STUDIES ON MECHANISM TO COPE WITH HARSH ENVIRONMENTS IN FILAMENTOUS CYANOBACTERIA

DISSERTATION

STUDIES ON MECHANISM TO COPE WITH HARSH ENVIRONMENTS IN FILAMENTOUS CYANOBACTERIA

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Summary

In this study, microscopic analysis of cyanobacterium *Nostoc* sp. Strain SO-36 which found from Antarctic revealed that morphology of the cells grown at 30°C was filamentous and differentiated heterocysts, the specialized cells for fixation of gaseous nitrogen, under nitrogen deprived conditions, indicating that the strain can grow diazotrophically. The cells grown at 10°C have smaller cell size and shortened filament length with decreased chlorophyll content per cell. At 10°C, cells also got aggregated with extracellular polysaccharides (EPS), which is a common mechanism to protect cells from UV. These results imply that the segmentation to short filaments was induced by photodamage at low temperature. To understand the adaptation mechanisms for low temperature in *Nostoc* sp. strain SO-36 more in detail, the author conducted next generation sequence analyses. The complete genome sequence of Antarctic cyanobacteria revealed that the strain has one main chromosome approximately 6.8 Mbp with 4 plasmids, including 6855 coding sequences (CDSs), 48 tRNA genes, 4 copies of rRNA operons and 5 CRISPR regions. Putative genes for EPS biosynthesis were found to conserved in *Nostocaceae* regardless of their habitat. Data provided in this study will be a basic information to understand the adaptation mechanisms to low temperature and the strain will be a model organism to analyzed adaptation to extreme environments. In most environmental stresses, signal transduction pathways are triggered by changes of a membrane physical state which can rigidify or fluidize under stress, for example changes in temperature or salinity. The membrane lipids could adapt to the stress due to the change its fatty acids composition inside the cell. Under conditions of low temperature, unsaturated fatty acids accumulated and the chain length of fatty acids in the glycerol moiety also decreased. The process that introduces a double bond between two carbons in the fatty acid chain was namely desaturation and the enzyme that took a role is *desaturase*. In cyanobacterial membrane lipids, C18 fatty acids are the most abundant at the *sn*-1 position and C16 at the *sn*-2 position of the glycerol backbone. In model unicellular cyanobacteria, *Synechocystis* 6803, contains four acyl lipid desaturases, DesA, DesB, DesC, and DesD, which catalyze the desaturation at the Δ12, Δ15, Δ9, and Δ6 positions of C18 fatty acyl chains in membrane lipids, respectively. DesC is the initial desaturase that introduces a double bond at Δ9 positions of both C16 and C18 fatty acids. It has been reported that *Nostoc* sp strain SO-36 has two DesC enzymes (DesC1 and DesC2). The model filamentous cyanobacterium *Anabaena* sp. PCC 7120 also has two *desC* genes (*all1599* and *all4991*) whose functions are still unknown. In this study the author first expressed the *desC* genes from *Nostoc* sp. strain SO-36 and *Anabaena* sp. PCC 7120 in a model unicellular cyanobacterium *Synechocystis* sp. PCC 6803 that only have sole *desC* gene. No obvious phenotype was observed under the optimum growth condition and the fatty acid composition of transformants shows that DesC1 accumulated C18:1 (9) at *sn*-1 position in all membrane lipid and DesC2 accumulated C16:1 (9) at *sn*-1 position in DGDG and PG, and in both *sn*- position in MGDG. Next, knocked out

the *Synechocystis desC* gene using the transformants described above. The transformants with *desC*1 gene was completely segregated but not with *desC*2 gene. The unsaturated fatty acids C16:1 (9) and C18:1 (9) of the mutant was lower compared to the wildtype. This result indicated that *desC2* is not enough to complement the original *desC* in *Synechocystis* sp. PCC 6803. On the other hand, *desC*1 that share more similarity in amino acid sequence with *desC* of *Synechocystis* sp. PCC 6803. Knock out mutant of *desC*1 and *desC*2 genes in *Anabaena* sp. PCC 7120 could not be isolated, suggesting that both *desC*1 and *desC*2 genes is essential in *Anabaena* sp. PCC 7120 and perhaps in filamentous cyanobacterium.

List of tables

Chapter II.....19
Table 2.1. 16S rRNA gene list with accession numbers used in phylogenetic tree 27
Table 2.2. General feature of the Nostoc sp. SO-36..... 33
Table 2.3. Copy number of genes involved in the polymerization chain length control and export of EPS in *Nostoc* sp. SO-36 and related species..... 38
Chapter III.....53
Table 3.1. List of plasmid and vector used in this study 62
Table 3.2. List of primers used in this study..... 69

List of figures

Chapter 1.....1
Figure 1.1. The mechanisms of bacterial community for cold adaptation..... 2
Figure 1.2. Blight field microscopy of *Synechocystis* and *Anabaena* 8
Chapter II.....19
Figure 2.1. Growth curve of *Nostoc* SO-36 and *Anabaena* 7120..... 29
Figure 2.2. Phenotypes of *Nostoc* SO-36 and *Anabaena* 7120 at low temperature 30
Figure 2.3. Genomic structure of chromosome and four plasmids..... 39
Figure 2.4. Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequences of *Nostoc* SO-36 and related species of Nostocacean cyanobacteria..... 40
Figure 2.5. Dot-plot matrix comparisons of the chromosomes of *Nostoc* SO-36 with closely related strains 41

Chapter III.....53
Figure 3.1. Genotype analysis of wild-type and transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120) 77
Figure 3.2. Genotype analysis of wild-type and mutants(Δ*desC/desC1*-7120/N36)..... 78
Figure 3.3. Physiological analysis of transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120) and mutants (Δ*desC/desC1*-7120/N36)..... 79
Figure 3.4. Lipid profile and fatty acid composition of the wild-type, (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120), and (Δ*desC/desC1*-7120/N36) 80
Figure 3.5. Positional distribution of wild-type, (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120), and (Δ*desC/desC1*-7120/N36)..... 81
Figure 3.6. Genotype analysis of the wild-type and candidates of 7120Δ*desC1* and Δ*desC2* 83
Figure 3.7. Genotype analysis of the wild-type and transformants 7120/*desC1/2-SO-36* 84
Figure 3.8. Genotype analysis of the wild-type and candidates of mutant Δ*desC1/2* -N36 85

Contents

Summary i

List of tables iii

List of figures iv

Chapter I 1

1.1 Bacterial adaptation to harsh environment 1

1.2 Cyanobacteria 5

1.3 Morphology of the cyanobacteria 6

1.4 Cyanobacteria under environmental stress 7

1.5 Synthesis of extracellular polysaccharide in cyanobacteria to protect the cells from environmental stresses. 9

1.6 Environmental stress could change the unsaturation of membrane lipids in cyanobacteria 10

1.7 Research objectives 12

1.8 Reference 14

Chapter II 19

2.1 Introduction 19

2.2 Materials and Methods 22

2.2.1. Cyanobacterial strains and growth conditions 22

2.2.2. Measurements of growth curve and pigment contents 22

2.2.3. Alcian blue staining and measurement of sugar content 22

2.2.4. Isolation of genomic DNA from *Nostoc* SO-36 23

2.2.5. Whole-genome sequencing strategy 24

2.2.6. Phylogenetic analysis 25

2.2.7. Data availability 25

2.3 Results 28

2.4 Discussion 42

2.4.1 Phylogenetic position of *Nostoc* SO-36 42

2.4.2 Genes for EPS biosynthesis and export pathways in *Nostoc* SO-36 43

2.4.3 Putative factors for psychrotolerant phenotype of Antarctic cyanobacteria 44

2.5 References 46
Chapter III 53
3.1 Introduction 53
3.2 Materials and method 58
3.2.1 Growth conditions of cyanobacteria strains 58
3.2.2 Expression of *Nostoc* and *Anabaena desC* genes and knock-out the original *desC gene* in *Synechocystis* 58
3.2.3 Isolation of knock out mutants of *desC* genes in *Anabaena* 60
3.2.4 Growth curve, chlorophyll content, oxygen evolution rate, and chlorophyll fluorescence measurement of *Synechocystis* transformants 63
3.2.5 Lipid and fatty acid composition analysis 63
3.3 Results 71
3.3.1 Complementation of Δ*desC/6803* with *desC1* and *desC2* from filamentous cyanobacteria 71
3.3.2 Physiological function of *desC1* and *desC2* 82
3.4 Discussion 86
3.4.1 DesC1 from filamentous cyanobacteria can complement DesC in *Synechocystis* 6803 86
3.4.2 Both DesC in filamentous cyanobacteria is essential 89
3.5 References 90
Chapter IV 96
General Discussion 96
4.1 Mechanism of psychrotolerant filamentous cyanobacteria upon environmental stress 96
4.2 Desaturation is essential to produce the unsaturated fatty acids and its possibility to cope in harsh environment 97
4.3 Reference 101

Abbreviations

Anabaena 7120	*Anabaena* sp. PCC 7120
bp	Base pair
BG-11	Blue green-11
C1	*Anabaena desC1* overexpressor
C2	*Anabaena desC2* overexpressor
desC	*Δ9 desaturase* gene
DesA	*Δ12 desaturase* protein
DesB	*Δ6 desaturase* protein
DesC1	*desC1* protein
DesC2	*desC2* protein
DesD	*Δ15 desaturase* protein
DGDG	Digalactosyldiacylglycerol
DNA	Deoxyribonucleic acid
E.coli	Eschericia coli
EPS	Exocellular polysaccharide
Em^r	Erythromycin resistance gene cassette
FA	Fatty acids
Fv/Fm	Maximum quantum yield of PSII at dark conditions
Fv'/Fm'	Maximum quantum yield of PSII at given light conditions

GC	Gas chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
Km^r	Kanamycin resistance gene cassette
MGDG	Monogalactosyldiacylglycerol
NC	Negative control
Neo^r	Neomycin resistance gene cassette
N-36	*Nostoc* sp. strain SO-36
PAM	Pulse-amplitude modulation
PCC	Pasteur culture collection
PCR	Polymerase chain reaction
PG	Phosphatidylglycerol
SD	Standard deviation
SO-36	*Nostoc* sp. strain SO-36
Spe^r	Spectinomycin resistance gene cassette
SQDG	Sulfoquinovosyldiacylglycerol
Synechocystis	*Synechocystis* sp. PCC 6803
WT	Wildtype
Δ	Deletion or position number of double bond (desaturation)

Chapter I

General introduction

1.1 Bacterial adaptation to harsh environment

Harsh environment described of term for the low chance to survive for some organism or even impossible to live with. In this study, the author uses this term to study the micro-organism which the origin was live in Antarctic, due to its cold environment. The ability of the micro-organism to live under cold environment is dependent on some strategies to survive from the stress factors which associated with cold environments, such as excessive UV, low or high pH, high osmotic pressure and low nutrient availability (De Maayer et al., 2014). The bacterial adaptation to harsh environment (in this study is cold adaptation) can be seen in **Figure 1.1.** The organism that could survive in cold environments have been subdivided into psychrophiles, which can grow optimally at less than 15^{o}C (up to 20^{o}C) and psychrotolerant organisms, which can survive at temperatures below 0^{o}C and can grow optimally at room temperature (Morita, 1975). Psychrophiles is predominating in marine ecosystem, where the oceanic waters are permanently cold (<5^{o}C), whereas psychrotolerant micro-organisms usually isolated in terrestrial environment considered as cold-adapted micro-organisms that can survive to extreme temperature fluctuations. Common mechanisms that took a role in cold adaptation have been described through maintenance of membrane fluidity, expression of cold shock proteins, presence of compatible solutes, oxidative metabolism, and structural adaptation of the enzymes and

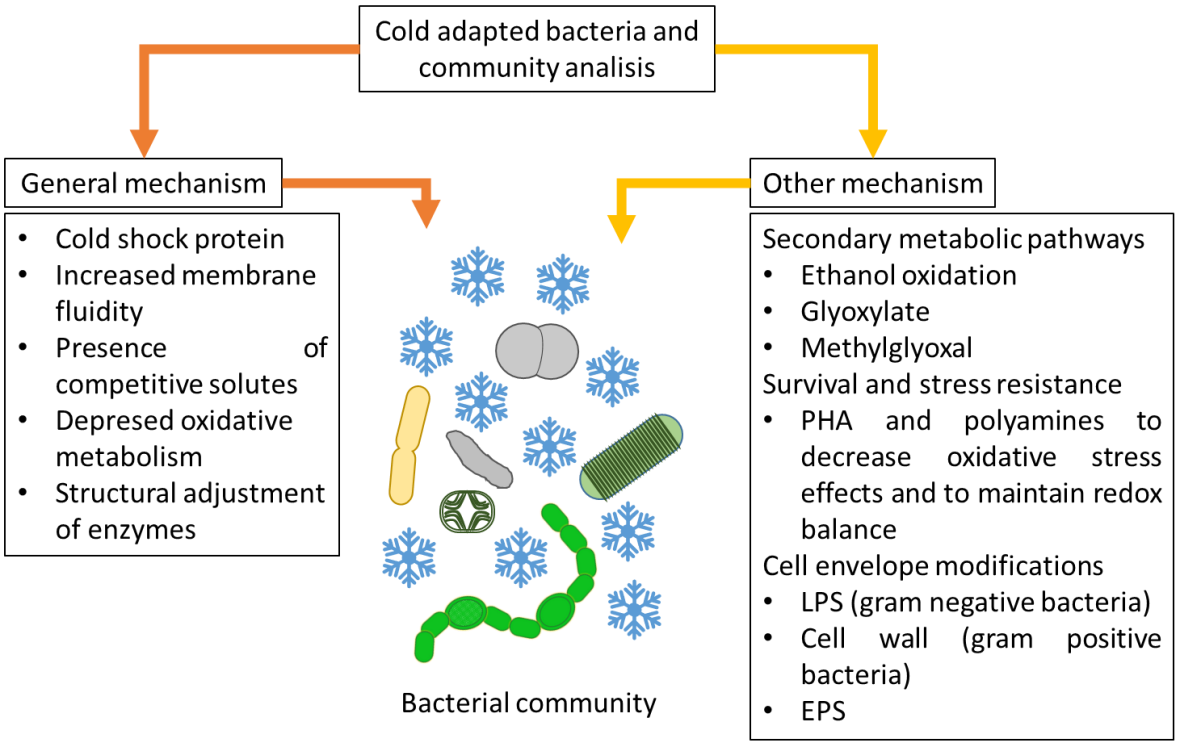

Figure 1.1 The mechanisms of bacterial community for cold adaptation. (Tribelli and Lopez, 2018)

adaptation of transcription and translation machinery (Barria et al., 2013). Cold shock protein has an important role in cold response environment. Cold-shock proteins took a main activity in the cold-response environment and consist of small nucleic acid-binding proteins around 65–76 amino acids (Graumann and Marahiel, 1996; Czapski and Trun, 2014) which are characterized by psychrophilic, mesophilic, thermophilic, and even hyperthermophilic bacteria (Phadtare and Inouye, 2004). Cold-shock response were aims to help its cells to overcome changes in cell membrane fluidity. This may affect protein secretion and active transport due to temperature reduction. In addition, stabilizing changes in the structure of RNA and DNA can reduce the efficiency of transcription and translation leading to protein folding and become inefficient, the ribosomes must adapt to cold temperatures before they can function properly (Phadtare and Severinov, 2010; Keto-Timonen et al., 2016).

Another mechanisms that active due to cold adaptation have been described through secondary metabolic pathways, survival and stress tolerance, and cell envelope alterations. Oxidative stress also occurred at low temperatures due to increased oxygen solubility, which affects the accumulation of ROS (reactive oxygen species). Therefore, oxidative metabolism such as tricarboxylic acid cycle (TCA), pentose phosphate pathway, glycolysis, and electron transport chain are generally suppressed at low temperatures (Piette et al., 2012). Overall, the TCA cycle and glycolysis appear to be inhibited during low temperature growth, whereas the production of intermediates by other secondary

metabolic pathways or alternative pathways bypassing the full metabolic pathway is critical for energy production. (Tribelli and Lopez, 2018).

Some bacteria can adapt to cold environments by synthesize polyhydroxyalkanoates (PHAs) which took a role in physiological changes. These polymers accumulate under stress conditions such as: excessive carbon source such as phosphorus and nitrogen, acting as a dynamic store of carbon and reducing equivalents (Lopez et al., 2015). PHAs enhance the resistance and survival of bacteria to various environmental stresses while also being ecologically relevant (Lopez et al., 1995; Handrick et al., 2000; Kadouri et al., 2003). Several studies reported that antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase, etc.) are induced under cold conditions (Lopez et al., 2015). Some of these enzymes rely on nicotinamide dinucleotides as cofactors (Cabiscol et al., 2000), providing an explanation for the PHA mechanism that regulates oxidative stress due to cold exposure. In *Pseudomonas* and *Janthinobacterium* which isolated from Antarctic soil showed higher polymer accumulation between 5°C and 20°C compared to higher temperatures. The presence of a medium-chain highly unsaturated PHA in Pseudomonas sp. UMAB-40, can survive in cold stress environment (Goh and Tan, 2012).

The Envelope structure and composition are important for cold adaptation as they provide a dynamic interface between cells and the environment, allowing them to survive environmental challenges (Tribelli and Lopez, 2018). Cold-adapted microorganisms can survive in cold environment by altered the lipid composition of the cell membrane in favor of shorter chains and altered the lipid saturation then one can maintain the membrane

fluidity while other can maintain the membrane rigidity at low temperatures. (De Maayer et al., 2014).

The importance of these components has also been recognized in Gram-positive bacteria, whose envelope consists of a cell wall and an inner membrane. Cell wall thickening can protect cells from ice formation and/or destruction by osmotic pressure generated at subzero temperatures (Rodrigues et al., 2008). In Gram-negative bacteria, the envelope consists of an inner and outer membrane separated by a thin layer peptidoglycan which formed a periplasmic space. The outer membrane is composed of phospholipids, proteins, and lipopolysaccharide (LPS). LPS contains an external O-polysaccharide, an intermediate oligosaccharide component (core), and membrane-anchored lipid A. Extracellular polysaccharides (EPS), which form a key part of the extracellular polymers that surround bacterial cells, are also thought to be key cellular functions for coping with cold environment (Tribelli and Lopez, 2018). Gram-negative cyanobacteria, have great potential to survive in harsh climatic conditions such as the Antarctic and Arctic by fixated the carbon and nitrogen. (Parnanen et al., 2015).

