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CYANOBACTERIAL QUINOMICS

STUDIES OF QUINONES IN CYANOBACTERIA

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Biochemistry, Microbiology, and Molecular Biology

by

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ABSTRACT

Roles and functions of isoprenoid quinones (phyloquinone, plastoquinone-9) and α-tocopherol were investigated in cyanobacteria. Comparative genome analyses of 14 cyanobacteria suggested that phyloquinone (PhyQ) biosynthesis in most but not all cyanobacteria occurs similarly to menaquinone biosynthesis in *Escherichia coli*. This was further supported by the discovery that two cyanobacteria, *Synechococcus* sp. PCC 7002 and *Gloeobacter violaceus* PCC 7421, synthesize menaquinone-4 (MQ-4). Targeted inactivation of the *menB*, *menF*, and *menG* genes resulted in the incorporation of plastoquinone-9 (PQ-9) and demethyl-MQ or demethyl-PhyQ into Photosystem I (PS I) complexes. In the PS I complexes containing demethyl-PhyQ, the rate of electron transfer from A₁ to the iron-sulfur clusters slowed by a factor of two, while the kinetics of the P700⁺ [F₅/F₆]⁻ backreaction increased by a factor of 3 to 4. These results were explained by a lowering of the equilibrium constant between Q⁻/Q and F₅/F₆ in the demethyl-PhyQ containing PS I complexes by a factor of ~10.

Populations of α-tocopherol mutants of the cyanobacterium *Synechocystis* sp. PCC 6803, previously isolated in the presence of glucose, were found to be phenotypically and genotypically heterogeneous. Newly isolated, “authentic” tocopherol mutants were unable to grow in the presence of glucose at pH 7.0; this was suggested to be due to a significant reduction of the amounts of *sigA* and *rbcL* transcripts in cells under these conditions. The *slr2031* product, which has been previously shown to be involved in sulfur, nitrogen, and carbon metabolism, and genes encoding inorganic carbon uptake mechanisms, were found to be constitutively down-regulated in the
“authentic” tocopherol mutants. The results indicate that \( \alpha \)-tocopherol is involved in the transcriptional regulation of these metabolic genes and plays an important role in the coordination of nitrogen, sulfur, and carbon metabolism in *Synechocystis* sp. PCC 6803.

The PQ-9 biosynthesis pathway was predicted to be similar to that for ubiquinone biosynthesis based on comparative genome analyses of 14 cyanobacteria. However, targeted inactivation mutagenesis of eight genes encoding putative methyltransferase genes similar to UbiE/MenG in *E. coli* did not affect PQ-9 biosynthesis in *Synechocystis* sp. PCC 6803. Based on the results obtained, a possible PQ-9 biosynthesis pathway is proposed.
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Cyanobacteria are a phylogenetically coherent group of organisms, yet morphologically, physiologically, and genetically they are very diverse (Bryant, 1994). They are found in a variety of terrestrial and aqueous environments such as open oceans, coastal waters, fresh water lakes, rivers, municipal reservoirs, ponds, and even kitchen sinks in domestic households. Some perform N$_2$ fixation, and some are capable of chemotaxis or phototaxis or both. While the majority are obligate photoautotrophs, a few are able to grow chemoheterotrophically. Genome sizes vary from 2 Mbp to ~15 Mbp. Despite these differences, there is one thing that is common among all cyanobacteria: the unique ability to perform oxygenic photosynthesis. All cyanobacteria perform oxygenic photosynthesis, which uses CO$_2$ as the source of carbon, water as the source electrons, and light as the source of energy, in order to produce biomass. As a result, molecular oxygen is produced as a byproduct (Barber and Anderson, 2002). Through the activity of oxygenic photosynthesis, cyanobacteria have affected, and are continuing to affect, our environment since their first appearance more than 3 billion years ago. This activity of cyanobacteria has transformed the atmosphere of our planet from an extremely reducing to a rather oxidizing environment, and this change has supported the evolution of diverse forms of life on Earth. In an era of global warming, these organisms attract more attention given that about half of the global CO$_2$ fixation known to occur on Earth is carried out by cyanobacteria (Bryant, 2003).
Much has been learned about the molecular mechanisms of oxygenic photosynthesis owing to decades of study, which have dissected and largely solved the mystery of water oxidation and the detailed functions of the photosynthetic apparatus primarily from the viewpoint of protein functions. Such a perspective, however, has somewhat overlooked the biological importance of non-proteineous molecules that are also involved in the process. A group of such molecules are the quinones. In cyanobacteria these are \textbf{phyloquinone} (PhyQ, 2-methyl-3-phytyl-1,4-naphthoquinone, also known as vitamin \textit{K}_1), \textbf{plastoquinone} (PQ-9, 2,3-dimethyl-5-solanyl-1,4-benzoquinone). These two compounds are isoprenoid quinones, which are composed of a quinonoid nucleus with a polyprenyl substituent (Fig 1.1). There are two types of quinone substructures: 1,4-naphthoquinone (1,4-NQ), and 1,4-benzoquinone (1,4-BQ). PhyQ is a methyl- and phytol-substituted 1,4-NQ, while PQ-9 is a methyl- and solanyl-substituted 1,4-BQ. The phytol substituent is a partially saturated tetra-isoprene unit (equivalent to 20 carbon atoms), while the solanyl substituent is a nona-isoprene unit (equivalent to 45 carbon atoms). \textbf{α-Tocopherol} (2,5,7,8-tetramethyl-6-chromanol, also known vitamin E) is a tetramethyl-substituted chromanol, another type of quinone nucleus that is derived from a phytol-substituted 1,4-BQ after ring closure (Fig 1.1).

These quinones are synthesized only in oxygenic phototrophs including cyanobacteria, algae and higher plants (Collins and Jones, 1981; Threlfall and Whistance, 1971), and their functions are tightly connected to oxygenic photosynthesis. PhyQ and PQ-9 are bound cofactors of the Photosystem I (PS I) and Photosystem II (PS II) complexes, respectively, and in this context they mediate electron transfer as one-electron carriers (see below). PQ-9 also serves as a membrane-associated two-electron and two-
proton carrier, both in the photosynthetic electron transport and the respiratory electron transport chains. α-Tocopherol, on the other hand, is thought to provide protection against oxidative stress in animals and plants, although its role(s) in cyanobacteria has not yet been demonstrated (see below).

**Cyanobacterial oxygenic photosynthesis**

Fig 1.2 illustrates the process of oxygenic photosynthesis in cyanobacteria. In general, photosynthesis is described in the context of two processes known as the light reactions and dark reactions. The light reactions are the processes in which the energy of photons is transformed into an electrochemical membrane potential that drives ATP synthesis and into a strong reducing power that ultimately generates NADPH. The dark reactions are the processes in which CO₂ is reduced or “fixed” into biomass at the expense of NADPH and ATP as the reducing power and energy source, respectively.

The light reactions are catalyzed by the photosynthetic electron transport chain that resides in the thylakoid membranes (Bryant, 1994; Hervás et al., 2003). This electron transport chain involves 3 membrane integral protein complexes (PS I, PS II, cytochrome b₆f complex) and 2 water-soluble electron carriers (cytochrome c₆ or plastocyanin, and ferredoxin or flavodoxin), as well as the lipid-soluble electron carrier PQ-9 (Fig 1.2). X-ray crystallographic structures of PS I, PS II, and cytochrome b₆f complexes are now available at resolutions of 2.5 Å (Jordan et al., 2001), 3.5 Å (Ferreira et al., 2004), and 3.0 Å (Kurisu et al., 2001), respectively. Upon illumination, photons absorbed by the peripheral antenna complexes known as phycobilisomes are transferred predominantly to the PS II complexes, in which they trigger photochemistry that drives the light-dependent
oxidation of water and the reduction of plastoquinone (Barry et al., 1994; Bricker and Ghanotakis, 1996; Britt, 1996; Diner and Babcock, 1996). Water oxidation occurs in the thylakoid lumen and results in the release of molecular oxygen and protons (2H₂O → O₂ + 4H⁺ + 4e⁻), while the reduction and protonation of PQ-9 occurs on the stromal side of the membrane and releases doubly reduced plastoquinone, or plastoquinol (PQH₂), into the membrane matrix (2 PQ-9 + 4 H⁺ + 2 e⁻ → 2 PQH₂). PQH₂ shuttles electrons to the quinol oxidation site (o) of the cytochrome b₆f complex in a diffusion-dependent fashion, where it is reoxidized and becomes available for a new round of photochemical reduction by the PS II complexes. At this step, the electron transfer bifurcates into two separate pathways within the cytochrome b₆f complex; one electron is transferred to the Reiske iron-sulfur protein and subsequently to a soluble electron carrier such as cytochrome c₆ or plastocyanin in the lumen with concomitant release of two protons into the lumen, while the other electron is transferred to heme b₆ and heme b₇ and reduces PQ-9 at the quinone reduction site (r) near the stromal surface of the thylakoid membranes (Kallas, 1994; Hauska et al., 1996). After two rounds of PQH₂ oxidation, one PQ-9 at the quinone reduction site becomes doubly reduced and is protonated by concomitant uptake of two protons from the stroma (cytoplasm). The resulting PQH₂ is released into the membrane matrix where it joins the pool of PQH₂ and participates in electron donation to the cytochrome b₆f complex (2PQH₂(o) + PQ (r) + 2H⁺_Lumen → 2PQ(o) + PQH₂(r) + 4H⁺_Stroma + 2e⁻). This process is known as the Q cycle, and the result is an amplification of the proton gradient across the membranes, which is coupled to ATP synthesis by ATP synthase (Michell, 1976). The final steps of the electron transport chain involve the PS I complex. Essentially, this light-dependent cytochrome c₆/plastocyanin-
ferredoxin/flavodoxin oxidoreductase shuttles an electron from cytochrome $c_6$ or plastocyanin in the lumen to ferredoxin or flavodoxin in the stroma (Golbeck, 1994; Brettel and Leibl, 2001). The resulting strong reductant donates an electron to ferredoxin-NADPH$^+$ oxidoreductase, which catalyzes the reduction of NADP$^+$ to NADPH (Morand et al., 1994).

It is noteworthy that the cyanobacterial photosynthetic electron transport chain shares the membrane-associated PQ-9 pool and cytochrome $b_{6f}$ complexes with the respiratory electron transport chain (Fig 1.2). In this case, electrons enter the electron transport chain through NADH dehydrogenase in the form of NAD(P)H that is generated through glycolysis and the TCA cycle, and the oxidative pentose phosphate pathway. The terminal electron acceptor is molecular oxygen, which is reduced and protonated by cytochrome $c$ oxidase resulting in generation of water (Schmetterer, 1994).

NADPH and ATP generated through the photosynthetic electron transport chain are used to fuel the dark reactions. A CO$_2$ molecule is first incorporated into the C$_5$ backbone of ribulose-1,5-bisphosphate, which is then split into two molecules of 3-phosphoglycerate (Tabita, 1987, 1994). This reaction is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which is probably the most abundant protein on Earth (Garrett and Grisham, 1999). In cyanobacteria, RuBisCO is housed within protein-encapsulated structures known as carboxysomes (Badger and Price, 2003), which play a role in locally increasing the CO$_2$ concentration. 3-Phosphoglycerate is reduced to 3-phosphoglyceraldehyde, which is processed through the reductive pentose phosphate pathway to regenerate ribulose-1,5-bisphosphate. The processes are known as the Calvin cycle and cause the net production of organic molecules that constitute the
metabolic precursors for the biosynthesis of proteins, nucleic acids, sugars, pigments, and other metabolites. The net production of biomass in the Calvin cycle can be expressed as the following equation:

$$6 \text{CO}_2 + 12 \text{NADPH} + 18 \text{ATP} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 12 \text{NADP}^+ + 18 \text{ADP} + 18 \text{P}_i$$

*PhyQ and Photosystem I*

The PS I complex consists of twelve protein subunits, 96 Chl $a$ molecules, 22 $\beta$-carotene molecules, two PhyQ molecules, three iron-sulfur clusters, and some structural lipids and water molecules (Jordan et al., 2001). Tightly bound electron transfer cofactors are found within the core of the complex formed by PsaA and PsaB proteins. These included a chlorophyll $a$ (Chl $a$) dimer (P700), two Chl $a$ monomers (A$_0$), two PhyQ molecules (A$_1$), and three [4Fe-4S] clusters (denoted as F$_X$, F$_A$, F$_B$). These cofactors are arranged in two branches related by $C_2$ symmetry. The spatial arrangement of these cofactors and their estimated *in situ* redox potentials are shown in Fig. 1.3. Upon photoexcitation, the primary donor P700 reduces the primary acceptor, A$_0$, resulting in the initial charge separated state [P700$^+$ A$_0^{-}$]. Subsequent electron transfer to the secondary acceptor A$_1$ stabilizes the charge-separated state [P700$^+$ A$_0$ A$_1^{-}$] and minimizes the rapid charge recombination that occurs between P700$^+$ and A$_0^{-}$. The electron is subsequently transferred to F$_X$ [P700$^+$ A$_0$ A$_1$ F$_X^{-}$], and then to the terminal acceptors F$_A$ [P700$^+$ A$_0$ A$_1$ F$_X$ F$_A^{-}$] and F$_B$ [P700$^+$ A$_0$ A$_1$ F$_X$ F$_A$ F$_B^{-}$], which are located in the peripheral PsaC subunit. The reduction of ferredoxin or flavodoxin takes place upon direct protein-protein interaction between these acceptors and the stromal proteins: PsaC, PsaD, and PsaE (Zhao et al., 1991, 1993; Li et al., 1991; Golbeck, 1994). On the lumenal side, the
photooxidized $\text{P700}^+$ accepts an electron from reduced cytochrome $c_6$ or plastocyanin (Durán et al., 2004).

Since the first discovery of PhyQ in oxygenic phototrophs by Dam in 1941 (cited in Hauska, 1988), the role of PhyQ in PS I as the secondary acceptor $A_1$ was obscure for more than two decades. After its initial discovery by Thornber and coworkers in the 1970’s, in the 1980s it was found to be exclusively associated with PS I (Thornber et al., 1976; Takahashi et al., 1985; Shoeder and Locau, 1986) and to participate in electron transfer as shown by spin-polarized EPR analysis (Petersen et al., 1987) and optical kinetic analysis (Brettel et al., 1987; see also reviews by Golbeck, 1994, 2003 and references therein). Further supporting evidence was derived from solvent extraction studies, in which extraction of PhyQ from PS I with hexane or ether resulted in a rapid, nano-second-scale charge recombination between $\text{P700}^+$ and $A_0^-$. Stable charge separation was restored by the addition of exogenous PhyQ (Biggins and Mathis, 1988; Itoh and Iwaki, 1989).

