博 士 論 文（要約）

Biosynthesis and regulation of extracellular polysaccharides in cyanobacteria.

（シアノバクテリアにおける細胞外多糖の合成・制御機構）

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Abstract

Extracellular polysaccharides (EPSs) of the microorganisms participate in many physiological functions such as adhesion, biofilm formation and stress tolerance, and are often related to pathogenicity of certain bacteria. In addition, EPSs themselves are beneficial natural polymers for food, thickener, medicine and fiber. Because of these utilities, EPSs and their various biosynthesis mechanisms have been well studied in many bacteria including pathogens, and various biosynthesis systems and their regulation for EPSs have been reported. On the other hand, the EPS biosynthesis mechanisms have been poorly studied in phototrophic cyanobacteria, though EPS related ecologies such as microbial mat formation, colony formation and tolerance against desiccation and other stresses have been observed in some cyanobacteria. In addition, production of EPS has been documented in many cyanobacteria. As cyanobacteria are the member of Bacteria, some molecular systems are very similar to bacterial ones. However, very little has been elucidated for the cyanobacterial EPS biosynthesis systems so far, even though genome data of many cyanobacterial species are available today. This may be, at least partly, due to variations unique to cyanobacteria. So I studied about biosynthesis and regulation of cyanobacterial EPS to elucidate their molecular mechanisms and to get some insights into physiological and ecological roles in my thesis.

In chapter I, I studied about the NDP-sugar pyrophosphorylases (PPases), which supply substrates (NDP-sugar) for the EPS biosynthesis. I studied two uncharacterized groups likely diverged from a common ancestor. One was a novel type of UDP-Glc PPase, which I named CugP (cyanobacterial UDP-Glc PPase). While the bacterial major UDP-Glc PPase (GalU) is not universal in cyanobacteria, CugP is found in almost all cyanobacteria and some bacteria. CugP and GalU may have been differently selected and lost in cyanobacteria and other bacteria in evolution. The other was identified as specific GDP-Glc PPase, which has not been reported in bacteria. This protein was also conserved in many cyanobacteria, so I designated CggP (cyanobacterial GDP-Glc PPase). The enzyme evolution and hypothetical functions are discussed.

In chapter II, I studied about extracellular cellulose biosynthesis and cell aggregation in a

thermophilic cyanobacterium XXXX under blue light and low temperature. I identified two novel key components of the cellulose biosynthesis, HlyD-like protein XXXX and endoglucanase-like XXXX based of the finding that the cell aggregation and cellulose production were abolished by their gene disruption. The transcriptional level of *xxxx* was markedly upregulated under low temperature. Overexpression of *xxxx* allowed production of cellulose and cell aggregation under normal growth conditions, when signaling pathway was activated. It was suggested that the upregulation of *xxxx* is a part of the low temperature induction of cellulose biosynthesis. I designated the cyanobacterial extracellular cellulose synthase as Xcs (extracellular cellulose synthase). In chapter II, I proposed a structural model for the Xcs cellulose synthase complex, which is distinct from the known bacterial cellulose synthase Bcs complex. In addition, I also presented re-classification of the prokaryotic cellulose synthases.

In chapter III, I found formation of bloom-like floating cell aggregates in XXXX and studied its molecular background, biosynthesis of sulfated EPS. Flotation of cell aggregates strictly depended on photosynthetic gas generation and accumulation of viscous polysaccharide released from cells. Because the floating cell aggregates are similar to the bloom formed by known cyanobacteria, I called them “bloom” in this thesis. I developed the procedure to collect the viscous EPS that is responsible for the bloom formation. By combination of determination of the viscous EPS and gene disruption of membrane-bound glycosyltransferase genes, I was able to identify a gene cluster that is essential for the production of viscous EPS and the bloom formation. Because this cluster, which was located on a large plasmid, consisted of a number of functionally related genes, most of them were disrupted and phenotype was evaluated. Chemical analysis revealed that the viscous EPS is a novel sulfated polysaccharide, which I named “synechan”. Based on the phenotype and the homology to known bacterial EPS synthase, I tentatively assigned genes involved in synechan biosynthesis and named them *xss* (extracellular sulfated polysaccharide synthase) genes (*xssA* ~ *xssS*). The predicted products include glycosyltransferases, sulfotransferases, polymerization protein, and secretion proteins, and regulatory proteins. Real-time qPCR analysis and other experiments suggested that the two-component

regulatory system transcriptionally regulates several genes in the cluster, leading to qualitative and quantitative regulation of the sulfated EPS production. This is the first report of bacterial sulfated EPS synthase gene cluster.

The results of all three chapters revealed the unique evolution of EPS biosynthesis in cyanobacteria. I believe that this study will open the avenue for the study of sulfated EPSs and other EPSs in cyanobacteria.

Abbreviations

AI	aggregation index
Ala	alanine
ATP	adenosine triphosphate
bp	base pair
cAMP	cyclic adenosine monophosphate
CBB	Coomassie brilliant blue R-250
CPS	capsular polysaccharide
CTP	cytidine triphosphate
c-di-GMP	cyclic-dimeric-guanosine monophosphate
Da	dalton
DCMU	3-(3, 4-dichlorophenyl)-1, 1-dimethylurea
Δ (delta)	deletion
DGDG	digalactosyldiacylglycerol
dTTP	deoxythymidine triphosphate
EPS	extracellular polysaccharide
Frc	fructose
Frc-1P	fructose 1-phosphate
Fuc	fucose
GAG	glycosaminoglycan
Gal	galactose
Gal-1P	galactose 1-phosphate
Glc	glucose
Glc-1P	glucose 1-phosphate
GlcA	glucuronic acid
GlcDG	glucosyldiacylglycerol
GlcNAc	N-acetylglucosamine
GlcNAc-1P	N-acetylglucosamine 1-phosphate
Gly	glycine
GTP	guanosine triphosphate
Gul	gulose
IPTG	isopropyl thiogalactoside
IS	insertion sequence
Man	mannose
ManA	mannuronic acid
Man-1P	mannose 1-phosphate
Man-6P	mannose 6-phosphate
NGS	next generation sequencer
NTP	nucleoside triphosphate

nRPS	non-viscous released polysaccharide
OD_{xxx}	optical density at xxx nm
OPX	outer membrane polysaccharide export protein
OX	overexpression
PAGE	polyacrylamide gel electrophoresis
PCP	polysaccharide co-polymerase
PCR	polymerase chain reaction
PPase	pyrophosphorylase
PPi	pyrophosphate
PTFE	polytetrafluoroethylene
Rha	rhamnose
RPS	released polysaccharide
SDS	sodium dodecyl sulfate
ST	sulfotransferase
UTP	uridine triphosphate
vRPS	viscous released polysaccharide
Xyl	xylose

General Introduction

Outline of the Introduction

In my thesis, I aimed to identify a wide range of genes involved in biosynthesis of the two typical cyanobacterial extracellular polysaccharides (EPSs): cellulose and a sulfated polysaccharide. My long-term goal is application of basic biology in laboratory to elucidation of EPS-mediated behaviors in ecology and industrial production of EPSs. Because EPSs are widely distributed with vast varieties, I would like to outline the General Introduction in this thesis, as follows: first, I defined EPSs in a broad sense, extending the notion of "extracellular" polysaccharides from bacteria to plants and animals. Second, I summarized current knowledge about biosynthesis of typical bacterial EPSs: cellulose, alginate, and xanthan. I also overviewed three general mechanisms for production of bacterial EPSs. Third, I briefly mentioned eukaryotic "EPSs", biosynthesis of which has, at least in part, common features with those of bacterial EPSs. Lastly, I summarized the current status of our understanding about biosynthesis of EPSs in cyanobacteria, before describing my results.

Definition of EPSs in this thesis

EPSs are generally defined as microbial polysaccharides which are secreted to extracellular environment (Schmid *et al.*, 2015). They are typically divided into released polysaccharides (RPSs) and capsular polysaccharides (CPSs). EPSs sometimes include outer membrane lipopolysaccharides (LPSs) of Gram-negative bacteria. Peptidoglycan may not be included in EPSs. However, cellulose of the cell wall in bacteria, algae and plants may be similar to each other. Sulfated polysaccharides may also be identified in not only cyanobacteria, but also algae and animals. Thus I extend the notion of "extracellular" polysaccharides from bacteria to include eukaryotic species. These similarities could give us profound insights into the details of EPS biosynthesis mechanisms and help us to identify unrevealed ones.

Biosynthesis of EPSs in bacteria

Bacteria, the major inhabitants of the Earth, are living in various environments such as sea, river, hotspring, soil, desert, and inside of the other large living organisms. They respond to various environmental changes by regulating their community behavior and cell surface conditions using EPSs. Cell aggregation, microbial mat formation, and adhesion to various objects, namely biofilm formation activities are typical EPS-mediated phenomena in bacteria. On the other hand, EPSs are also crucial for bacterial movement as a lubricant or a rail (Zhao *et al.*, 2013). These motility and sessility control via EPSs form the basis of infection of the pathogenic species (Ma *et al.*, 2009; Mika *et al.*, 2014). In addition to the physiological and medical importance, EPSs themselves are industrially beneficial as food, stabilizer, suspension, viscosity control agent, and biomaterial (Becker *et al.*, 1998). Thus, the characteristics, biosynthesis, regulation mechanisms and physiological roles of various bacterial EPSs have been well documented (Schmid *et al.*, 2015). Some typical examples are as follows: cellulose in *Komagataeibacter* (*Acetobacter*), *Salmonella* and other bacteria (Ross *et al.*, 1987; McNamara *et al.*, 2015), alginate in *Pseudomonas* (Linker *et al.*, 1966; Ramsey *et al.*, 2005), xanthan in *Xanthomonas campestris* (Jansson *et al.*, 1975; Becker *et al.*, 1998) (Fig. GI-1A). I will overview the structure and biosynthesis of these EPSs below.