1.2 Cyanobacteria

Modern cyanobacteria constitute a well-diversified and ancient bacterial phylum with a unique complex cellular differentiation and morphologies. Cyanobacteria took a role in food webs as primary producers by performing oxygenic photosynthesis. Cyanobacteria are photoautotrophic prokaryotes that form oxygen-evolving photosynthesis with water as

the primary electron donor. Cyanobacteria have very simple nutrient requirements e.g., light, water, carbon dioxide, and inorganic salts that allow these organisms to occupy highly diverse ecological niches. Cyanobacteria are found in almost all terrestrial freshwater, and marine habitats. Some cyanobacteria form symbiotic associations with hosts, such as fungi (lichens), bryophytes, cycads, mosses, fern (*Azolla*), and one angiosperm *Gunnera* (Rai et al., 2000; Arima et al., 2012). Due to similarity with chloroplast it is generally accepted that cyanobacteria and plant chloroplast share a common ancestor (Awai, 2016). Cyanobacteria also played a major role in early biogeochemical fluxes and in Life and Earth evolution. Cyanobacteria are the only prokaryotic organisms that perform oxygenic photosynthesis and are thus generally held responsible for the rise of oxygen in the atmosphere and oceans around 2.4 Ga (billion years ago), during the so-called Great Oxidation Event (GOE), facilitated by geological processes (Bekker et al., 2004; Demoulin et al., 2019).

1.3 Morphology of the cyanobacteria

Cyanobacteria are described as algae and have been called "cyanophyta" or "cyanobacteria". In the late 1970s, Stanier and his colleagues found the prokaryotic nature of cyanobacteria and proposed to follow the international nomenclature of bacteria (Stanier et al., 1978). According to Bergey's Manual of Systematic Bacteriology and the approach of Stanier and Rippka et al. (1979) Concepts (Rippka et al., 1979) classified the cyanobacteria into two he groups. Five subdivisions based on morphological criteria corresponding to the five previous orders of cyanobacteria: *Chroococcales* and *Pleurocapsales*, which divide by binary fission in one or more plans and produce unicellular

cells arranged singly or in colonies. After division by multiple fissures, *Pleurocapsales* can generate "baocytes" (small easily dispersed cells). Among multicellular filamentous cyanobacteria, *Oscillatoriales* have only filamentally arranged vegetative cells, whereas *Nostocales* and *Stigonemateles* are able to generate specialized cells called heterocytes, allowing her N_2 fixation in anoxic compartments. Alternatively, they may represent environmental stress-tolerant cells called "Acinetes" (Demoulin et al., 2019).

1.4 Cyanobacteria under environmental stress

By colonizing soils (which include deserts) and aquatic ecosystems (fresh and marine waters), cyanobacteria are unavoidably uncovered to a couple of stresses inclusive of sun ultraviolet radiations and versions in mild depth and quality, salinity, inorganic-nutrient availabilities, drought, temperatures (excessive and low), pH (acidic and primary), and pollutants (heavy metals and herbicides). Consequently, it isn't always sudden that cyanobacteria have advanced as extensively various organisms, which can be of excessive hobby for primary and carried out research. Thus, cyanobacteria are desirable version organisms to look at the effect of environmental situations at the physiology, metabolism, and morphology of microbial cells. Furthermore, cyanobacteria synthesize a huge type of bioactive metabolites, lots of which can be an interest to human health (Dittmann et al., 2015; Cassier-Chauvat et al., 2017; Demay et al., 2019; Pereira et al., 2019), and cyanobacteria appeared as promising molecular factories to provide chemicals (fuels and biodegradable bioplastics) from resources such as: sun energy, water, CO_2, and minerals (Cassier-Chauvat et al., 2021).

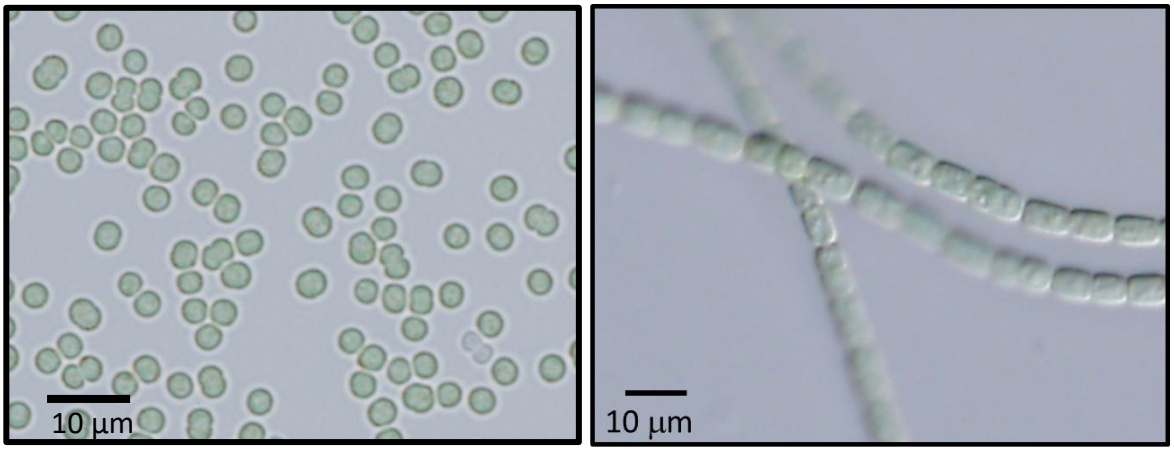

Figure 1-2. Model of (**A**) unicellular *Synechocystis* sp. PCC 6803 and (**B**) filamentous cyanobacteria *Anabaena* sp. PCC 7120.

1.5 Synthesis of extracellular polysaccharide in cyanobacteria to protect the cells from environmental stresses.

Among bacteria, cyanobacteria end up great applicants to provide polysaccharide polymers considering the fact that produce extracellular polymeric substances (EPS), especially composed of heteropolysaccharides, with a specific set of industrially suitable functions including (i) robust anionic nature, (ii) presence of sulfate groups, (iii) excessive sort of feasible structural conformations, and (iv) amphiphilic behavior (Pereira et al., 2009). Using cyanobacteria and microalgae to produce extracellular polymeric substances (EPS) molecules with a top-notch ecological importance for the manufacturing organisms, serving in a big selection of organic approaches and growing the organism`s tolerance to environmental stresses. In addition, because of their specific chemical, and rheological properties, and their organic activity, those macromolecules may want to discover software in industrial, pharmaceutical, and scientific fields (Rossi and De Philippis, 2016). The use of cyanobacteria as molecular factories gets rid of the want for carbon feedstocks considering the fact that their photoautotrophic metabolism lets in a low-price manufacturing even as contributing to carbon dioxide sequestration. Hence, a deeper understanding of the EPS biosynthetic pathways of cyanobacteria is needed, to both enhance the engineer compositional and structural and productivity (Pereira et al., 2019).

In this study, microscopic analysis of cyanobacterium *Nostoc* sp. Strain SO-36 which found from Antarctic revealed that morphology of the cells grown at 30^{o}C was filamentous and differentiated heterocysts, the specialized cells for fixation of gaseous nitrogen, under

nitrogen deprived conditions, indicating that the strain can grow diazotrophically. The cells grown at 10^{o}C have smaller cell size and shortened filament length with decreased chlorophyll content per cell. At 10^{o}C, cells also got aggregated with extracellular polysaccharides (EPS), which is a common mechanism to protect cells from UV. These results imply that the segmentation to short filaments was induced by photodamage at low temperature. To understand the adaptation mechanisms for low temperature in *Nostoc* sp. strain SO-36 more in detail, the author conducted next generation sequence analyses. The complete genome sequence of Antarctic cyanobacteria revealed that the strain has one main chromosome approximately 6.8 Mbp with 4 plasmids, including 6855 coding sequences (CDSs), 48 tRNA genes, 4 copies of rRNA operons and 5 CRISPR regions. Putative genes for EPS biosynthesis were found to conserved in *Nostocaceae* regardless of their habitat. Data provided in this study will be a basic information to understand the adaptation mechanisms to low temperature and the strain will be a model organism to analyzed adaptation to extreme environments.

1.6 Environmental stress could change the unsaturation of membrane lipids in cyanobacteria

Cyanobacterial molecular mechanisms responsible for cellular responses to stresses such as intense light, temperature, osmotic pressure, and oxidative stress involve a two-component signaling system consisting of a group of transmembrane receptors (histidine kinase, Hik33) and response regulators. Its action leads to the induction or repression of specific genes. At most stresses, signaling pathways are triggered by adaptation to change

the physical state of the membrane, which can fluidize or rigidify under stress such as temperature changes (Kanesaki et al., 2007; Mironov et al., 2018). Cold-dependent phosphorylation of Hik33 leads to expression of multiple genes, including fatty acid desaturases (FADs). FAD participate in unsaturated fatty acid (FA) synthesis, which, when included in membrane lipids, increase the overall membrane fluidity. The enhanced action of FADs is one of the cellular responses to a stress with a rigidifying component such as low temperature (Murata and Los, 2006).

In model unicellular cyanobacteria, *Synechocystis* 6803, 4 acyl lipid desaturases were discovered as, DesC, DesA, DesB, and DesD, which catalyze the desaturation at the Δ9, Δ12, Δ15, and Δ6 positions of C18 fatty acyl chains in membrane lipids, respectively (Murata and Wada, 1995; Chintalapati et al., 2006). Desaturation is the process that introduces a double bond between two carbons in the fatty acid chain, that causes the change of saturated fatty acids into unsaturated fatty acids. Environmental factors especially temperature shifts are the main causes of desaturation in cyanobacteria. Under low temperature conditions, unsaturated fatty acids increase and the chain length of fatty acids in the diacyl glycerol moiety decreases. DesC is the initial desaturase that introduces a double bond at Δ9 positions of both C16 and C18 fatty acids. It has been reported that *Nostoc* sp. strain SO-36 has two DesC enzymes (DesC1 and DesC2). DesC1 was found to desaturate stearic acid to oleic acid and DesC2 desaturate palmitic acid to palmitoleic acid. This DesC1 activity has also been experimentally confirmed in *Anabaena variabilis*, *Synechocystis* sp. PCC 6803, and a thermophilic cyanobacterium *Thermosynechococcus*

vulcanus. The model filamentous cyanobacterium *Anabaena* sp. PCC 7120 also has two *desC* genes (*all1599* and *all4991*) whose functions are still unknown. In this study the author first expressed the *desC* genes from *Nostoc* sp. strain SO-36 and *Anabaena* sp. PCC 7120 in a model unicellular cyanobacterium *Synechocystis* sp. PCC 6803 that only have sole *desC* gene. No obvious phenotype was observed under the optimum growth condition and the fatty acid composition of transformants shows that DesC1 accumulated C18:1 (9) at *sn*-1 position in all membrane lipid and DesC2 accumulated C16:1 (9) at *sn*-1 position in DGDG and PG, and in both *sn*- position in MGDG. Next, knocked out the *Synechocystis desC* gene using the transformants described above. The transformants with *desC*1 gene was completely segregated but not with *desC*2 gene. The unsaturated fatty acids C16:1 (9) and C18:1 (9) of the mutant was lower compared to the wildtype. This result indicated that *desC2* is not enough to complement the original *desC* in *Synechocystis* sp. PCC 6803. On the other hand, *desC*1 that share more similarity in amino acid sequence with *desC* of *Synechocystis* sp. PCC 6803. Knock out mutant of *desC*1 and *desC*2 genes in *Anabaena* sp. PCC 7120 could not be isolated, suggesting that both *desC*1 and *desC*2 genes is essential in *Anabaena* sp. PCC 7120 and perhaps in filamentous cyanobacterium.

1.7 Research objectives

As described above, In most environmental stresses, signal transduction pathways are triggered by changes of a membrane physical state which can rigidify or fluidize under stress, in this case is the cold-adapted cyanobacteria *Nostoc* sp. strain SO-36. These filamentous cyanobacteria could adapt to the temperature stress due to the modification

in cell envelope which is produced EPS and change its fatty acids composition inside the cell membrane. In accordance with this hypothesis, the author studied as follows: in Chapter II, to understand the adaptation mechanisms for low temperature in *Nostoc* sp. strain SO-36 more in detail, the author conducted next generation sequence. Also, growth rate and sugar content of *Nostoc* sp. strain SO-36 was analyzed under different temperature. Due to the indication that fatty acid desaturation is essential for low temperature tolerance, in Chapter III, the author analyzed the essentiality of two kinds of first desaturation enzyme which is *desC1* and *desC2* In *Nostoc* sp. strain SO-36 and model filamentous cyanobacteria *Anabaena* sp. PCC 71720. In chapter IV, the author discussed the genomic data obtained and the correlation with coping in harsh environment and the desaturation as a producer of unsaturated fatty acids in cyanobacteria.

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Chapter II

Possible involvement of extracellular polysaccharides in adaptation to harsh environments in the Antarctic cyanobacterium *Nostoc* sp. strain SO-36.

2.1 Introduction

Cyanobacterium is a globally distributed microorganism which performs oxygenic photosynthesis. Their habitat is mainly in the hydrosphere of both fresh water and sea water, and also found in soil. Cyanobacteria can also adapt to various temperatures. In hot springs, thermophilic cyanobacteria, such as *Thermosynechoccocus elongatus* can grow at higher than 50°C (Dyer and Gafford 1961). Vice versa, cyanobacteria can also survive at low temperatures, including glaciers, frozen soil, and polar areas, such as Antarctica. Antarctica is well known as one of the most extreme conditions on earth with the coldest and driest climates. Furthermore, in the periods of winter, Antarctica has continuous darkness and in the period of summer, it has sun light continuously. However, a number of cyanobacterial species classified into several cyanobacterial genera, Leptolyngbya, Oscillatoria, Phormidium, and Nostoc, have been isolated in exposed soils, the ice-covered lake, and surface on ice in Antarctic area (Cavatini 2001; Ohtani 1986; Ohtani et al. 1991). Most Antarctic cyanobacteria are classified into psychrotolerant or psychrotrophic species because of their viability near 0ºC and their optimum temperature for growth are usually higher than 15ºC (Singh and Elster 2007).

Since the first exploration in 1895 to Antarctica, many countries have dispatched Antarctic research expeditions to investigate ecosystems in the Antarctic area, structure of ice core, mechanism of aurora generation, and so on. A Japanese permanent research station, Syowa Station was established on East Ongul Island in Antarctica in 1957 and research teams have isolated several cyanobacterial species from cyanobacteria-moss association on sunny slopes of exposed rocks in Antarctica. *Nostoc* sp. strain SO-36 (hereafter *Nostoc* SO-36) was isolated from a cyanobacteria-moss community in Padda island located 80 km in a South-West direction from Syowa station in 1988. Since most polar cyanobacteria are psychrotolerant, not psychrophiles (Tang et al. 1997), *Nostoc* SO-36 was also regarded as a psychrotolerant strain. *Nostoc* SO-36 has been used in several studies on cloning of acyl-lipid desaturase genes and lipid composition analysis (Chintalapati et al. 2006; Chintalapati et al. 2007). However, it has yet to be elucidated that Nostoc SO-36 is a truly psychrotolerant strain, and if so, what is the difference to the strains sensitive to low temperature.

Basic understandings about the mechanisms of cold shock response in cyanobacteria has been investigated using mesophilic species such as *Synechocystis* sp. PCC 6803 (reviewed in Los and Murata (1999); Morgan-Kiss et al. (2006)). The mechanisms include maintaining fluidity of membrane, unwinding secondary structures of RNA, and so on. It was also reported that the expression of a number of genes were induced by cold shock treatment (Suzuki et al. 2001). However, it is still insufficient to understand how cyanobacteria adapt to the extreme conditions such as Antarctica. One of the reasons is

accessibility to the cyanobacterial samples thrived in polar regions and usually their slow growth or contamination of other bacteria. The other reason is that the cyanobacteria thrived in Antarctica are subjected to not only cold stress, but also to desiccation, irradiation, and chronic nutrient depletion. Ecological relationships between other bacteria, fungi and plants is also important to understand stress resistance. For example, production of extracellular polymeric substrates (EPS) has been supposed to play a role in protection from extreme cold and dry environments. In general, EPS act as a reservoir of water and nutrients in the microenvironment surrounding bacteria, and it may reduce the water loss of the cells, like a gelatinous barrier (Roberson and Firestone 1992). EPS also contribute to form stable biofilm formation (Rossi and De Philippis 2015; Zippel and Neu 2011) and to endure freeze-thaw treatment (Tamaru et al. 2005). However, genomic sequences and information of EPS biosynthesis-related genes of Antarctic cyanobacteria are also insufficient so far.

Here, the author report that *Nostoc* SO-36 can survive but cannot proliferate less than 10°C, in dicating that the strain is not psychrophilic but a psychrotolerant strain. The author also deciphered the complete genome sequence of this legacy strain isolated more than 30 years ago by Antarctic Research Expedition and tried to identify phylogenetic position and EPS-biosynthesis related genes. The genomic information contributes to further studies on the genetic backgrounds of psychrotolerant mechanisms of antarctic cyanobacteria.

2.2 Materials and Methods

2.2.1. Cyanobacterial strains and growth conditions

Nostoc sp. strain SO-36 and *Anabaena* sp. PCC 7120 were grown in a liquid medium of $BG11_0$ (BG11 without nitrate) (Stanier et al. 1971) at 30°C in the light (30-50 μmol m^{-2} s^{-1}) on a rotary shaker (120 rpm) as described previously (Awai et al. 2007). For low temperature condition experiments, cells were first grown in the $BG11_0$ medium at 30°C to an optical density at 730 nm (OD_{730}) of approx. 1.0 and inoculated into fresh $BG11_0$ to OD_{730} of approx. 0.1. Then the new diluted culture was incubated at 10°C under illumination as described above.

2.2.2. Measurements of growth curve and pigment contents

The growth curve of *Nostoc* sp. strain SO-36 and *Anabaena* sp. PCC 7120 were constructed by diluting cells in fresh liquid $BG11_0$ medium. The initial optical density of 0.1 was measured at a wavelength of 730 nm. Cell density was measured every 24 h for 1 week by UV-2600 (Shimadzu, Japan). The chlorophyll and total carotenoid contents of cells were measured by extraction with 100% methanol (Arnon et al. 1974; Wellburn 1994).