This *in vitro* reconstitution method was subsequently used to study the structural and thermodynamic requirements for $A_1$ by introducing diverse quinones of abiotic origin into the $A_1$ site, including benzoquinones, naphthoquinones, and anthraquinones with various ring substitutions (Iwaki and Itoh, 1989, 1991; Itoh and Iwaki, 1991; see also review by Itoh 2001). These authors concluded that the successful restoration of stable charge separation occurs only when quinones with redox potentials between those of $A_0$ and $F_X$ are used. They additionally found that neither the structure of the head group nor the presence of an alkyl substituent is important. This conclusion contradicted earlier studies, in which the presence of an alkyl substituent was shown to be essential when
hexane/hexane-methanol-extracted PS I complexes were studied (Biggins and Mathis, 1998). These contradictory interpretations imply that different solvent-extraction methods affect the architecture of PS I differently; hence, the inconsistency in conclusions may reflect artifacts of the methods employed. Such artifacts can be avoided if “biologically” or “physiologically” modified materials are used. Materials that have not gone through harsh extraction processes, which result in co-extraction of other important PS I constituents such as water, Chl a, carotenoid, and lipid molecules (Biggins and Mathis, 1988; Itoh and Iwaki, 1989), should produce more consistent and interpretable results. Some of these molecules are located in the vicinity of the quinone-binding site; in particular, as revealed by 2.5Å-resolution crystallographic structure (Jordan et al., 2002), β-carotenes and chlorophylls form hydrophobic contacts with the phytol side chain of PhyQ and seem to provide structural stability for this important electron carrier.

Genetic manipulation of the PhyQ biosynthetic pathway was first introduced by Chitnis and colleagues (Johnson et al., 2000) and provided an alternative and very powerful experimental system, by which the selective elimination of PhyQ from PS I could be achieved in vivo. Based on the menaquinone biosynthetic pathway in Escherichia coli (Meganathan, 2001), a part of the PhyQ biosynthetic pathway in Synechocystis sp. PCC 6803 was first predicted. Targeted insertional mutagenesis of genes encoding dihydroxynaphthoate phytyltransferase (MenA) and dihydroxynaphthoate-CoA synthase (MenB) successfully interrupted the PhyQ biosynthesis in the mutant strains (Johnson et al., 2000). The PS I complexes isolated from these mutants completely lacked PhyQ and accumulated PQ-9 (Johnson et al., 2000; Zybailov et al., 2000; Semenov et al., 2000). As confirmed by continuous-wave EPR,
electron spin-polarized transient EPR, and electron spin-echo modulation experiments (Zybailov et al., 2000), the orientation of the carbonyl bonds relative to the membrane normal and the distance between PQ-9 and P700$^+$ were the same as with PhyQ in the wild-type PS I complexes. In the PS I complexes isolated from the mutants, PQ-9 was shown to participate in electron transfer from A$_0$ to F$_X$ (Zybailov et al., 2000; Semenov et al., 2000), although the rate of forward electron transfer from A$_1$ to F$_X$ slowed at least by a factor of 100. The estimated in situ redox potential of PQ-9 differs from that of PhyQ by ca. 130 mV (Shinkarev et al., 2002), which is consistent with the faster rate of charge recombination between P700$^+$ and FeS$^-$ in PS I (Semenov et al., 2000). Despite this significant defect in the efficiency of forward electron transfer, the PS I complexes containing PQ-9 supported nearly 85% of the wild-type activity of PS I, as measured by the rate of cytochrome c$_6$:flavodoxin oxidoreduction (Johnson et al., 2000). More recent studies have shown that PS I complexes are functional when the A$_1$ site is occupied by PhyQ, PQ-9 and even anthraquinones with various substituents (Golbeck et al., 2001; Zybailov, 2003). The combined results indicate that PS I has an innate capacity to accommodate and use a wide range of redox-active quinones with various structures and substituents.

**PQ-9 and Photosystem II**

Cyanobacterial Photosystem II is a light-driven, water:plastoquinone oxidoreductase and is composed of at least 19 protein subunits, seven carotenoid molecules, two pheophytin molecules, two PQ-9 molecules, two bicarbonate molecules, one non-heme Fe, one heme $b$, and one heme $c$ (Zouni et al., 2001; Ferreira et al.,...
Four manganese atoms and one calcium atom are bound to the oxygen-evolving complex that is attached to the lumenal surface of the PS II complex and is responsible for water oxidation. As shown in Fig. 1.4, the electron transfer cofactors are arranged in two branches that are related by a pseudo-C2 symmetry axis within the core of the PS II complex that is formed by the D1 and D2 proteins. Aside from the differences in the specific types of the cofactors and their arrangement, the principle of the photochemistry is largely the same in PS II as in PS I. Upon photoexcitation of a Chl a monomer (P680, the primary donor P680), an electron is transferred to a pheophytin molecule (PheoD1) (Danielius et al., 1987). This initial charge separation [P680+ Pheo−] is further stabilized by electron transfer to a tightly bound PQ-9 molecule (QA) [P680+ Pheo QA−], which is then followed by transfer to a second PQ-9 molecule (QB) [P680+ Pheo QA QB−] (Renger, 1992; Barry et al., 1994). The second electron transfer doubly reduces QB, which, after protonation, dissociates from the complex into the thylakoid membrane matrix (Bouges-Bocquet, 1973; Velthuys and Amesz, 1974; Govindjee and van Rensen, 1993). The oxidized P680+ is reduced by the redox-active tyrosine Z (TyrZ, Y161 in the D1 protein) (Gerken et al., 1988; Diner and Babcock, 1996), which is then reduced by a tetramanganese cluster that catalyzes the abstraction of electrons from water molecules (Bricker and Ghanotakis, 1996). With the exception of QA, all redox-active cofactors that are involved in electron transfer are in principle bound by subunit D1 (PsbA) (Ferreira et al., 2004).

Prolonged exposure to high light intensity is known to cause irreversible damage to PS II, and this phenomenon is generally known as photoinhibition (see review by Aro et al., 1993, and references therein). Under such conditions, QA becomes doubly reduced.
and leaves the binding pocket (Vass et al., 1992). This leads to rapid charge recombination between P680$^+$ and Pheo$^-$ and to formation of the triplet P680$^T$. P680$^T$ can readily react with molecular oxygen in its vicinity and generate singlet oxygen (O$_2^*$). Singlet oxygen, and other reactive oxygen species generated from it, are responsible for the photodamage. Photoinhibition can also occur under normal light intensity, when the donation of electrons to P680 occurs slower than their removal by the acceptor side (Chen et al., 1995; Aro et al, 1993; Anderson and Chow, 2002). In such cases, highly oxidizing Tyr $\cdot$ and P680$^+$ cation radicals are formed, which can extract electrons from the surrounding environment and also cause irreversible damage to PS II. In either case, the D1 protein is rapidly degraded (Gong and Ohad, 1991; Philbrick et al., 1991; Aro et al., 1993).

Genetic manipulation of the quinone species present in the PS II quinone-binding sites has not been demonstrated. The PQ-9 biosynthesis pathway has been intensively studied, and the complete pathway is known in higher plants (Threlfall and Whistance, 1971). The synthesis occurs from the precursor homogentisate after two enzymatic steps involving homogentisate solanyltransferase and 2-methyl-6-solanyl-1,4-benzoquinone (MSBQ) methyltransferase (Soll et al., 1980, 1985; Collakova and DellaPenna, 2001; Cheng et al., 2003; also see review by Threlfall and Whistance, 1971) (Fig. 1.5). This pathway in plants overlaps with the $\alpha$-tocopherol biosynthesis pathway, since both utilize homogentisate as a precursor, and both pathways share the step that introduces the second methyl group, a reaction which is catalyzed by MPBQ/MSBQ methyltransferase (Cheng et al. 2003) (Fig 1.5). Recent studies have shown that the cyanobacterial PQ-9 biosynthetic pathway involves neither homogentisate (Dänhardt et al., 2002) nor MPBQ
activity (Cheng et al., 2003). Therefore, PQ-9 seems to be synthesized by a completely different pathway in cyanobacteria.

**Role of α-Tocopherol**

α-Tocopherol was first discovered by Evans and Bishop (1922) as a factor essential for reproduction in rats. Forty years later, its antioxidant activity was recognized by Epstein and colleagues (Epstein et al., 1966). Because α-tocopherol is an essential component of our diet, much work since then has been done on its significance in animal systems. Studies in animals, animal cell cultures, and artificial membranes have shown that tocopherols scavenge or quench various reactive oxygen species and lipid oxidation by-products that would otherwise propagate lipid peroxidation chain reactions in membranes (Kamal-Eldin and Appelqvist, 1996). Upon interaction with a prooxidant, α-tocopherol can undergo one-electron transfer and form a relatively stable tocopheroxyl cation radical, which is then recycled back to α-tocopherol by an antioxidant network consisting of ascorbate, glutathione, and NADPH/NADH (see review by Packer et al., 2001). α-Tocopherol can also undergo two-electron transfer and form α-tocopherylquinone. In higher plants, this antioxidant function of α-tocopherol has been discussed in connection with protection against oxidative stress caused by various environmental factors (Munné-Bosch and Leonor, 2000). In the eukaryotic green algae *Chlamydomonas reinhardii*, the herbicide-mediated interruption of the α-tocopherol biosynthetic pathway rendered PS II more susceptible to oxidative stress induced by an
extreme high light illumination (Trebst et al., 2002). Under these conditions, the D1 protein was rapidly degraded.

In addition to these antioxidant functions, other “non-antioxidant” functions related to modulation of signaling and transcriptional regulation in mammals have also been reported (Chan et al., 2001; Azzi et al., 2002; Ricciarelli et al., 2002). For example, α-tocopherol has been shown to bind to phospholipase A2 specifically at the substrate-binding pocket and to act as a competitive inhibitor, which thereby decreased the release of arachidonic acid for eicosanoid synthesis (Chandra et al., 2002). α-Tocopherol has also been suggested to modulate the phosphorylation state of protein kinase Cα in rat smooth-muscle cells, possibly via phosphorylation of protein phosphatase 2A (Ricciarelli et al., 1998). α-Tocopherol is also directly involved in transcriptional regulation in animals, including the expression of the genes encoding liver collagen αI, α-tocopherol transfer protein, and α-tropomyosin collagenase (Yamaguchi et al., 2001; Azzi et al., 2002). Whether α-tocopherol in cyanobacteria performs one or both of these roles has not yet been demonstrated.

**Cyanobacterial Quinomics**

The goal of this study was to develop experimental systems in which the type and amounts of the quinone species found in cyanobacteria could be manipulated *in vivo*. Such systems would allow the functions of the complexes in which they occur to be studied. This has been accomplished by using comparative genome analyses, combined with a reverse genetic approach, to predict and verify the biosynthetic pathways of PhyQ,
α-tocopherol, and PQ-9 (Chapter 2, 3, 6). Consequences of altering the nature of these quinone species are studied in the context of PS I function (Chapter 3), stress responses (Chapter 4), and gene regulation (Chapter 5). To express the relatively wide scope of and inclusiveness of these studies, I introduce the term “quinomics” to describe my investigations of the cyanobacterial “quinome”.
ABBREVIATIONS

BQ  benzoquinone
Chl $a$  chlorophyll $a$
DHNA  1,4-dihydroxy-2-naphthoate
MPBQ  2-methyl-6-phytlyl-1,4-benzoquinone
MSBQ  2-methyl-6-solanyl-1,4-benzoquinone
NQ  naphthoquinone
PhyQ  phylloquinone
PQ-9  plastoquinone-9
PQH$_2$  plastoquinol
PS I  Photosystem I
PS II  Photosystem II
RuBisCO  ribulose-1,5-bisphosphate carboxylase/oxygenase
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Fig 1.1: Structures of phyloquinone (PhyQ), plastoquinone-9 (PQ-9), and α-tocopherol.
**Fig 1.2: Scheme for oxygenic photosynthesis in cyanobacteria.**

ATPase, ATP synthase; COX, cytochrome c oxidase; Cyt b₆f, the cytochrome b₆f complex; Cyt c₆, cytochrome c₆; Fd, ferredoxin; Fl, flavodoxin; FNR, ferredoxin:NADP⁺ oxidoreductase; NDH, NADH dehydrogenase; PBS, phycobilisome; PC, plastocyanin; PS I, Photosystem I; PS II, Photosystem II.
Fig 1.3: The spatial arrangement and redox potentials of the electron transfer cofactors in Photosystem I.

The soluble electron donor cytochrome $c_6$ (Cyt$c_6$) and electron acceptor ferredoxin (Fd) are also shown.
Fig 1.4: The electron transfer cofactors in Photosystem II.

(A) Illustration of electron transfer between the cofactors, (B) the spatial arrangement of the cofactors obtained from the 3.5-Å resolution X-ray crystal structure, adapted from Ferreira KN, Iverson TM, Maghlaoui K, Barber J, Iwata S (2004) Science 303: 1831-1838.
Fig 1.5: Biosynthetic pathway of PQ-9 and α-tocopherol in higher plants.

