylate cyclase containing the GGDEF domain and degraded into pGpG or two GMP by a phosphodiesterase containing the EAL or HD-GYP domain[19]. In previous studies, comparative microarray analysis of ZM401, a flocculent strain isolated by chemical mutagenesis, confirmed that the ZMO1055 gene encoding diguanylate cyclase and phosphodiesterase was up-regulated compared to the wild type ZM4. In addition, it was found that the 526 amino acid position of ZMO1055 EAL domain was converted from amino acid alanine to valine[15]. Our group previously

constructed ZM4*ΔZMO1055*^{EAL} and ZM401*ΔZMO1055*^{EAL*} strains that were inactivated the ZMO1055 EAL domain gene in *Z. mobilis*. These recombinant strains showed higher c-di-GMP levels than the wild type strain ZM4, and similar cell flocculation phenotype to cell flocculent ZM401[18]. Four genes consisting of the GGDEF and/or EAL domains, which function similarly to the ZMO1055 gene were additionally found in the *Z. mobilis* genome through a bioinformatic analysis. Therefore, it was required to understand the difference of 5 genes involved in c-di-GMP turnover in the cell flocculation strain ZM401 compared with parent strain ZM4. Also, it was required to understand the enzymatic characteristics and activity of the ZMO1055 EAL domain protein, which is presumed to play a major role in the cell floc formation of ZM401.

We confirmed by qRT-PCR transcription analysis that these five genes involved in c-di-GMP turnover were 2 to 3 times up-regulated in ZM401 compared with ZM4. Several c-di-GMP interact with numerous c-di-GMP effector proteins or riboswitches, so regulate its target genes related to the cell processes[19]. The c-di-GMP-mediated riboswitches are found in the upstream of genes involved in c-di-GMP metabolism and can promote or inhibit mRNA transcription upon c-di-GMP binding[33]. In addition, other studies reported that c-di-GMP-mediated riboswitches regulate the expression of its downstream genes in the response to changing concentration of c-di-GMP in particular other bacteria such as *Bacillus thuringiensis*[34]. Typically, riboswitches induce mRNA transcription termination

by forming the Rho-independent terminator structure[35]. To determine the functional RNA motifs and sites in five genes involved in the synthesis and degradation of c-di-GMP of *Z. mobilis* ZM4, we searched at the upstream positions of these genes by bioinformatic analysis with RegRNO 2.0 software. The c-di-GMP-mediated riboswitches were not identified, but one or more Rho-independent terminators were identified in the upstream region of these five genes. It is possible to predict that the interaction of c-di-GMP and functional RNA motifs will induce the up-regulation of genes involved in c-di-GMP turnover in cell flocculation mutant strain ZM401.

The cell flocculent ZM401 was experimentally confirmed to maintain higher c-di-GMP levels than parent strain ZM4 and exhibited phenotypically distinct cell flocculation with ZM4, despite the up-regulation of genes including the EAL domain related to c-di-GMP degradation. The point mutation in the ZMO1055 EAL domain of ZM401 was presumed to be one of the major factors in these outcomes. Thus, we performed the analysis of substrate specificity and enzyme activity of the phosphodiesterase encoded by the ZMO1055 EAL domain. The PDE enzyme assay showed that ZMO1055 EAL domain protein of ZM4 showed 1.38 times higher hydrolytic activity against c-di-GMP than that against c-di-AMP. In addition, the total PDEs and purified ZMO1055^{EAL*} (the mutant type) of ZM401 were 0.8- and 0.75-times lower activities than those of wild type strain ZM4. These results indicate that the ZMO1055^{EAL} protein is a c-di-GMP specific-

phosphodiesterase and acts as one of key functional enzymes that regulates the intracellular levels of c-di-GMP in *Z. mobilis*. In addition, it is suggested that the single point mutation in the ZMO1055 EAL domain of ZM401 is associated with a decrease in PDE activity. To determine how the point mutation affects the ZMO1055 PDE enzyme activity, it was necessary to search for the secondary structure of ZMO1055 based on the amino acid sequence.