2.2.3. Alcian blue staining and measurement of sugar content

Nostoc sp. strain SO-36 and *Anabaena* sp. PCC 7120 cells were observed under an upright microscope (BX53, Olympus, Tokyo, Japan) at 1000x magnification and a stereo microscope (SZX7, Olympus). Heterocyst and/or carboxylic polysaccharide in the culture

were stained with 1% Alcian blue in 3% acetic acid (pH 3.0) solution and incubated for 10 min before the observation as previously described (Di Pippo et al. 2013).

Total sugar content was analyzed with the phenol-sulfate method as previously described (DuBois et al. 1956). In brief, 200 µL of the sample was diluted with 200µL of 5% (w/w) phenol and vortexed for 30 s. Then 1 mL of sulfuric acid was added and the tube was immediately vortexed for another 30s and then kept in a water bath at room temperature for 10 minutes. Total sugar contents were measured colorimetrically by measuring absorption at 490 nm using a UV-2600 (Shimadzu, Japan). Standard curve was determined by dilution of glucose at various concentrations (0, 0.2, 1, 2, 10, 20, 100, and 200 nmol/mL).

2.2.4. Isolation of genomic DNA from *Nostoc* SO-36

Cells from 100 mL of 7-14 days culture in BG11 (OD_{730} is approx. 1.0-1.5) were collected by centrifugation and the cell pellet was transferred to a mortar. The cell pellet was then frozen by liquid nitrogen and macerated to be a fine powder using a pestle. The powder was suspended in lysis buffer (10 mM Tris-HCl pH 9.5, 10 mM EDTA, 100 mM KCl, 500 mM Sucrose, 4mM spermidine, 0.1% 2-mercaptoethanol, 0.5% Triton X-100) (Nishi et al. 2019). Then, the suspension was centrifuged and the supernatant was discarded. For removal of polysaccharides, the precipitate was resuspended with sorbitol buffer (Souza et al. 2012) with slight modification (100 mM Tris-HCl, pH 8.0, 350 mM sorbitol, 5 mM EDTA, 1% Polyvinylpyrrolidone-10, 1% 2-mercaptoethanol) and centrifuged again to obtain a precipitate. Using this precipitate, genomic DNA was extracted and purified according to

(Nishi et al. 2019) with the aid of Qiagen Genomic-tip 20/G (Qiagen). Concentration of the samples was determined by NanoDrop (Thermo Fisher Scientific, Tokyo, Japan).

2.2.5. Whole-genome sequencing strategy

The sequencing library was synthesized by PacBio Sequel Microbial Library Construction Kit (Pacific Biosciences of California, Inc., Menlo Park, CA) with manufacturer's protocol. Whole-genome sequencing was performed by a massive parallel sequencer Sequel (Pacific Biosciences of California, Inc.) via the sequencing service of Macrogen Inc. (Macrogen Japan, Tokyo, Japan). A total length of 366 Mb of sequencing reads were obtained. Number of subreads, subread N50 value and average read length were 46,289, 10,409 bases and 7,914 bases, respectively. Sequencing reads were assembled *de novo* using Flye v.2.4.2 (Kolmogorov et al. 2019) with following settings: Selected minimum overlap: 2000; Predicted genome-size: 8 Mbp. Assembly results were checked with QUAST v.5.0.2 (Gurevich et al. 2013) and Bandage v.0.8.1 (Wick et al. 2015), both with default settings and also by BLASTn search of the sequences. The single chromosome and four plasmid assemblies were circularized by removing the terminal overhanging region. No contamination of SMRTbell adaptor sequence in these super-contigs was confirmed by BLASTn search. The nucleotide sequence of the constructed circular genome was corrected by mapping the reads generated by the other massively parallel sequencer MiSeq (Illumina, Inc.). Miseq reads were prepared with a read length of 302 bp and an average insert size of 700 bp. Reads were filtered for quality values of phred score 30 with the read-through adaptor trimming option using the CLC Genomics Workbench v.11.0. (Qiagen, Venlo, The

Netherlands) with following parameters: mismatch cost: 2, indel cost: 3, length fraction: 0.95, similarity fraction: 0.95, allow to read mapping to multi locus: ignored. Gene prediction and functional annotation of the complete genome sequence were performed using the DFAST pipeline v.1.1.4 (Tanizawa et al. 2018) with an option using CyanoBase (Fujisawa et al. 2017) as the main annotation resource. The start codon of the *dnaA* gene was defined as the +1 position of the chromosome.

Nucleotide sequence similarity between the chromosome of *Nostoc* SO-36 and that of closely related species was compared by constructing a diagonal dot plot by Genome Traveler v.3.0.25 with default settings. (In Silico Biology, Inc., Yokohama, Japan). The dot plot patterns were also confirmed by D-Genies v1.3.0 (Cabanettes and Klopp 2018).

2.2.6. Phylogenetic analysis

Phylogenetic analyses were performed by the maximum-likelihood (ML) algorithms in MEGA-X software ver. 10.1.7 (Kumar et al. 2018) with following parameters; Number of bootstrap replication: 1000; Substitution model: Tamura-Nei model (Tamura and Nei 1993); Rates among sites: Uniform rates; Gaps/Missing data treatment: Complete deletion; ML heuristic methods: NNI; Initial Tree for ML: Default (NJ/BioN); Branch Swap Filter: Moderate. The tree topology was also checked by comparing with that obtained by neighbor-joining (NJ) algorithms. A list of accession numbers of 16S ribosomal RNA sequence used in this study was shown in **table 2-1**.

2.2.7. Data availability

Original sequencing reads were deposited in the DRA/SRA/ENA database with the following accession numbers (DRR337996 and DRR337997). The accession numbers of the complete genome sequence were as follows; chromosome: AP025732; pANSO36A: AP025733; pANSO36B: AP025734; pANSO36C: AP025735; pANSO36D: AP025736.

Table 2-1. 16S rRNA gene list with accession numbers used in phylogenetic tree

Strain name	accession No.	Types of habitat Free-living or Symbiotic (Host information)
Gloeobacter violaceus PCC 7421	NC_005125	Free-living
Thermosynechococcus elongatus BP-1	NC_004113	Free-living
Fischerella sp. NIES-3754	NZ_AP017305	Free-living
Anabaena sp. PCC 7120	NC_003272	Free-living
Nostoc punctiforme PCC 73102	NC_010628	Zamia, Various hosts
Nostoc sp. SO-36	in this work	Bryophyta
Nostoc sp. SO-42	AB098071	Bryophyta
Nostoc sp. TLC240-02	CP040094	Leiosporoceros dussii
Nostoc linckia NIES-25	AP018223	Free-living
Nostoc cycadae WK-1	NZ_DF978488	Cycas revoluta
Nostoc sp. PCC 7107	NC_019676	Free-living
Nostoc sp. HK-01	AP018318	Free-living
Nostoc sp. HK-02	AP018326	Free-living
Nostoc azollae 0708	CP002059	Azolla
Desmonostoc sp. PCC 7422	HG004586	Cycas sp.
Nostoc sp. Cc3	HG004580	Cycas circinalis
Nostoc sp. KVJ2	EU022712	Blasia pusilla
Nostoc sp. KVJ10	EU022708	Blasia pusilla
Anabaena sp. PCC 7108	AJ133162	Free-living
Nostoc sp. GT138	KF494240	Gunnera tinctoria
Nostoc sp. 'Peltigera membranacea cyanobiont'	JX181775	Peltigera membranacea
Nostoc sp. ATCC 53789	CP046703	☒chen
Nostoc piscinale CENA21	CP012036	Free-living
Desmonostoc muscorum PCC 7906	AB325908	Free-living
Nostoc commune NIES-4072	GCA_003113895	Free-living
Nostoc sphaeroides Kutzing En	GCA_003443655	Free-living
Nostoc sp. NIES-2111	LC322125	Free-living
Nostoc sp. NIES-2110	LC228974	Free-living
Nostoc sp. 'Lobaria pulmonaria-5183 cyanobiont'	CP026692	Lobaria pulmonaria
Nostoc sp. ATCC53789	AF062638	Lichen thallus
Nostoc sp. PCC 7524	CP003552	Free-living
Anabaena cylindrica PCC 7122	NR_102457	Free-living
Anabaena sp. WA102	CP011456	Free-living
Nostoc sp. PCC9709 'Peltigera membranacea cyanobic	AF027654	Peltigera membranacea
Nostoc sp. A39	KF494247	Anthoceros punctatus
Nostoc sp. 'Peltigera membranacea cyanobiont N6'	JX975209	Peltigera membranacea
Nostoc sp. SKSL1	EU022726	Peltigera canina
Nostoc sp. C057	NZ_CP040281	Phaeoceros
Nostoc punctiforme BKP_SS6	MW383844	Free-living
Anabaena variabilis ATCC 29413	CP000117	Free-living

2.3 Results

2.3.1. *Nostoc* SO-36 is a psychrotolerant cyanobacterium

Because *Nostoc* SO-36 was isolated in Antarctica, it was expected that the strain can grow (psychrotrophic) or at least survive (psychrotolerant) at low temperature. To test this, the author grew the strain at 10℃ and compared with the representative filamentous cyanobacterium, *Anabaena* sp. PCC 7120 (hereafter *Anabaena* 7120) (**Figure 2-1**). At 10℃, *Nostoc* SO-36 could not grow but kept its OD for at least 7 days. On the other hand, OD of *Anabaena* 7120 decreased constantly and became ten times lower than that of the beginning after 7 days of culture. Because *Nostoc* SO-36 could survive but not grow at low temperature, the author examined its growth rate at higher temperature i.e. 30℃. At 30℃, *Nostoc* SO-36 grew exponentially and *Anabaena* 7120 as well. These results suggest that *Nostoc* SO-36 is a psychrotolerant but not psychrotrophic cyanobacterium.

Nostoc SO-36 grew in a filamentous shape very similar to that of *Anabaena* 7120 at 30℃ (**Figure 2A** and **C**). Formation of heterocyst, which is a specialized cell for fixation of gaseous nitrogen, was observed in the medium without fixed nitrogen source ($BG11_0$ medium), indicating that *Nostoc* SO-36 can grow diazotrophically. As for pigment content, the low temperature treatment decreased the content of chlorophyll, but not total carotenoid (**Figure 2-2I** and **J**). At 10°C, filament became much shorter compared to the cells grown at 30℃ (**Figure 2E**). Moreover, the cell got aggregated to make a small particle

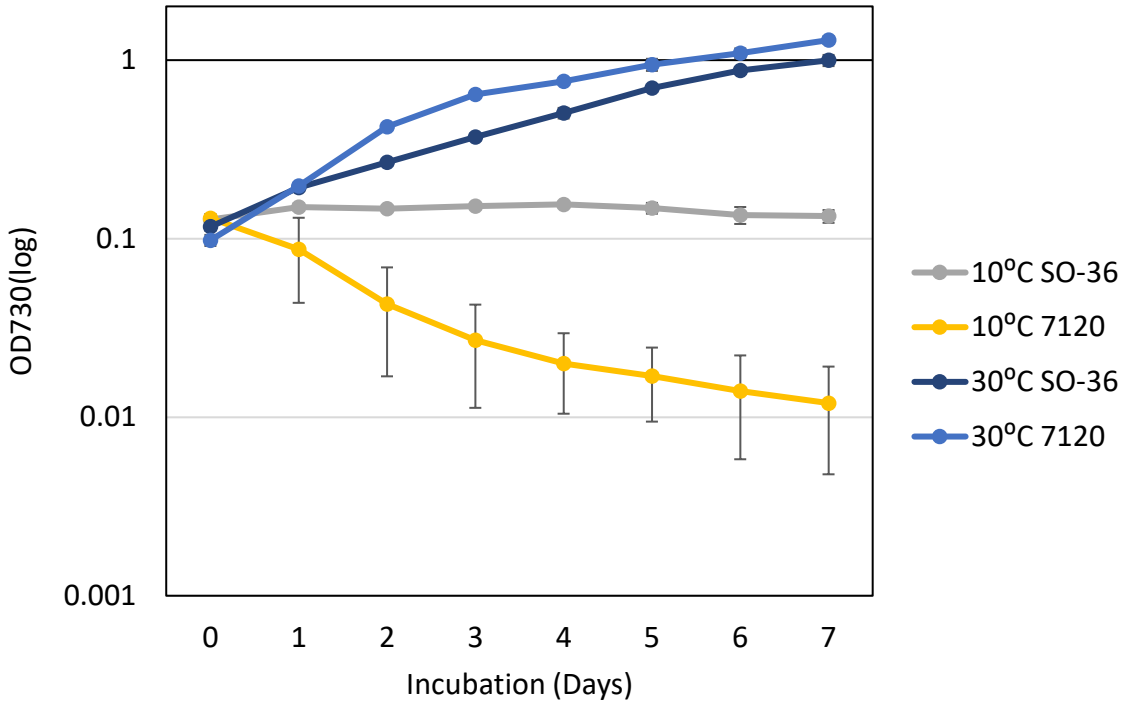

Figure 2-1. Growth curve of *Nostoc* SO-36 and *Anabaena* 7120. Both were cultured in BG110 at 10℃ (dotted line) or 30℃ (solid line). Black lines are for *Nostoc* SO-36 and gray lines *Anabaena* 7120. Error bars indicate the SD based on three independent experiments.

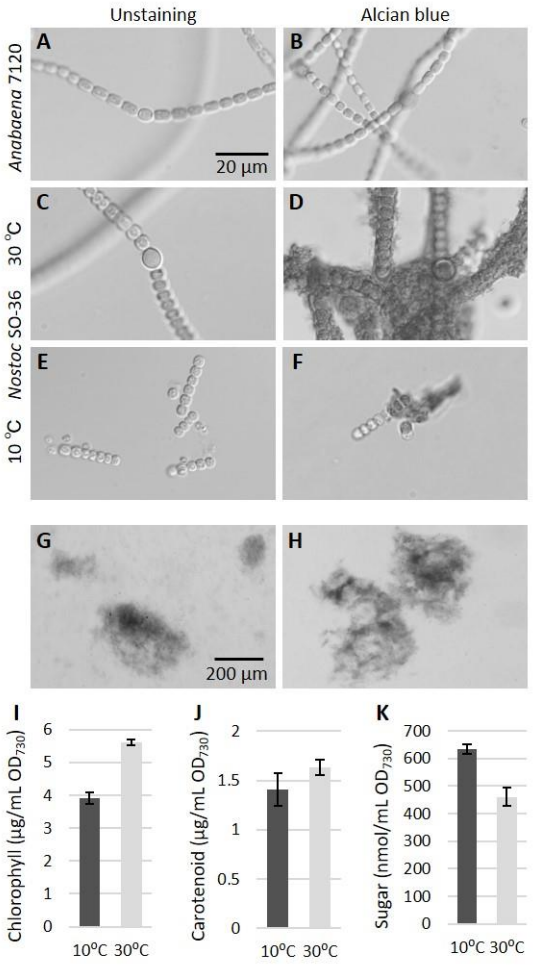

Figure 2-2. Phenotypes of *Nostoc* SO-36 and *Anabaena* 7120 at low temperature. **A**-**F**. Cell shapes and accumulation of extracellular polysaccharides of *Nostoc* SO-36 (**C**-**F**) and *Anabaena* (**A**, **B**). **A**, **C**, **E**: Cell shapes observed under a blight field microscope. **B**, **D**, **F**: Culture stained with Alcian blue. **A**-**D**: Grown at 30℃, E, F: Grown at 10℃. Size bar = 20 μm. **G**, **H**. Morphology of *Nostoc* SO-36 grown at 10℃ observed under a stereomicroscope. Size bar = 200 μm. **I**. Chlorophyll content per OD_{730}. J. Total carotenoid content per OD_{730}. Total sugar content per OD_{730}. Error bars indicate the SD based on three independent experiments.

(**Figure 2G** and **H**, about 100 to 500 μm in a diameter) with some components, possibly extracellular polysaccharides (EPS).

To see whether EPS content increased by low temperature, the author first stained cells of both *Nostoc* SO-36 and *Anabaena* 7120 with Alcian blue after 7 days of culture at 30℃. Figure 2B and 2D show that even at 30℃, *Nostoc* SO-36 accumulated more oligosaccharides outside of the cells compared with *Anabaena* 7120. The shorter filaments of *Nostoc* SO-36 grown at 10℃ showed accumulation of EPS. The amount of EPS per OD_{730} was higher in the cells grown at 10℃ compared to that grown at 30℃. Since *Nostoc* SO-36 survived extreme environments of Antarctica such as freezing, desiccation, high light in summer and low light in winter, there must be mechanisms to adapt low temperature. Genomic sequence is a fundamental information to understand those mechanisms (Chrismas et al. 2018), and the author first developed a method to isolated genome DNA from *Nostoc* SO-36 at a grade enough for NGS analysis.

2.3.2. Isolation of genomic DNA from *Nostoc* SO-36 and initial trial for NGS analysis

Because *Nostoc* SO-36 accumulates extracellular polysaccharide, it is important to remove these compounds from genomic DNA for NGS analysis. The author first tried to isolate the genomic DNA with an ordinary phenol/chloroform-based method followed by CsCl purification. However, polysaccharide could not be removed, and the author finally

adopted the DNA purification method with sorbitol (Nishi et al. 2019; Souza et al. 2012), followed by purification with Qiagen Genomic-tip.

Nostoc SO-36 is isolated from a moss community on a southwest slope in Padda island, and it should have a mixture of other organisms. Before the author started this research, the author tried to purify the strain by agar plate method. The obtained strain has no visible contamination under a microscope. The author then put the isolated genome to NGS analysis using TruSeq Nano DNA Library Prep kits (Illumina, San Diego, CA) with 8 cycles of PCR amplification. The library with approximately 800 bp of averaged insert DNA size was sequenced by MiSeq with the manufacturer's protocol. Because this protocol and Illumina sequencing procedure includes PCR for amplification of fragments for the analysis, the author found that almost a half of the reads were the sequence from other microorganisms. It is probably due to GC-bias (More suitable GC content tends to have more Illumina reads.) between the contaminated microorganisms and the *Nostoc* SO-36. The author then used the PacBio system because this method does not include many cycles of PCR steps.

2.3.3. Genomic feature of *Nostoc* SO-36

The complete genome sequence of *Nostoc* SO-36 consists of a 7,408,262 base pairs (bp) with a single circular chromosome and four plasmids named pANSO36A, pANSO36B, pANSO36C and pANSO36D (**Table 2-I**). The total length of the circular chromosome was 6,802,717 bp with an average G+C content of 41.41%. Lengths and G+C content of four plasmids were as follows; pANSO36A: 271,158 bp, 40.81%; pANSO36B: 228,652 bp, 40.47%;

Table 2-2. General feature of the Nostoc sp. SO-36.