BQ, benzoquinone; HDDP, 4-hydroxyphenylpyruvate dioxygenase; HTP, homogentisate phytoltransferase; MSBQ/MPBQ MT, 2-methyl-6-solanyl-1,4-benzoquinone/2-methyl-6-phytanyl-1,4-benzoquinone methyltransferase; SAM, S-adenosyl-L-methionine; TC, tocoferol cyclase; γ-TMT, γ-tocopherol methyltransferase.
Chapter 2

Comparative Genome Analysis and Identification of Menaquinone-4 Biosynthetic Pathways in Cyanobacteria

Publications:

Yumiko Sakuragi, Boris Zybailov, Gaozhong Shen, Ramakrishnan Balasubramanian, Bruce A. Diner, Irina Karygina, Yulia Pushkar, Dietmar Stehlik, Donald A. Bryant and John H. Golbeck, Recruitment of a foreign quinone into the A1 site of Photosystem I. Spectroscopic characterization of a menB rubA double deletion mutant in Synechococcus sp. PCC 7002 containing plastoquinone-9 but devoid of F_X, F_B, F_A, in preparation

Yumiko Sakuragi and Donald A. Bryant, a review article ‘Genetic Manipulation of the Quinone Pathway in Photosystem I’ in Photosystem I: the Plastocyanin:Ferredoxin Oxidoreductase in Photosynthesis’, Golbeck JH (ed), in preparation
Phylloquinone (PhyQ, 2-methyl-3-phytyl-1,4-naphthoquinone) is an electron transfer cofactor at the A1 site of Photosystem I and is synthesized only in oxygenic phototrophs such as cyanobacteria, algae, and higher plants. The biosynthetic pathway of PhyQ was investigated by means of comparative genomics using the menaquinone (MQ) biosynthesis genes of *Escherichia coli* as queries. The homologs of *menA, menB, menC, menD, menE, menF, menG* and *menH* were found to be conserved in most of the cyanobacteria. In some of these organisms, these genes formed clusters similar to those found in *Escherichia coli, Bacillus subtilis, and Chlorobium tepidum*, suggesting that the PhyQ biosynthetic pathway has evolved from that for MQ. In support of this hypothesis it was demonstrated that *Gloeobacter violaceus* and *Synechococcus* sp. PCC 7002 wild-type strains synthesize MQ-4 (2-methyl-3-geranylgeranyl-1,4-naphthoquinone) that functions at the A1 site in Photosystem I complexes. Targeted insertional mutagenesis of *menB, menF*, and *menG* in *Synechococcus* sp. PCC 7002 results in the complete interruption of MQ-4 biosynthesis and demonstrates that the predicted pathway indeed functions in this cyanobacterium. Phylogenetic analysis of MenA (dihydroxynaphthoate phytlytransferase) showed that the MenA sequences of the plant *Arabidopsis thaliana* and *Oryza sativa* group with cyanobacterial sequences, whereas MenB (dihydroxynaphthoate-CoA synthase) and MenD (2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase) do not. This leads to the hypothesis that PhyQ biosynthesis is a result of mosaic evolution derived from a non-cyanobacterial MQ
pathway together with a cyanobacterial-type MenA, possibly derived from endosymbiosis.

**ABBREVIATIONS**

- Chl $a$: chlorophyll $a$
- DHNA: dihydroxynaphthoate
- HPLC: high-performance liquid chromatography
- MQ-4: menaquinone-4
- PCR: polymerase chain reaction
- PhyQ: phylloquinone
- PQ: plastoquinone-9
- PS I: Photosystem I
INTRODUCTION

Phylloquinone (PhyQ, 2-methyl-3-phytyl-1,4-naphthoquinone), also known as vitamin K₁, is synthesized only in oxygenic phototrophs such as cyanobacteria, algae, and higher plants. PhyQ plays a crucial role in photosynthesis as a cofactor in Photosystem I by mediating electron transfer between the primary acceptor A₀ (Chl a) and the terminal acceptor Fₓ (4Fe-4S cluster) after photoexcitation of the primary donor P700 (Golbeck, 1994, 2003; Brettel and Leibl, 2001). Two molecules of PhyQ are found within the protein environments of the PS I complex, which is composed of 12 protein subunits, 96 Chl a molecules, 22 β-carotenes, 3 [4Fe-4S] clusters, and 4-5 structural lipids (Jordan et al., 2001). PS I is a light-driven cytochrome c₆/plastocyanin:ferredoxin/flavodoxin oxidoreductase and is responsible for providing electrons that are ultimately used for the generation of NADPH, the reducing power for cellular anabolism. The quantum efficiency of this enzyme is largely dependent on the stability of a charge-separated state between P700⁺ and Fₓ⁻, and the presence of PhyQ ensures an optimal rate of transfer between these cofactors.

Biosynthesis of PhyQ has been studied by isotope tracer and direct enzymatic activity assays in higher plants (see a review by Pennock and Threlfall, 1981). The results have indicated that PhyQ biosynthesis resembles menaquinone (MQ) biosynthesis in E. coli (see reviews by Meganathan 1996, 2001). MQ biosynthesis occurs through eight enzymatic steps as summarized in Fig 2.1. The first committed step is the isomerization of chorismate to isochorismate by isochorismate synthase (MenF), followed by the
condensation of isochorismate and thiamine-pyrophosphate-succinic semialdehyde by 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase (MenD). Dehydration of SHCHC is catalyzed by 6-succinylbenzoate (OSB) synthase (MenC) and the subsequent CoA thioesterification by OSB-CoA synthetase (MenE); the ring cyclization reaction is catalyzed by 1,4-hydroxynaphthoyl-CoA synthase (MenB), and deesterification by 1,4-hydroxynaphthoyl-CoA thioesterase (MenH) results in 1,4-dihydroxy-2-naphthoate (DHNA). DHNA polyprenyltransferase (MenA) catalyzes the condensation of DHNA and polyprenyl diphosphate, yielding 2-polyprenyl-1,4-naphthoquinone (demethylmenaquinone). The final methylation at the C2 position is carried out by 2-polyprenyl-1,4-naphthoquinone methyltransferase (MenG) with S-adenosyl-methionine as a methyl donor, resulting in the production of MQ. PhyQ and MQ have the same general molecular structure except that PhyQ has a partially saturated, C20 phytol substituent and MQ found in other bacteria has an unsaturated polyprenyl substituent typically consisting of 30 to 50 carbon units (Collins and Jones, 1981).

The targeted inactivation of the menA, menB, menD, and menE homologs in Synechocystis sp. PCC 6803 was shown to result in the complete interruption of PhyQ biosynthesis. This demonstrated that a MQ-like pathway is responsible for PhyQ biosynthesis in this organism (Johnson et al., 2000; Johnson et al., 2002). The following questions remained to be answered: is this MQ-like PhyQ biosynthesis pathway ubiquitous among cyanobacteria and higher plants; what is its evolutionary origin; how has it evolved among the various oxygenic phototrophs? In this study, MQ biosynthesis was investigated in 14 cyanobacteria whose genomes have been or are currently being
sequenced. The results indicate that the MQ-like pathway is conserved among most, although not all, of the cyanobacteria and higher plants. A detailed analysis of the gene arrangements and phylogenetic reconstruction lead to the hypothesis that the PhyQ biosynthetic pathway has evolved from the MQ pathway in both cyanobacteria and higher plants.
RESULTS

As of April 26, 2004, complete genomic sequences of 8 cyanobacteria are available: *Synechocystis* sp. PCC 6803 (Kaneko et al., 1996), *Nostoc* PCC 7120 (Kaneko et al., 2001), *Thermosynechococcus elongatus* BP-1 (Nakamura et al., 2002ab), *Gloeobacter violaceus* (Nakamura Y et al., 2003), *Prochlorococcus marinus* MED4 and *Prochlorococcus marinus* MIT9313 (Rocap et al., 2003), *Prochlorococcus marinus* SS120 (Dufresne et al., 2003), *Synechococcus* sp. WH8102 (Palenik et al., 2003); and incomplete sequences exist for *Synechococcus* sp. PCC 7002 (Jürgen Marquardt, Tao Li, Jindong Zhao, and Donald A. Bryant, unpublished), *Nostoc punctiforme* (Gene Bank accession number: NZ_AAAY00000000), *Trychodesmium erythraeum* (Gene Bank accession number: NZ_AABK00000000), *Synechococcus elongatus* sp. PCC 7942 (Gene Bank accession number: NZ_AADZ01000001), *Anabaena variabilis* ATCC 29413 (Gene Bank accession number: NZ_AAEA01000001), and *Crocosphaera watsonii* WH 8501 (Gene Bank accession number: NZ_AADV01000004). Similarity searches with the MenA through MenH protein sequences of *E. coli* as queries were performed against each genome, and the results are summarized in Table 2.1. Of 14 cyanobacteria examined, 11 were shown to possess the complete set of 8 Men protein homologs. Similarities based on amino acid sequences were typically in the range of 30-70%, although the MenB sequences were very highly conserved among a wide range of organisms including cyanobacteria, the γ-proteobacterium *E. coli*, the green sulfur bacterium *Chlorobium tepidum*, the Gram-positive bacterium *Bacillus subtilis*, and the archeon *Halobacterium* sp. NRC-1 (similarity >70%). In the genome of *G. violaceus,*
homologs of only MenA and MenG were detected. When the *Synechocystis* sp. PCC 6803 MenB sequence was used to search the *G. violaceus* genome database, the best hit was Gll2549 with only 38% similarity. Further database searching has revealed that Gll2549 is highly similar to 6-oxo camphor hydrolase in *Rhodococcus* sp. NCIMB 9784 (69%). When *Synechocystis* sp. PCC 6803 MenC, MenD, MenE, and MenF sequences were used to search the *G. violaceus* database, the best hits were Gll3099 (35%), Gll2804 (44%), Glr1146 (45%), and Gll0757 (43%), respectively. These ORFs also showed higher similarities to other hypothetical proteins: Gll3099 is similar to chloromuconate cycloisomerase in *Clostridium acetobutylicum* (57%); Gll2804 is similar to acetolactate synthase in *Synechocystis* sp. PCC 6803 (Sll1981, 86%); Glr1146 is similar to a long-chain fatty-acid CoA ligase in *Bacillus cereus* (54%); and Gll0757 is similar to anthranilate synthase component I in *Synechocystis* sp. PCC 6803 (Slr1979, 66%). No homologs of MenD (*A. variabilis*) or MenC and MenF (*C. watsonii*) have been identified to date, but these genomes are not yet completely sequenced.

Some of the *men* genes involved in PhyQ biosynthesis are found to form clusters in several cyanobacterial genomes. A cluster composed of *menF, menA, menC,* and *menE* is conserved in *T. erythraeum, N. punctiforme, Nostoc* sp. PCC 7120, *Synechococcus* sp. WH 8102, and all three *Prochlorococcus* species (Fig 2.2). In a separate region of the genome, *menD* and *menB* are also found to form a cluster. The observed gene arrangements in these cyanobacteria are similar to parts of the gene clusters found in *E. coli, C. tepidum, B. subtilis* and *Halobacterium* sp. NRC-1 (Fig 2.2). Combined with the conservation in the amino acid sequences among Men proteins, the conserved
arrangements of the men genes are strong indications that PhyQ biosynthesis in cyanobacteria evolved from the MQ pathway of other bacteria.

Given that oxygenic photosynthesis is believed to have originated in cyanobacteria and that PhyQ is associated exclusively with PS I, one may expect that the common ancestor of higher plants and algae acquired the entire PhyQ biosynthetic pathway from cyanobacteria upon endosymbiosis. Interestingly, the men gene arrangement in Arabidopsis thaliana does not resemble that in cyanobacteria (Fig 2.2). Lack of conserved gene arrangements does not necessarily indicate the absence of an evolutionary relationship; however, it is sufficient to cast doubt about it. Thus the evolutionary relatedness between cyanobacterial and higher plant PhyQ biosynthetic genes was evaluated further. As shown in Table 2.1, A. thaliana contains the whole set of men genes in its nuclear chromosomes, which indicates that PhyQ biosynthesis in A. thaliana is similar to that in cyanobacteria and to MQ synthesis in other prokaryotes. In a phylogenetic tree based on the MenA sequences, the two higher plants, A. thaliana and Oryza sativa, form a clade within a domain composed entirely of cyanobacteria, which suggests that MenA in higher plants is evolutionarily more related to MenA in cyanobacteria than to MenA of other eubacteria (Fig 2.3A). On the contrary, in trees based on the MenB and MenD sequences, these two higher plants form a clade outside the cyanobacterial domain. This suggests that MenB and MenD in higher plants are more closely related to their homologs in other eubacteria than to those in cyanobacteria (Fig 2.3B and C). Trees based on the MenC, MenE, and MenF sequences showed the topology similar to those observed for the MenB and MenD sequences; however, the detailed branching orders within the cyanobacterial domain were variable probably due to low sequence conservation of these proteins (< ~50%). The observed variation in the
phylogenetic relationships therefore suggests that not all men genes in higher plants originated from cyanobacteria.

By comparative genome analyses and phylogenetic reconstructions, it was so far hypothesized that PhyQ biosynthesis in cyanobacteria and higher plants evolved from that of MQ, which suggests that early cyanobacteria synthesized and utilized MQ for the PS I complexes. This hypothesis was supported by the discovery that G. violaceus and Synechococcus sp. PCC 7002 synthesize MQ-4 instead of PhyQ. Fig 2.4A shows the HPLC profile of the solvent extract obtained from the PS I complexes isolated from Synechocystis sp. PCC 6803 wild type. PhyQ, which typically elutes at 22.4 min, was shown to be present. In the HPLC profiles of the solvent extracts obtained from the PS I complexes isolated from Synechococcus sp. PCC 7002 wild type and from the G. violaceus whole cells, no such peak was present as judged by the absence of UV-absorbing compounds at this retention time (Fig 2.4B and C). Further examination of the chromatograms led to the discovery of a new peak at ca. 14 min that had an intense UV-absorption with maxima at 248, 263, 270, and 332 nm (Fig 2.5B and C), which coincide well with the absorption spectrum of phylloquinone obtained from Synechocystis sp. PCC 6803 (Fig 2.5A). This spectrum is characteristic of a 1,4-naphthoquinoid compound with two alkyl substitutions at the C2 and C3 positions (Dunphy and Brodie, 1971). The absence of PhyQ in G. violaceus was further confirmed by the absence of the PhyQ absorption in the eluate at 22.5 min (Fig 2.5C). The same results were obtained for Synechococcus sp. PCC 7002. Mass spectrometric analysis of the solvent extracts of PS I complex isolated from Synechococcus sp. PCC 7002 showed that the compound eluting at ca. 14 min has a m/z of 444, as opposed to a m/z of 450 for PhyQ (Figure 2.6). This difference is best explained by the presence of a geranylgeranyl substituent with four unsaturated isoprenoid units. Thus, Synechococcus sp. PCC 7002 synthesizes MQ-4
instead of PhyQ. The absence of PhyQ was confirmed by the absence of any component with a \(m/z\) of 450 at 22.5 min (Fig 2.6). The solvent extracts from the PS I complexes isolated from *Synechococcus* sp. PCC 7002 revealed the presence of 2.3 MQ-4 molecules per 100 Chl \(\alpha\) molecules, demonstrating that MQ-4 plays a role as the A\(_1\) cofactor in the PS I complexes in this cyanobacterium. Similar quantitative analysis for *G. violaceus* has not yet performed.