In the homology search for amino acid sequence of ZMO1055, the *P. aeruginosa* MorA protein showed the highest sequence similarity with ZMO1055. In the EAL domain of MorA, DDFGTG(YSS)motif and EAL-α6 helix involved in EAL domain dimerization affect formation of active site. The dimerization of EAL domain induces formation of c-di-GMP binding site by expanding and stabilizing the helix loop of the DDFGTG(YSS) motif[29]. We performed multiple alignments between GGDEF-EAL hybrid proteins of other strains and ZMO1055. The EAL motif and DDFGTG (YSS) motifs were conserved in the ZMO1055, and the α13 helix in the ZMO1055 EAL domain is similar to the amino acid sequence of the EAL-α6 helix of *P. aeruginosa* MorA. The point mutation in the ZMO1055 EAL domain of cell flocculation mutant strain ZM401 converts one of the amino acids(A526 to V526) constituting α13 helix, which is presumed to be involved in the dimer formation of EAL domain. Other studies reported that the changes in a helix located at the dimeric interface of the EAL domain could be associated with local structural changes in DDFGTG(YSS) motif, which is a functional active

site[36]. Therefore, these results suggest that this point mutation reduced the enzymatic activity of c-di-GMP specific phosphodiesterase of ZMO1055 by affecting the formation of c-di-GMP binding site.

In this study, we determined the characteristics and enzymatic activity of ZMO1055 PDE which induce cell flocculation through regulation of intracellular c-di-GMP levels in *Z. mobilis*. In addition, the differences to gene expression involved in c-di-GMP turn over in flocculation strain ZM401 and parent strain ZM4 was confirmed. Therefore, the present study provides an increased understanding of the mechanisms for bacterial cell flocculation and developing economically efficient strains for bioethanol production in this bacterium.

5. 국문 초록

*Zymomonas mobilis*는 목질계 원료를 활용한 연료 에탄올 생산 공정에 유용한 산업적 발효 균주로 연구되어왔다. 이전 연구를 통해 분리된 세포 응집 특성을 가진 *Z. mobilis* ZM401 균주는 에탄올 생산에 중요한 특성인 에탄올 및 발효 저해 물질에 대한 높은 내성을 가진다고 보고되었다. 이러한 이유로, 세포 응집 균주 ZM401에 대한 세포 응집 관여 인자를 규명하기 위해 transcriptomic microarray 및 genome sequencing 분석이 이루어졌으며 그 결과는 세포 응집 균주 ZM401의 ZMO1055 EAL 도메인에 암호화된 phosphodiesterase(PDE)에 발생한 단일 점 돌연변이(A526V)가 세포 응집의 주요 원인 중 하나임을 암시하였다. PDE는 박테리아 2차 신호 전달 물질인 c-di-GMP의 세포 내 수준을 조절함으로써 세포 응집에 관여하는 주요 단백질로 알려져있다. 따라서, 본 연구에서는 c-di-GMP 합성 및 분해에 관여하는 유전자의 발현 정도를 규명하고 heterologous expression을 통해 획득한 ZMO1055 PDE의 효소 기능 및 해당 단백질의 단일 점 돌연변이(A526V) 영향을 분석하였다. qRT-PCR 분석 결과, 세포 응집 균주 ZM401의 ZMO1055 유전자를 포함한 c-di-GMP 합성 및 분해에 관여하는 5 개의 유전자가 모 균주 ZM4에서 보다 2-3 배 상향 조절됨을 확인하였다. PDE 효소 분석은 세포 응집 돌연변이 균주 ZM401의 세포 내 총 PDEs 및 정제된 ZMO1055 PDE가 모 균주 ZM4의 해당 단백질보다 각각 0.8 및 0.75 배 낮은 가수 분해 활성을 가짐을 확인하였다. 이러한 결과는 세포 응집 균주 ZM401의 ZMO1055 EAL 도메인에 발생한 점 돌연변이(A526V)가 해당 균주의 PDE 활성 감소에 영향을 미침으로써 c-di-GMP의 세포 내 수준이 모 균주 ZM4에서보다 높게 유지되는 것과 관련 있음을 시사하였다. 또한, ZMO1055 EAL 도메인 서열 비교 및 2차 구조 분석으로 ZMO1055 EAL 도메인의 점 돌연변이가 α13 나선의 하나의 아미노산 잔기를 대체하며 해당 아미노산을 포함하는 α13 나선이 PDE 이량체 형성에 관여함을 확인하였다. PDE의 이량체 형성은 EAL 도메인의 c-di-GMP 결합 부위인 DDFGTG(YSS)모티프의 나선 루프의 확장 및 안정화와 관련하여 해당 모티프를 활성 형태로 유도하는 것으로 알려져 있다. 결과적으로, 이 연구는 ZMO1055 PDE가 c-di-GMP의 조절에 관여하는 주요 기능 효소이며, ZMO1055 PDE의 효소적 활성은 이합체 형성을 통한 c-di-GMP 결합 부위의 활성 유도에 의해 결정된다는 것을 제시한다.