Features	length (bp)	G+C content	number of CDS	number of tRNA	number of rRNA operon	number of CRISPR
chromosome	6,802,717	41.41	6252	48	4	5
pANSO36A	271,158	40.81	279	0	0	0
pANSO36B	228,652	40.47	207	0	0	0
pANSO36C	69,903	41.25	87	0	0	0
pANSO36D	35,832	39.98	30	0	0	0

pANSO36C: 69,903 bp, 41.25%; and pANSO36D: 35,832 bp, 39.98%. Functional annotation revealed a total of 6855 coding sequences (CDSs), 48 tRNA genes, 4 copies of rRNA operons and 5 CRISPR regions. The genome is composed of coding sequences with the coding ratio, 78.0%. Probably due to the quality problem of the NGS reads, small number of indels or frameshifts remained in ORFs in the current version of the genome sequence. Completeness score and strain heterogeneity score of the genome by CheckM program v.1.0.18 (Parks et al. 2015) were 97.96% and 0.00%, respectively.

According to a BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with default settings using the 16S rRNA gene sequence of the *Nostoc* SO-36 revealed 100%, 98.99% and 98.79% similarity with that of *Nostoc* sp. strain SO-42 also isolated from Antarctica (GenBank accession number: AB098071.1) by Dr. S. Ohtani (Arima et al. 2012), *Nostoc* sp. GT138 (accession number: KF494240.1) isolated from symbiotic gland tissue of *Gunnera tinctoria*, and *Nostoc* sp. TCL240-02 (accession number: CP040094.1) isolated from the hornwort *Leiosporoceros dussii*, respectively. A number of symbiotic or host-associated species were listed in the blastn results with high similarity scores (both e-value and identity score). To address a phylogenetic position of the strain SO-36, the author constructed the phylogenetic tree by the Maximum-likelihood (ML) method using 16S rRNA sequences of various Nostoc species and some outgroup species (**Figure 2-3**). The tree shape and division of clades were similar with previous reports about phylogenetic analysis of the genus Nostoc (Rajaniemi et al. 2005; Řeháková et al. 2007). As a result of the phylogenetic analysis of 16S rRNA genes, the author identified the strain SO-36 belongs to the typical clade of

Nostoc sensu stricto (Bagchi et al. 2017; Řeháková et al. 2007) which also includes *Nostoc punctiforme* PCC 73102, *Nostoc commune* NIES-4072, and many symbiotic Nostoc species. The Nostoc strains isolated as a symbiont or from a host-associated environment were marked with black squares in **Figure 2-4**. As several researchers reported, symbiotic strains belonging to the genus Nostoc were identified as a polyphyletic group (Bell-Doyon et al. 2020). As shown in **Figure 2-4**, a number of free-living strains were also included in the clade of *Nostoc sensu stricto*.

Among these closely related strains, whole genome sequences of the five species such as *Nostoc* sp. TCL240-02, *Nostoc* sp ATCC 53789, *Nostoc* sp. '*Peltigera_membranacea* cyanobiont' N6, *Nostoc* sp. C57 and *Nostoc punctiforme* PCC 73102 were available, so far. All these strains showed higher than 98.5% identity of the 16S rRNA sequence with that of the *Nostoc* SO-36. The author compared nucleotide sequences between the chromosome of the *Nostoc* SO-36 and other closely related strains by dot plot matrix with a window length as 1kb and blast e-value < 10. However, there were no large genomic islands with high similarity between the chromosome of the *Nostoc* SO-36 and other five species (**Figure 2-5**). In contrast, dot plot analysis revealed that the chromosome of *N. punctiforme* PCC 73102 showed several highly homologous genomic islands with other closely related species except the strain N6. These results indicated that chromosomal structure and sequence organization of the *Nostoc* SO-36 was not similar with that of closely related species whose genome sequences were deciphered.

2.3.4. Genes for extracellular oligosaccharide synthesis conserved in *Nostocaceae*

Since low temperature treatment enhance EPS production and aggregation of the cells, the author surveyed for conserved genes for the EPS synthesis pathway among *Nostoc* SO-36 and closely related species with complete or high-quality draft genome sequences. The EPS synthesis pathways of Gram-negative bacteria are known to include the Wzx/Wzy pathway, the ABC transporter-dependent Kps pathway, the synthase-dependent pathway and extracellular sucrase pathways (Pereira et al. 2013; Schmid et al. 2015). Whether the synthase-dependent and extracellular sucrase pathways were functional in cyanobacteria has not been proposed yet. Since heterocyst-forming cyanobacteria have relatively large size of genome and multicopy genes for EPS biosynthesis in general, genes for the Wzx/Wzy pathway were found in all strains. Only in the *Nostroc azollae* 0708, the *wzx* gene was missing. In the *Nostoc* SO-36, the *wzx* gene and the *wzb* gene were found as a single copy gene. Relatively small copy number of the genes in Wzx/Wzy pathways is a characteristic feature of *Nostoc* SO-36. By some possibility, EPS-biosynthetic mechanisms in this strain likely simpler than that of other related species. Recently, the EPS synthesis gene cluster of the Wzx/Wzy pathway encoded by the plasmid pSYSM of a unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Hereafter *Synechocystis* 6803) was shown to be involved in the Synechan synthesis (Maeda et al. 2021), but the ortholog gene of the *wzy* gene in the pSYSM cluster was not conserved in the strains examined here. But the ortholog gene of the other putative *wzy* gene conserved in the chromosome of *Synechocystis* 6803 was found in all strains. On the other hand, the ortholog gene of the *wzz* (PCP-2a) gene conserved in the pSYSM of *Synechocystis* 6803 was found among all strains examined. Multicopy feature of

these genes indicated the complexity of regulatory system for EPS biosynthesis in various Nostocaceae species.

Four genes, KpsD (OPX family), KpsE (PCP family), KpsM, and KpsT, which are core components of the ABC transporter-dependent pathway, were found in all strains, suggesting that this pathway would be functional in these cyanobacteria. However, other accessory protein groups such as KpsC/KpsS and KpsU were not conserved: in *Nostoc* SO-36. The genes for the synthase-dependent pathway was not found in the genome of *Nostoc* SO-36.

Table 2-3 Copy number of genes involved in the polymerization chain length control and export of EPS in *Nostoc* sp. SO-36 and related species

Gene Name	Product	Nostoc sp. SO-36	Nostoc punctiforme PCC 73102	Nostoc sp. ATCC 53789	Nostoc sp. TCL240-02	Nostoc sp. C057	Nostoc sp. cyanobiont N6	Nostoc cycadae WK-1	Nostoc sp. 7107	Anabaena sp. PCC 7120	Nostoc piscinale CENA21	Nostroc azollae 0708
Wzx	polysaccharide flippase	1	4	3	2	3	3	2	3	5	2	nf
Wzy	polysaccharide polymerase, slr0728-like	2	5	4	4	4	4	4	3	5	1	2
Wza/KpsD	outermembrane EPS exporter, OPX	2	2	2	2	2	2	2	2	3	2	2
Wzb	low-molecular weight protein-tyrosine-phosphatase	1	2	2	1	1	2	2	2	4	2	2
Wzz/Wzc/KpsE	polysaccharide biosynthesis tyrosine autokinase, PCP	4	7	5	4	5	5	3	3	7	2	4
ExoD	CPS/EPS-related protein	2	2	2	2	2	2	2	2	2	1	nf
KpsM	Capsule polysaccharide transport permease	3	5	3	5	5	2	4	2	8	3	3
KpsT	Capsule polysaccharide transport ATP binding component	1	1	1	1	1	1	1	1	1	1	1
KpsC/KpsS	Capsule polysaccharide export protein	nf	nf	nf	nf	nf	nf	nf	nf	nf	nf	nf
KpsF	arabinose 5-phosphate isomerase	2	2	2	2	2	2	2	2	2	2	2
KpsU	3-deoxy-manno-octulosonate cytidylyltransferase	nf	2	nf	1	1	nf	nf	nf	nf	nf	nf
		nf: not found										

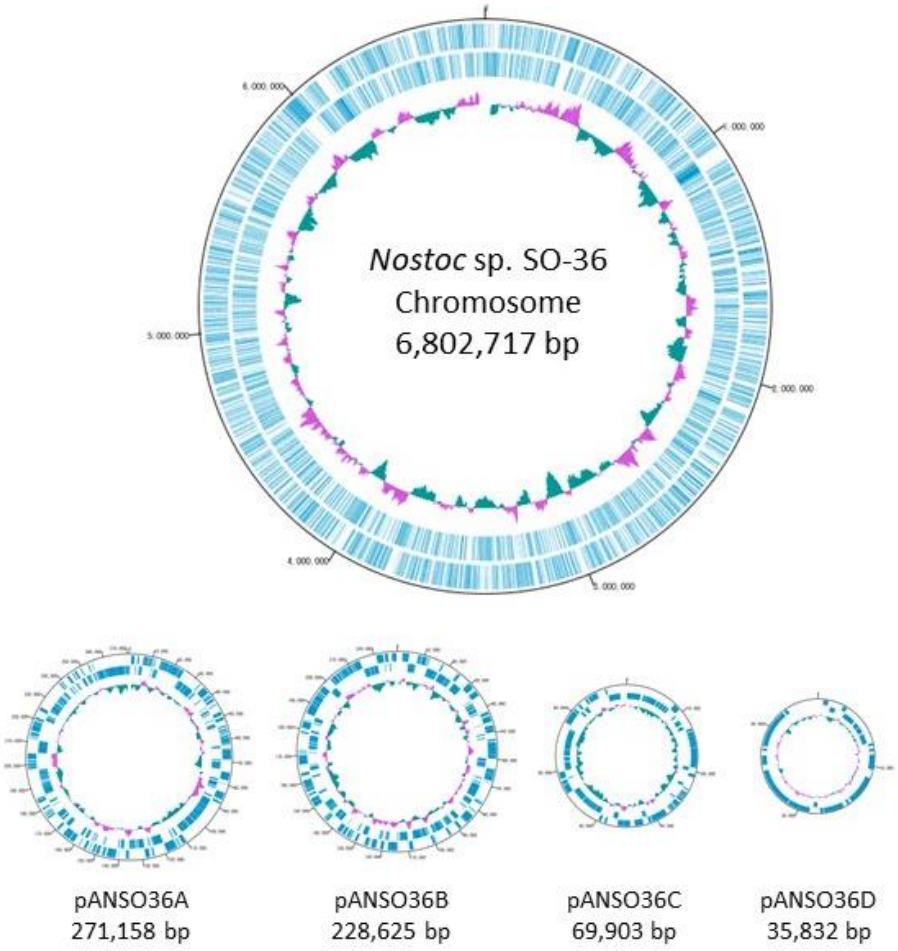

Figure 2-3. Genomic structure of chromosome and four plasmids. The circles from outer to inner represent strand information. The outer black circle shows the scale bars in Mbps. In the second outer circle, blue bars indicate genes transcribed clockwise direction. In the third outer ring, blue bars indicate genes transcribed counter-clockwise direction. Inner ring shows the GC skew, (G−C)/(G+C). GC skew was calculated with a 10-kb window at a step size of 100 bp. Similar to other species in the genus *Nostoc*, GC skew was not clearly observed in this species.

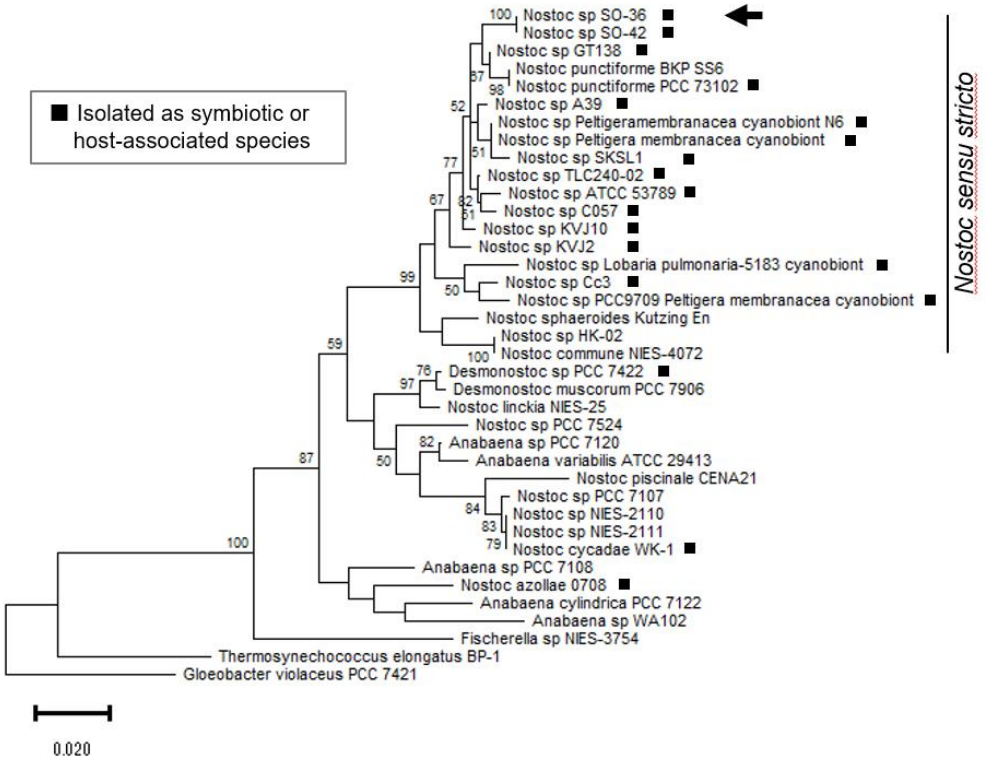

Figure 2-4. Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequences of *Nostoc* SO-36 and related species of Nostocacean cyanobacteria. The bootstrap values obtained are displayed at the nodes when it is higher than 50%. GenBank sequence accession numbers were listed in supplemental table I. *Nostoc* SO-36 was indicated by an arrow. The strains isolated as symbiotic or host-associated species were indicated with black square.

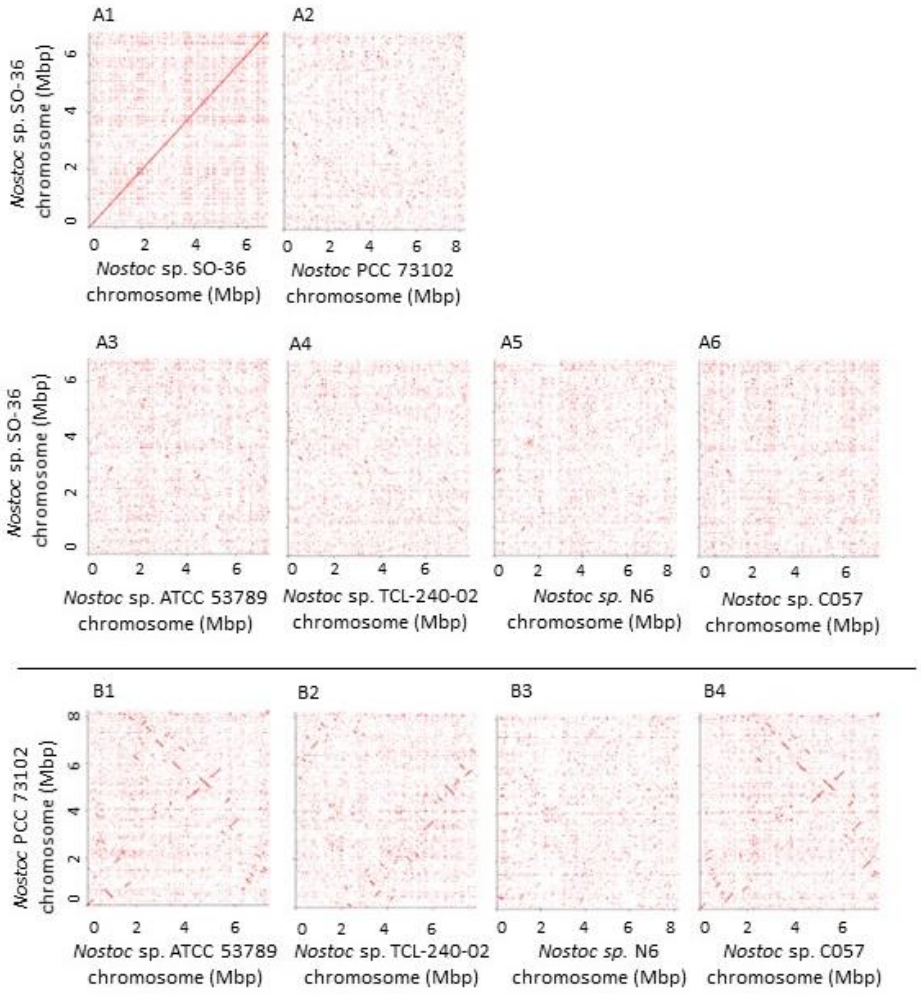

Figure 2-5. Dot-plot matrix comparisons of the chromosomes of *Nostoc* SO-36 with closely related strains. Conserved regions in the dot plot are represented by red dots. Continuous red dots indicate highly conserved genomic regions. (A) Comparison of *Nostoc* SO-36 with itself and other strains. (B) Comparison of *Nostoc punctiforme* PCC 73102 with other strains.

2.4 Discussion

2.4.1 Phylogenetic position of *Nostoc* SO-36

As stated in the results section, BLASTn search of 16S rRNA of *Nostoc* SO-36 showed a number of symbiotic Nostoc species as closely related species. So far, a number of symbiotic or host-associated cyanobacteria have been identified from various plants and fungus, such as lichen, azolla, cycad and gunnera. Most of these symbiotic relationships with plants and fungus were established by nitrogen-fixing cyanobacterial species in an order, Nostocales. Although the strains isolated as symbiotic species were polyphyletic among Nostoclaes, a number of symbiotic strains belonged to a phylogenetic clade of *Nostoc sensu stricto* (Bell-Doyon et al. 2020; Papaefthimiou et al. 2008). Many researchers tried to identify common genomic features for host-specificity of these strains, but persuasive explanations have not been found yet, except nitrogen fixation or hormogonium formation. Recently, Hashidoko et al. (2019) identified a Nostoc strain is able to form hormogonium in response to a diacylglycerol compound which was extracted from leaf litter of cycad (Hashidoko et al. 2019). Since *Nostoc* SO-36 was isolated from the Bryophyte community on the sunny slope in Antarctica, it might be possible that this strain is also able to respond to some metabolites released from these plants to establish the host-associated habitat to endure the extremely cold and dry climate. Interestingly, the phylogenetic clade of *Nostoc sensu stricto* includes a lot of strains isolated from both temperate or subtropical climates and also polar areas. Furthermore, the fact that a number of strains from various cyanobacterial genera were

isolated from the Antarctic area indicate that the psychrophilic or psychrotolerant phenotypes would be a polyphyletic feature in evolutionary history in cyanobacteria.