Targeted insertional inactivation of *menB*, *menF*, and *menG* in *Synechococcus* sp. PCC 7002 was performed to verify the involvement of these gene products in the MQ-4 biosynthesis (Fig 2.7). The *menF* and *menG* mutations were introduced into the wild type, whereas the *menB* mutation was introduced into the *rubA* mutant for the purpose of generating PS I complexes that contain plastoquinone-9 but lack iron-sulfur clusters for future studies concerning electron transfer kinetics and thermodynamics (Y. Sakuragi, B. Zybailov, G. Shen, R. Balasubramanian, B. A. Diner, I. Karygina, Y. Pushkar, D. Stehlik, D. A. Bryant and J. H. Golbeck, manuscript in preparation). The full segregation of the mutated alleles from the respective wild-type alleles was analyzed by PCR as shown in Fig 2.8. In the *rubA* mutant, PCR using the designed primers that are targeted to the *menB* region resulted in a product of 1.0 kb, which is expected based on the restriction map, as shown in Fig 2.7. Likewise, in the wild type, PCR using the designed primers that are targeted to the *menF* and *menG* regions resulted in products of 1.7 kb, and 1.8 kb, respectively. No products with corresponding sizes were detected for the mutants; instead products with larger sizes were detected. In the *menB rubA* and the *menF* mutants, the size of the products were ca. 2.1 kb and 2.8 kb, respectively. The difference between the PCR products from the wild type and the mutants corresponds to the size of the 1.1-kb gentamicin-resistance cartridge derived from pMS266. In the *menG* mutant a product of 2.2 kb was observed. The difference between the PCR products from the wild type and
the menG mutant is 0.3 kb, which corresponds to the difference between the 1.1-kb gentamicin-resistance cartridge derived from pMS266 and the 0.8-kb deletion in the N-terminal region (Fig 2.7C). These results show that the menB rubA double mutant and menF and menG single mutants are free of the respective wild-type alleles and therefore homozygous in the menB::GmR, menF::GmR, and menGB::GmR alleles, respectively.

When the pigment extracts from the PS I complexes isolated from the menB rubA and the menG mutants were analyzed, no peak with UV-absorption was detected at ca. 14 min, demonstrating that the menB and menG homologs are indeed required for MQ-4 synthesis in Synechococcus sp. PCC 7002 (Fig 2.9). In extracts of PS I complexes from the menF mutant, a small peak was detected at this retention time; however, the absorption properties of the component eluted at this retention time were significantly different from those of MQ-4 with an absorption maximum at 292 nm (Fig 2.10B). This component is virtually absent in the wild type and its chemical nature has not yet been determined. These results confirm that the interruption of the menF gene also results in the complete loss of MQ-4 and that MenF is required for MQ-4 synthesis in Synechococcus sp. PCC 7002. In the menF and the menB rubA mutants, a peak at 35.1 min was detected, which is absent in the extracts of PS I complexes in the wild type (Fig 2.9). The component eluted at this retention time showed absorption properties with a single peak at 256 nm (Fig 2.10C), which is characteristic of PQ-9 (Crane and Dilley, 1963). Mass spectrometric analysis of the component has shown a m/z of 748, which is characteristic of PQ-9. These results are in good agreement with a previous study in which the interruption of PhQ biosynthesis in Synechocystis sp. PCC 6803 resulted in the incorporation of PQ-9 in PS I (Johnson et al., 2000). Likewise, no MQ was detectable in the chromatogram of PS I complexes isolated from the menG mutant (Fig 2.9D). A new peak was detected at 11.9 min, which showed a strong UV-absorption with a maximum at
248 nm and a shoulder at 265 nm. Because the loss of a methyl group is expected to lower the hydrophobicity and size of a molecule, it was expected that demethylphyloquinone would elute earlier than MQ-4 in the reverse phase HPLC analysis. Therefore, it is highly plausible that the component eluting at 11.9 min is due to demethylmenaquinone. Combined with the sequence similarities to E. coli counterparts, these results confirm that menB, menG, and menF encode dihydroxynaphthoic acid-CoA synthase, 2-geranylgeranyl-1,4-naphthoquinone methyltransferase, and isochorismate synthase, respectively, in Synechococcus sp. PCC 7002. Together with the previous identification of menA, menB, menD, menE, and menG in Synechocystis sp. PCC 6803, it is concluded that PhyQ/MQ-4 biosynthesis in cyanobacteria requires MenF, MenD, MenE, MenB, MenA, and MenG.
DISCUSSION

Comparative genome analyses were conducted on 14 cyanobacteria, *E. coli*, *B. subtilis*, *Halobacterium* sp. NRC-1 and *A. thaliana*. Based on the results, it was concluded that PhyQ biosynthesis in cyanobacteria is similar to the MQ biosynthesis pathway in *E. coli*. Supporting this hypothesis is the discovery that *G. violaceus* and *Synechococcus* sp. PCC 7002 synthesize MQ-4 instead of PhyQ and that *Synechococcus* sp. PCC 7002 utilizes it as the A1 cofactor in PS I complexes. *G. violaceus* is thought to be one of the earliest branching lineages among the cyanobacteria (Nelissen et al., 1995). The fact that this organism synthesizes MQ-4 is a strong indication that PhyQ biosynthesis probably evolved from MQ biosynthesis pathway in other bacteria. It is therefore highly plausible that the early cyanobacteria synthesized MQ-4, and PhyQ biosynthesis evolved from the MQ-4 biosynthesis in cyanobacteria. This is consistent with the fact that the occurrence of PhyQ biosynthesis is restricted to cyanobacteria and to algae and higher plants, which are evolutionarily related to cyanobacteria. A recent study has shown that the red alga *Cyanidium caldarium* also synthesizes MQ-4 and utilizes it in its PS I complexes (Yoshida et al., 2004). Therefore, MQ-4 synthesis may be a widely occurring phenomenon among oxygenic phototrophs.

Based on phylogenetic reconstructions, it was concluded that not all men genes in higher plants are of cyanobacterial origin. It is believed that plastids, and hence the ability to perform oxygenic photosynthesis, in eukaryotic phototrophs were acquired from a cyanobacterium upon endosymbiosis (Margulis, 1970). Therefore, the presence of a gene
with strong similarity to the cyanobacterial menA in the plant nuclear chromosome is probably a result of gene transfer from the plastids. This gene is presumably derived from the endosymbiotic cyanobacterium and would have been transferred to a host nuclear chromosome during endosymbiotic interaction (Douglas, 1998; Delwiche and Palmer, 1997; Martin et al., 1998). On the other hand, the presence of the non-cyanobacterial-type menB and menD in the plant nuclear chromosome suggests they originated in other organisms. One possibility is that menB and menD were already present in the eukaryotic host cells before the endosymbiotic cyanobacterium was incorporated into them. It is believed that mitochondria in eukaryotic cells originated from a proteobacterium, possibly in the α-subdivision (Olsen et al., 1994; Andersson et al., 1998; Martin and Müller, 1998; Emelyanov 2001; Horner et al., 2001; Emelyanov, 2003), through an endosymbiosis that preceded the acquisition of plastids. Given that some, although not all, proteobacteria synthesize both ubiquinone and MQ (Collins and Jones, 1981), it is possible that the endosymbiotic proteobacterium brought the ability to synthesize MQ into the eukaryotic host cells. Another possibility is that menB and menD genes were acquired via horizontal gene transfer from bacteria to plant cells. Interestingly, the men genes in the plastid genomes of the red algae Cyanidium caldarium and Cyanidioschyzon merolae form a cluster (menD-menF-menB-menA-menC-menE), which is very similar to the cluster found in a variety of prokaryotes (Fig 2.2). Phylogenetic analyses show that neither the MenA nor the MenB sequences of the red algae Cyanidioschyzon merolae forms a cluster with the cyanobacterial sequences (Fig 2.3). These observations therefore support the occurrence of horizontal gene transfer from bacteria other than cyanobacteria to these eukaryotic phototrophs.
Targeted mutagenesis of the \textit{menB}, \textit{menF}, and \textit{menG} homologs in \textit{Synechococcus} sp. PCC 7002, encoding for DHNA synthase, isochorismate synthase, and 2-methyl-1,4-naphthoquinone methyltransferase, respectively, resulted in the expected interruption of PhyQ biosynthesis. This confirms that the pathway responsible for MQ-4 synthesis in \textit{Synechococcus} sp. PCC 7002 is similar to that in other bacteria.

PhyQ mediates electron transfer between $A_0$ and $F_X$ of PS I during the charge separation process upon photoexcitation of P700. The optimal rate of electron transfer from $A_0$ to $F_X$ is ensured by the thermodynamically downhill arrangement of these cofactors. The molecular structures of PhyQ and MQ-4 are identical except for the level of saturation of the C$_3$ polyprenyl side chain, and thus the redox potentials of the two quinones are also expected to be identical. Therefore, it is not surprising that MQ-4 can function at the $A_1$ site in the PS I complexes. The prenylation reaction of DHNA in \textit{Synechococcus} sp. PCC 7002, \textit{G. violaceus}, and \textit{C. caldarium} is likely to be different from that in PhyQ-synthesizing species either by i) higher substrate specificity of the MenA protein for geranylgeranyl diphosphate compared to phytanyl diphosphate; and/or ii) substantially higher intracellular concentration of geranylgeranyl diphosphate than phytanyl diphosphate in these cells.

The whole-genome analysis of \textit{G. violaceus} seems to suggest that \textit{menB}, \textit{menC}, \textit{menD}, \textit{menE}, and \textit{menF} homologs are absent in this organism. ORFs that show similarity to the proteins encoded by these \textit{men} genes are more related to other proteins that do not play roles in MQ-4 biosynthesis. One example is the MenB-like protein Gll2549, which shows high similarity to 6-oxo camphor hydrolase. This enzyme is a member of the
crotonase superfamily that catalyzes the cleavage of carbon-carbon bonds. Therefore, it is possible that Gll2549 participates in the PhyQ pathway and catalyzes the ring cyclization reaction of o-succinylbenzoate. However, MenB is a very highly conserved enzyme among a wide variety of organisms (Table 2.1), and the absence of its homolog together with four other Men proteins in *G. violaceus* suggests the presence of an alternative pathway that can lead to the synthesis of DHNA. Alternatively, it was also possible that *G. violaceus* does not synthesize PhyQ, MQ-4 or related compounds at all. Previous studies have shown that PhyQ-less mutants recruit PQ into the A₁ site of PS I, and, despite a slightly lower catalytic efficiency, as measured by the flavodoxin reduction rate, the cells are able to grow photoautotrophically (Johnson et al., 2000). However, HPLC analyses performed in this study demonstrated that MQ-4 is present in the whole cell extracts of *G. violaceus* (see above). This result largely rules out the possibility of PQ-9 dependent PS I function in this organism. It also suggests that a part of the MQ-4 biosynthetic pathway in this organism is very different from those in the rest of the oxygenic phototrophs analyzed in this study.

In this study, a comparative genomic approach was shown to be useful in predicting the entire biosynthetic pathway for a secondary metabolite. Combined with reverse genetics, one can now genetically modify the quinone species in PS I in cyanobacteria. The advantage of this approach is that one can study the biochemical and biophysical properties of PS I, as well as the physiology of the cells, by using a biologically intact system. Further examples are provided in the following chapters.
SUMMARY

*Synechococcus* sp. PCC 7002 and *G. violaceus* were found to synthesize MQ-4 instead of PhyQ. Targeted inactivation of the *menF*, *menB*, and *menG* genes in *Synechococcus* sp. PCC 7002 demonstrated that these genes are required for MQ-4 biosynthesis. The PS I complexes isolated from the *menB rubA* and *menF* mutants contained PQ-9, while the PS I complexes isolated from the *menG* mutant contained an UV-absorbing compound, which may be demethyl-MQ-4. Comparative genome analyses of 14 cyanobacteria and four eukaryotic phototrophs suggested that PhyQ biosynthesis in cyanobacteria evolved from MQ biosynthesis in bacteria and that this pathway in higher plants may have developed as a result of mosaic evolution.
MATERIALS AND METHODS

Comparative Genome Analyses

The Men protein sequences of \textit{E. coli} were used as queries to detect the presence of homologs in cyanobacteria using the Blast-P search method. Retrieved sequences were named after their locus tags in the genomes. Sequence similarities between the homologs were obtained based on pairwise alignment by BLOSUM30 using ClustalW. Multiple amino acid sequence alignments were also generated by BLOSUM30 using ClustalW. For the phylogenetic reconstructions based on the MenA, MenB, and MenD sequences, unambiguous regions in the alignments were removed. The resulting “trimmed” multiple alignments were subjected to phylogenetic reconstruction by the Parsimony method using PAUP*4.0 beta. The bootstrap values are results of 10,000 repetitions.

Generation of Mutant and Verification of Segregation

Wild type and mutant strains of \textit{Synechococcus} sp. PCC 7002 were grown as described previously (Frigaard et al., 2004). A DNA fragment containing the \textit{menB} coding region was amplified from the isolated genomic DNA by PCR using the primer B1 (5’-CGTCGGGACACATCTTTCAC-3’) and the primer B2 (5’-CGGTATCTACTCTACCAAATTGGG-3’). The resulting fragment was digested with \textit{HindIII} and \textit{BamHI}, and inserted into the \textit{HindIII-BamHI} sites of pUC19 (pUC19menB). A fragment containing the \textit{menF} coding region was amplified by PCR using the primer F1 (5’-TTTGGGCAGCATCCCGGGTAAGCTGG-3’) that contains the engineered \textit{KpnI} cleavage site and the primer F2 (5’-GGAACCTATGAAGTTAGTCTGCGTTTTCG-3’). The resulting fragment was digested with \textit{KpnI} and \textit{HindIII}, and inserted into the \textit{KpnI-}
HindIII fragment of the pUC19 cloning vector (pUC19menF). A fragment containing the menG coding region was amplified by PCR using the primer G3 (5’-GATGGTATTGTCAACACCGC-3’) and the primer G4 (5’-CACCACCCTATGGTACCAGGCG-3’). The resulting fragment was digested (pUC19menG). The accC1 gene, derived from the plasmid pMS266 and conferring gentamycin resistance, was inserted into the unique PstI site and SmaI cleavage sites in the plasmids pUC19menB and pUC19menF, respectively. The accC1 gene was inserted into the EcoRV sites of pUC19menG, resulting in a deletion of 740 bp encompassing the N-terminal and the upstream region of the menG gene. The menB::accC1 construct was used to transform the rubA mutant of Synechococcus sp. PCC 7002 as previously described (Shen et al., 2002). The menF::accC1 construct was used to transform a psaB strain. After achieving complete segregation of the menF::accC1 locus, the wild type psaB gene was introduced into the menF::accC1 psaB mutant to generate the menF::accC1 single mutant by homologous recombination at the psaB locus. The menG::accC1 construct was introduced into the wild-type strain. The full segregation of each allele was tested by PCR using the primers B1 and B2 for the menB::accC1 transformants, the primers F1 and F2 for the menF::accC1 transformant, and the primers G1 (5’-CGTTTCAATTTCCAGGCAAAGTC-3’) and G2 (5’-CCGAGGAAGTTAACCGCATATTC-3’) for the menG::accC1 transformant.
**Isolation of Thylakoid Membranes and PS I Particles**

Thylakoid membranes were isolated from cells in the late-exponential growth phase as described previously (Shen et al., 1993). Cells were broken at 4°C by three passages through a French pressure cell at 124 MPa. The thylakoid membranes were resuspended and solubilized for 2 h at 4°C in the presence of 1% (w/v) *n*-dodecyl-β-D-maltoside. PS I complexes were separated from other membrane components by centrifugation on 5-20% (w/v) sucrose gradients with 0.05% *n*-dodecyl-β-D-maltoside in the buffer. Further purification was achieved by a second centrifugation on sucrose gradients in the buffer in the absence of *n*-dodecyl-β-D-maltoside. Trimeric PS I complexes were used in these studies (Golbeck, 1995).