Cellulose (β-1,4-glucan), the most abundant biopolymer on the earth (Somerville, 2006), is produced by a wide range of organisms including plants, animals, bacteria and cyanobacteria. Cellulose is excreted and deposited in the cell wall or released as microfibrils to form extracellular matrix. Bacterial cellulose is the most studied EPS. In a pioneering research, scientists first identified the cellulose synthase four-gene operon, *bcsABCD*, in *Komagataeibacter (Acetobacter) xylinus* (Wong *et al.*, 1990), which forms pellicle that consists of pure cellulose fibers on the surface of liquid culture medium. Subsequently, similar bacterial cellulose and its biosynthesis genes have been reported in a wide variety of bacteria, including the nitrogen-fixing plant symbiont *Rhizobium leguminosarum*, soil bacteria (e.g. *Pseudomonas putida*), plant pathogens (e.g. *Dickeya dadantii*), tumor-producing

Agrobacterium tumefaciens, and the well-known model organisms *Escherichia coli* and *Salmonella enterica*, and the early essential analyses were mainly performed in the last two species (Zogaj *et al.*, 2001). The biosynthesis of bacterial cellulose was intricately regulated in various transcriptional and post-transcriptional ways. The most essential factor is the cyclic bis-(3′-5′)-diguanylic acid (c-di-GMP), which proved to be a universal bacterial second messenger. In early research, c-di-GMP was found to activate the cellulose synthase (Ross *et al.*, 1987), and subsequently, c-di-GMP biosynthesis and degradation enzymes (Tal *et al.*, 1998), and a c-di-GMP–binding PilZ domain in the catalytic subunit BcsA (Amikam *et al.*, 2006; Morgan *et al.*, 2014) were found. The physiological roles of bacterial cellulose are diverse: biofilm formation, bacterial mat formation, floating, and infection.

To date, many variations of the bacterial cellulose synthase gene clusters have been documented and they were classified into three types based on their gene composition (Romling *et al.*, 2015). Here, I summarized them in Fig. GI-1B. In brief, BcsA, the cellulose synthase catalytic protein, and BcsB, a largely periplasmic protein, are essential for the polymerization of cellulose in all types, whereas other accessory genes are needed but differentially conserved in each type. BcsZ, an endoglucanase, is also essential for the cellulose production, though its precise role still remains elusive (Nakai *et al.*, 2013). BcsC and related proteins may be located on the outer membrane of Gram-negative bacteria, serving as a membrane pore to facilitate excretion of cellulose chain (Fig. GI-1C) (Romling *et al.*, 2015; McNamara *et al.*, 2015). The crystal structure of the cellulose synthase BcsA/BcsB complex and other works provided insights into mechanism of cellulose biosynthesis (Morgan *et al.*, 2013; Omadjela *et al.*, 2013). The polymerization of the cellulose chain occurs at the catalytic site of BcsA on the cytoplasmic side. BcsA also mediates the export of the cellulose chain from the catalytic site to the periplasmic space. BcsB tightly attaches to BcsA activating the BcsA activity and may also guide for transport of the cellulose chain through the periplasmic space. The PilZ domain of BcsA is located on the cytoplasmic side, being a receptor for the second messenger c-di-GMP.

Alginate, or alginic acid, is made of variable amounts of β-1,4-linked mannuronic acid and its C5-epimer guluronic acid (Fig. GI-1A). The alginate polymer are separated into three blocks: continuous mannuronic acid residues (M-blocks), guluronic acid residues (G-blocks), and combination residues (MG-blocks). Alginates have been extensively studied in *Pseudomonas*, and industrially taken from brown algae. They are viscous acidic polymers and utilized in many ways for food industry, medical field, and industrial uses (Rehm *et al.*, 1997). Their biosynthesis genes form a cluster in *Pseudomonas* (Fig. GI-1B), and their products probably assemble into a synthase complex much like the bacterial cellulose synthase complex (Fig. GI-1C) (Hay *et al.*, 2013). In the alginate synthase complex, alginate synthase catalytic protein, Alg8, is activated by c-di-GMP via PilZ domain of Alg44, a large periplasmic protein. The substrate of Alg8 is only GDP-mannuronic acid and after polymerization, some of residues are epimerized to guluronic acids by epimerase, AlgG, at the periplasmic space. The polysaccharide chain is further decorated with acetyl groups by AlgJ and AlgX, and finally secreted through the outer membrane pore, consisting of AlgE proteins. The alginate lyase AlgL, which is known to degrade alginate, is also essential for the alginate biosynthesis like BcsZ in the bacterial cellulose synthase complex (Monday *et al.*, 1996).

Xanthan consists of a cellulose like backbone β-1,4-glucan and a side chain made of two mannose molecules and one glucuronic acid molecule (Fig. GI-1A). Xanthan is synthesized by a plant pathogen *Xanthomonas campestris* (Jansson *et al.*, 1975). Because xanthan has one uronic acid residue and one substituted residue with pyruvate, so this polysaccharide is negatively charged and has viscosity. This biopolymer is produced industrially by microbial fermentation and utilized as stabilizer, suspension, and thickener in practical application. In *X. campestris*, biosynthesis of xanthan is indirectly regulated by c-di-GMP (Ryan, 2013). Xanthan biosynthesis genes form a gene cluster (Fig. GI-1B), and the gene products probably assemble into a synthase complex like other synthases mentioned above (Fig. GI-1C) (Rehm, 2010). As the first step of xanthan biosynthesis, “priming glycosyltransferase” GumD transfers glucose 1-phosphate (Glc-1P) from UDP-Glc to phosphorylated

lipid linker (polyisoprenol, C55). Then four soluble glycosyltransferases, GumM, GumH, GumK, GumI, sequentially transfer monosaccharides to the membrane anchored sugar chain. The resulting polysaccharide unit is modified (acetylation and addition of pyruvate by GumF, GumG, and GumL), and flipped from the cytoplasmic side to the periplasmic side by the flippase GumJ. Finally, the pentasaccharide units are combined by "xanthan polymerase" (GumE and GumC), and then secreted to extracellular space through the outer membrane pore consisting of GumB proteins.

The EPS biosynthesis mechanisms in bacteria are full of diversity, but they can be categorized into three groups by their secretion mechanism and components: the synthase type, the Wzx/Wzy type, and the ABC transporter type (Schmid *et al.*, 2015). The synthase type includes the bacterial cellulose synthase complex and the alginate synthase complex. In this type of complex, the polysaccharide chain is directly synthesized from NDP-sugar by the synthase subunit, and subsequently, the chain is guided through the periplasm and outer membrane pore to the extracellular space by periplasmic component(s) and outer membrane pore protein(s). The second Wzx/Wzy type is typified by xanthan synthase complex in *Xanthomonas campestris*. An oligosaccharide unit is synthesized on the membrane-anchored lipid linker by the priming glycosyltransferase and several glycosyltransferases at the cytoplasmic side, and then flipped to the periplasmic side by Wzx (equivalent to GumJ/flippase). Subsequently, the repeat units are combined by the polymerase Wzy (equivalent to GumE/polymerase) and PCP (polysaccharide co-polymerase) protein (equivalent to GumC/exporter), and finally secreted to extracellular space via OPX (outer membrane polysaccharide export) protein (equivalent to GumB). The third type, ABC transporter type, includes various CPS (capsular polysaccharides, or cell attached polysaccharides) synthase complexes in *E. coli* (Willis *et al.*, 2013). This type slightly resembles the Wzx/Wzy type, but has some differences. The oligosaccharide chain is synthesized on the membrane anchored lipid linker, but connected to the linker via poly-2-keto-3-deoxyoctulosonic acid (Kdo). The nascent chain of polysaccharide is guided to the outer space by the ABC transporter instead of flippase. The polysaccharides synthesized by this type are anchored to the cell surface.

Biosynthesis of "EPSs" in eukaryotes

The eukaryotic "EPSs" include cellulose and other polysaccharides in plants, glycosaminoglycans (GAGs) in mammals, sulfated EPSs in algae, etc. Of these, the plant cellulose and mammalian GAGs have been well studied. Their biosynthesis mechanisms may resemble those of bacterial EPSs to some extent and I overviewed them briefly.

Cellulose microfibrils in higher plants are stronger and more stable than the cellulose filaments in bacteria, because the microfibrils consist of a bundle of twisted cellulose chains. Plant cellulose synthase protein, CESA, partially resembles the bacterial BcsA, but its cellulose biosynthesis mechanism is different from bacterial one (Pear *et al.*, 1996; Kudlicka *et al.*, 1997; Holland *et al.*, 2000). There are many variations in CESA proteins in plants, and they may assemble differently to form various cellulose synthase complexes (CSCs). In *Arabidopsis thaliana*, for example, CESA1, CESA3, and CESA6 are specific for the primary cell wall cellulose biosynthesis, while CESA4, CESA7, and CESA8 are specific for secondary cell wall cellulose biosynthesis. These CESAs form the cellulose synthase complex in the plasma membrane, and the complex has been observed as a hexameric rosette structure through freeze-fracture transmission electron microscopy (Mueller *et al.*, 1980; Kimura *et al.*, 1999; Hill *et al.*, 2014). Recent studies suggested that the rosette is probably made of hexamer of trimeric CESA proteins, which produce 18 chains of cellulose in a bundle (Vandavasi *et al.*, 2016; Kumar *et al.*, 2017). CESA proteins are larger than BcsA in a way that CESAs possess several additional regions. These additional regions are engaged in oligomerization so that the huge cellulose synthase complex enables production of the bundle of cellulose chains, microfibrils. Recent studies also revealed that an endoglucanase KORRIGAN plays important roles in the trafficking of the cellulose synthase complexes in plants (Lei *et al.*, 2014; Vain *et al.*, 2014). This endoglucanase may take part in modification of cellulose chain like the endoglucanase BcsZ in the bacterial cellulose biosynthesis.