2.4.2 Genes for EPS biosynthesis and export pathways in *Nostoc* SO-36

Generally, in bacteria, four pathways are known for EPS biosynthesis; 1) Wzx/Wzy dependent pathway, 2) ABC transporter-dependent pathway, 3) Synthase-dependent pathway, and a minor pathway 4) Extracellular synthesis by single sucrase protein (see review. Schmid et al. (2015)). Since orthologous genes for the alginate polymerase gene for the pathway 3) and fructansucrase protein for the pathway 4) were not found in the complete genome sequence of the strains SO-36 and closely related species in Table 2-3, EPS biosynthesis in these species would depend on the pathway 1) and 2). Recently, Maeda et al. (2021) identified that proteins encoded by a cluster of Wzx/Wzy pathway genes on the plasmid pSYSM play a primary role in EPS synthesis in *Synechocystis* 6803. *Synechocystis* 6803 has an additional Wzy-like gene *sll0923* in the main chromosome. The putative Wzy genes found in the *Nostoc* SO-36 and closely related species were more similar to the chromosomal gene in *Synechocystis* 6803. In case of Wzz (PCP-2a) gene, *Synechocystis* 6803 also has two homologous genes, *sll5052* on the plasmid and *sll0923* on the chromosome. *Nostoc* SO-36 and closely related species possess only homologous gene of the plasmid type (*sll5052*) in *Synechocystis* 6803. This information suggests that acquire, loss or complex horizontal transfers of a group of Wzx/Wzy dependent pathway genes have occurred and resulted in the diverged EPSs in this phylum. In case of the ABC transporter-dependent pathway, genes for only core complex are conserved in *Nostoc* SO-36 and also in closely

related species. Other accessory proteins or the genes for polymer unit synthesis were not found, except the *kpsF* gene. It has not been clear yet, whether Wzx/Wzy dependent pathway and ABC transporter-dependent pathway operate the EPS export coordinately or selective use of these pathways under stress conditions. Disruption of the single copy genes in Table 2.3 might be effective strategy to propose the function of these pathways. Further investigations about the temperature-specific expression of the genes for EPS synthesis pathway would contribute to understand the functional importance of them.

2.4.3 Putative factors for psychrotolerant phenotype of Antarctic cyanobacteria

Biondi et al. (2008) collected 51 Antarctic cyanobacterial strains which include six *Nostoc* strains from benthic mat of frozen lake. They reported that although photosensitivity varied among the strains, five *Nostoc* strains showed slight or strong photosensitivity with changing yellowish color when cells were cultivated under 30-40 µmol m^{-2} s^{-1} irradiation in their laboratory conditions. The author observed shorter filaments of *Nostoc* SO-36 when the author grew the cells at 10℃ (Fig. 2). This is probably due to the photoinhibitory effect induced by low temperature. Filamentous cyanobacteria are known to form shorted filaments, most likely cleavage at the site between heterocyst and vegetative cells. In line with this hypothesis, the author observed decrease of chlorophyll content and did not observe heterocyst in the cells grown at 10℃. Biondi et al. (2008) also observed that a Nostoc strain produced high amount of polysaccharide. It is possible that the formation of EPS under low temperature is to form large aggregates of the *Nostoc* SO-36 cells and contribute to reduce light irradiance to the cells. To ascertain that EPS is

involved in protection mechanisms from excess light in *Nostoc* SO-36, it is crucial to analyze knock-out mutants of EPS synthetic genes. The author have tried to knock-out several genes in *Nostoc* SO-36 using the triparental method used for *Anabaena* 7120 (Elhai and Wolk 1988), but it was not successful. The author could not isolate even a partial knock-out mutant of the multicopy of cyanobacterial genome, indicating that *Nostoc* SO-36 has different mechanism for the restriction-modification system with that of *Anabaena* 7120. Further study on the photosensitivity of the *Nostoc* SO-36 would clear the function of EPS under low temperature.

It is also well known that fatty acid desaturation is essential for low temperature tolerance through keeping membrane fluidity and recovery of photosynthesis from low-temperature photoinhibition (Gombos et al. 1994; Wada et al. 1990). Chintalapati et al. (2007) reported that when the growth temperature shifted from 25℃ to 10℃, *Nostoc* SO-36 showed an increase in the content of tri-unsaturated fatty acid [C18:3(9,12,15)] of membrane lipids. They also revealed that transcript levels of genes for fatty acid desaturase, *desA*, *desB*, *desC*, and *desC2* are constitutively expressed regardless of the growth temperature. These results suggest that the cold adaptation in *Nostoc* SO-36 is not simply regulated at the transcription level, but also at post-transcriptional or translation level. These types of regulations might be important not only for the fatty acid desaturation, but also for other mechanisms in the *Nostoc* SO-36 to acquire phychrotolerance.

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Chapter III

Desaturation of fatty acids in both *sn*-1 and *sn*-2 positions is essential in the filamentous cyanobacteria

3.1 Introduction

Cyanobacteria are gram-negative bacteria that have a characteristic membrane layer, thylakoid membrane, inside of the cells. According to the morphology, cyanobacteria could be divided into 2 parts, namely unicellular and filamentous types. The morphology of unicellular cyanobacteria is similar to that of chloroplasts in higher plants and algae. This resemblance is not just the morphology; cyanobacteria have photosynthetic functions that are similar to the chloroplasts. The membrane that took a role in photosynthetic reactions is the thylakoid membrane. The function of this membrane system is influenced by membrane lipids and proteins contained therein (Awai, 2016). Thylakoid and cytoplasmic membranes in cyanobacteria are composed of 4 major glycerolipids namely monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylglycerol (PG) (Wada and Murata, 1998). MGDG in cyanobacteria occupies around 50% of thylakoid membranes, and DGDG, SQDG, and PG are around 5-25%, depending on the type of cyanobacteria. Cyanobacteria also have minor glycerolipids monoglucosyldiacylglycerol (GlcDG) as an intermediate of MGDG synthesis (Awai et al., 2006; Awai et al., 2014).

One of the factors making difference between cyanobacteria and other bacteria is the composition of fatty acids in the membrane lipids. Most cyanobacteria synthesize fatty acids with carbon chain lengths 16-18. Among them, cyanobacteria contain saturated and unsaturated fatty acids. For saturated fatty acids, cyanobacteria have C16:0 (palmitic acid) and C18:0 (stearic acid), but some cyanobacteria are capable of synthesizing C14:0 (myristic acid) (Saito et al., 2018). For unsaturated fatty acids, cyanobacteria generally synthesize C16:1 Δ9 (palmitoleic acid), C18:1 Δ9 (oleic acid), C18:1 Δ11(vaccenic acid), C18:2 Δ9,12 (linoleic acid), C18:3 Δ6,9,12 (γ-linolenic acid), C18:3 Δ9,12,15 (α-linolenic acid), and C18:4 Δ6,9,12,15 (stearidonic acid) (Murata et al., 1992).

Based on the composition of fatty acids, cyanobacteria have been classified into four groups. Group 1 has mono-unsaturated fatty acids, palmitoleic, and oleic acid. Cyanobacteria strains in group 1 are including *Thermosynechococcus vulcanus, Thermosynechococcus elongatus, Synechococcus* sp PCC 6301, *Synechococcus elongatus* PCC 7942, and *Prochlorothrix hollandica*. Group 2 has palmitoleic acid at the *sn*-2 position of the glycerol backbone and *α*-linolenic acid at the *sn*-1. Strains for group 2 are including *Anabaena variabilis*, *Anabaena* sp. PCC 7120 (hereafter *Anabaena*), *Gloeobacter violaceus, Nostoc punctiforme*, *Synechococcus* sp. PCC 7002 and *Trichodesmium erythraeum*. Group 3 has *γ*-linolenic acid at the *sn*-1 position. Strains for group 3 are including *Prochlorococcus marinus* and *Arthrospira* (*Spirulina*) *platensis*. Group 4 has similar fatty acid compositions to Group 3. However, group 4 additionally has *α*-linolenic acid and stearidonic acid at the *sn*-1

position. Strain for group 4 is including *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) (Kenyon, 1972; Kenyon et al., 1972; Los and Mironov, 2015).

Desaturation is the process that introduces a double bond between two carbons in the fatty acid chain, that causes the change of saturated fatty acids into unsaturated fatty acids. The enzyme taking this role is desaturase. The type of desaturase in cyanobacteria is acyl-lipid desaturase because it desaturates fatty acids esterified to glycerolipids. In general, saturated fatty acids are synthesized through *de novo* fatty acid biosynthesis and desaturation of fatty acids occurs after the esterification of fatty acids into glycerolipids (Wada and Murata, 2006). There are 4 types of desaturase enzymes, and the first cyanobacterial desaturase is DesC, which introduces double bonds in the chain of saturated fatty acids at Δ9 positions, namely, converting palmitic acid to palmitoleic acid, and stearic acid to oleic acid. The second is DesA, which introduces a double bond at Δ12 positions after the DesC reaction and forms the second double bond in the fatty acids chain, synthesizing linoleic acid. The third is DesD, which introduces double bonds at Δ6 position and forms γ-linolenic acid, and DesB, which introduces double bonds in the ω3 position and forms α -linolenic acid (Los and Murata, 1999).

Environmental factors especially temperature shifts are the main causes of desaturation in cyanobacteria. Under conditions of low temperature, unsaturated fatty acids accumulated and the chain length of fatty acids in the glycerol moiety also decreased (Russell, 1984). As the report by Sato and Murata (1979) under low-temperature conditions, membrane lipids MGDG and DGDG in *Anabaena variabilis* increased concentrations of α-

linolenic acid at the *sn*-1 position and C16:2 Δ9,12 at the *sn*-2 position. In cyanobacterial membrane lipids, C18 fatty acids are the most abundant at the *sn*-1 position and C16 at the *sn*-2 position of the glycerol backbone. In *Synechococcus* and *Anabaena variabilis*, which belonged to groups 1 and 2 respectively, C16:0, C16:1 Δ9, and C16:2 Δ9,12 are esterified at the *sn*-2 position, while groups 3 and 4 have only C16:0 at the *sn*-2 position (Chi et al., 2008).

DesC, as described above, is the first desaturase that introduces a double bond at Δ9 positions of both C16 and C18 fatty acids. Chintalapati et al. (2006) reported that *Nostoc* sp strain SO-36, which belongs to group 2, has two DesC enzymes, DesC1 and DesC2. DesC1 desaturate stearic acid to oleic acid and DesC2 desaturate palmitic acid to palmitoleic acid. This DesC1 activity has also been experimentally confirmed in *Anabaena variabilis* (group 2) (Sato et al., 1979), *Synechocystis* sp. PCC 6803 (group 4) (Sakamoto et al., 1994), and a thermophilic cyanobacterium *Thermosynechococcus vulcanus* (group 1; Kiselva et al., 2000). The model group 2 cyanobacterium *Anabaena* sp. PCC 7120 also has two *desC* genes (*all1599* and *all4991*) whose functions are still unknown. To identify their exact roles, the author expressed both *desC* genes from *Nostoc* and *Anabaena* into *Synechocystis* sp. PCC 6803 and analyzed substrate and position specificity of these two enzymes.

In this chapter, the author reveals the function of DesC1 and DesC2 as the first desaturation from *Nostoc* SO-36 and *Anabaena* 7120 by the isolated mutant in *Synechocystis* 6803. DesC in *Synechocystis* 6803 can be complemented by DesC1 from filamentous cyanobacteria *Nostoc* SO-36 and *Anabaena* 7120 and not by DesC2. The significant phenotype is the alteration of unsaturated fatty acids production and likely

altered the growth rate, and oxigenic activity. The author confirmed that this phenomenon is likely due to the complementation of desaturation pathways and not induced either. It also revealed that both DesC in filamentous cyanobacteria is essential and has a specific function to desaturate C18:0 and C16:0 at *sn*-1 and *sn*-2 respectively.

3.2 Materials and method

3.2.1 Growth conditions of cyanobacteria strains

Cyanobacteria *Nostoc* sp. strain SO-36, *Anabaena* sp. PCC 7120, and *Synechocystis* sp. PCC 6803 were grown at 30^{o}C in BG-11 medium (Stainer et al. 1971) supplemented with 22 mM TES buffer (pH 7.5). These cells were incubated under continuous light from a fluorescent lamp at 30 μmol photon m^{-2} s^{-1} on a rotary shaker at 120 rpm as described previously (Awai et al., 2007).

3.2.2 Expression of *Nostoc* and *Anabaena desC* genes and knock-out the original *desC gene* in *Synechocystis*

DNA sequences of both *desC1* (accession number AJ121244) and *desC2* (accession number AJ621247) of *Nostoc* strain SO-36 were obtained from NCBI database. *Anabaena desC1* (*all1599*) and *desC2* (*all4991*) genes, and *Synechocystis desC* (*sll0541*) gene were obtained from Cyanobase (genome.microbedb.jp/cyanobase/). The vector for *Nostoc* and *Anabaena desC1* and *desC2* overexpression in *Synechocystis* was constructed as the following sequence: both DNA fragments of *desC1* and *desC2* were amplified from genomic DNA of wild-type *Nostoc* and *Anabaena* as a template using primer pairs (#1/5 and #2/6 for *desC1* and #3/7 and #4/8 for *desC2*). Both fragments were subcloned separately into the vector from the previous study, pSEM3(Matsumoto and Awai, 2020), a vector that introduces the desired gene into neutral site 2 of *Synechocystis* (*slr2031*). The DNA fragments were introduced into the *Sma*I site by the hot fusion cloning system (Fu et al.,

2014) and the nucleotide genome by sequence were confirmed. These vectors were used to generate overexpressors in *Synechocystis* and introduced by double homologous recombination. For that, wildtype of *Synechocystis* and constructed vectors were mixed and incubated for 6 hours at 30°C under the illumination of fluorescence lamp as described (Kufryk et al., 2002). For isolation of candidates, the mixture was plated on a nitrocellulose membrane (HATF 08520, Merck Millipore Ltd., Tokyo, Japan) on the solid agar medium of BG-11 and incubated at 30°C under continuous light for 2 days. After the incubation, the membrane was transferred onto the solid agar medium of BG-11 containing erythromycin (20 μg/mL) to isolate candidates of the mutants. Resistant colonies were segregated on BG-11 plates with erythromycin (Xu et al., 2015). After 5 times of segregations, genome DNA was extracted from the candidates by glass bead methods (Kindle et al., 1991) and genotyped by PCR using the primers in **Table 3-2**.

To knock out the original *desC* gene (*sll0541*) in *Synechocystis*, plasmid pMobΩ1/*desC* was constructed as follows. DNA fragments upstream of *desC* were amplified by PCR using KOD polymerase (Takara Bio, Shiga, Japan) using *Synechocystis* wild-type genome as a template with specific primer pair 12 and 13. The PCR product were subcloned into *SmaI* site of pMobΩ1 (Saito and Awai, 2020) to make pMobΩ1/*desC* 5'using the hot fusion and the sequence was confirmed. DNA fragments downstream of both genes were amplified by PCR using as described above with specific primer pairs 14 and 15. The PCR products were subcloned into *ApaI* site of pMobΩ1/*desC* 5' using the hot fusion and sequence was confirmed. Resultant plasmids that contain flanking region of *desC* gene were

designated as pMobΩ1/*desC*. This vector then introduced into transformants *Synechocystis*/*desC1* and *Synechocystis*/*desC2* use same method as described previously for overexpression of *Nostoc* SO-36 and *Anabaena* 7120 *desC* genes.

The genomic DNA of the wildtype and candidates were used as templates for PCR genotyping with the primers described below and Hybridpol DNA polymerase (Bioline, London, UK). Genotyping of *desC1*/*2* insertion was performed using the primer pair # 12 and # 13 for the amplification of neutral site I and the primer pair # 1/3, 5/7 and # 2/4, 6/8 for *desC1* and *desC2* insertion of *Nostoc* SO-36 and *Anabaena* 7120 respectively. Genotyping of internal *desC* in *Synechocystis* 6803 was performed using the primer pair # 9/11 and # 18 for the amplification of full length of *desC*, the primer # 9/11 and # 10 for the detection of deletion of the central part of *desC* and the primer pair # 1/3, 5/7 and # 2/4. 6/8 for the amplification of external *desC1* and *desC2* gene from *Nostoc* SO-36 and *Anabaena* 7120 respectively.

3.2.3 Isolation of knock out mutants of *desC* genes in *Anabaena*

The author tried to knock out the *desC1*/*desC2* in *Nostoc* sp. strain SO-36, however, still no null mutant cannot be isolated. Then, to study the essentiality of both *desC* genes, the author try to to knock out the *desC1* (*all1599*) and *desC2* (*all4991*) gene in *Anabaena* which share similarity with *desC* genes in *Nostoc* sp. strain SO-36 and a model of filamentous cyanobacteria. The knock out vector of *desC* genes was constructed as follows. DNA fragments upstream of *desC1/2* were amplified by PCR using KOD polymerase (Takara Bio,

Shiga, Japan) using *Anabaena* wild-type genome as a template with primer pair #19/#23 and #20/#24. Amplicon were subcloned into *SmaI* site of pMobΩ1 to make pMobΩ1/*desC1/2* 5' using hot fusion and the sequence was confirmed. DNA fragments downstream were amplified by using as described above with primer pair #21/#25 and #22/#26. Amplicon were subcloned into *ApaI* site of pMobΩ1/*desC1*/*2* 5' using the hot fusion, the sequence then confirmed. Resultant plasmids that contain flanking region of *desC* genes were designated as pMobΩ1/*desC1* and pMobΩ1/*desC2*. The constructed plasmid vectors were introduced consecutively into the WT by the tri-parental mating method. This method was done by mixing the *E.coli* strain HB101 (pRL623) containing pMobΩ1/*desC1* or pMobΩ1/*desC2* and *E.coli* J53 (RP4) with wild-type *Anabaena* as described (Elhai and Wolk, 1988). After the mating process of *E.coli* and *Anabaena*, the mixture was plated on a nitrocellulose membrane on solid agar medium of BG-11 and incubated at 30°C under continuous light for 2 days. After the incubation, the membrane was transferred onto solid medium of BG-11 containing spectinomycin (20 μg/mL) and 5% sucrose to isolate candidates of the mutants. Resistant colonies were segregated on the BG-11 agar plates with spectinomycin (Xu et al., 2015). After 5 times of segregation, genome DNA was extracted from the candidates by glass bead methods (Kindle et al., 1991) and genotyped by PCR using the primers which described in **Table 3-2**.