**Quinone and Chlorophyll Analysis**

Isolated PS I was either lyophilized or dried under N₂ gas before extraction with acetone:methanol (7:2 v/v). Pigments and quinones were extracted with cold acetone:methanol (7:2 v/v) after brief ultrasonication. Alternatively, 20 µL of the PS I preparations were extracted with 400 µL of acetone:methanol. After centrifugation and filtration through a PTFE filter membrane with a 0.2-µm pore size (Whatman International Ltd., Maldstone, UK), the extract in the organic phase was directly injected into an Agilent Technology 1100 series HPLC system (Agilent Technology, Palo Alto, CA, USA) equipped with a SUPELCO (Sigma-Aldrich Corp., St. Louis, MO, USA) Discovery® C₁₈ column (25 cm x 4.6 mm, 5 µm). Analyses were carried out using the following protocol: 80%A/20%B from 0 to 10 min, a linear change to 20%A/80%B in 40 min, and 20%A/80%B for 5 min, where solvent A and solvent B are 100% methanol and 100% isopropanol, respectively. The flow rate was 0.75 ml min⁻¹. Detection of eluates was performed with a diode array detector (Agilent 1100 series). The chlorophyll *a*,...
phylloquinone, and plastoquinone contents of each sample were determined based on integrated peak areas and their molar absorption coefficients, which are 17.4 mM$^{-1}$ cm$^{-1}$ at 618 nm, 18.9 mM$^{-1}$ cm$^{-1}$ at 270 nm (Dunphy and Brodie, 1971), and 15.2 mM$^{-1}$ cm$^{-1}$ at 254 nm (Crane and Dilley, 1963), respectively. The absorption coefficient of chlorophyll a at 618 nm was calculated from the ratio of the absorption peaks at 618 nm and 666 nm in methanol-isopropanol (6:4, v/v) and from its absorption coefficient at 666 nm in methanol (MacKinney, 1941). The peak assignments were confirmed by mass spectrometry using atmospheric-pressure chemical ionization with a Quattro II time-of-flight mass spectrometer (Micromass, Beverly, MA, USA) operated in the negative-ion mode.
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Table 2.1: Whole genome analyses for the MQ/PhyQ biosynthesis enzymes in 14 cyanobacteria, *E. coli, B. subtilis, C. tepidum*, and *Halobacterium* sp. NRC-1. Similarity to *Synechocystis* sp. PCC 6803 proteins are shown in parentheses (%). The similarity is based pairwise alignment of the entire sequences constructed by BLOSUM30. (Table continues on next page)

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S. 8102, *Synechococcus* sp. WH8102; P.MED4, *Prochlorococcus marinus* MED4; P.MIT, *Prochlorococcus marinus* MIT9313; P.SS120, *Prochlorococcus marinus* SS120; Arabidopsis, *Arabidopsis thaliana*; E. coli, Escherichia coli; C. tep, *Chlorobium tepidum*; B. sub, *Bacillus subtilis*. "N-terminal sequence was truncated."
**Fig 2.1: Menaquinone biosynthetic pathway in *E. coli*.**

DHNA, dihydroxynaphthoate; DMQ, demethylmenaquinone; OSB, o-succinylbenzoate; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; TPP-SS, thiamine-pyrophosphate-succinic semialdehyde.
Fig 2.2: The *men* gene arrangements.

**Fig 2.3:** Phylogenetic trees based on the amino acid sequences of (A) MenA, (B) MenB, and (C) MenD.

The multiple alignments were generated by BLOSUM30 using Clustal W, and the trees were constructed by the Parsimony method using PAUP*4.0 beta. Cyanobacteria, red algae and higher plants are indicated with black circles, black diamonds, and stars, respectively. The locus tags used to indicate organisms are summarized in Table 2.1, except for the following: Cymecp095, Cymecp092, Cymecp094 for MenA, MenB, and MenD in *C. merolae*, respectively; Oj1217B09.18 and P0671D0118 for MenA and MenB in *Oryza sativa*, respectively. AF2036 and AF0435 are hypothetical proteins in the archeon *Archeoglobus fulgidus* DSM4304. MI2270 is a MenD homolog in *Mycobacterium leprae*. Bootstrap values were obtained after 10,000 repetitions and those larger than 50 are shown.
Fig2.4: HPLC profiles of solvent extracts from the PS I complexes and whole cells.

Chromatograms of reverse-phase HPLC analysis of solvent extracts obtained from (A) PS I complexes isolated from the wild-type *Synechocystis* sp. PCC 6803 (B) PS I complexes isolated from wild-type *Synechococcus* sp. PCC 7002, and (C) whole cells of the wild-type *G. violaceus*. Chromatograms were recorded at 270 nm. Chl *a*, chlorophyll *a*; β-car, β-carotene; MQ-4, menaquinone-4.
Fig 2.5: Absorption spectra of solvent-extracted components analyzed by HPLC.

Components eluting (A) at 22.5 min from the PS I complex of the wild-type *Synechocystis* sp. PCC 6803, (B) at 14.5 min from the PS I complex of the wild-type *Synechococcus* sp. PCC 7002, and (C) at 14.5 min from the whole cell of the wild-type *G. violaceus* (solid line) (see Fig 2.4). A component eluting at 22.5 min in *G. violaceus* is also shown by dotted line and does not appear to be a quinone.
Fig 2.6: LC-mass spectrometric analysis of the solvent extracts from *Synechococcus* sp. PCC 7002.

(A) Chromatograms at the m/z of 444.3 (top) and 450.3 (bottom), (B) mass spectra at the retention time at 30 min (top) and 12.4 min (bottom). PQ-9 elutes at 30 min.
Fig 2.7: Restriction maps of the *menB* (A), *menF* (B), and *menG* (C) coding regions in *Synechococcus* sp. PCC 7002.

Short black arrows show the primers used for verification of segregation by PCR.
Fig 2.8: PCR analyses on genomic DNAs isolated from the *menB* *rubA*, *menF*, and *menG* mutants of *Synechococcus* sp. PCC 7002.

Primers used for the analyses are shown by the black arrows in Fig 2.7. Control amplifications using template DNA from the *rubA* and wild-type (WT) strains are also shown.
Fig 2.9: Reverse-phase HPLC analyses of the solvent extracts from PS I complexes.

(A) wild type, (B) the menB rubA mutant, (C) the menF mutant, and (D) the menG mutant of *Synechococcus* sp. PCC 7002. The retention time for MQ-4 is indicated by the black arrows.
Fig 2.10: Absorption spectra of eluting components analyzed for PS I complexes isolated from *Synechococcus* sp. PCC 7002 using the reverse-phase HPLC analysis (see Fig 2.9).

(A) MQ-4 at 13.5 min in wild type, (B) unidentified component eluting at 13.5 min in the *menF* mutant, (C) PQ-9 at 35.1 min in the *menB rubA* mutant, (D) unidentified component (possibly demethyl-MQ-4) eluting at 11.9 min in the *menG* mutant.
Chapter 3

Recruitment of a Foreign Quinone into the $A_1$ Site of Photosystem I.

Interruption of $menG$ in the Phylloquinone Biosynthetic Pathway of $Synechocystis$ sp.

PCC 6803 Results in the Incorporation of 2-Phytyl-1,4-Naphthoquinone and Alteration of the Equilibrium Constant between $A_1$ and $F_X$

Publication:

ABSTRACT

A gene (*menG*), encoding a methyltransferase was identified in *Synechocystis* sp. PCC 6803 as responsible for transferring the methyl group to 2-phytyl-1,4-naphthoquinone (demethylphyloquinone) in the biosynthetic pathway of phylloquinone, the secondary electron acceptor in Photosystem I (PS I). Mass spectrometric measurements showed that targeted inactivation of the *menG* gene prevents the synthesis of phylloquinone and leads to the accumulation of 2-phytyl-1,4-naphthoquinone in PS I complexes. Growth rates of the wild-type and the *menG* mutant strains under photoautotrophic and photomixotrophic conditions were virtually identical. The chlorophyll a content in whole cells of the *menG* mutant was similar to that in the wild type when the cells were grown at a light intensity of 50 µE m⁻² s⁻¹ but slightly lower when grown at 150 µE m⁻² s⁻¹. Low temperature chlorophyll fluorescence emission measurements showed a larger increase in the ratio of PS II to PS I in the *menG* mutant strain relative to the wild type as the light intensity was elevated from 50 µE m⁻² s⁻¹ to 300 µE m⁻² s⁻¹. CW EPR studies at 34 GHz and transient EPR studies at multiple frequencies showed that the quinone radical in the *menG* mutant has the same overall linewidth as in the wild type, but the pattern of hyperfine splittings showed two lines in the low-field region consistent with the presence of an aromatic proton at ring position 3. The spin polarization pattern indicated that 2-phytyl-1,4-naphthoquinone is in the same orientation as phylloquinone, and out-of-phase, spin-echo modulation spectroscopy showed the same P700⁺ to Q⁻ center-to-center distance in the two samples. Transient EPR studies indicated that forward electron transfer from Q⁻ to F_X is slowed from
290 ns in the wild-type to 600 ns in the \textit{menG} mutant. The redox potential of 2-phytyl-1,4-naphthoquinone was estimated to be 50 to 60 mV more oxidizing than phylloquinone in the $A_1$ site, which translates to a lowering of the equilibrium constant between $Q^-/Q$ and $F_X^-/F_X$ by a factor of ca. 10. The kinetics of the $P700^+ [F_A/F_B]^-$ backreaction increased from 80 msec in the wild-type to 20 msec in the \textit{menG} mutant strain, thus supporting a thermally-activated uphill electron transfer through the quinone rather than a direct route for charge recombination between $[F_A/F_B]^-$ and $P700^+$. 
ABBREVIATIONS

bp  basepair(s)
Chl  chlorophyll
CW  continuous wave
ENDOR  electron nuclear double resonance
EPR  electron paramagnetic resonance
HEPES  4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC  high performance liquid chromatography
kb  kilobase
MS  mass spectrometry
NQ  naphthoquinone
ORF  open reading frame
PCR  polymerase chain reaction
PhyQ  phylloquinone
PQ-9  plastoquinone-9
PS  photosystem
Tricine  N- [2-hydroxy-1,1-bis(hydroxymethylethyl)]glycine
INTRODUCTION

Photosystem I (PS I) in cyanobacteria is a membrane-integral pigment-protein complex consisting of 12 protein subunits (PsaA through PsaF, PsaI through PsaM, and PsaX) and a variety of cofactors, including 96 molecules of chlorophyll \( a \) (Chl \( a \)), two molecules of 2-phytyl-3-methyl-1,4-naphthoquinone (phylloquinone, PhyQ), three [4Fe-4S] clusters, and about 22 molecules of \( \beta \)-carotene (Jordan et al., 2001). After the absorption of a photon by one of the antenna molecules, charge separation takes place between a special pair of Chl \( a \) molecules (P700) and a Chl \( a \) monomer (A0). The charge-separated state is stabilized on the donor side by electron transfer from plastocyanin or cytochrome \( c_6 \) to P700\(^+\), and on the acceptor side by electron transfer to PhyQ in the A1 site, through the iron-sulfur clusters at F\(_X\), F\(_A\) and F\(_B\), and ultimately to ferredoxin or flavodoxin. This light-driven enzyme therefore functions as cytochrome \( c_6 \)/plastocyanin:ferredoxin/flavodoxin oxidoreductase that ultimately provides reducing power to the cell in the form of NADPH.

Two molecules of PhyQ are present per P700 in PS I complexes of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 (Biggins and Mathis, 1988; Malkin, 1986; Schoeder and Lockau, 1986). Both quinones have been located on the 2.5 Å-resolution electron density map of *Synechococcus elongatus* PS I complexes (Jordan et al., 2001), and there is a good agreement with EPR studies that provide distance (Bittl et al., 1997) and orientation (Kamlowski et al., 1998; Zech et al., 2000) information for the EPR-active quinone. For example, the same orientation of the carbonyl oxygen bonds of PhyQ, tilted
ca. 30 degrees from the membrane plane, is deduced from the orientation of the magnetic
$g_{xx}$ [(Zech et al., 2000) and earlier references therein] and the electron density map (Jordan
et al., 2001). PhyQ is structurally related to menaquinone-9 (2-
nonaprenyl-3-methyl-1,4-naphthoquinone). Both have 3-methyl-1,4-naphthoquinone core
structures with a poly-isoprenoid chain at the C$_2$ ring position: menaquinone has an
unsaturated C$_{45}$ isoprenoid chain, while PhyQ has a partially saturated C$_{20}$ phytyl chain.
Because of their similarities in structure, it is likely that the PhyQ biosynthetic pathway is
similar to the menaquinone biosynthetic pathway in *Escherichia coli* (Johnson et al., 2000,
see also Chapter 2). The biosynthetic pathway of PhyQ is interesting for two reasons.
Firstly, the genes and enzymes of this pathway have not yet been completely characterized
in cyanobacteria. Secondly, knowledge of this pathway should enable one to devise
biological and chemical strategies to modify the quinone present in the A$_1$ site.