Generally, glycosaminoglycans (GAGs) are highly sulfated polysaccharides such as heparan sulfate, dermatan sulfate and chondroitin sulfate. They exist as viscous supportive materials in

connective tissues of mammalian bodies. They are unbranched polymer of the disaccharide repeats that consist of an amino sugar and an uronic acid, and usually exist as the polysaccharide parts of proteoglycans. Their biosynthesis mechanism in Golgi apparatus and modification in extracellular space have been revealed (Li *et al.*, 2016). In brief, the polysaccharide chain is synthesized on a glucuronosyl-galactosyl-galactosyl-xylosyl tetrasaccharide linker anchored to the serine residue of proteoglycan core proteins. Subsequently, the polysaccharide chain is modified by several epimerases and sulfotransferases in Golgi apparatus. After excretion into the extracellular space, these GAGs are sometimes further modified by degrading enzymes and sulfatases (Li *et al.*, 2016). These modifications are often linked to functionality of GAGs and many modification genes have been identified with their substrate specificities (Tran *et al.*, 2012). Nevertheless, large-scale production has not been established in *E. coli* or other microorganisms, which is important for industrial purposes, because of the complexity of the biosynthesis pathway for GAGs.

Various sulfated EPSs are found in macroalgae: carrageenan and agaran in red algae, ulvan in green algae and fucan (fucoidan) in brown algae (Wijesekara *et al.*, 2011; Ramus, 1972). These sulfated EPSs function as intercellular matrix like ones in the mammalian connective tissues. Although the chemical characteristics of these algal EPSs and their pharmacological activities have been well reported, the biosynthesis mechanisms of these EPSs are still unclear. This may be partly because of insufficient genetic and molecular information despite the genome data. In silico approach suggested the involvement of some hypothetical sulfotransferases in the biosynthesis of fucoidan (Ho, 2015). Interestingly, these sulfotransferases are classified in the same protein family (Pfam: PF00685, PF03567, and PF13469, Sulfotransfer families) as the sulfotransferases of GAG biosynthesis in mammals.

The eukaryotic “EPSs” may somewhat resemble each other and also bacterial ones in structure and physiological roles, although their biosynthesis pathways might highly diverge the bacterial ones. However, it would be still possible to screen related genes such as those for sugar modifications and cellulose synthase. Especially, sulfated polysaccharides are commonly found in

mammalian GAGs, algal EPSs and cyanobacterial EPSs, but not yet found in any bacterial EPSs to date. Because cyanobacterial EPSs are positioned at the junction between bacterial EPSs and eukaryotic EPSs, molecular studies on some critical EPSs in cyanobacteria would give us useful insights into biosynthesis mechanisms, regulation and application of EPSs in general.

Biosynthesis of EPSs in cyanobacteria

Cyanobacteria are oxygenic photosynthetic bacteria inhabiting aquatic and terrestrial environments, namely almost everywhere on the Earth. They are unicellular or filamentous, often forming colonies, which are planktonic or attached on the solid surfaces, likely to optimize phototrophic performance. Many natural species accumulate wide varieties of EPSs as biofilm matrix, capsule or slime (De Philippis *et al.*, 1998). Many species shows EPS-mediated adhesion (Fisher *et al.*, 2013), cell aggregation (Kawano *et al.*, 2011), bacterial mat formation (Stolyar *et al.*, 2014) and colony formation (Fujishiro *et al.*, 2004). Formation of harmful cyanobacterial blooms may also be coupled with EPSs (Dervaux *et al.*, 2015). In addition to these physiological and environmental importance, the cyanobacterial EPSs attract industrial attention because of their medical effects, biosorption activities, and photosynthetic production (Ohki *et al.*, 2014). However, very little has been elucidated for EPS biosynthesis mechanisms in cyanobacteria.

There are a few studied examples of EPSs and producing cyanobacteria: spirulan, sacran or "Suizenji-Nori", and cellulose. Spirulan is a sulfated rhamnosyl polysaccharide in *Arthrospira platensis*, known as "Spirulina". The chemical structure and medical effects have been well studied (Hayashi, 2008). Complete genome of *A. platensis* NIES-39 was determined and a possible pathway for rhamnose biosynthesis was discussed (Fujisawa *et al.*, 2010). *A. platensis* accumulates some EPSs upon stimulation with externally added second messenger molecule, cyclic AMP (Ohmori *et al.*, 1992). However, genes for biosynthesis of spirulan or other EPSs have not been identified yet, because this species was not amenable to the molecular studies due to infeasibility of genetic transformation except very recent report (Jeamton *et al.*, 2017). Another famous cyanobacterial EPS is sacran or

"Suizenji-Nori" in *Aphanothece sacrum*. This acidic polysaccharide consists of many kinds of monosaccharides including sulfated muramic acid (Ngatu *et al.*, 2012). Sacran is famous for traditional food in Japan as "Suizenji-Nori" and is a valuable biomaterial due to its high capacity to absorb metals, high moisturizing effect, etc. However, molecular mechanism of its biosynthesis is still unknown because the genome of *Aphanothece sacrum* has not yet been determined. Many other cyanobacterial EPSs have also been reported, but their biosynthesis mechanisms have not been comprehensively revealed.

The extracellular cellulose biosynthesis in *Thermosynechococcus vulcanus* is the rare example of the well-studied cyanobacterial EPS biosynthesis at molecular level. This thermophilic cyanobacterium exhibits cellulose-mediated cell aggregation that is induced by light and physiologically low temperature; i.e., ~30–35ºC for aggregation vs. ~45–57ºC for proliferation (Hirano *et al.*, 1997). One of the putative cellulose synthase genes, *tll0007*, is responsible for cellulose production and cell aggregation under the induced condition (Kawano *et al.*, 2011). Light-induced cell aggregation is mediated by the principal blue-light receptor, SesA, and two accessory photoreceptors, SesB and SesC (Enomoto *et al.*, 2014; Enomoto *et al.*, 2015). These photoreceptors regulate the cellular level of c-di-GMP, and in turn, c-di-GMP activates the cellulose synthase catalytic subunit, Tll0007, via the binding of c-di-GMP to its PilZ domain, leading to accumulation of extracellular cellulose. On the other hand, details concerning the cellulose synthase complex and its low-temperature induction remain unclear. More specifically, no apparent homologs for *bcsB*, *bcsC*, or *bcsZ*, which are required for the typical bacterial cellulose synthase, have been found in the *Thermosynechococcus* genome, although Tll0007 protein is homologous to the bacterial BcsA. I presumed that there should be yet unknown key factors in the cellulose biosynthesis in this cyanobacterium.

Aim of the study in this thesis

Because cyanobacteria not only belong to Bacteria, but also gave rise to chloroplasts in plant

cells, they may have the unique nature of the EPS biosynthesis among eukaryotes and prokaryotes. The goal of my research is to identify the biosynthesis mechanism and its regulation of various cyanobacterial EPSs. I also tried to understand the cyanobacterial survival strategy by extrapolating my results to the natural environment. I believe that the EPSs are highly diversified among species. I would like to understand diversity and unity in evolution of phototrophic organisms on the basis of the EPSs. In addition, I am interested in application of the basic research of beneficial EPSs such as cellulose and sulfated EPS to utilization of photosynthetic production.

In this thesis, there are three different subjects and purposes about the cyanobacterial EPS biosynthesis. In chapter I, I studied about cyanobacterial NDP-sugar PPases that supply substrates to the polysaccharide synthase. This is suitable for my thesis, because their variation is a good case study to focus on divergence and evolution of simple enzymes. In chapter II, I studied about genetic factors critical for cellulose biosynthesis in *Thermosynechococcus vulcan*us on the basis of heritage in my laboratory. I also studied on temperature regulation of the cellulose biosynthesis, looking for a possibility to correlate the regulation with the fitness to the natural environment such as hot springs, where *T. vulcanus* was derived from. In chapter III, I studied about biosynthesis and regulation of a sulfated EPS produced by *Synechocystis* sp. PCC 6803. This study was started during screening of the strains suitable for heterologous expression of the cellulose synthase genes I found in chapter 2. To my surprise, the accumulation of the sulfated EPS led to the flotation of the aggregated cells, which highly resembled the bloom formation of some cyanobacteria. I aimed to identify the chemical composition, synthase gene cluster, regulatory mechanism and physiological roles for this EPS comprehensively.