Another aproach to study the complementation of both *desC* genes, a vector for *Nostoc* SO-36 *desC1* and *desC2* overexpression in *Anabaena* was constructed as the following sequence: DNA fragments of *desC1* and *desC2* were amplified from genomic DNA of wild-

type *Nostoc* SO-36 as a template using primer pairs (#31 and #32 for *desC1* and #33 and #34 for *desC2*). Both fragments were subcloned separately into the vector pSU101, a vector that introduces the desired gene by single homologous recombinant into neutral site *cyaA* of *Anabaena* (*all1118*) (Katayama and Ohmori, 1997). The DNA fragments of promoter *psbA* from *Amaranthus hybridus* (Elhai, 1993) were introduced into the *Bam*HI site and the *desC1*/*2* were introduced into *Kpn*I site by the hot fusion, then the sequence was confirmed. Resultant plasmids that contain *desC* genes from *Nostoc* SO-36 were designated as *pSU101*/*36desC1* and *pSU101*/*36desC2* and introduced into wildtype using tri-parental method which is previously described. After 5 times segregation in BG-11 solid agar medium with neomycin (20 μg/mL) and 5% sucrose, the genome DNA was extracted by glass bead methods (Kindle et al., 1991) and genotyped by PCR using the primers which is described in **Table 3-2**.

After the genome of 7120/36*desC1*/*2* was confirmed by PCR genotyping, the internal *desC* genes in 7120 then introduced by the knock out vector which is previously described in this section. After 5 times of segregation, genome DNA was extracted from the candidates by glass bead methods (Kindle et al., 1991) and genotyped by PCR.

The genomic DNA of the wildtype and candidates were used as templates for PCR genotyping with the primers described below and Hybridpol DNA polymerase (Bioline, Londone, UK). Genotyping of 7120/36*desC1*/*2* was performed using primers # 29 and # 30 for the amplification of *desC* genes from *Nostoc* in plasmid vector *pSU*101. Genotyping of gene disruption was performed using primers # 19 / # 23 and # 22 / #26 for amplification of

full length of *Anabaena desC1*/*desC2*, primer pair # 27 and # 28/29 for amplification of the spectinomycin resistance gene into *Anabaena* (WT and 7120/36*desC1*/*2*).

3.2.4 Growth curve, chlorophyll content, oxygen evolution rate, and chlorophyll fluorescence measurement of *Synechocystis* transformants

A spectrophotometer (UV-2600, Shimadzu) was used for growth curve analysis and chlorophyll content measurement. For growth curve analysis, both wildtype and transformant cells were moved into fresh BG-11 at an initial optical density of 0.1 with a wavelength of 730 nm. For chlorophyll content, cells were extracted by 100% methanol and the contents were calculated as described (Arnon et al., 1974).

Photosynthetic activity was analyzed by oxygen evolution rate using 2mL cell suspension at an initial optical density of 0.1 with the Clark electrode (Hansatech Instruments, Norfolk, UK). *Synechocystis* cells were illuminated with a LED lamp with infrared cutoff filters. The fluorescence of chlorophyll was measured by a Dual-Pam system (Heinz Walz GmbH, Germany) in cell suspensions at OD_{730} of 1.0. All the samples were acclimated to the dark environment for 30min-1 hour before the measurement. The Fv/Fm and Fv'/Fm' parameters were measured after 2-min illumination of either 34 μmol/m^2s (OL) or 212 μmol/m^2s (HL) (Kobayashi et al., 2020).

3.2.5 Lipid and fatty acid composition analysis

Total lipids of cyanobacteria were extracted by using chloroform:methanol (2:1) solution for 30 minutes as described by Bligh and Dyer (1959). Lipid profiles were analyzed

by TLC with a solvent system chloroform/methanol/25%ammonium hydroxide (65:35:5) for cyanobacteria as described by Sato and Murata (1988). These separated lipids were stained with the primuline solution (0.01g primuline diluted in 80% acetone) and observed under UV lights. For positional analysis of fatty acids, each lipid was isolated and treated with lipase from *Rhizopus oryzae* (Sigma Aldrich) and the lipid profiles were analyzed with a solvent system chloroform/methanol/dH_2O (170:30:2) as described by Sato and Murata (1998). For visualization of lipids of cyanobacteria, TLC plates were sprayed with the anthrone solution (a mixture of 0.05 g anthrone and 1 g thiourea diluted in 100 ml of 66% sulfuric acid) and heated at 120^{o}C at 5 min. Fatty acid composition of isolated lipids was analyzed with a gas chromatography-flame ionization detector (GC-FID) on a capillary column (BPX90, 60 m x 0.25 mm, SGE Analytical Science) as described by Awai et al. (2014).

Table 3.1. List of plasmid and vector used in this study

No.	Plasmid	Description	Reference
1	pMobΩ1	Vector containing spectinomycin / streptomycin antibiotic resistant cassette	(Saito and Awai, 2020)
2	pMobΩ1/*desC*	Vector constructed from pMobΩ1 with insertion of upstream (*Sma*I site) and downstream (*Apa*I site) fragments of *sll0541* into knock out *desC* gene of *Synechocystis*	This study
3	pMobΩ1/*desC1*	Vector constructed from pMobΩ1 with insertion of upstream (*Sma*I site) and downstream (*Apa*I site) fragments of *all1599* to knock out *desC1* gene in *Anabaena*	This study
4	pMobΩ1/*desC2*	Vector constructed from pMobΩ1 with insertion of upstream (*Sma*I site) and downstream (*Apa*I site) fragments of *all4991* to knock out *desC2* gene in *Anabaena*	This study
5	pSU101-*cyaA*	Vector containing kanamycin/neomycin-resistant cassette for expression in *Anabaena*	(Higo et al., 2018)

6	pSU101-*cyaA/desC*	Vector constructed from pSU101-*cyaA* with insertion of *desC* gene (*sll0541*) from *Synechocystis*	This study
7	pSU101-*cyaA/desC1*	Vector constructed from pSU101-*cyaA* with insertion of *desC1* gene from *Nostoc* SO-36	This study
8	pSU101-*cyaA/desC2*	Vector constructed from pSU101-*cyaA* with insertion of *desC2* genes from *Nostoc* SO-36	This study
9	pSEM3	Vector containing erythromycin-resistant cassette for strong expression in *Synechocystis*	(Matsumoto and Awai, 2020)
11	pSEM3*desC1*	Vector constructed from pSEM3 with insertion of *desC1* gene from *Nostoc* SO-36 or (*all1559*) *Anabaena* 7120	This study
12	pSEM3*desC2*	Vector constructed from pSEM3 with insertion of *desC2* gene from *Nostoc* SO-36 or (*all4991*) *Anabaena* 7120	This study

Table 3.2. List of primers used in this study

No.	Primer	Sequence (5'→3')
1	*Nostoc* SO-36 *desC1* Fw	ATTATAACCAAATGCCCATGACAATTGCTACTTCAACC
2	*Nostoc* SO-36 *desC1* Rv	TCGAATTCCTGCAGCCCTAACTACTTTTTGTTGGGG
3	*Nostoc* SO-36 *desC2* Fw	ATTATAACCAAATGCCCATGACCGCAAATTTTGGGGCG
4	*Nostoc* SO-36 *desC2* Rv	TCGAATTCCTGCAGCCCTTAGCCGTGAGTTGCACCTTG
5	*all1599 desC1* Fw	ATTATAACCAAATGCCCATGACAATTGCTACTTCAAC
6	*all1599 desC1* Rv	TCGAATTCCTGCAGCCCTTACTGCTTTTTGTCTGCTAG
7	*all4991 desC2* Fw	ATTATAACCAAATGCCCATGACCGTAAAACATCTGGC
8	*all4991 desC2* Rv	TCGAATTCCTGCAGCCCTCAACCTTGATTCATTCTTTGTG
9	*sll0541* desC1 Fw	GCTACCGGGCTGCAGCTAGGCTTTGTTGGCCATCG
10	*sll0541* desC1&desC2 Rv	TGCAAGCTTGATATCGTTGGTAAATCCCGGCGATG
11	*sll0541* desC2 Fw	CAGAGTGGGCTGCAGCTAGGCTTTGTTGGCCATCG

12	Neutral site *Synechocystis* Fw	ACTAGTGGATCCCCCGAGTTGTAGTCGGCA
13	Neutral site *Synechocystis* Rv	GCGAATTGGGTACCGCAGAACGACCAATG
14	Neutral site *Synechocystis* mid	TTGGACATTGCTGCCCAGTG
15	*desC* neutral site upstream Fw	GAACTAGTGGATCCTGGGGCAGTCAATCTATAGG
16	*desC* neutral site upstream Rv	TCGAATTCCTGCAGCATCGCCGGGATTTACCAAC
17	*desC* neutral site downstream Fw	CGTCGACCTCGAGGGGATGGCCAACAAAGCCTAG
18	*desC* neutral site downstream Rv	GGGCGAATTGGGTACCAAAACTTCTCCGGATTGC
19	*desC1* neutral site upstream Fw	GAACTAGTGGATCCCCCAGTTGTAGCGATGAGGGTG
20	*desC1* neutral site upstream Rv	TCGAATTCCTGCAGCCCGGTAGCTGTCCTAGTGTTTG

21	*desC1* neutral site downstream Fw	CGTCGACCTCGAGGGGGTAATACCCAAACCACCAG
22	*desC1* neutral site downstream Rv	GGGCGAATTGGGTACCGTCCAGCTACATAACCACCAG
23	*desC2* neutral site upstream Fw	GAACTAGTGGATCCCCCGACGCTCTCTATAACAGGAACG
24	*desC2* neutral site upstream Rv	TCGAATTCCTGCAGCCCACTCTGCCACCCATATGTGG
25	*desC2* neutral site downstream Fw	CGTCGACCTCGAGGGGGCAACAGCAAACCCAATGCTG
26	*desC2* neutral site downstream Rv	GGGCGAATTGGGTACCGGCGGTAAATCGGCTACTTC
27	Spectinomycin site Fw	CGAGGCCAAGCGATCTTC
28	*desC1* Spectinomycin site Rv	TGTAAAACGACGGCCAG

28	*desC2* Spectinomycin site Rv	ACTAGTGGATCCCCCATGCAGAAATAATTCCAC
29	pSU101 site Fw	GTCTGACGCTCAGTGGAAC
30	pSU101 site Rv	TCACAATGCGGGTGTCTGG

3.3 Results

3.3.1 Complementation of Δ*desC/6803* with *desC1* and *desC2* from filamentous cyanobacteria

The desaturation at *sn*-1 position was generally catalyzed by the *desC* gene in all groups based on fatty acid composition of cyanobacteria. However, group 1 (e.g., *Synechococcus* sp. PCC 6301 and *Synechococcus* sp. PCC 7942) was found to desaturate both *sn*- position (Murata and Wada, 1995). DesC in Group 2 or in filamentous cyanobacteria (e.g., *Nostoc* and *Anabaena*) has a two *desC* genes. In *Nostoc* SO-36 it was reported that DesC2 has the specific function to desaturate C16:0 to C16:1 (9) at *sn*-2 position and DesC1 desaturate C18:0 to C18:1 (9) at *sn*-1 position, also they reported there is an alteration of C16:0 at *sn*-1 position in the transformants *Synechocystis* sp. PCC 6803 after insertion of *desC2* gene (Chintalapati et al., 2006). DesC in group 3-4 (e.g., *Spirulina platensis* and *Synechocystis* sp.) was found to desaturate only at *sn*-1 position (Sakamoto et al., 1994; Murata and Wada, 1995). To confirm the specific function of DesC in Group 4, firstly, the author expressed the *desC* genes from *Nostoc* SO-36 and *Anabaena* 7120 in *Synechocystis* sp. PCC 6803 under strong promoter *psbA2* (Matsumoto and Awai, 2020). The *desC1* and *desC2* genes of *Nostoc* SO-36 and *Anabaena* 7120 were inserted into genome of *Synechocystis* sp. PCC 6803 which is confirmed in **Figure 3-1**. A PCR analysis using primers external to *desC* resulted in two PCR fragments of different sizes. The wildtype produce 1426-bp fragment, and the transformants produced a larger 3389-bp / 3386-bp for *desC1* and 3422-bp / 3425-bp for *desC2* from *Nostoc* SO-36 and *Anabaena* 7120 respectively because of the presence of the

erythromycin resistance cassette. PCR with primer that annealed to the middle region of *desC* produce an amplicon only from the wild type genome. These results indicated that the *desC* genes is introduced in all genome copies of the transformant cells. Isolated transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120) showed no obvious phenotype indicating that introduction of *desC1* or *desC2* is not toxic to *Synechocystis* 6803. The author use this transformants to knocked out the internal *desC* gene in *Synechocystis* 6803. After numerous rounds of segregations with antibiotics, null mutant of Δ*desC/*6803 were segregated as shown in **Figure 3-2**. A PCR analysis uising primers extrernal to *desC* resulted in two PCR fragments of different sized. The wild type produced a 1841-bp fragment, and a mutants produced a larger 2985-bp and 2981-bp for *desC1* from *Nostoc* SO-36 and *Anabaena* 7120, and not in *desC2* from each candidates because of the presence of the spectinomycin/streptomycin resistant cassette. PCR with primer that annealed to the middle region of *desC* #9/11 and #10 produced an amplicon only from the wildtype genome, as the necessary region was not present in the mutant genome. PCR with primer # 1/2 and # 5/6 produced an amplicon from external gene *desC1* from *Nostoc* SO-36 and *Anabaena* 7120. Those results indicate that no wild type copies of *desC* were present in the mutant cells. The isolated transformants (Δ*desC/desC1*-7120/N36) also showed no obvious phenotype indicating that replacement with external *desC1* can complemented the function of *desC* in *Synechocystis* sp. PCC 6803 but not *desC*2 from both strains of *Nostoc* SO-36 and *Anabaena* 7120.

In **Figure 3-3A** and **3B**, growth rate and chlorophyll content was analyzed in wildtype, transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120), and mutants (Δ*desC/desC1*-7120/N36), the results showed no significant different between the strain, there is a slight accumulation in transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120), and slight retardation in the knockout *desC* mutants (Δ*desC/desC1*-7120/N36) as shown in the figure but the difference is not significant. Due to the different of growth rate and chlorophyll content between mutant cells and wildtype, the author analyzed the photosynthetic variables such as oxygen evolution and chlorophyll fluorescence of wildtype, transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120), and mutants (Δ*desC/desC1*-7120/N36). The rates of photosynthetic oxygen evolution results were monitored to understand the overall physiological process in the cultures after genomic change of wildtype, transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120), and mutants (Δ*desC/desC1*-7120/N36). As a results in **Figure 3-3C**, transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120) showed a slightly higher oxygenic rate compared to the wildtype and mutants (Δ*desC/desC1*-7120/N36) showed a slight retarded of oxygenic rate but the difference is not significant. These results suggest that mutants (Δ*desC/desC1*-7120/N36) has some defects inside its cells but is still can managed to grow photoautotrophically. Further analysis by comparison of chlorophyll fluorescence parameter that the maximum quantum yield of PSII (Fv/Fm) (**Figure 3-3D**) in dark-adapted shows that transformants 6803/*desC2*-7120 and mutants (Δ*desC/desC1*-7120/N36) shows less accumulation compared to wildtype, which indicates the photoinhibition was occurred

inside the cells. This results was further proved by the value of quantum yield of PSII (Fv'/Fm') parameter at the different light conditions which suggest that the stress by light irradiation can be observed even at the optimum growth condition. Based on these results., the addition of external gene and complementation can affect the photochemical balance which leads to the sensitivity of cells to light irradiation. Then, photosynthetic activity of mutant and transformants was showed that no significant difference while the difference between transformants with external DesC both from *Anabaena* 7120 and *Nostoc* SO-36. This results, indicating that mutants (Δ*desC/desC1*-7120/N36) still can complement the internal *desC* gene in *Synechocystis* and transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120) can change the photochemical balance inside the cyanobacteria's cells. These results remain a question, is external gene change the desaturation of fatty acid composition inside the transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120) and mutants (Δ*desC/desC1*-7120/N36) cell?

To answer the previous question, fatty acid composition of each membrane lipids in *Synechocystis* WT, transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120) and mutants (Δ*desC/desC1*-7120/N36) were also quantified by GC-FID. In **Figure 3-4**, membrane lipids in transformants 6803/*desC2*-7120 shows an alteration of C16:0 in MGDG and higher composition of C16:1 (9) in major galactolipids MGDG and DGDG. The accumulation of C16:1 (9) also occurred in minor lipid, PG which is previously observed to take a role in the stabilization of the photosystem. To obtain the specific function of both *desC* genes, a lysolipid analysis used *Rhizopus lipase* was done to show the fatty acid composition at *sn*-1

and *sn*-2 positions **Figure 3-5**. Mutants' strains (Δ*desC/desC1*-7120/N36) showed the fatty acid composition at C16:1 of candidates was lower than wildtype *Synechocystis* 6803 and transformant in all membrane lipids.

Figure 3-5A showed wild-type *Synechocystis* 6803 and negative control do not synthesize unsaturated fatty acid at the *sn*-2 position. The alteration of saturated fatty acid C16:0 and accumulation C16:1 (9) showed that DesC proteins also took a role in the synthesis of unsaturated fatty acids at *sn*-2 position especially DesC2 (Chintalapati et al., 2006). In **Figure 3-5B** DGDG also showed the accumulation of C16:1 (9) specifically in the *sn*-2 position. At DGDG the accumulation of C18:1 (9) can be shown especially at the *sn*-1 position. These results can explain the major function of DesC protein which is to introduce 1 double bond at C18 (Murata and Wada, 1995). In **Figure 3-5C,** SQDG, no significant difference at both *sn*-position, SQDG was previously observed (Pittera *et al.*, 2018) which has a quite different fatty acid composition from the other two galactolipids, MGDG and DGDG. In **Figure 3-5D**, PG, the accumulation of C16:1 (9) at *sn*-2 position occurred at transformant *desC2* 7120. It was previously observed that C16:1 (9) in PG has an important role in photosynthetic activity both in PSII and PSI due to its function to stabilize the complexes in thylakoid membrane (Kobayashi et al., 2017) due to the interaction to phytol chains of chlorophyll and hydrophobic domains of proteins in the complexes especially fatty acids chain at *sn*-2 position (Endo *et al.*,2021). Also, C18:1 (9) in transformant *desC2* 7120 and *Nostoc* SO 36 were accumulated. As for the mutants, it was analyzed that desaturation

activity to synthesize the C16:1 (9) was lower compared to the transformant at the *sn*-2 position and the fatty acid composition of both candidates was not significantly different.