A pathway of PhyQ biosynthesis in *Synechocystis* sp. PCC6803 was recently
proposed and included the genes *menA*, *menB*, *menC*, *menD*, *menE*, *menF*, and *menG*
(Johnson et al., 2000) (see Fig 2.1). Inactivation of the *menA* and *menB* genes, predicted to
encode 1,4-dihydroxy-2-naphthoic acid phytyltransferase and 1,4-dihydroxy-2-naphthoate
synthase, respectively, prevented the synthesis of PhyQ and led to the incorporation of
plastoquinone-9 (PQ-9) into the A$_1$ site of PS I (Johnson et al., 2000). PQ-9 is the
secondary quinone acceptor in Photosystem II (PS II) and additionally functions as the
molecule that shuttles electrons to the cytochrome $b_{6f}$ complex. It was reported that PQ-9 is
present in the A$_1$ site of PS I with the same orientation and asymmetric spin-density
distribution as PhyQ in wild type PS I complexes (Zybailov et al., 2000). PQ-9 is a
benzoquinone with a C45 alkyl tail and is structurally and functionally only distantly related to PhyQ. The fact that PQ-9 could be inserted into the A₁ site and could function in lieu of PhyQ indicates that the binding site is not highly specific for PhyQ. Rather, the redox properties of the quinone appear to be conferred largely by the protein environment of the A₁ site rather than by the identity of the quinone (Semenov et al., 2000).

The object of this study is the protein encoded by ORF sll1653, which was originally annotated as gerC2, a spore germination protein in Bacillus subtilis. This ORF is homologous to menG, which codes for a methyltransferase enzyme in E. coli and other bacteria. In Synechocystis sp. PCC 6803, this gene is anticipated to encode a 2-phytyl-1,4-naphthoquinone (2-phytyl-1,4-NQ) methyltransferase enzyme (Johnson et al., 2000). 2-phytyl-1,4-NQ differs from PhyQ only by the absence of the methyl group at ring position C₃ and by a higher redox potential (Depew and Wan, 1988). The relationship between the molecular structure of this quinone and the axes of the molecular g-tensor is depicted in Fig 3.1.

This study has two aims. The first is to confirm that sll1653 codes for the methyltransferase responsible for synthesizing 2-phytyl-3-methyl-1,4-NQ from 2-phytyl-1,4-NQ. Indeed, it was found that inactivation of ORF sll1653 resulted in the synthesis of PS I complexes containing 2-phytyl-1,4-NQ in the A₁ site. The second is to study the roles of the methyl group in quinone binding and electron transfer in PS I. Here, it was shown that 2-phytyl-1,4-NQ is present in the A₁ site with the same orientation and asymmetric spin-density distribution as PhyQ in native PS I. It was also reported that
2-phytyl-1,4-NQ functions in forward electron transfer with some attendant alteration of the PS I activity and electron-transfer kinetics.
RESULTS

Analysis of the Genotype of the menG Mutant Strain

As shown in Fig 3.2A, the menG gene (ORF sll1653) was inactivated by insertion of a 1.3-kb DNA cassette, encoding the aphII gene and conferring resistance to kanamycin, in the unique KpnI site within the coding sequence. Full segregation of the menG and menG::aphII alleles was confirmed by PCR amplification and by Southern blot-hybridization analysis of the genomic DNA. For PCR analysis, the primers flanking the menG coding sequence (Fig 3.2A small arrows) were used to amplify the DNA fragments from the wild-type and the menG mutant genomic DNAs. As expected, a 1.2-kb fragment was amplified by PCR for wild type (Fig 3.2B). For the menG::aphII mutant strain a 2.5-kb fragment was amplified by PCR; no DNA fragment with a size equal to that expected from the wild-type menG allele was detected. This result indicates that the menG mutant is homozygous for the menG::aphII gene interruption. To verify the menG mutation further, the genomic DNA isolated from the wild-type and the menG mutant strains were digested with restriction enzymes BglII and HindIII (see in Fig 3.2A) for the Southern-blot hybridization analysis. Hybridization was performed using the PCR product from the wild type genome as a probe. A single band with the size of 2.3 kb was observed for the wild type strain (Fig 3.2C). For the menG mutant, two bands with the sizes of 2.7 kb and 1.5 kb were observed due to the presence of the BglII site in the Km’ cartridge. All of these results confirmed that the menG::aphII allele has fully segregated from the wild-type gene.
**High Performance Liquid Chromatography/Mass Spectrometry Analysis**

To determine whether the \textit{menG} gene is involved in PhyQ biosynthesis, solvent-extracted quinone fractions from PS I complexes isolated from the wild-type and the \textit{menG} mutant strains were subject to orthogonal acceleration/time-of-flight mass spectrometry. Pigments and quinones extracted from the isolated PS I trimers were separated by reverse-phase HPLC, and the molecular mass of each eluate was determined by mass spectrometry. In the wild-type complexes, PhyQ was detected by its characteristic retention time, its UV absorption spectrum, and its \textit{m/z} of 450 (Fig 3.3A). In PS I from the \textit{menG} mutant strain, no compound absorbing at 270 nm eluted at this retention time, nor was a \textit{m/z} of 450 detected. Rather, a UV-absorbing component eluted near the retention time of Chl \textit{a} that had a \textit{m/z} of 436, which corresponds to the mass of 2-phytyl-1,4-NQ (Fig 3.3B).

**Growth Rate under Photoautotrophic and Photomixotrophic Conditions**

As indicated by HPLC and mass spectrometry, mutation of the \textit{menG} gene resulted in the replacement of PhyQ by 2-phytyl-1,4-NQ in PS I complexes. To measure the effectiveness of 2-phytyl-1,4-NQ in supporting photosynthesis, growth rates of the wild-type and \textit{menG} mutant strains were compared. The results are summarized in Table 3.1. Growth of the wild-type and \textit{menG} mutant strains were virtually the same under low (50 µE m\(^{-2}\) s\(^{-1}\)) and moderate (150 µE m\(^{-2}\) s\(^{-1}\)) illumination conditions, in either the presence or absence of glucose (Table 3.1). When cells were grown photoautotrophically, doubling times of both the wild-type and the \textit{menG} mutant strains were ca. 10 h under low light intensity conditions and ca. 8 h under moderate light intensity conditions. When cells were
grown photomixotrophically, doubling times of the wild-type and the menG mutant strains were both ca. 8 h under low light intensity conditions, and ca. 6 h under the moderate light intensity conditions. The observed differences in the growth rates between the wild-type and the menG mutant strains were all within the range of error. These results suggest that under these illumination regimes, replacement of PhyQ with 2-phytyl-1,4-NQ has no significant effect on growth.

**PS II/PS I Ratio Measured by 77 K Fluorescence Emission Spectra**

The relative ratios of PS II to PS I were compared by measuring 77 K fluorescence emission spectra of wild-type and the menG mutant cells grown at various light intensities. The results are summarized in Fig 3.4. When cells were grown under low-light conditions, which is typical for Synechocystis sp. PCC 6803 (Fig 3.4A), the emission amplitude from PS I at 721 nm was much greater than that from PS II at 695 nm. When cells were grown at higher light intensities, an obvious decrease at 721 nm was found in the emission spectra of the menG mutant cells when compared to wild-type cells (Fig 3.4B and 3.4C). In wild-type cells the emission peak at 721 nm due to Chl a associated with PS I decreased approximately 10% in cells grown at a light intensity of 300 µE m$^{-2}$ s$^{-1}$ relative to cells grown at a light intensity of 50 µE m$^{-2}$ s$^{-1}$, while emission peaks at 685 and 695 nm due to Chl a associated with PS II remained nearly constant. However, the peak amplitude at 721 nm decreased substantially in the menG mutant cells grown at higher light intensities. The amplitude of this peak in cells grown at a light intensity of 300 µE m$^{-2}$ s$^{-1}$ was less than 70% that for cells grown at 50 µE m$^{-2}$ s$^{-1}$. The PS II/PS I ratio is slightly higher in the menG mutant than in wild type when grown at 50 µE m$^{-2}$ s$^{-1}$ and significantly higher when
grown at 300 \mu E \text{ m}^{-2} \text{ s}^{-1}. These results suggest that there may be either a defect in the assembly of PS I complexes or an accelerated degradation of PS I complexes in the \textit{menG} mutant cells grown at high light intensities.

\textit{Chlorophyll Content of Whole Cells Grown at Various Light Intensities}

The chlorophyll contents of wild-type and the \textit{menG} mutant cells grown under various light intensities were also determined (Table 3.2). When cells were grown under low or moderate light intensities (50 or 150 \mu E \text{ m}^{-2} \text{ s}^{-1}), the chlorophyll content of the \textit{menG} mutant cells was similar to that of the wild-type cells. However, when the light intensity was increased to 300 \mu E \text{ m}^{-2} \text{ s}^{-1}, the chlorophyll content in the wild-type cells decreased to approximately 70\% of the initial value (Table 3.2). A significantly lower value, approximately 58\% of the value obtained at lower light intensities, was observed for \textit{menG} mutant cells grown at a light intensity of 300 \mu E \text{ m}^{-2} \text{ s}^{-1}. This value is only 80\% that of the wild-type strain under the same conditions, and this decrease can be correlated with the loss of fluorescence emission from PS I observable in the decreased emission at 721 nm in Fig 3.4. In combination, the results from Table 3.2 and Fig 3.4 suggest that content of PS I complexes is lower in the \textit{menG} mutant strain when cells were grown at high light intensity. This difference could be due to a defect either in biogenesis or in stability of the PS I complexes in the mutant strain.

\textit{Polypeptide Composition of Isolated PS I Trimers}

To analyze whether the interruption of the \textit{menG} gene has any effect on the PS I subunit composition, PS I trimers were analyzed by SDS-polyacrylamide gel electrophoresis. Polypeptide bands were visualized by silver staining. All of the eleven
polypeptides detected in the wild-type, PsaA though F and PsaI, J, L and M, were found to be present in the menG mutant strain (Fig 3.5). No additional polypeptides were observed in PS I complexes isolated from the menG mutant strain. These results indicate that the replacement of PhyQ by 2-phytyl-1,4-NQ does not affect the subunit composition of the PS I complexes in the menG mutant strain.

EPR spectroscopy of Q− and P700+ Q−

Fig 3.6A shows continuous wave EPR spectra measured at 34 GHz (Q-band) of the photoaccumulated semiquinone radical (Q−) in PS I complexes isolated from the wild-type (solid line, top) and the menG mutant strains (solid line, bottom). The PhyQ anion radical is characterized (Fig 3.6A, solid line) by principal g-tensor values (Zybailov et al., 2000) and the principal hyperfine A-tensor for the three protons in the methyl group at the C2 position (Rigby et al., 1996) as summarized in Table 3.3. The hyperfine splitting due to the remaining aromatic hydrogen atoms and the methylene group of the phytyl chain at the C3 position remain unresolved and are included in the inhomogeneous line broadening using linewidths of 3.0, 6.0, and 4.0 G for each of the three g-tensor components (Fig 3.6B, dotted line). The deviations of the experimentally obtained spectrum (Fig 3.6B, solid line) from the simulated spectrum (Fig 3.6A, dotted line) seen in the mid- and high field regions are due to contamination by A0−.

The Q-band spectrum of the photoaccumulated semiquinone radical in the PS I complexes isolated from menG mutant strain is shown in Fig 3.6B (solid line). The spectrum has about the same overall width as that of wild type, which indicates that the g-tensor of the semiquinone radical is about the same as that of the PhyQ anion radical. The
pattern of hyperfine splitting, however, is different in the *menG* mutant sample. The usual four hyperfine lines are missing and instead, only two lines are present in the low-field region. Such properties are expected if the semiquinone radical in the A₁ site is replaced with a 2-phytyl-1,4-NQ that lacks the methyl group at ring position 3 (see below). Indeed, a satisfactory simulation of the spectrum of the *menG* mutant strain was obtained by modifying the wild-type simulation, with the A-tensor of the methyl group replaced by that of an aromatic C-H fragment with the same tensor components evaluated from the spin polarized spectra described in the next section (Fig 3.6B, dotted line).

**Transient EPR Spectroscopy**

Fig 3.7 compares transient, spin-polarized EPR spectra (X-, Q-, W-band) of the transient charge separated P700⁺ Q⁻ state in the PS I complexes isolated from the wild-type (dashed line) and the *menG* mutant strains (solid line). The two sets of spectra coincide quite well in the high-field region, which is dominated by the P700⁺ contribution, but are quite different in the low-field region. The W-band spectrum indicates a slightly larger $g$-anisotropy for the *menG* mutant than that of the wild type PS I complex. The best fit yields $g_{xx} = 2.00633$ (*menG*) versus 2.00622 (wild type) while $g_{yy} = 2.00507$ and $g_{zz} = 2.00218$ remain unchanged from the wild type (Zech et al., 2000) as summarized in Table 3.3.

Closer inspection reveals that the main difference in the low field region concerns the partially resolved hyperfine pattern. It is sufficiently resolved to permit an initial, qualitative analysis directly from the spectra. For wild-type, the characteristic hyperfine pattern due to the 1:3:3:1 quartet associated with a methyl group in ring position 2 is
particularly obvious in the Q-band spectrum. The fact that it is partially resolved results from the increased spin density at the carbon ring position to which the methyl group is attached (specific to the semiquinone radical ion in the A1 site). The components of the axially symmetric hyperfine tensor have been determined by ENDOR to be $A_{11} = 12.8$ MHz and $A_{\perp} = 9.0$ MHz (see Rigby et al., 1996, for summary and further references; see also Kamlowski et al., 1998; Zech et al., 2000). In the menG mutant, the CH$_3$ group is expected to be replaced by an aromatic C-H fragment. Indeed, the quartet hyperfine pattern due to the CH$_3$ group is missing in the menG mutant spectrum. Instead it is replaced by a doublet (menG), the center position of which is shifted down field with respect to the center of the quartet (wild type). Such a shift is expected with an altered orientation of the dominant hyperfine principal axis with respect to the molecular axis frame. As concluded from the observed shift direction, the dominant hyperfine axis will point more in the direction of the $g_{xx}(Q)$ tensor axis in the menG mutant while it points more in the direction of the $g_{yy}$ tensor axis in the wild type. Since the dominant hyperfine axis $A_{11}$ of the CH$_3$ hyperfine tensor is usually almost parallel to the C-CH$_3$ bond axis, its direction is closer to (about 30 degrees off) the $g_{yy}$ tensor axis. In contrast, the direction of the dominant hyperfine axis of the aromatic C-H fragment (which is in-plane and perpendicular to the C-H bond, see Carrington and MacLachlan, 1967) is closer to (about 30 degrees off) the $g_{xx}(Q)$ tensor axis. The associated downfield shift of the center of the hyperfine doublet is therefore in agreement with the experimental result.