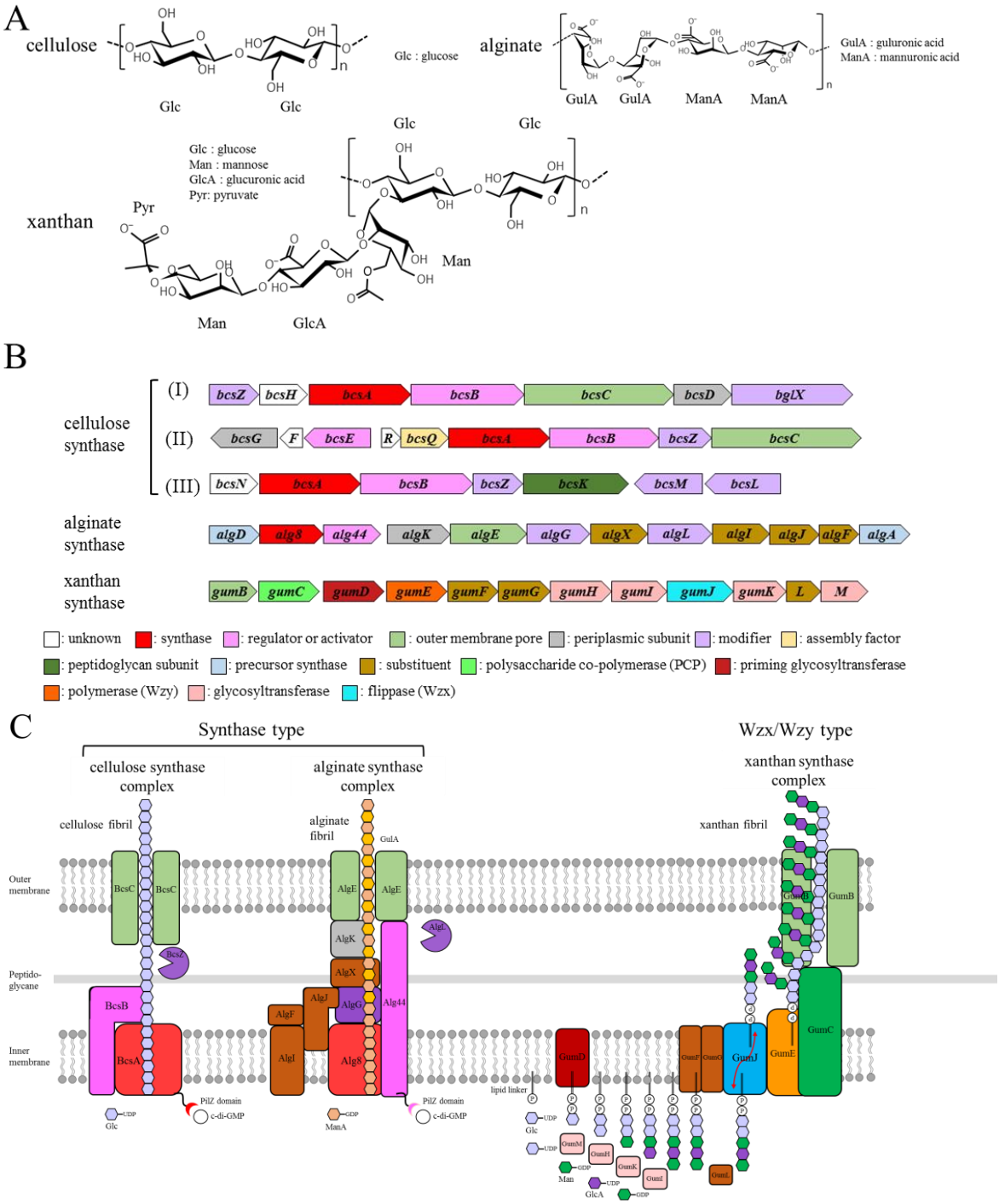

Fig. GI-1. Chemical structures, synthase gene clusters and structural models of the synthase complex for typical bacterial EPSs: cellulose, alginate and xanthan.

A, chemical structures of the three bacterial EPSs. The brackets indicate repeat units. B, gene clusters of cellulose synthase, alginate synthase and xanthan synthase. Cellulose synthase *bcs* clusters of type I, *Komagataeibacter xylinus*; type II, *Salmonella enterica*; type III, *Agrobacterium fabrum*. The alginate synthase *alg* cluster, *Pseudomonas aeruginosa*. The xanthan synthase *gum* cluster, *Xanthomonas campestris*. C, structural models of cellulose synthase complex, alginate synthase complex, and xanthan synthase complex. Gene names and color labels are same as in B.

Chapter I

NDP-sugar pyrophosphorylases in cyanobacteria.

I - a

Identification of a novel ubiquitous cyanobacterial UDP-glucose pyrophosphorylase, CugP.

Abstract

UDP-glucose pyrophosphorylase (UDP-Glc PPase) synthesizes UDP-Glc from UTP and Glc-1P, and exists in almost all species. Most bacteria possess a GalU-type UDP-Glc PPase, whereas many cyanobacteria species do not. In certain cyanobacteria, UDP-Glc is used as a substrate for synthesis of EPS cellulose in spite of the absence of GalU-type UDP-Glc PPase. Therefore, there should be an uncharacterized UDP-Glc PPase in cyanobacteria. Here I show that all cyanobacteria possess a non-GalU-type bacterial UDP-Glc PPase, i.e., CugP, a novel family in the nucleotide triphosphate (NTP) transferase superfamily. The expressed recombinant *Synechocystis* sp. PCC 6803 CugP had PPase activity that was highly specific for UTP and glucose 1-phosphate. The fact that the CugP gene cannot be deleted completely in *Synechocystis* sp. PCC 6803 suggests its central role as the substrate supplier for galactolipids synthesis. Galactolipids are major constituents of the photosynthetic thylakoid membrane and important for the photosynthetic activity. Based on the phylogenetic analysis, this CugP-type UDP-Glc PPase may have recently been horizontally transferred to certain non-cyanobacteria.

Introduction

Glycosylation, which is catalyzed by various types of glycosyl transferases, is important for the biosynthesis of many biological molecules, e.g., polysaccharides, glycoproteins and glycolipids. Nucleotide diphosphate (NDP)-sugars, which are the substrates for glycosylation, are mostly synthesized by nucleotidyl transferase that use a nucleotide triphosphate (NTP) and a sugar 1-phosphate (sugar-1P) as substrates. Various NDPs and sugars are combined into NDP-sugars *in vivo*. Of these, UDP-glucose (UDP-Glc) is used for the biosynthesis of cellulose (Romling, 2002) and glycogen (Alonso *et al.*, 1995); galactose metabolism (Holden *et al.*, 2003); and addition of the Glc moiety in heteroglycans, glycoproteins (Roth, 1995; Verbert, 1995; Silbert *et al.*, 1995) and glycolipids (Sandhoff *et al.*, 1992). Other NDP-sugars are utilized in the biosynthesis of other polysaccharides and in various glycosylation reactions.

UDP-Glc and the byproduct inorganic pyrophosphate (PPi) are produced from UTP and Glc-1P by the UDP-Glc pyrophosphorylases (PPases) (EC 2.7.7.9) in the reversible reaction UTP + Glc-1P ⇆ UDP-Glc + PPi. This enzyme is ubiquitous, although its typical bacterial form, the GalU-type, is widely distributed throughout Bacteria including Proteobacteria (Bosco *et al.*, 2009), Firmicutes (Ma *et al.*, 2011) and Actinobacteria (Lai *et al.*, 2008; Diez *et al.*, 2012) and is distinct from its eukaryotic enzyme in amino acid sequence and three-dimensional structure (Mollerach *et al.*, 1998; Flores-Diaz *et al.*, 1997; Mollerach *et al.*, 2000). The best-known bacterial UDP-Glc PPase is GalU from *Escherichia coli* (Weissborn *et al.*, 1994; Hossain *et al.*, 1994). The crystal structures and the reaction mechanism of various GalU-type homologs have been elucidated (Thoden *et al.*, 2007b; Thoden *et al.*, 2007a; Kim *et al.*, 2010). These bacterial GalU-type UDP-Glc PPases belong to a large superfamily of nucleotidyl transferases that also includes UDP-*N*-acetyl glucosamine (GlcNAc) PPase, ADP-Glc PPase, CDP-Glc PPase, dTDP-Glc PPase, GDP-mannose (Man) PPase, and some yet uncharacterized enzymes. These enzymes probably diverged from a common ancestor in evolution (Kawano *et al.*, 2013).

There are many Glc-containing polysaccharides such as glycogen and Glc-enriched EPSs in

cyanobacteria (Philippis *et al.*, 1998). Certain cyanobacteria also accumulate cellulose (β-1,4-glucan) (Nobles *et al.*, 2001; Kawano *et al.*, 2011). Consistently, cyanobacterial genomes have been shown to encode many putative nucleotidyl transferases that might supply NDP-sugars (Nakamura *et al.*, 2002; Kaneko *et al.*, 2001). Recently the GalU-type protein All3274 from the filamentous cyanobacterium *Anabaena* sp. PCC 7120 was biochemically revealed to be a UDP-Glc PPase (Kawano *et al.*, 2013). However, such a GalU-type enzyme is not present in all cyanobacteria. Specifically, although *Thermosynechococcus* is reported to possess a cellulose synthase to produce cellulose under conditions of light and low temperature (Kawano *et al.*, 2011), a GalU-type gene that could supply UDP-Glc to this cellulose has not be found in its genome (Nakamura *et al.*, 2002). Given these observation, I hypothesized that an uncharacterized non-GalU-type UDP-Glc PPase may exist in such cyanobacteria. To test this hypothesis, I first focused on a common sequence feature that is shared only by GalU-type UDP-Glc PPases, UDP-GlcNAc PPases, and a putative enzyme family that is present in every species of cyanobacteria. Herein, I report that the members of this previously uncharacterized family are cyanobacterial UDP-Glc PPases (thus denoted CugP).

Results and discussion

Sequence features of nucleotidyl transferases

The phylogeny of the nucleotidyl transferase superfamily (Pfam: NTP_transferase) in cyanobacterial species was analyzed based on alignment of full-length amino acid sequences of the transferases (Fig. I-1). It is of note that GalU-type UDP-Glc PPase is found in only a few species (*Anabaena* sp. PCC 7120, *Arthrospira platensis* NIES-39 and *Nostoc punctiforme* ATCC 29133). On the other hand, many other enzymes such as dTDP-Glc PPase, CDP-Glc PPase, ADP-Glc PPase, UDP-GlcNAc PPase and as yet uncharacterized nucleotidyl transferases are found in most species of cyanobacteria. I searched for common residues in the GalU-type UDP-Glc PPase and UDP-GlcNAc PPase sequences. As reported previously, the G-x-G-t-R motif is highly conserved in all sequences as a part of the (d)NTP-binding site. I found that one residue just prior to the motif is characteristically conserved: Ala is shared in GalU-type UDP-Glc PPase, UDP-GlcNAc PPase and one uncharacterized family (provisionally annotated ”GDP-Man PPase”), whereas Gly is shared in all of the others (Fig. I-2). Since this residue was the only one candidate that might be responsible for substrate discrimination, I expressed and characterized the activities of Sll1558, the putative *Synechocystis* sp. PCC 6803 ”GDP-Man PPase”, and its Ala-8-Gly mutant A8G-Sll1558, which I chose because that cyanobacterium is a well-studied model for cyanobacteria in general.