Fatty acid compositions of all strains showed that mutants *desC*/6803 has lower unsaturated fatty acid content compared to wildtype these results might answer the reason of alteration in chl content and photosynthetic activity in previous question. It can be concluded external *desC* genes in mutants *desC*/6803 can maintain the complementation of desaturation inside the cell.

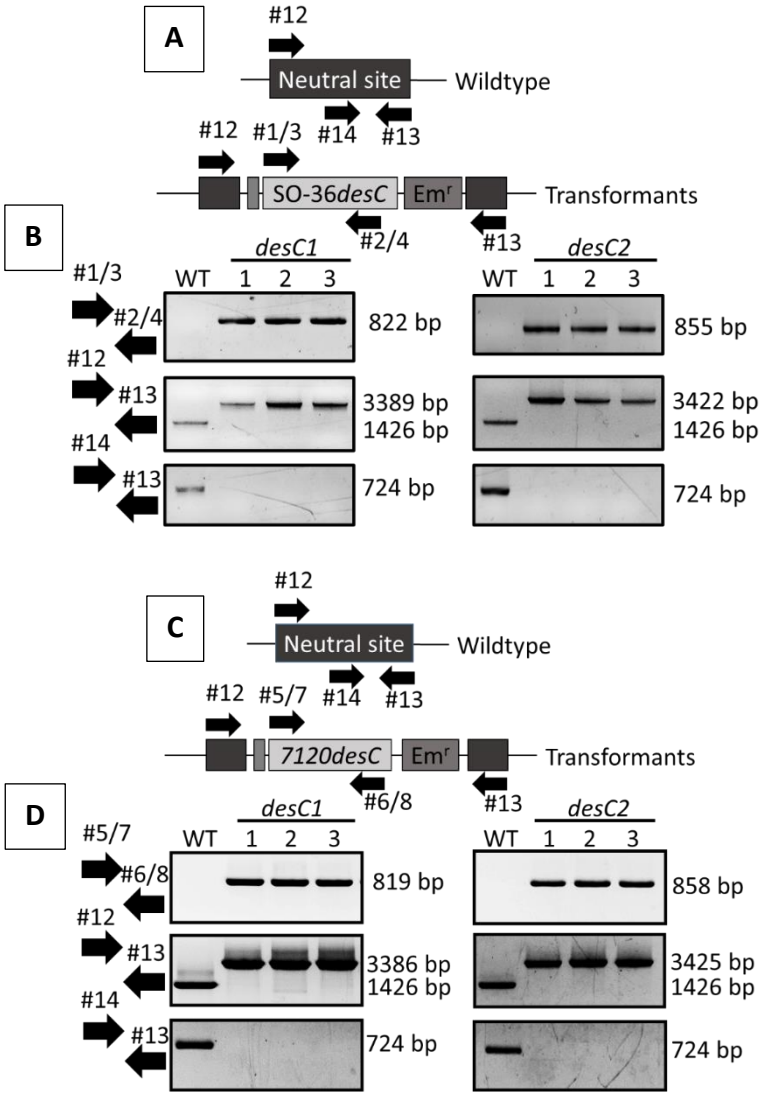

Figure 3-1. (A/C) Schematic representation of the insertion site in *Synechocystis* sp. PCC 6803. **(B/D)** Genotype analysis of transformants. Each fragment was amplified by PCR with the indicated primer pairs (numbers and arrow in **A**/**C** and **B**/**D**) and visualized by agarose gel electrophoresis.

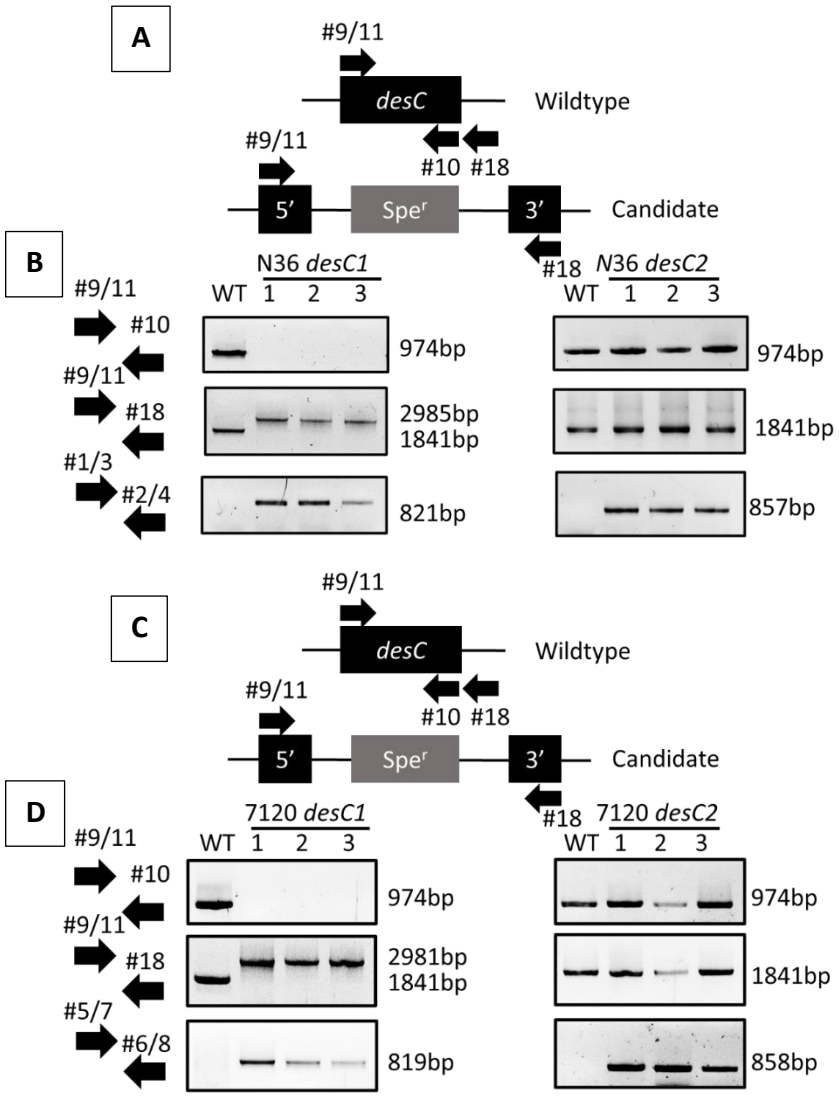

Figure 3-2. (A/C) Schematic representation of the insertion site in *Synechocystis* sp. PCC 6803. **(B/D)** Genotype analysis of mutants. Each fragment was amplified by PCR with the indicated primer pairs (numbers and arrow in **A/C** and **B/D**) and visualized by agarose gel electrophoresis.

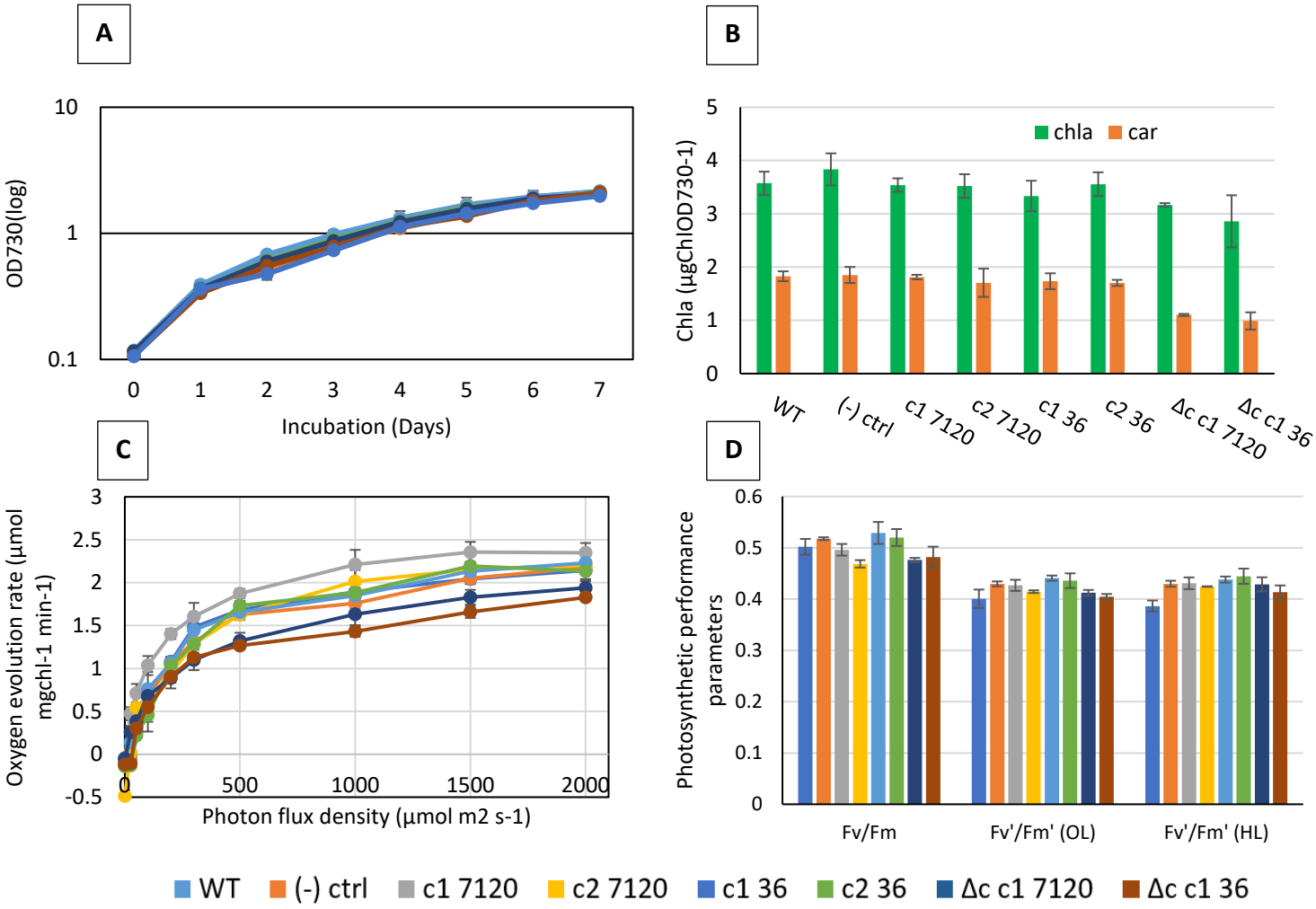

Figure 3-3. Physiological analysis of transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120) and mutants (Δ*desC/desC1*-7120/N36) (**A**) Growth rate under photoautotrophic conditions. Initial optical density of 730 nm was set to 0.1. (**B**) Cellular chlorophyl content was extracted with methanol. (**C**) Oxygen evolution rate as function of light intentsity. (**D**) maximum efficiency of PSII photochemistry of dark-adapted cells (Fv/Fm) and light-adapted cells (Fv'/Fm') at each given light conditions. Error bars indicate the SD based on three independent experiments.

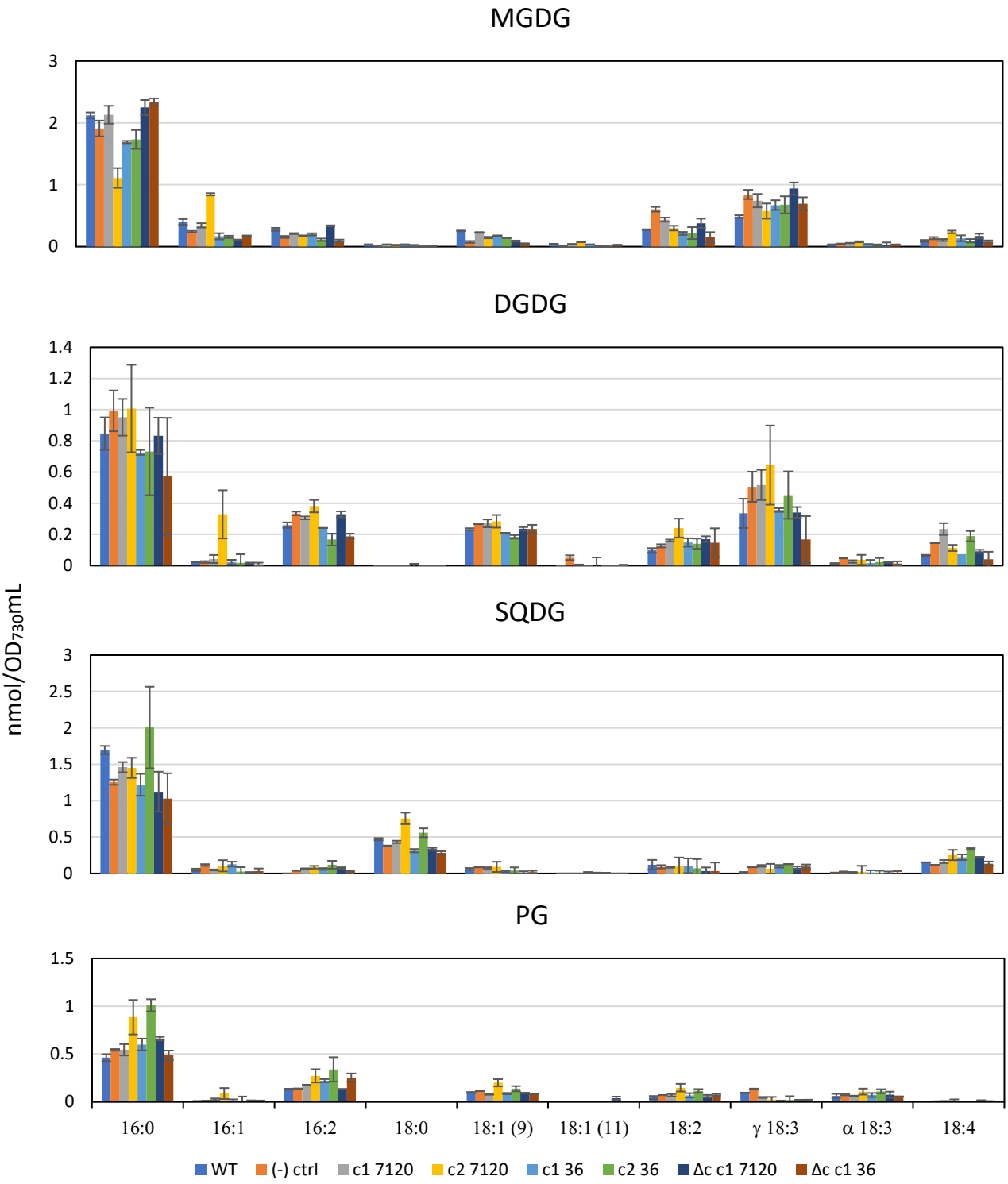

Figure 3-4. Fatty acid composition of wildtype *Synechocystis* 6803 and the transformants

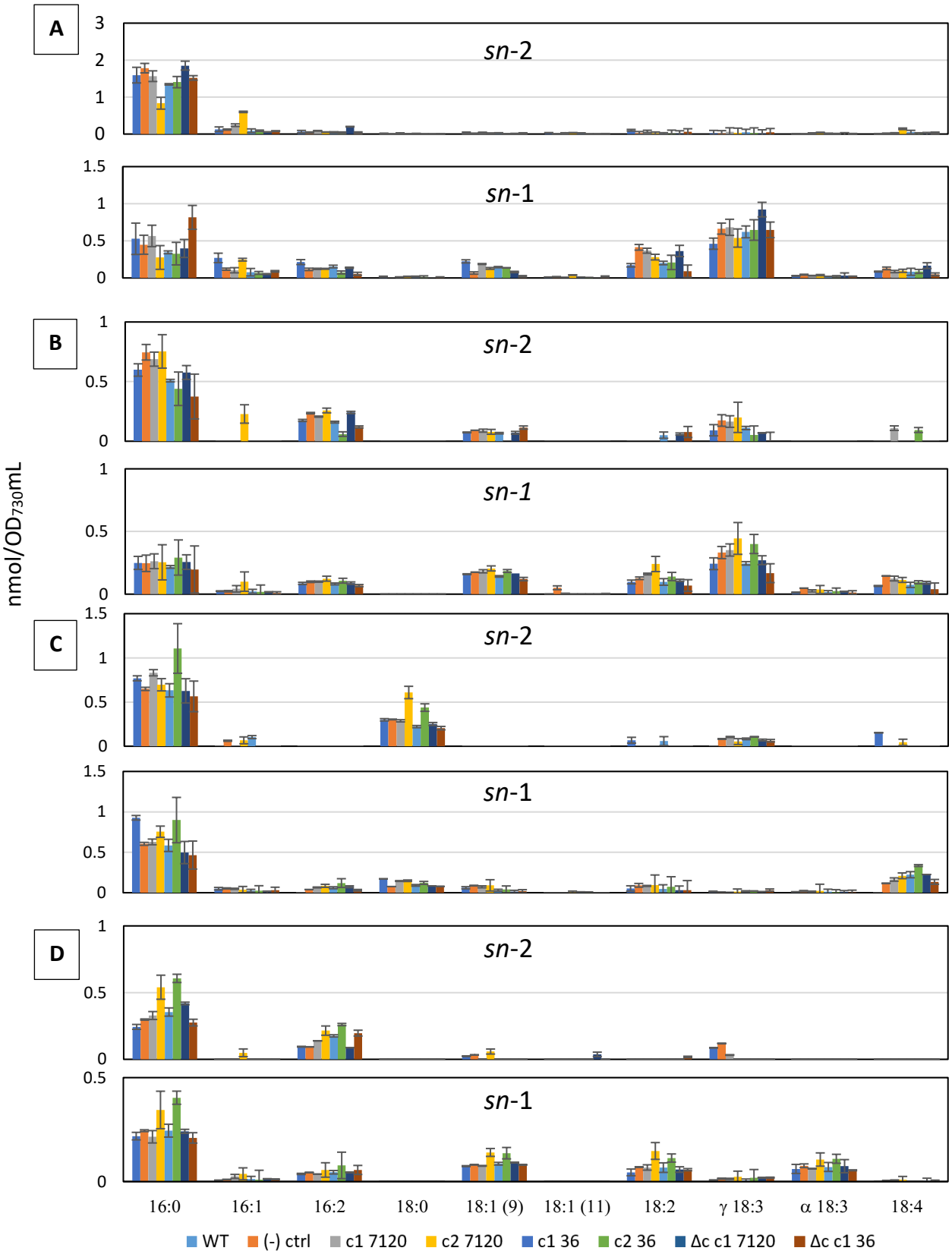

Figure 3-5. Lysolipid analysis of (**A**) MGDG (**B**) DGDG (**C**)SQDG and (**D**) PG.