To prepare for a quantitative simulation of the spectra, the following findings were taken into account. The spin polarization pattern shows that 2-phytyl-1,4-NQ has the
same orientation in the $A_1$ site as native PhyQ (Fig 3.7). The same P700 to $A_1$
center-to-center distance has been verified by out-of-phase, spin-echo modulation
spectroscopy (data not shown). An independent determination of the hyperfine tensor
components of the C-H fragment has been obtained from pulsed ENDOR spectroscopy of
the same P700$^+$ Q$^-$ state in the menG mutant (data not shown). The principal hyperfine
tensor components are given in Table 3.3. While the two large components, $A_{11}$ and $A_{22}$ are
clearly resolved, the third component is part of the line manifold in the center part of the
ENDOR spectrum and cannot be evaluated. With the reasonable assumption that the spin
density of the relevant carbon ring position does not change with respect to wild type
(corresponding to $A_{iso} = -10.3$ MHz with the appropriate McConnell relation) one can
estimate the third hyperfine principal component to be $A_{33} = -3.6$ MHz.

Although the axis associated with the largest hyperfine component is expected to
be in-plane and perpendicular to the C-H bond, this direction can be influenced by
significant spin densities on the neighboring molecular atoms, such as the spin density that
is known to reside on the carbonyl groups of the semiquinone radical. Therefore,
simulations of the Q-band spectrum (Fig 3.8) were carried out for various angles between
the largest principal axis $A_{11}$ and the $g_{xx}$ (Q) axis (in-plane along the carbonyl bond
direction). The $A_{11}$ axis is perpendicular to the C-H bond direction when this angle is 30°.
Indeed, of the simulations shown, the one for this value (Fig 3.8, bottom, long dashed
curve) comes closest to the experimental spectrum (Fig 3.8, top). A slightly smaller angle
than 30 degrees improves the simulation result to some degree. This indicates a weak
influence from spin density on neighboring atoms. The only significant spin density is
expected for the oxygen atom of the carbonyl group in ring position 1, as concluded from the alternating spin density distribution due to asymmetric H-bonding to the opposite carbonyl group in position 4 (see Kamlowski et al., 1998; Zech et al., 2000, and references therein). However, the oxygen atom is already quite distant from the proton spin of the C-H fragment, and thus the deviation of the C-H fragment hyperfine tensor from the (3:2:1) ratio of the undisturbed C-H fragment is small, as indeed is observed here.

**Kinetics of Forward Electron Transfer from A_1\(^-\) to F_X**

Forward electron transfer kinetics from the quinone to the iron sulfur clusters in PS I complexes isolated from the wild-type and the menG mutant are compared in Fig 3.9 and Fig 3.10, which show spin-polarized EPR transients and decay-associated spectra, respectively. The transients in Fig 3.9 labeled a, b and c were taken at the corresponding field positions indicated with arrows in Fig 3.10. In wild-type PS I complexes, two consecutive spectra are observed as electron transfer from A_1\(^-\) to F_X occurs. At early times (< ~1 µs) an emission/absorption/emission (EAE) pattern due to P700\(^+\) A_1\(^-\) is observed; this pattern changes to an emissive spectrum due to P700\(^+\) in the state P700\(^+\) FeS\(^-\) at later times (> ~1 µs). The identity of the iron-sulfur cluster partner to P700\(^+\) in the state giving the late spectrum requires some discussion. It has been shown that electron transfer proceeds via F_X (van der Est et al., 1994), which suggests the spectrum is due to P700\(^+\) F_X\(^-\). However, there is evidence (Leibl et al., 1995) that electron transfer from F_X\(^-\) to F_A/F_B is faster than the transfer from A_1 to F_X. In this case, the late spectrum would be due to P700\(^+\) F_A\(^-\) and/or P700\(^+\) F_B\(^-\). Calculations (Kandrashkin et al., 1998) show the polarization patterns corresponding to P700\(^+\) F_X\(^-\), P700\(^+\) F_A\(^-\), and P700\(^+\) F_B\(^-\) show only minor
differences. Thus, the state giving the late spectrum P700$^+$ FeS$^-$ is labeled P700$^+$ FeS$^-$ and the identity of the iron sulfur cluster(s) is left open. In the kinetic traces (Fig 3.9), the electron transfer is seen most clearly at field position b. The positive (absorptive) signal at early times (<~50 ns) is due to P700$^+$ Q$, while the negative (emissive) signal at late times (<~1 μs) is due to P700$^+$ FeS$. The electron transfer dominates the decay of the early signal while relaxation of the spin polarization causes the decay of the late signal. At positions a and c the intensity of the late signal is weak and thus the transients decay primarily with the electron transfer rate. As can be seen at all three field positions shown in Fig 3.10, the rate of electron transfer is considerably slower in the mutant than in the wild type sample. For the mutant PS I complexes, a fit of the entire data set as described in (van der Est, 1994) yields an average electron transfer lifetime of 600 ± 100 ns. This compares with a value of 290 ± 70 ns for the wild-type complexes.

**Kinetics of Charge Recombination Measured by Optical Spectroscopy**

The reduction of P700$^+$ was monitored at 811 nm after a brief laser flash in PS I trimers isolated from the wild-type and the *menG* mutant strains (Fig 3.11). Because no terminal electron acceptors such as ferredoxin or flavodoxin were present, the reduction rate of P700$^+$ represents the rate of charge recombination between [F$_{A/B}$] to P700$^+$. The kinetics of P700$^+$ reduction in the wild-type PS I complexes were fitted by one stretched exponent with (1/e) lifetimes of 0.70, 30.7, and 94.7 ms (Fig 3.11A). The latter two values are similar to the two lifetime components found previously for the P700-F$_{A/B}$ complexes from *Synechocystis* sp. PCC 6803 and are attributed to charge recombination between [F$_{A/B}$] and P700$^+$ (Vassiliev et al., 1997). The first value is probably due to charge
recombination between P700$^+$ and F$_X^-$ in damaged PS I complexes that lack an intact PsaC subunit. The kinetics of P700$^+$ reduction in PS I complexes from the menG mutant (Fig 3.11B) were also fitted by two exponentials with $(1/e)$ lifetimes of 10.3 and 29.3 ms, These values are attributed to charge recombination between [F$_A$/F$_B$]$^-$ and P700$^+$ and are ca. 3 times faster than the wild-type rates. Even though the reason for the heterogeneity in P700$^+$ reduction is not understood, it is noteworthy that the two kinetic phases attributed to charge recombination between [F$_A$/F$_B$]$^-$ and P700$^+$ are equally slowed in the menG mutant samples.

**Steady-State Rates of Flavodoxin Reduction**

Steady-state rates of flavodoxin reduction as a function of light intensity were measured for PS I complexes isolated from the wild-type and menG mutant strains to assess the relative efficiencies of forward electron transfer. The rates at saturating light intensity were determined by treating light as a substrate in a Michaelis-Menten kinetic analysis (Fig 3.12). The maximal rate of flavodoxin reduction was found to be $1527 \pm 247$ µmol (mg Chl a)$^{-1}$ h$^{-1}$ for the wild-type PS I complexes and $1845 \pm 399$ µmol (mg Chl a)$^{-1}$ h$^{-1}$ for the menG mutant PS I complexes. Assuming that 100 Chl a molecules are present per P700 in all PS I complexes, these maximal rates of electron transfer correspond to $37.9 \pm 6.2$ e$^{-}$ PS I$^{-1}$ s$^{-1}$ in the wild-type PS I complexes and $45.8 \pm 8.4$ e$^{-}$ PS I$^{-1}$ s$^{-1}$ in the menG mutant PS I complexes. Although the value obtained for the PS I complexes from the menG mutant was slightly higher than that for the wild-type, this difference is within the margin of error and therefore is not statistically significant (Fig 3.12). Hence, the steady-state rate of electron transfer for the PS I complexes of the menG mutant, which
contain 2-phytyl-1,4-NQ, was virtually identical to the steady-state rate of electron transfer for the PhyQ-containing PS I complexes of the wild-type.
DISCUSSION

The menG gene, encoding the 2-phytyl-1,4-NQ (demethylphyloquinone) methyltransferase, was identified by targeted inactivation of ORF sll1653 in Synechocystis sp. PCC 6803. This open reading frame was originally assigned as gerC2, which encodes the spore germination protein C2 in Bacillus species and other spore-former bacteria (Kaneko et al., 1996). In fact, the deduced amino acid sequence for ORF sll1653 shows only 27% sequence identity to the menG gene product of E. coli. Targeted inactivation of ORF sll1653 was accomplished by insertion of a kanamycin resistance cartridge in the coding region, which was confirmed by PCR amplification of the region containing the ORF and by Southern-blot hybridization analysis. The presence of 2-phytyl-1,4-NQ in PS I complexes was confirmed by high performance liquid chromatography and mass spectrometry, and its function in electron transfer was demonstrated by CW and transient EPR spectroscopy. The orientation and distance of 2-phytyl-1,4-NQ relative to P700 was confirmed to be the same as for PhyQ in wild-type PS I complexes by transient EPR, out-of-phase, spin-echo modulation, and pulsed ENDOR spectroscopy. It can be safely concluded that ORF sll1653 in Synechocystis sp. PCC 6803 is menG; that it encodes 2-phytyl-1,4-NQ methyltransferase, the enzyme required for the last step of the PhyQ biosynthetic pathway; and that 2-phytyl-1,4-NQ functionally replaces PhyQ at the A1 site in PS I complexes.

Despite the absence of PhyQ and the presence of 2-phytyl-1,4-NQ in PS I complexes, the growth rates of the wild-type and menG mutant cells were identical. As observed for many other cyanobacteria (Fujita, 1997), the ratio of PS II to PS I is known to
change in *Synechocystis* sp. PCC 6803 as a function of the light intensity during cell growth (G. Shen, J. H. Golbeck, and D. A. Bryant, unpublished observation). An interesting observation was that the change in the ratio of PS II to PS I was more pronounced in the menG mutant strain than in the wild type as the light intensity was increased from 50 µE m\(^{-2}\) s\(^{-1}\) to 300 µE m\(^{-2}\) s\(^{-1}\). In wild-type cells, the PS II to PS I ratio increased by a factor of 1.1, while in the menG mutant cells the PS II to PS I ratio increased by a factor of 2 as estimated from the amplitudes of the fluorescence emission spectra at 77 K. It is noteworthy that the overall chlorophyll content in the wild-type and menG mutant cells were similar at light intensities of 50 and 150 µE m\(^{-2}\) s\(^{-1}\) and differed only at 300 µE m\(^{-2}\) s\(^{-1}\). Therefore, the difference in the PS II to PS I ratio between wild type and the menG mutant strain can be primarily attributed to a change in the content of PS I, i.e. the number of PS I complexes declines in the menG mutants to a greater extent than in the wild-type cells as the light intensity is raised. By way of comparison, the menA and menB mutant strains, which contain plastoquinone-9 in the A\(_1\) site, have been observed to grow significantly more slowly and can be grown only under very low light intensity conditions (< 20 µE m\(^{-2}\) s\(^{-1}\)). The ratio of PS II to PS I is already greater than 2:1 at low light intensity, and might be expected to be even greater as the light intensity is increased (the cells, however, do not survive). Thus, it appears that *Synechocystis* sp. PCC 6803 must exceed ca. 2:1 ratio of PS II to PS I before light becomes toxic to the growing cells.

To understand further the behavior of 2-phytyl-1,4-NQ in electron transfer, one must consider the redox potential of this quinone in the A\(_1\) site. In aqueous solution, the \(E(Q/Q^-)\) of 2-methyl-1,4-NQ is \(-203\) mV vs. NHE (normal hydrogen electrode) and the
E(Q/Q') of 2,3-dimethyl-1,4-NQ is –240 mV vs. NHE (Swallow, 1982). The E(Q/Q') of 2-phytyl-3-methyl-1,4-NQ (PhyQ) in aqueous solution (containing 5 M 2-propanol and 2M acetone) is reported to be –170 mV. The presence of the second alkyl group, ortho to the first, therefore lowers the redox potential of the quinone by ca. 37 mV. However, the A₁ site is highly hydrophobic (Iwaki and Itoh, 1991, 1994), and reduction potentials of quinones in organic solvent are probably more relevant to their properties in PS I complexes. In dimethylformamide (DMF), the E₁/₂ value for 2-methyl-1,4-NQ has been reported as –650 mV vs. SCE and the E₁/₂ value for 2,3-dimethyl-1,4-NQ has been reported to be –746 mV (Prince et al., 1983) vs. SCE (standard calomel electrode). The lower redox potential for the dimethyl quinone is logical chemically; a methyl group is an electron donor and should therefore destabilize the semiquinone radical in either aqueous solution or in organic solvent. A prenyl substituent on the quinone ring is not as effective as a second alkyl group at electron donation and lowers the E₁/₂ value by only 50 to 60 mV (Prince et al., 1983). For example the E₁/₂ of menaquinone-2 (2-all-trans-polyprenyl-3-methyl-1,4-NQ) is –746 mV vs. SCE (to the best of our knowledge, the reduction potential of 2-phytyl-1,4-NQ in DMF is not available). Using the redox potentials for 2-methyl-1,4-NQ and 2,3-dimethyl-1,4-NQ as models for 2-phytyl-1,4-NQ and PhyQ (both differ by the addition of one methyl group), the addition of a second methyl group ortho to the first should lower the redox potential by ca. 96 mV. If one further employs the concept of an ‘acceptor number’ that was utilized for PQ-9 in the menA and menB mutants (Semenov et al., 2000), the redox potential of 2-phytyl-1,4-NQ in the A₁ site would be 61 mV more oxidizing than PhyQ. An acceptor number corrects the
E_{1/2} value for the degree of the nucleophilic (donor) or electrophilic (acceptor) properties of the solvent (Gutmann, 1976) and was employed initially by Itoh (Iwaki and Itoh, 1994) to estimate the redox potential of PhyQ in the A₁ site of PS I. It is a dimensionless number that expresses the acceptor properties of a solvent relative to SbCl₅.