Purified Sll1558 has a specific UDP-Glc PPase activity

I expressed Sll1558 as a His-tagged protein, which I purified by nickel-affinity chromatography (Fig. I-3). According to my SDS-PAGE study, the molecular mass of Sll1558 is that predicted by the translated sequence of *sll1558* (45kDa). The enzyme activity of this product was measured through the liberation of a byproduct pyrophosphate using a combination of various NTPs and sugar-1Ps. I found that Sll1558 showed activity only in the presence of UTP and Glc-1P (Table I-1). Notably, it showed practically no activity towards any other substrates or combinations, including

Man-1P, even though Sll1558 and related homologs had provisionally been considered to be a GDP-Man PPase as annotated in CyanoBase. dTTP does not substitute for UTP in reactions with Sll1558, a finding that contrasts with the ability of dTTP to act as a substrate of the GalU-type UDP-Glc PPase from *Xanthomonas campestris* (Bosco *et al.*, 2009) and *Sphingomonas elodea* ATCC 31461 (Silva *et al.*, 2005).

Activity and substrate affinity comparison between Sll1558 and A8G-Sll1558

To further examine my prediction about nucleoside discrimination at the Ala residue, I evaluated the possible role of the Ala-8 residue of Sll1558 by site-directed replacement of this residue with Gly. After purification, the substrate specificity and enzyme kinetics of A8G-Sll1558 were measured. A8G-Sll1558 also exhibited the PPase activity only for a combination of UTP and Glc-1P, as with wild type Sll1558 (Table I-1).

According to Lineweaver-Burk plots (Fig. I-4), the values of K_m(UTP) and K_m(Glc-1P) for A8G-Sll1558 were 0.352 mM and 0.0882 mM, and for Sll1558 were 0.181 mM and 0.0855 mM, respectively (Table I-2). Thus, the K_m for UTP was found to be approximately 2-fold higher than wild type, and the K_m for Glc-1P was the same degree to wild type. This finding suggests that the Ala-8 residue does not so critically affects specific binding to the uridyl moiety, but have a slight effect on. Notably, the V_{max} and k_{cat} values for A8G-Sll1558 were comparable to the wild type enzyme (Table I-2). The K_m values for Sll1558 were similar to those of GalU-type enzymes from bacteria other than cyanobacteria, whereas the V_{max} and k_{cat} were about 100-1000 fold lower than these counterpart enzymes (Ma *et al.*, 2011; Lai *et al.*, 2008; Bosco *et al.*, 2009). On the other hand, the kinetics parameters of Sll1558 were found to be similar to those of All3274, the GalU-type UDP-Glc PPase from *Anabaena* sp. PCC 7120 (Kawano *et al.*, 2013).

Distribution of the GalU-type and CugP-type enzymes, two types of UDP-Glc PPase

Herein, I designated Sll1558 and its homologs as CugP-type UDP-Glc PPase after

cyanobacterial UDP-Glc PPase. Because all cyanobacterial genomes examined to date encode a CugP-type UDP-Glc PPase, this enzyme appears to be present in all cyanobacteria (Fig. I-1), whereas the GalU-type UDP-Glc PPase is present only in certain species (Kawano *et al.*, 2013). As shown in Figure I-1, a phylogram of all related enzymes in the cyanobacterial nucleotidyl transferase superfamily confirmed such a distribution in cyanobacteria. It must be mentioned also that the cyanobacterial species that possess the GalU-type also possess the CugP-type UDP-Glc PPase.

According to the structure of the phylogram, the GalU-type and CugP-type enzyme sequences diverged early on from each other or from other related enzymes during the evolution of the nucleotidyl transferase superfamily. It is thus possible that CugP-type UDP-Glc PPases evolved independently within this superfamily. When Blast search was performed for the whole NCBI database, the GalU-type PPases were found to be widely distributed throughout the bacterial kingdom, whereas the CugP-type could be found in only very limited species other than cyanobacteria. These limited species included certain, but not all, of the beta- and gamma-Proteobacteria. The sequence similarity among the cyanobacterial and proteobacterial CugP-type enzymes is much greater (E value 1e-122 to -147) than that of their GalU-type counterparts (E value 1e-28 to -32). Notably, the proteobacterial sequences have clearly diverged from the inside of the cyanobacterial CugP cluster (e.g. *Yersinia intermedia*, *Methylotenera mobilis* and *Vibrio ichthyoenteri*; see Fig. I-1), suggesting that the proteobacterial CugP-type enzymes have been acquired by horizontal gene transfer from cyanobacteria rather recently. Conversely, although the GalU-type enzymes were, in general, widely distributed in Bacteria, they were found in only some filamentous cyanobacteria and may, therefore, have also been acquired through another horizontal transfer from bacteria (e.g. *Poribacteria* and *Anaerolinea thermophila*) to the limited species of cyanobacteria or may have been lost in many cyanobacterial lineages. I speculate that the GalU-type enzyme may supply UDP-Glc to certain glycosyltransferases specific for cell differentiation in those filamentous cyanobacteria.

Structural implications of the motif for substrate specificity

According to the crystal structure (the crystal structure of CugP does not exist) and sequence similarities, the overall structure of the whole catalytic domain seems to be conserved between GalU-type UDP-Glc PPase, CDP-Glc PPase, dTDP-Glc PPase and other subfamilies of the nucleotidyl transferases including CugP-type UDP-Glc PPase. These enzymes bind (d)NTP and sugar-1P sequentially in the substrate-binding pocket, which can be divided into a (d)NTP-binding site and a sugar-binding site (Thoden *et al.*, 2007a; Silva *et al.*, 2005). In the former site, three highly conserved residues directly interact with the pyrimidine/purine base of (d)NTP by hydrogen bonds (Fig. I-5 and I-6). However, these hydrogen bonds are mostly derived from main chain amide of Ala or Gly residue and, therefore, the specificity determination for (d)NTP has not clearly been elucidated. For example, the amide NH of Gly-117 appears to discriminate uracil of UTP in UDP-Glc PPase from cytosine of CTP (Fig. I-6), but is also conserved in other nucleotidyl transferases. The Ala residue that is highlighted in this study as a possible marker for the UTP-utilizing enzymes directly interacts by hydrogen bonding with C-2 carbonyl of uracil, but very similar hydrogen bonding is found between Gly-8 and C-2 carbonyl of cytosine in CDP-Glc PPase (Fig. I-6). This similarity may account for my less pronounced result of A8G mutagenesis in CugP. Thus, the precise mechanism to determine the specificity for UTP still remains elusive. However, it must be mentioned that the spatial arrangement of Gly residue (Gly-21/Gly-9/Gly-9 in GalU/CugP/CDP-Glc PPase) next to the mutated Ala position is markedly different, resulting in distinct hydrogen bonding to cytosine in CDP-Glc PPase or ribose in GalU-type UDP-Glc PPase (Fig. I-5 and I-6). This may be the reason why the K_m of A8G-Sll1558 for UTP slightly increased. Thus, it is still possible that the highlighted Ala residue, which is exclusively conserved in the UTP-utilizing transferases, is somehow involved in discrimination of uracil of UTP as a substrate. To test this hypothesis, it will be needed to study the substrate discrimination of the UTP-utilizing transferases by thorough mutagenesis survey.

Role of CugP-type UDP-Glc PPase in cyanobacteria

To better understand the role of cyanobacterial CugP-type UDP-Glc PPases, I attempted to disrupt Sll1558 in the *Synechocystis* sp. PCC 6803 genome. However, I could not produce such a mutant, because cyanobacteria typically contain multiple copies of their chromosomes and, consequently, the mutated *Synechocystis* sp. PCC 6803 did not cleanly segregate, suggesting that the CugP-type UDP-Glc is essential for survival of *Synechocystis* (Fig. I-7). In this context, it must be mentioned that a galactolipid, monogalactosyldiacylglycerol (MGDG), which is essential in *Synechocystis*, is supplied from UDP-Glc via monoglucosyldiacylglycerol (Awai *et al.*, 2006). The galactolipids MGDG and digalactosyldiacylglycerol (DGDG) are major constituents of the photosynthetic thylakoid membrane in cyanobacteria and in plant chloroplasts. They bind to the membrane-spanning photosynthetic complexes and thereby maintain them in a functional state (Sakurai *et al.*, 2007). Hence, the role of UDP-Glc PPases in providing UDP-Glc must be essential for growth and survival in cyanobacteria. In this non-segregated mutant, the chlorophyll (Chl) content was 3.47 ± 0.514 μg Chl / OD_{730}, and that of wild type was 4.22 ± 0.0436 μg Chl / OD_{730} (n = 3). This difference may reflect the weakened thylakoid membrane in the partially Sll1558-deleted mutant. Because galactolipids are specific to photosynthetic membranes, the unique CugP-type enzyme, found mainly in cyanobacteria, may be the result of an evolutionary event in oxygenic photosynthesis. Certain EPSs secreted by cyanobacteria including *Synechocystis* often contain Glc as the major constituent (Philippis *et al.*, 1998). However, it is unknown if they are essential for survival. Cellulose is selectively deposited in a thermophilic cyanobacterium *Thermosynechococcus* under conditions of low temperature and light (Kawano *et al.*, 2011). So, a CugP-type Tlr0611 appears to be the sole enzyme to provide UDP-Glc for biosynthesis of cellulose and might be induced under such conditions in this cyanobacterium. Regional distribution of GalU-type PPases in certain cyanobacteria may account for variation in the requirement of EPSs. Further functional studies of the CugP-type and GalU-type enzymes are needed to understand the role of UDP-Glc in cyanobacteria.