6. Acknowledgement

석사 과정을 마무리하며 긴 시간동안 크고 작은 갈림길 앞에서 헤매는 저에게 지혜와 따뜻한 마음을 나눠준 많은 분들께 이 자리를 빌려 감사의 인사를 드리고자 합니다.

먼저 언제나 연구에 대한 아낌없는 지도를 해주시는 전용재 교수님께 깊은 감사를 드립니다. 실수가 잦아도 넓은 마음으로 받아 주시고 연구 방법 뿐만 아니라 연구자의 태도 등 많은 것들을 가르쳐 주신 덕분에 제대로 된 연구 활동을 할 수 있었습니다. 그리고 바쁘신 일정에도 불구하고 부족한 제 논문을 지도해주신 이명숙 교수님과 김군도 교수님께도 감사드립니다. 석사 과정동안 많은 가르침을 주신 송영환 교수님, 김영태 교수님, 최태진 교수님과 김경호 교수님께도 감사드립니다.

실험실 생활하는 동안 많은 도움과 조언 그리고 즐거운 추억을 나눈 실험실 식구들에게도 감사의 마음을 전합니다. 같은 길을 걷고 있는 사람들이 있다는 것이 저에게 얼마나 큰 힘이 되었는지 모릅니다. 긴 시간 함께 해온 동료, 부족한 저를 언니로, 누나로 의지하고 챙겨준 동생들, 타지에서 본인의 목표를 향해 열심히 노력하고 있는 언니와 동기들, 다들 고맙고 항상 응원합니다.

그리고, 10년 가까이 함께 한 나를 가장 잘 아는 내 소중한 친구들, 사회로 첫 걸음을 하며 자주 만나지 못하지만 전화로 나누는 소소한 얘기들이 여전히 즐겁고 내가 힘들어 할 때면 찾아와 나를 다독이고 웃게 해줘서 항상 고마워.

마지막으로, 언제나 아낌없는 사랑과 성원을 보내주시며 저를 삶의 가장 큰 기쁨으로 여겨 주시는 부모님께 사랑을 가득 전합니다. 누나의 든든한 버팀목이 되어주는 하나뿐인 동생에게도 사랑을 전합니다.

저에게 대학원 생활은 실험이 계속해서 잘 안될 때는 그렇게 힘들 수가 없다 가도 또 좋은 결과가 나오면 그렇게도 기쁠 수가 없었고, 미래와 진로에 대한 고민으로 가득했지만 결과적으로 가장 많은 것을 배운 시기였습니다. 졸업을 시작으로 앞으로 더 나아가기 위해 아낌없이 노력하겠습니다. 모두 감사드립니다.

7. References

1 Othman, M. F., Adam, A., Najafi, G. & Mamat, R. (2017) Green fuel as alternative fuel for diesel engine: A review. Renewable and Sustainable Energy Reviews 80:694-709

2 Lynd, L. R. & Wang, M. Q. (2003) A Product-Nonspecific Framework for Evaluating the Potential of Biomass-Based Products to Displace Fossil Fuels. Journal of Industrial Ecology 7:17-32

3 Sarkar, N., Ghosh, S. K., Bannerjee, S. & Aikat, K. (2012) Bioethanol production from agricultural wastes: An overview. Renewable Energy 37:19-27

4 Saladini, F., Patrizi, N., Pulselli, F. M., Marchettini, N. & Bastianoni, S. (2016) Guidelines for emergy evaluation of first, second and third generation biofuels. Renewable and Sustainable Energy Reviews 66:221-227