3.3.2 Physiological function of *desC1* and *desC2*

To know the specific function of *desC1* and *desC2* in *Nostoc* SO-36, the author tried to knock out *the* genes using tri-parental mating method (Elhai and Wolk, 1988), however the knock-out was not successful and partial knock-out mutant cannot be isolated. This might be due to the contamination in the strains. Another approach for this analysis was done by knockout *desC1* and *desC2* in model filamentous cyanobacteria *Anabaena* sp. PCC 7120 due to its similarity with *Nostoc*. *Anabaena* share 80.0% similarity of amino acid sequence of these proteins with *Nostoc*. However, the results showed that after several times of segregation, knock out ouf each *desC* gene is accomplished only in some genome copies of each candidate and these results showed that both *desC* genes in *Anabaena* is essential (**Figure 3-6**). Another approach was to introduce the external *desC* gene in wildtype *Anabaena* 7120 which is previously described. In **Figure 3-7**, *desC1* and *desC2* from *Nostoc* SO-36 was introduced into *KpnI* site of pSU101 (Higo et al., 2018; Higo and Ehira, 2019) unders strong promoter *psbA* from *Amaranthus Hybridus* (Elhai, 1993). External *desC* genes was expressed under normal growth condition which is confirmed in **Figure 3-8**. It showed that both *desC* gene cannot complement each other and *desC1* from *Nostoc* still cannot be complement with the internal *desC1 in Anabaena* 7120. (*desC2* from *Nostoc* can complement with the internal *desC1* in *Anabaena* 7120).

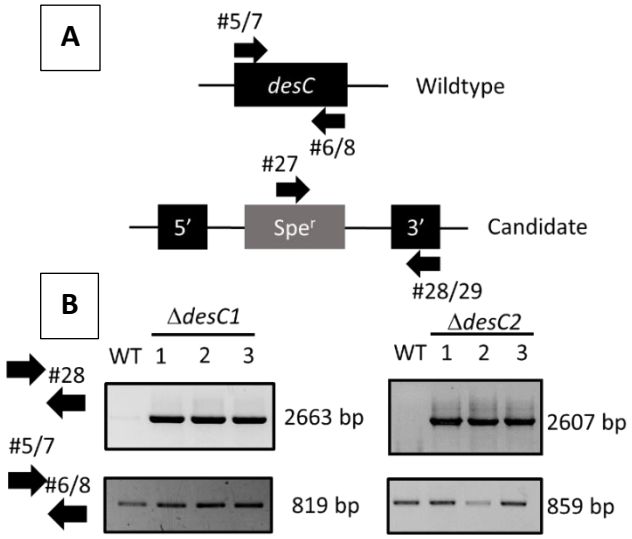

Figure 3-6. (A) Schematic representation of the knock out in *Anabaena* sp. PCC 7120. **(B)** Genotype analysis of candidates. Each fragment was amplified by PCR with the indicated primer pairs (numbers and arrow in **A** and **B**) and visualized by agarose gel electrophoresis.

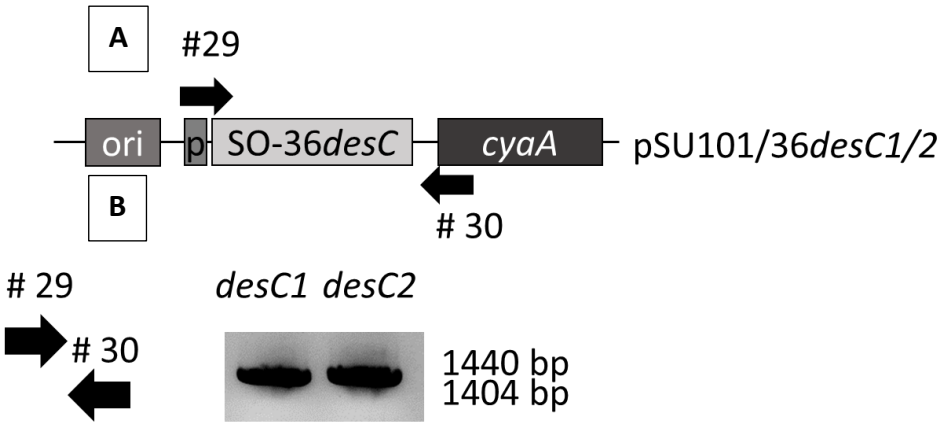

Figure 3-7. (A) Schematic representation of the insertion site in *Anabaena* sp. PCC 7120. **(B)** Genotype analysis of candidates. Each fragment was amplified by PCR with the indicated primer pairs (numbers and arrow in **A** and **B**) and visualized by agarose gel electrophoresis.

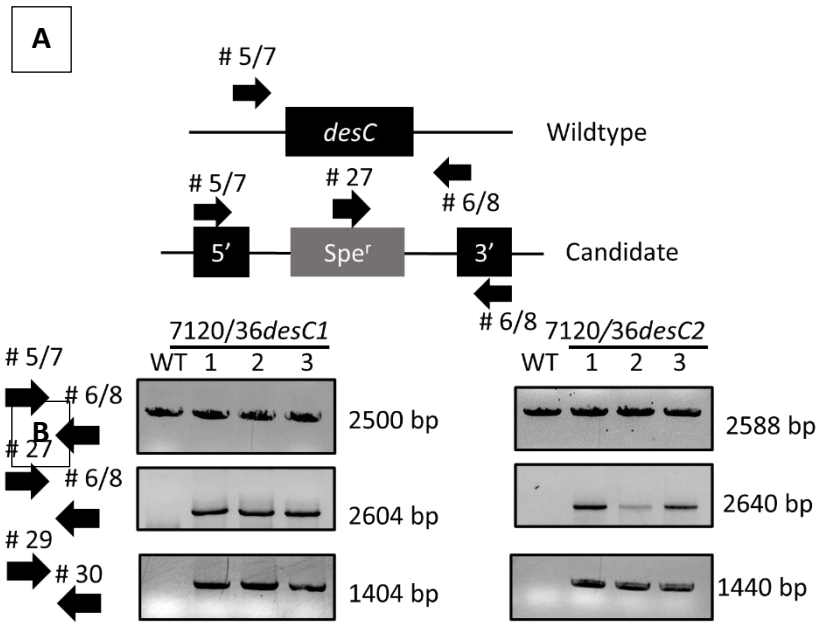

Figure 3-8. (A) Schematic representation of the site transgene candidates of mutant in *Anabaena* sp. PCC 7120. **(B)** Genotype analysis of *desC1* and *desC2* in candidates of mutant *Anabaena* 7120 after insertion of external *desC* gene from *Nostoc* SO-36. Each fragment was amplified by PCR with the indicated primer pairs (numbers and arrow in A and **Figure 8A**) and visualized by agarose gel electrophoresis.

3.4 Discussion

3.4.1 DesC1 from filamentous cyanobacteria can complement DesC in *Synechocystis* 6803

It was previously studied that inactivating of DesC in *Synechocysits* 6803 suggested to eliminate an unsaturated fatty acids process from membrane lipids (Sakamoto et al., 1994; Tasaka et al., 1996). In this study, the author analyze the specific function of both DesC protein in *Nostoc* SO-36 and *Anabaena* 7120, by knock out the gene in the wild type *Synechocystis* 6803 and its transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120). In wild type, after introduced by a knock out vector, *desC* gene band in genotyping by PCR even after segregated several times was observed. These phenomena are occurred due to inhibition of unsaturated fatty acid synthesis (Sakamoto et al., 1994). So, *desC* gene in Group 4 (*Synechocystis* 6803) was essential and cannot be knock out. In transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120), introducing a knock out vector was purposed to identify neither *desC* gene origin in *Synechocysits* 6803 can be replaced by both *desC*1 and *desC*2 from *Anabaena* 7120 or *Nostoc* SO-36. The candidates of mutant in transformant were completely segregated with *desC*1 gene from *Anabaena* 7120 or *Nostoc* SO-36 and not with *desC*2 gene. From this result, it was purposed that DesC from *Synechocystis* 6803 can be replaced by DesC1 from *Anabaena* 7120 or *Nostoc* SO-36. Also, due to the similarity in amino acid sequence, these two proteins share some similar functions to desaturate saturated fatty acids at *sn*-1 position especially for C18:0 to C18:1 (9). Since it was observed that transformant 6803/*desC2*-N36/7120 cannot replace the *desC*

in *Synechocystis* 6803. It might be due to different functions between *desC1* and *desC2* which cannot be complemented to each other. This is the first example, to the author knowledge, of totally effective manipulation of the study of essentiality of the unsaturation of fatty acids by genetic engineering. The mutated strains that the author produced are useful tools to example the study of essentiality of DesC genes by the complementation.

The growth rate and chlorophyll/carotenoid analysis between wildtype, transformants, and mutants in *Synechocystis* 6803, showed no significant difference due to the insertion of external *desC* and slightly retarded after the inactivation of original *desC* in *Synechocystis* transformants (6803/*desC1*-N36/7120). The measurements of oxygen evolution rate can provide an indication of how well of the PSII function and how many electrons are being released into the Z-scheme at a particular light intensity (Schuurmans et al., 2015). Compared to the wildtype, the oxygen evolution rate of transformants was higher and the mutants showed slightly retarded. Those results showed in the transformants 6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120 has an improvement while in mutants (Δ*desC/desC1*-7120/N36) showed that the functions were slightly retarded. Another analysis to estimate the relative efficiency of oxygenic phototropic growth on light was the measurement by Dual-PAM analysis. The photosynthetic activity of mutant and transformants was showed no significant difference while the difference between transformants with external DesC both from *Anabaena* 7120 and *Nostoc* SO-36 show higher quantum efficiency both in PSII and PSI, while the mutant whose original *desC* in 6803 was deleted, showing complimentary with wildtype and lower quantum efficiency.

It can be concluded that both DesC1 from *Anabaena* 7120 and *Nostoc* SO-36 can complement the function of the original disc in *Synechocystis* 6803. The difference in fatty acid content in MGDG has no impact on PSII and PSI activity, it might be the remaining MGDG is sufficient to maintain the structure and function of the photosystem. As previous reports in *Arabidopsis mgd1-1* mutant showed a decrease of PSII activity (F_V/F_M) by 40% has no impact on PSII activity (Aronsson et al., 2008).

From the fatty acid analysis of transformants and mutants, it showed that 6803/*desC2*-7120 can desaturate C16:0 into C16:1 (9) at the *sn*-2 position at galactolipids and *sn*-1 position at MGDG as major lipids in cyanobacteria. These results could be compared to previous research in transformants *desC2* in *Synechocystis* 6803. Also, at PG, transformants 6803/*desC2*-7120 showed an accumulation of C16:1 (9) which is related to the stabilization of the complexes in thylakoid membrane (Endo et al., 2022). These results might be an answer for the improvement of photosynthetic activity as previously described in this research. However, in this research, when the author tried to compare with the transformant 6803/*desC1/2*-N36, it showed different results from the previous research (Chintalapati et al., 2006). The author speculates that the growth conditions of the cultures used in this study had affected their fatty acid composition, leading to synthesis/absence of C16:0;C18:0 to C16:1;C18:1. However, they also approached that DesC2 from *Nostoc* SO-36 can also catalyze the desaturation at the *sn*-1 position which is also found in this research.

3.4.2 Both DesC in filamentous cyanobacteria is essential

Several studies tried to knock out the null mutant of *desC* neither in *Synechococcus* sp. PCC 7002 or *Synechocystis* sp. PCC 6803 (Tasaka et al., 1996; Mendez-Perez et al., 2014). In this study, the author found that both DesC1 and DesC2 in model filamentous cyanobacteria *Anabaena* sp. PCC 7120 is essential. When the attempt to knock out each gene the author cannot succeed to get the null mutant. In another word, both DesC cannot complement each other. These results showed in **Figure 3-8** that each *desC* gene cannot complement each other. In conclusion that *desC1* and *desC2* have a specific function that is responsible for unsaturation in substrate specificity in *sn*-1 and *sn*-2 respectively.

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Chapter IV

General Discussion

4.1 Mechanism of psychrotolerant filamentous cyanobacteria upon environmental stress

In cyanobacteria, iron limitation, light stress, salt stress, and nitrogen limitation among others all initiate expression of groups of specific genes in order to alleviate the cellular stress that these environmental pressures incur. (Billis et al., 2014; Kopf et al., 2015; Choi et al., 2016). Since most cyanobacteria from cryosphere do not grow preferentially at low temperatures, they are likely to be experiencing constant stress in their environment. The mechanism allowing cold tolerance are common throughout the cyanobacterial phylum e.g. the production of EPS (Pereira et al., 2015) and fatty acid desaturation (Chi et al., 2008), differences may exist in the way these shared characteristics are regulated to account for the increased levels of expression required in cold environments.

In this study, the author used filamentous cyanobacteria *Nostoc* sp. strain SO-36 which was isolated from Bryophyte community on the sunny slope in Antartica and has a possibility to uptake some metabolites released from Bryophyte plants in order to survive in harsh environment. The author also found that within a low temperature, *Nostoc* SO-36 can survive and excrete a polysaccharide, which is possible it was an EPS. EPS are molecules with a great ecological significance related to both organism survival and ecological fitness. These polysaccharide excretions represent a physical barrier protecting cells from harmful

agents and/or environmental constrains and serve in a wide array of physiological process including cell adhesion, cell-to-cell interaction and biofilm formation.(Rossi and De Philippis, 2016).

The author found the morphology of *Nostoc* SO-36 strains was make a clamping under microscope. This probably due to the cell stress which can excrete the polysaccharide. Nutrient availability is a paramount factor of EPS production. The combination of light intensity and temperature have a synergistic effect as temperature affects light inhibition. Light intensity is positively correlated to carbohydrate synthesis in cyanobacteria(Dephilippis et al., 1992; Carvalho et al., 2009). Temperature affects nutrient uptake, cell membranes, and oxygen evolution activity and thus supposedly also biosynthetic process. Temperature may have influenced the molecular weight of the secreted polymers(Lu and Vonshak, 2002).

In this study, the author sequenced the complete genome of Antarctic cyanobacterium *Nostoc* sp. strain SO-36. By the identifying key genes of interest within the genome, transcriptomic studies can be targeted towards genes of interest and their putative regulatory network to establish how cold tolerant cyanobacteria might be reacting to their environment at molecular level.

4.2 Desaturation is essential to produce the unsaturated fatty acids and its possibility to cope in harsh environment

In previous section, It is also well known that fatty acid desaturation is essential for low temperature tolerance through keeping membrane fluidity and recovery of photosynthesis from low-temperature photoinhibition (Gombos et al., 1991,1994; Wada et al., 1994). Chintalapati et al. (2007) reported that when the growth temperature shifted from 25℃ to 10℃, *Nostoc* SO-36 showed an increase in the content of tri-unsaturated fatty acid [C18:3(9,12,15)] of membrane lipids. They also revealed that transcript levels of genes for fatty acid desaturase, *desA*, *desB*, *desC*, and *desC2* are constitutively expressed regardless of the growth temperature. These results suggest that the cold adaptation in *Nostoc* SO-36 is not simply regulated at the transcription level, but also at post-transcriptional or translation level. These types of regulations might be important not only for the fatty acid desaturation, but also for other mechanisms in the *Nostoc* SO-36 to acquire phychrotolerance. In Antarctic *Nostoc* sp., constitutive expression of desaturase gene has been observed rather than being upregulated upon temperature reduction as is seen in template lineages (Chintalapati et al., 2007). Identifying how these processes are regulated there for the key to explaining mechanism of long-term cold tolerance.

In this study, the author analyzes the first desaturation in cyanobacteria DesC. It was observed that *desC* gene in Group 4 (*Synechocystis* 6803) was essential and cannot be knock out. These phenomena are occurred because the synthesis of unsaturated fatty acid will be inhibited (Sakamoto et al., 1994). To analyze the specific function of both DesC protein in *Anabaena* 7120 and *Nostoc* SO-36, the *desC* from *Synechocystis* 6803 was disrupted by knock out the gene in the transformants. This analysis also purposed to identify

neither *desC* gene origin in *Synechocysits* 6803 can be replaced by both *desC*1 and *desC*2 from *Anabaena* 7120 or *Nostoc* SO-36. The candidates of mutant in transformant were completely segregated with *desC*1 gene from *Anabaena* 7120 or *Nostoc* SO-36. and not with *desC*2 gene. From this result, it was purposed that DesC from *Synechocystis* 6803 can be replaced by DesC1 from *Anabaena* 7120 or *Nostoc* SO-36. Also, due to the similarity in amino acid sequence, these two proteins share some similar functions. Physiological change in *Synechocystis* WT, transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120), and mutants (Δ*desC/desC1*-7120/N36), electron and oxygen are required to activate the insertion of lipid desaturase enzyme into a catalytic site which comprises a histidine-rich box including a nonheme iron center. it can be demonstrated that the initial rate of photodamage linearly increases with light intensity even above the saturation of photosynthetic electron transport. The wildtype and transformants (6803/*desC1*-N36/7120 and 6803/*desC2*-N36/7120) shows no significant difference, the transformants 6803/*desC2*-N36/7120, and knocked out *desC* mutant Δ*desC/desC1*-7120/N36 shows lower activity might be due to limited light condition. As for the respiratory activity, the large variability among the respiration values is due to the lack of statistically significant differences regarding the measured respiration among the light/dark intervals at any given light. These results might be explained that *Synechocystis* share components of the photosynthetic and the respiratory electron transport chain which is previously (Avendano-Coletta and Schubert, 2005). The lower O_2 activity in the transformants and mutant can be related to the lower fatty acid content as shown in the fatty acid analysis. The author was tried to

isolate null mutant in *Nostoc* SO-36 but still not succeeded. This probably due to different mechanism with *Anabaena* 7120.

4.3 Reference

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