Materials and methods

Sequence alignment and phylogram

The sequences of the cyanobacterial proteins categorized as belonging to the bacterial nucleotidyl transferase superfamily (Pfam: PF00483, *NTP_transferase*) (http://pfam.sanger.ac.uk) were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and CyanoBase (http://genome.microbedb.jp/cyanobase) databases. Sequence alignment was performed by neighbor-joining method using ClustalX2 (Larkin *et al.*, 2007)**.**

Cloning of sll1558 and single amino acid substitution

sll1558 from *Synechocystis* sp. PCC 6803, which encodes the hypothetical UDP-Glc PPase Sll1558 was PCR amplified using PrimeSTAR® Max DNA Polymerase (Takara Bio, Otsu, Japan) and then directly cloned into the expression vector pET-28a(+) (Merck, Darmstadt, Germany) using an In-Fusion HD Cloning kit reagents (Takara) to produce *sll1558*-pET28a (The primers were pET28a-1F; 5′-GGA TCC GAA TTC GAG CTC CGT C-3′, pET28a-2R; 5′-CAT ATG GCT GCC GCG CGG CAC-3′, sll1558-1F28a; 5′-CGC GGC AGC CAT ATG AAA GCC ATG ATT TTG GCC-3′, and sll1558-2R28a; 5′-CTC GAA TTC GGA TCC TTA TTC CGG CTG GAG AAG-3′). To generate a single amino acid substitution within the Sll1558 protein product, the plasmid was amplified by two sets of primers (sll1558-3FA8G, 5′-GCC GGT GGC AAG GGC ACT CGG GTC AGA CCA ATC-3′, and sll1558-4RA8G, 5′-GCC CTT GCC ACC GGC CAA AAT CAT GGC TTT CAT-3′) and self-combined using the aforementioned cloning reagents. These proteins were His-tagged from the pET28a vector. The resulting DNA constructs were verified by dideoxy sequencing. These proteins were N-terminally His-tagged, derived from the pET28a vector.

Expression, purification and SDS-PAGE analysis of recombinant proteins

E. coli strain C41 (DE3) carrying *sll1558*-pET28a or *sll1558*(A8G)-pET28a were cultured in

1 L of LB medium at 37˚C. When each culture reached an OD_{600} of 0.4 - 0.8, 1mM isopropyl β-d-1-thiogalactopyranoside was added (final 100 μM) and the cells were grown at 18˚C for 16 h to achieve induction. The cells from each culture were then collected by centrifugation at 4,220 × *g* for 15 min and suspended in 20 mM HEPES (pH 7.5) containing 100 mM NaCl and 10 % (w/v) glycerol and homogenized three times with a French press at 1500 kg cm^{-2}. The soluble proteins were collected by centrifugation at 50,000 × *g* for 30 min. His-tagged Sll1558 and A8G-Sll1558 were purified by Ni-affinity column chromatography (HisTrap™ HP; GE Healthcare, Little Chalfont, UK) with the eluent being a gradient of 30-430 mM imidazole in the aforementioned buffer system. Proteins in each fraction were subjected to SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining. Low Molecular Mass Calibration kit standards (GE Healthcare) served as the molecular mass markers. The fraction enriched in each targeted protein was dialyzed against the aforementioned buffer to remove the imidazole. Protein concentration was assayed using the Bradford method (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

Nucleotidyl transferase activity assay

Nucleotidyl transferase activities were assayed at 37˚C in the direction of NDP–sugar formation from NTP and sugar-1P. The reaction rate measurements were determined as the change in the PP_i concentration with time and measured using the EnzChek Pyrophosphate Assay kit (Molecular Probes, Life Technologies, Carlsbad, CA). The basic reaction medium contained 50 mM Tris-HCl (pH7.5), 8 mM $MgCl_2$, 200 μM 2-amino-6-mercapto-7-methylpurine ribonucleoside, 1 U purine nucleoside phosphorylase, 30 mU inorganic pyrophosphatase, and substrates. To reduce contaminating inorganic phosphate found in the substrate preparations, each reaction solution was preincubated for 20 min at 22°C and for 10 min at 37°C prior to the addition of the protein. For measurements of substrate specificity, all substrates were included in the reactions at a 200 μM concentration and the purified enzymes at 0.66 μM.

Structure prediction based on previous studie*s*

The available Protein Data Bank (PDB) data of GalU-type UDP-Glc PPase (PDB ID, 2PA4) (Thoden *et al.*, 2007a), CDP-Glc PPase (PDB ID, 1TZF) (Koropatkin *et al.*, 2004) and dTDP-Glc PPase (PDB ID, 1H5T) (Zuccotti *et al.*, 2001) were used to compare the structure and substrate-binding region of Sll1558.

Gene disruption and mutant analysis

The *sll1558* gene was replaced with the chloramphenicol resistance cassette by the transformation plasmid made by In-Fusion method. Mutants were generated by transformation of *Synechocystis* cells with this DNA and selected on BG11 plates containing 20 μg $m1^{-1}$ chloramphenicol. The segregation was confirmed by PCR with the two sets of primers (sll1558-5F, 5′-GTG TGA TTG AGT TTG AGG -3′, and sll1558-6R, 5′-TTT CCC CCA GTT CTC TTC-3′). Chlorophyll content was calculated after extraction with 100% methanol as described (Midorikawa *et al.*, 2009).

Table I-1. Substrate specificity of Sll1558 and A8G-Sll1558.

	Sll1558 / A8G-Sll1558			
sugar-1P / **nucleotide**	**Glc-1P**	**GlcNAc-1P**	**Gal-1P**	**Man-1P**
UTP	**+ / +**	**0 / 0**	**0 / 0**	**0 / 0**
dTTP	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**
CTP	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**
ATP	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**
GTP	**0 / 0**	**0 / 0**	**0 / 0**	**0 / 0**

Plus sign indicates the activity. 0 indicates the value is lower than the detection limit (0.01 U / mg protein).

Table I-2. Kinetic parameters for Sll1558 and A8G-Sll1558.

Protein	Substrate	V_{max} (U mg^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
Sll1558	**UTP**	**0.63**	**0.18**	**28**	**160**
	Glc-1P	**0.61**	**0.085**	**27**	**320**
A8G-Sll1558	**UTP**	**0.64**	**0.35**	**29**	**83**
	Glc-1P	**0.64**	**0.088**	**29**	**330**

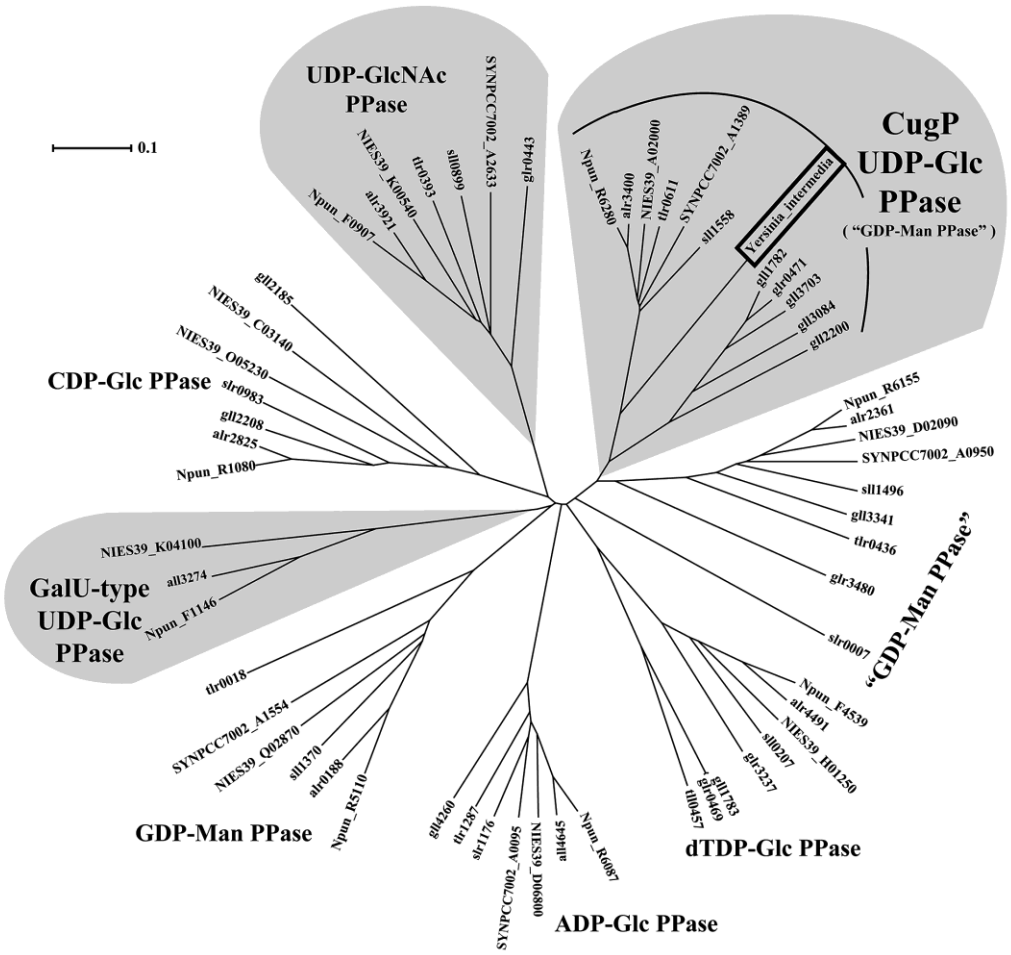

Fig. I-1. Phylogram of cyanobacterial nucleotidyl transferases.