5 Binod, P., Gnansounou, E., Sindhu, R. & Pandey, A. (2018) Enzymes for second generation biofuels: Recent developments and future perspectives. Bioresource Technology Reports

6 Ullah, K., Sharma, V. K., Ahmad, M., Lv, P., Krahl, J., Wang, Z. & Sofia (2018) The insight views of advanced technologies and its application in bio-origin fuel synthesis from lignocellulose biomasses waste, a review. Renewable and Sustainable Energy Reviews 82:3992-4008

7 Mohagheghi, A., Dowe, N., Schell, D., Chou, Y. C., Eddy, C. & Zhang, M. (2004) Performance of a newly developed integrant of *Zymomonas mobilis* for ethanol production on corn stover hydrolysate. Biotechnol Lett 26:321-325

8 Joachimsthal, E., Haggett, K. D., Jang, J.-H. & Rogers, P. L. (1998) A mutant of *Zymomonas mobilis* ZM4 capable of ethanol production from glucose in the presence of high acetate concentrations. Biotechnology Letters 20:137-142

9 Pampulha, M. E. & Loureiro, V. (1989) Interaction of the effects of acetic acid and ethanol on inhibition of fermentation in *Saccharomyces cerevisiae*. Biotechnology Letters 11:269-274

10 Rogers, P. L., Lee, K. J., Skotnicki, M. L. & Tribe, D. E. (1982) Ethanol production by *Zymomonas mobilis.* Springer Berlin Heidelberg :37-84

11 Swings, J. & De Ley, J. (1977) The biology of *Zymomonas*. Bacteriological Reviews 41:1-46

12 Mohagheghi, A., Evans, K., Chou, Y.-C. & Zhang, M. (2002) Cofermentation of glucose, xylose, and arabinose by genomic DNA-integrated xylose/arabinose fermenting strain of *Zymomonas mobilis* AX101. Applied Biochemistry and Biotechnology 98:885-898

13 Kim, I. S., Barrow, K. D. & Rogers, P. L. (2000) Kinetic and nuclear magnetic resonance studies of xylose metabolism by recombinant *Zymomonas mobilis* ZM4(pZB5). Appl Environ Microbiol 66:186-193

14 Lee, J. H., Skotnicki, M. L. & Rogers, P. L. (1982) Kinetic studies on a flocculent strain of *Zymomonas mobilis*. Biotechnology Letters 4:615-620

15 Jeon, Y. J., Xun, Z., Su, P. & Rogers, P. L. (2012) Genome-wide transcriptomic analysis of a flocculent strain of *Zymomonas mobilis*. Appl Microbiol Biotechnol 93:2513-2518

16 Nikolić, S., Mojović, L., Pejin, D., Rakin, M. & Vukašinović, M. (2010) Production of bioethanol from corn meal hydrolyzates by free and immobilized cells of *Saccharomyces cerevisiae var. ellipsoideus*. Biomass and Bioenergy 34:1449-1456

17 Kourkoutas, Y., Bekatorou, A., Banat, I. M., Marchant, R. & Koutinas, A. A. (2004) Immobilization technologies and support materials suitable in alcohol beverages production: a review. Food Microbiology 21:377-397

18 정원겸 (2018) 신호 전달 물질 cyclic-di-GMP 매개에 의한 *Zymomonas mobilis*의 세포 자가 응집 유도. 부경대학교 국내석사학위논문

19 Jenal, U., Reinders, A. & Lori, C. (2017) Cyclic di-GMP: second messenger extraordinaire. Nature Reviews Microbiology 15:271

20 Goodman, A. E., Rogers, P. L. & Skotnicki, M. L. (1982) Minimal medium for isolation of auxotrophic *Zymomonas* mutants. Appl Environ Microbiol 44:496-498

21 Luria, S. E. & Delbruck, M. (1943) Mutations of Bacteria from Virus Sensitivity to Virus Resistance. Genetics 28:491-511

22 Sanger, F., Nicklen, S. & Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America 74:5463-5467

23 Winer, J., Jung, C. K., Shackel, I. & Williams, P. M. (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 270:41-49

24 Letunic, I. & Bork, P. (2018) 20 years of the SMART protein domain annotation resource. Nucleic acids research 46:D493-D496