The phylogram of the nucleotidyl transferases from *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, *Arthrospira platensis* NIES-39, *Synechococcus* sp. PCC 7002, *Thermosynechococcus elongatus* BP-1, *Nostoc punctiforme* ATCC 29133 and *Gloeobacter violaceus* PCC 7421 nucleotidyl transferase sequences. The families those have Ala just before the G-x-G-t-R motif are shown in gray backgrounds. The gene of a proteobacteria, *Yersinia intermedia*, is highlighted by black frame.

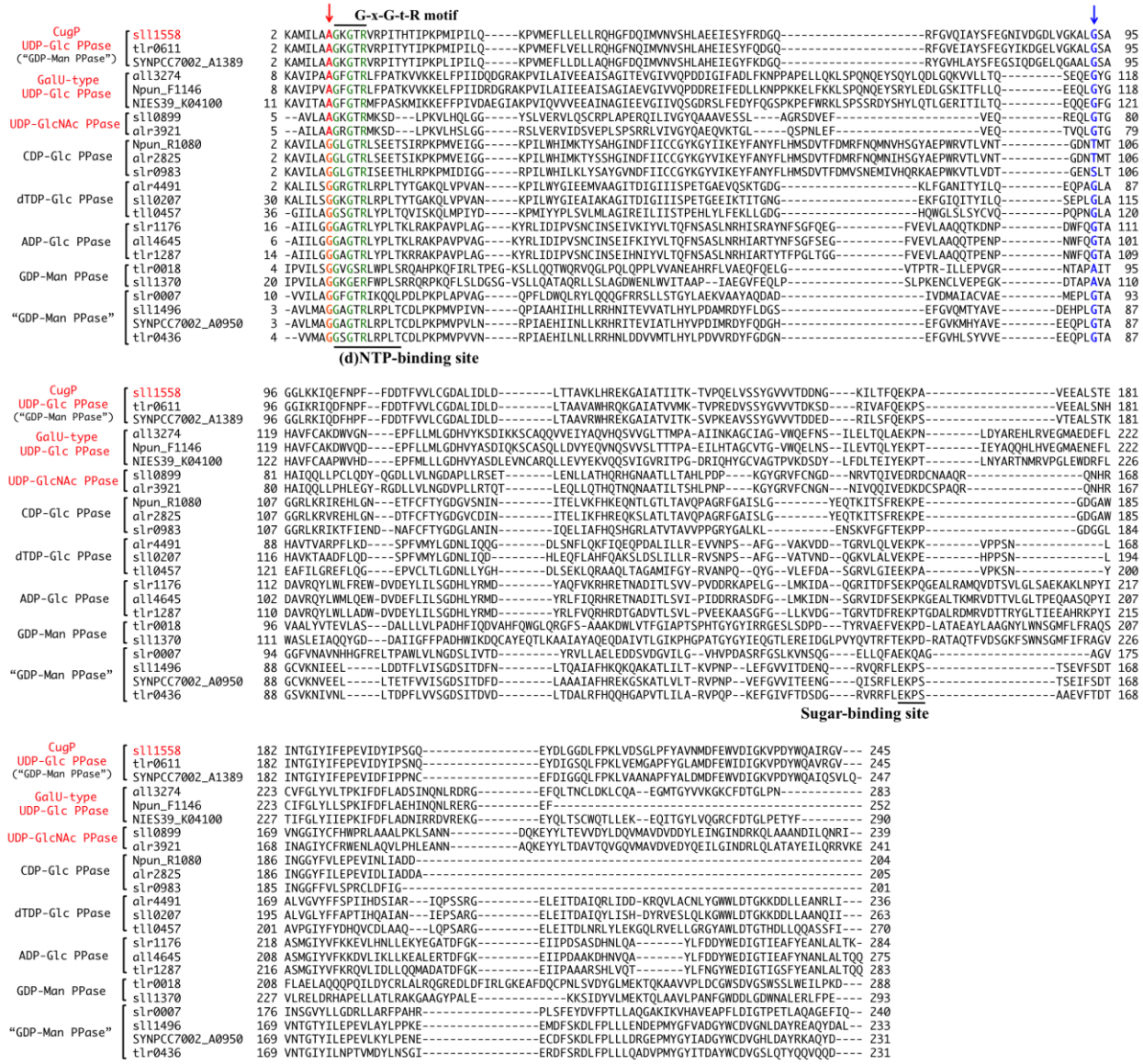

Fig. I-2. Sequence alignment of the NTP_transferase domain of the nucleotidyl transferases in Fig. I-1.

The highly conserved residues of the G-x-G-t-R motif are indicated in green. The residues that correspond to position 8 in Sll1558 are highlighted in red (Ala) and orange (Gly). The residues that are aligned with Gly-93 in Sll1558 are in blue type. Gly-93 in Sll1558 corresponds to Gly-117 in the GalU-type UDP-Glc PPase from *Corynebacterium glutamicum*, as shown in Fig. I-6.

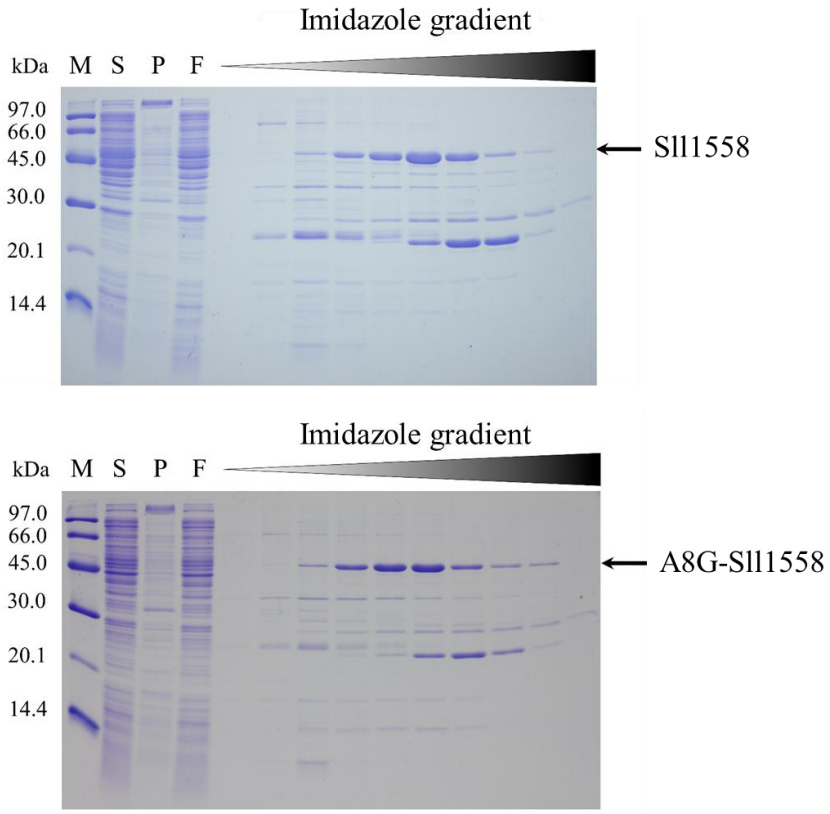

Fig. I-3. SDS-PAGE after Sll1558 and A8G-Sll1558expression and purification.

M, marker; S, supernatant of centrifugation; P, purified protein; F, chromatography flow through.

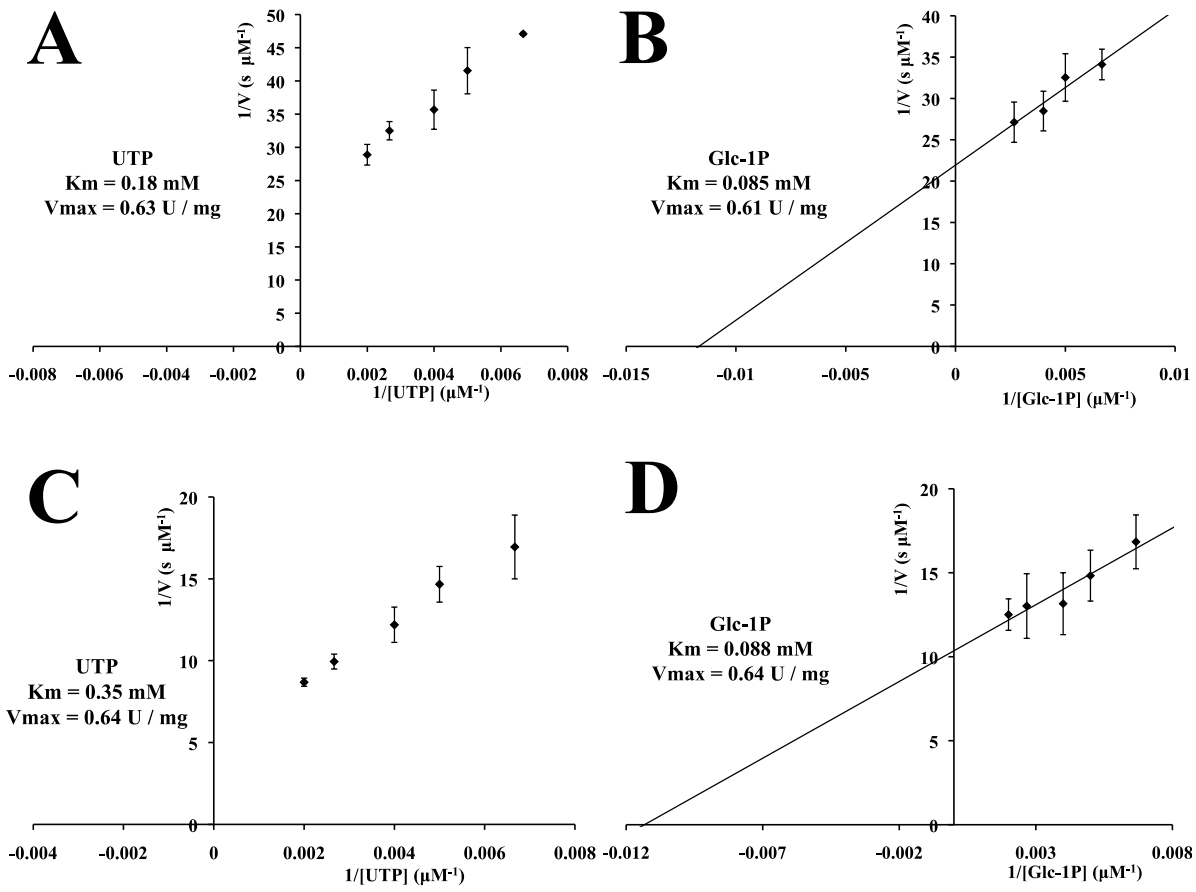

Fig. I-4. Lineweaver-Burk plots of the enzyme activity of Sll1558 (A, UTP; B, Glc-1P) and A8G-Sll1558 (C, UTP; D, Glc-1P).

As a fixed substrate, 1 mM Glc-1P was included in A and C, or 2 mM UTP was included in B and D. Protein concentration: A and B, 4.5 µg ml^{-1}; C, 18.0 µg ml^{-1}; D, 9.0 µg ml^{-1}. The error bars were based on SD (A, B, and C, n = 3; D, n = 6).

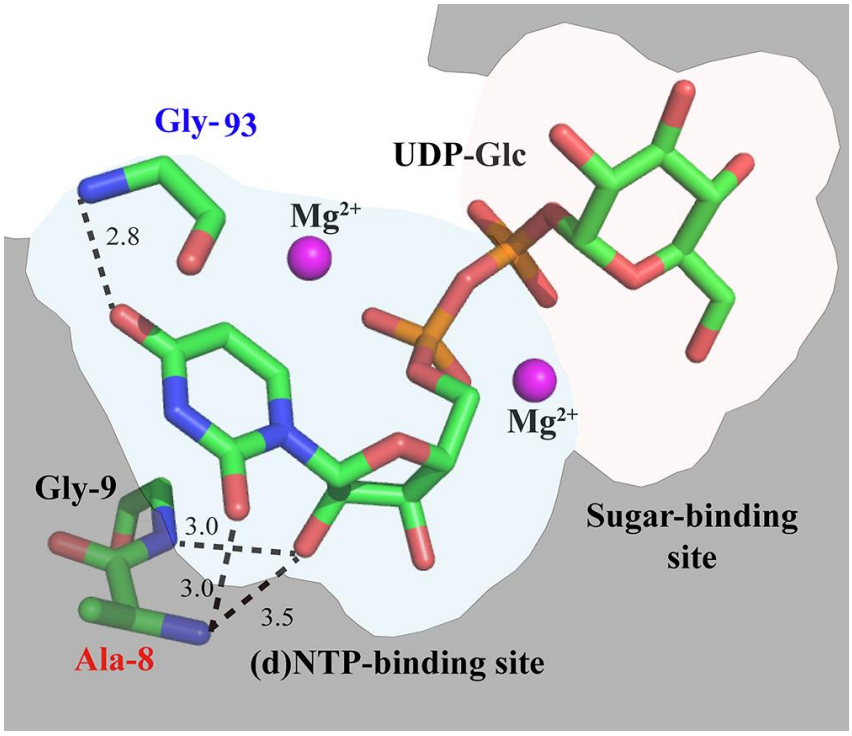

Fig. I-5. A predicted model of substrate-binding in Sll1558 (CugP-type UDP-Glc PPase).

This model is based on the crystal structure of GalU-type UDP-Glc PPase from *Corynebacterium glutamicum* in complex with magnesium and UDP-Glc (2PA4) (Thoden *et al.*, 2007a). Only a few key residues and substrates are shown (color indication: carbon, green; nitrogen, blue; oxygen, red; and phosphorus, orange). The broken lines denote hypothetical hydrogen bonds.

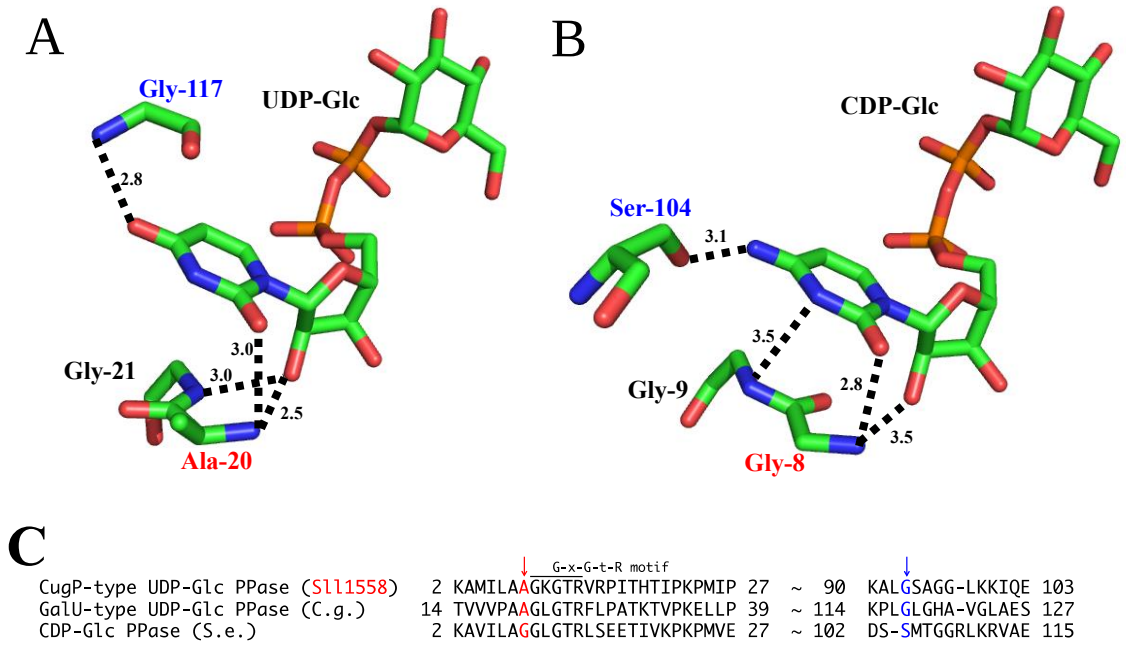

Fig. I-6. Key residues in the active sites of two nucleotidyl transferases and alignment of the active-site sequences of these two enzymes with the corresponding Sll1558 sequences. A, key substrate-binding residues in the active site of the GalU-type UDP-Glc PPase from *Corynebacterium glutamicum* (PDB ID, 2PA4). B, key substrate-binding residues in the active site of the CDP-Glc PPase from *Salmonella enterica* (PDB ID, 1TZF) (color code: carbon, green; nitrogen, blue; oxygen, red; and phosphorus, orange). The broken lines denote possible hydrogen bonds between transferase residues and the NDP moieties. C, sequence alignment of Sll1558, the GalU-type UDP-Glc PPase (*C.g.*) and CDP-Glc PPase (*S.e.*). Residues corresponding to Ala8 in Sll1558 are colored red. Residues corresponding to position 93 in Sll1558 are colored blue.

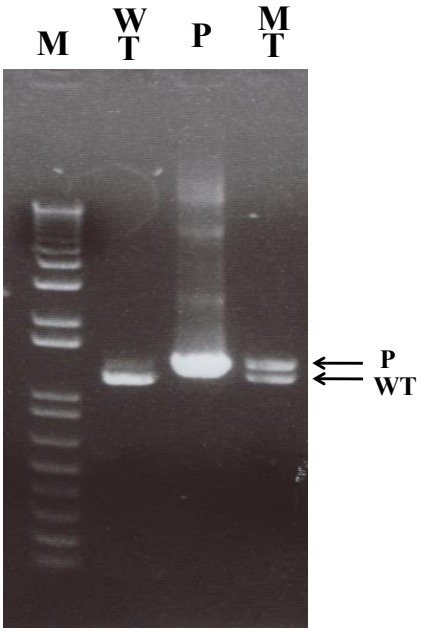

Fig. I-7. Agarose gel electrophoresis to check segregation of transformant.

M; marker, WT; wild type, P; transformation plasmid, MT; mutant. The band from wild type genome is 1.2 kbp (lower arrow), and that from transformation plasmid is 1.4 kbp (upper arrow).

I - b

Identification of various NDP-sugar pyrophosphorylases in cyanobacteria and bacteria.

将来的に学術雑誌に掲載される予定であるため除外

Chapter II

Extracellular cellulose biosynthesis in a thermophilic cyanobacterium *Thermosynechococcus vulcanus*.

将来的に学術雑誌に掲載される予定であるため除外

Chapter III

Biosynthesis and regulation of the viscous sulfated EPS in *Synechocystis* sp. PCC 6803 substr. PCC-P.

将来的に学術雑誌に掲載される予定であるため、及び特許申請予定であるため除外

General Discussion

将来的に学術雑誌に掲載される予定であるため、及び特許申請予定であるため除外

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