25 Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E. L. L., Tate, J. & Punta, M. (2014) Pfam: the protein families database. Nucleic acids research 42:D222-D230

26 Rose, P. W., Prlic, A., Altunkaya, A., Bi, C., Bradley, A. R., Christie, C. H., Costanzo, L. D., Duarte, J. M., Dutta, S., Feng, Z., Green, R. K., Goodsell, D. S., Hudson, B., Kalro, T., Lowe, R., Peisach, E., Randle, C., Rose, A. S., Shao, C., Tao, Y. P., Valasatava, Y., Voigt, M., Westbrook, J. D., Woo, J., Yang, H., Young, J. Y., Zardecki, C., Berman, H. M. & Burley, S. K. (2017) The RCSB protein data bank: integrative view of protein, gene and 3D structural information. Nucleic acids research 45:D271-D281

27 Lowe, E. C., Baslé, A., Czjzek, M., Firbank, S. J. & Bolam, D. N. (2012) A scissor blade-like closing mechanism implicated in transmembrane signaling in a Bacteroides hybrid two-component system. Proceedings of the National Academy of Sciences of the United States of America 109:7298-7303

28 Bellini, D., Horrell, S., Hutchin, A., Phippen, C. W., Strange, R. W., Cai, Y., Wagner, A., Webb, J. S., Tews, I. & Walsh, M. A. (2017) Dimerisation induced

formation of the active site and the identification of three metal sites in EAL-phosphodiesterases. Scientific reports 7:42166-42166

29 Phippen, C. W., Mikolajek, H., Schlaefli, H. G., Keevil, C. W., Webb, J. S. & Tews, I. (2014) Formation and dimerization of the phosphodiesterase active site of the *Pseudomonas aeruginosa* MorA, a bi-functional c-di-GMP regulator. FEBS letters 588:4631-4636

30 Barends, T. R., Hartmann, E., Griese, J. J., Beitlich, T., Kirienko, N. V., Ryjenkov, D. A., Reinstein, J., Shoeman, R. L., Gomelsky, M. & Schlichting, I. (2009) Structure and mechanism of a bacterial light-regulated cyclic nucleotide phosphodiesterase. Nature 459:1015-1018

31 Chan, C., Paul, R., Samoray, D., Amiot, N. C., Giese, B., Jenal, U. & Schirmer, T. (2004) Structural basis of activity and allosteric control of diguanylate cyclase. Proceedings of the National Academy of Sciences of the United States of America 101:17084-17089

32 Li, F., Zhao, X. Q., Ge, X. M. & Bai, F. W. (2009) An innovative consecutive batch fermentation process for very high gravity ethanol fermentation with self-flocculating yeast. Appl Microbiol Biotechnol 84:1079-1086

33 Sudarsan, N., Lee, E. R., Weinberg, Z., Moy, R. H., Kim, J. N., Link, K. H. & Breaker, R. R. (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. Science (New York, N.Y.) 321:411-413

34 Tang, Q., Yin, K., Qian, H., Zhao, Y., Wang, W., Chou, S.-H., Fu, Y. & He, J. (2016) Cyclic di-GMP contributes to adaption and virulence of *Bacillus thuringiensis* through a riboswitch-regulated collagen adhesion protein. Scientific Reports 6:28807

35 Bordeleau, E., Purcell, E. B., Lafontaine, D. A., Fortier, L.-C., Tamayo, R. & Burrus, V. (2015) Cyclic di-GMP riboswitch-regulated type IV pili contribute to aggregation of *Clostridium difficile*. Journal of bacteriology 197:819-832

36 Rao, F., Qi, Y., Chong, H. S., Kotaka, M., Li, B., Li, J., Lescar, J., Tang, K. & Liang, Z.-X. (2009) The functional role of a conserved loop in EAL domain-based cyclic di-GMP-specific phosphodiesterase. Journal of bacteriology 191:4722-4731

37 Zhao, N., Bai, Y., Zhao, X.-Q., Yang, Z.-Y. & Bai, F.-W. (2012) Draft genome sequence of the flocculating *Zymomonas mobilis* strain ZM401 (ATCC 31822). Journal of bacteriology 194:7008-7009