The estimated redox potential of 2-phytyl-1,4-NQ in the A₁ site can now be compared to estimates based upon the experimentally measured rate of electron transfer. Forward electron transfer is ca. 3-times slower when 2-phytyl-1,4-NQ rather than PhyQ occupies the A₁ site. According to the Moser-Dutton formulation (Moser et al., 1992), the rate of electron transfer in proteins depends on the Gibbs free energy between the donor-acceptor pair, the edge-to-edge distance between the donor-acceptor pair, and the reorganization energy. The distance between the 2-phytyl-1,4-NQ anion radical and P700⁺ was experimentally determined here to be the same as the center-to-center distance between the PhyQ anion radical and P700⁺. The orientation of 2-phytyl-1,4-NQ relative to the membrane plane was also shown to be identical to that of PhyQ. Hence, the edge-to-edge distance between 2-phytyl-1,4-NQ and Fₓ is likely to be the same as the distance between PhyQ and Fₓ. It seems reasonable to assume similar reorganization energies when 2-phytyl-1,4-NQ and PhyQ occupy the A₁ site, given that the only difference between the two quinones is the presence or absence of a single methyl group. Using the values of 11.3 Å for the edge-to-edge distance between the quinone and Fₓ (Klukas et al., 1999), and a value of 0.7 eV for the reorganization energy, a (1/e) lifetime of ca. 290 ns for Q⁻ in the wild-type translates to an 81 mV difference in Gibbs free energy between Q⁻/Q and Fₓ⁻/Fₓ. Retaining the same values for the distance and reorganization...
energy, a \((1/e)\) lifetime of 600 ns for \(Q^-\) in the \(menG\) mutant translates to a 28 mV difference in the Gibbs free energy between \(Q^+/Q\) and \(F_X^-/F_X\). According to this analysis, the addition of methyl group to 2-phytyl-1,4-NQ lowers the redox potential of the quinone in the \(A_1\) site by 53 mV. This value is only a rough estimate, especially given the uncertainties in the edge-to-edge distance between the cofactors and given the assumed (unchanged) reorganization energy of the site. Nevertheless, this value agrees quite well with the difference in redox potentials of 2-methyl-1,4-NQ and 2,3-dimethyl-1,4-NQ in organic solvents, especially after correction for the site’s “acceptor number”. The replacement of PhyQ by 2-phytyl-1,4-NQ would therefore lower the equilibrium constant between \(Q^+/Q\) and \(F_X^-/F_X\) from 27 to 3, a factor of ca. 10. Estimating the absolute redox potential of 2-phytyl-1,4-NQ in the \(A_1\) site is somewhat more difficult because the redox potential of \(A_1\) is not known with certainty. Three values have been published for the redox potentials of the \(Q^+/Q\) couple [-810 mV vs. NHE (Vos and Gorkom, 1990), -800 mV vs. NHE (Chamrovoskii and Cammack, 1983) and -754 mV vs. NHE (Iwaki and Itoh, 1994)]. If one uses the lower redox potentials, the replacement of PhyQ by 2-phytyl-1,4-NQ would therefore raise the redox potential of the \(Q^+/Q\) couple to ca. –750 mV, a value that is probably still lower than the redox potential of the \(F_X^-/F_X\) couple.

The kinetics of reduction of \(P700^+\) after a single flash represents dissipative charge recombination between \(P700^+\) and terminal electron acceptors \([F_{A/F_B}]^-\) in the absence of an acceptor such as ferredoxin or flavodoxin. It is not yet clear whether electron transfer occurs directly from \([F_{A/F_B}]^-\) to \(P700^+\), or whether the electron travels backward
by a thermally activated, uphill electron transfer through the quinone. The latter presupposes that each redox pair (Q\(^{-}/Q\) \(\leftrightarrow\) F\(_X^-/F_X\); F\(_X^-/F_X\) \(\leftrightarrow\) F\(_B^-/F_B\); F\(_B^-/F_B\) \(\leftrightarrow\) F\(_A^-/F_A\)) is described by an equilibrium constant that can be determined from the midpoint potentials of the acceptors. The large distance between P700 and the terminal iron-sulfur clusters [46 Å center-to-center distance from P700 to F\(_A\) (Schubert et al., 1998)] argues against the direct recombination mechanism, although experimental data to support thermally activated uphill electron transfer have been lacking. If the charge recombination between [F\(_A^-/F_B\)]\(^-\) and P700\(^+\) were to occur through backward electron transfer, then a change in the equilibrium constant between Q\(^{-}/Q\) and F\(_X^-/F_X\) should logically affect the rate. The equilibrium constant between Q\(^{-}/Q\) and F\(_X^-/F_X\) would be correspondingly higher as the redox potential of Q\(^{-}/Q\) were driven more negative, thereby resulting in a depopulation of reduced quinone. Conversely, the equilibrium constant between Q\(^{-}/Q\) and F\(_X^-/F_X\) would be correspondingly lower as the redox potential of Q\(^{-}/Q\) were driven more positive, thereby resulting in a repopulation of reduced quinone. Consequently, the back-reaction kinetics in the mutant would be faster than in wild-type PS I complexes.

The latter description is precisely the behavior observed. The forward electron-transfer kinetics from Q\(^{-}\) to F\(_X\) becomes correspondingly slower as PhyQ \([1/e\) lifetime of 200 ns (van der Est et al., 1994)], 2-phytyl-1,4-NQ \([1/e\) lifetime of 600 ns], and PQ-9 \([1/e\) lifetime of 15 µs (Semenov et al., 2000)] occupy the A\(_1\) site. Conversely, the charge recombination kinetics between [F\(_A^-/F_B\)]\(^-\) and P700\(^+\) become more rapid as PhyQ \([1/e\) lifetime of 80ms (Vassiliev et al., 1997)], 2-phytyl-1,4-NQ \([1/e\) lifetime of 20 ms] and PQ-9 \([1/e\) lifetime of 3 ms (Semenov et al., 2000)] occupy the A\(_1\) site. The trend among the
wild type, *menA* or *menB*, and *menG* mutants therefore strongly supports the backward, through-carrier route of electron transfer for charge recombination. Since the redox potential of the quinone in the A1 site influences the rate of charge recombination from the terminal electron acceptors to P700$^+$ as well as the forward electron transfer between the quinone and the terminal iron-sulfur clusters, this suggests that charge recombination occurs by thermally activated, uphill electron transfer through the secondary quinone acceptor in PS I.

Finally, although the kinetics of electron transfer within PS I are altered when PhyQ is replaced by 2-phytyl-1,4-NQ, these changes do not affect the growth rate of the organism. An explanation for this apparent discrepancy is that the slower rate of forward electron transfer from A1$^-$ to FX is still not the rate-limiting step in the overall electron transfer reaction to flavodoxin (or probably to ferredoxin). Indeed, the maximal rate of flavodoxin reduction of the PS I complexes isolated from the *menG* mutant was virtually identical to that observed for wild-type PS I complexes. Analysis of the rate of electron donation from PS I to flavodoxin is complicated by the two-electron reduction using this acceptor. Since the potentials at pH 7 for the oxidized flavodoxin/flavodoxin semiquinone and the flavodoxin semiquinone/hydroquinone couple are −212 and −436 mV (Schubert et al., 1998), respectively, it is likely that only the latter couple is physiologically relevant. Studies show that the reduction of flavodoxin semiquinone is biphasic; the fast, first-order phase corresponds to electron transfer within a preformed complex, but the slower phase is concentration-dependent, with a second-order rate constant of $1.7 \times 10^8$ M$^{-1}$ s$^{-1}$. Given the nearly wild-type electron-transfer rates from cytochrome $c_6$ to flavodoxin in the *menG*
mutant, it is clear that forward electron transfer continues to out-compete the faster back reaction rate in the mutant. Hence, PS I complexes are remarkably robust and are tolerant to changes caused by replacement of wild-type components with “suboptimal” ones. This robustness still allows for a remarkable level of functionality of the bound electron-transfer cofactors under suboptimal conditions.
SUMMARY

The *menG* gene, encoding the 2-phytyl-1,4-NQ (demethylphyloquinone) methyltransferase, was identified in *Synechocystis* sp. PCC 6803. The PS I complexes isolated from the *menG* mutant accumulated 2-phytyl-1,4-NQ, which functioned as the A₁ cofactor in the electron transfer. The rate of forward electron transfer from A₁ to the iron-sulfur clusters was slowed by a factor of two, while the rates of the P700⁺ [FA/FB]-backreaction increased by a factor of 3 to 4. These results were explained by the lowering of the equilibrium constant between Q⁻/Q and Fₓ⁻/Fₓ by a factor of ~10. Despite the defect in the forward electron transfer, the PS I complexes containing 2-phytyl-1,4-NQ showed catalytic activity similar to those of PS I complexes containing phylloquinone, which demonstrates that PS I complexes are remarkably robust and can function with presumably “suboptimal” electron transfer cofactors.
MATERIALS AND METHODS

Generation of the menG Mutant Strain of Synechocystis sp. PCC 6803

To generate the construct for inactivation of the menG gene, a 1.2-kb DNA fragment containing the sll1653 ORF was amplified through PCR from the genome of the Synechocystis sp. PCC 6803. For cloning convenience, a new EcoRI site was created in the 3'-end primer with one nucleotide change. The PCR-amplified DNA fragment was cloned into pUC19 vector and confirmed by sequencing. To inactivate the menG gene, a 1.3-kb kanamycin-resistance cartridge encoded by the aphII gene was inserted into the KpnI site in the middle of the menG gene. The resulting plasmid was linearized by digestion with EcoRI and was used to transform the Synechocystis sp. PCC 6803 wild-type cells. The transformation and the transformant screening were carried out as described (Shen et al., 1993). After several rounds of restreaking to single colonies, full segregation of the menG::aphII from the menG alleles was verified by PCR and Southern blot-hybridization analyses.

Growth of Synechocystis sp. PCC 6803

Wild-type Synechocystis sp. PCC 6803 was grown in medium B-HEPES. This medium is prepared by supplementing BG-11 medium (Stainer et al., 1971) with 4.6 mM of HEPES [4-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)]-KOH and 18 mg L⁻¹ ferric ammonium citrate. The menG mutant cells were grown in medium B-HEPES supplemented with 50 µg mL⁻¹ kanamycin. The growth temperature was maintained at 32°C and the light intensity was adjusted to 50, 150, and 300 µE m⁻² s⁻¹ by adding fluorescent
lamps or by shielding with paper as required. For photomixotrophic growth, the medium was supplemented with 5 mM glucose. Growth of the cells was monitored by measuring the absorbance at 730 nm using a Cary-14 spectrophotometer that had been modified for computerized data acquisition by On-Line Instruments, Inc (Bogart, GA). Cells from a liquid starter culture in the exponential phase (OD$_{730\text{nm}} < 0.7$) were adjusted to the same initial cell density (OD$_{730\text{nm}} = 0.05$) for growth-curve measurements. The liquid cultures were bubbled with 1% (v/v) CO$_2$ balanced with air.

**DNA Isolation, PCR, and Southern Blotting**

Chromosomal DNA from *Synechocystis* sp. PCC 6803 wild-type and mutant strains was isolated as previously described (Shen, et al., 1993). PCR primers used to amplify the menG DNA fragment for evaluation of the menG alleles were positioned as shown in the Fig 3.2. The sizes of the PCR products from the wild type and menG mutant strains were determined by agarose gel electrophoresis. For Southern blot-hybridization analysis, the chromosomal DNAs were subjected to restriction enzyme digestion, agarose gel electrophoresis, and capillary transfer to nitrocellulose membranes. Hybridization probes were generated by labeling the wild-type menG PCR fragment with the random-primed DNA labeling kit (Boehringer-Mannheim, Indianapolis, IN). Hybridization conditions and detection were as previously described (Shen and Bryant 1995)
Chlorophyll Extraction and Analysis

Chlorophyll was extracted from whole cells, thylakoid membranes and PS I particles with 100% methanol, and its concentration was determined spectrophotometrically as described (MacKinney, 1941).

Isolation of Thylakoid Membranes and PS I Particles

Thylakoid membranes were isolated from cells in the late exponential growth phase as described previously (Shen et al., 1993). Cells were broken at 4°C by three passages through a French pressure cell at 124 MPa. The thylakoid membranes were resuspended and solubilized for 2 h at 4°C in the presence of 1% (w/v) n-dodecyl-β-D-maltoside. PS I complexes were separated from other membrane components by centrifugation on 5-20% (w/v) sucrose gradients with 0.05% n-dodecyl-β-D-maltoside in the buffer. Further purification was achieved by a second centrifugation on sucrose gradients in the buffer in the absence of n-dodecyl-β-D-maltoside. Trimeric PS I complexes were used in these studies (Golbeck, 1995).

SDS-Polyacrylamide Gel Electrophoresis Analysis

Methods used for SDS-polyacrylamide gel electrophoresis were identical to those previously described (Shen and Bryant, 1995). A Tricine/Tris discontinuous buffer system was used to resolve the polypeptide composition of the PS I complexes prepared from the wild type and the menG mutant strains of Synechocystis sp. PCC 6803 (Schägger and van
The separating gel contained 16% acrylamide and 6 M urea, and the resolved proteins were visualized by silver staining (Blum et al., 1987).

**Analysis of Quinones by High Performance Liquid Chromatography and Mass Spectrometry**

For quinone extraction, PS I particles isolated from the wild-type and the *menG* mutant strains were exchanged into distilled water by dialysis for 4 h and lyophilized. The pigments were extracted from the lyophilized samples with acetone:methanol, 1:1 (v/v) at 4 °C, vacuum dried at room temperature in the dark, and resuspended in 100% methanol. The extracts were separated by reverse-phase high performance liquid chromatography (HPLC) using a C$_{18}$ column as previously described (Johnson et al., 2000). Eluates were analyzed using atmospheric-pressure chemical ionization with a Micromass Quattro II mass spectrometer operated in the negative-ion mode.

**Flavodoxin Photoreduction**

Steady-state rates of flavodoxin reduction were measured for the PS I trimers isolated from the wild-type and the *menG* mutant strains as previously described (Jung et al., 1995). Cyanobacterial flavodoxin was overexpressed in *E. coli* and purified as described (Fillat et al., 1991). PS I trimers were resuspended to a final concentration of 5 µg Chl mL$^{-1}$ in 25 mM Tris-HCl buffer, pH 8.3, containing 50 mM MgCl$_2$, 15 µM cytochrome c$_6$, 15 µM flavodoxin, 6.0 mM sodium ascorbate, and 0.05 % *n*-dodecyl-β-D-maltoside. Measurements were made by monitoring the rate of change in the absorption at 467 nm using a modified Cary 219 spectrophotometer fitted with a 465 nm interference filter on the surface of the photomultiplier. The 4-sided clear cuvette was
illuminated from two sides using high intensity, red light-emitting diodes (LS1, Hansatech Ltd., Norfolk, UK). Initial rates of the reaction were recorded under various light intensities and plotted as a function of the light intensity to assess the relative efficiency of forward electron transfer to flavodoxin.

77 K Fluorescence Emission Spectra

Fluorescence emission spectra were measured at 77 K using an SLM 8000C spectrofluorometer as previously described (Shen and Bryant, 1995). Cells from the exponential phase of growth were harvested and resuspended in 25 mM HEPES/NaOH, pH 7.0, containing 60% glycerol. Samples were adjusted to equal cell density (OD $730\text{nm} = 1.0$) prior to freezing in liquid nitrogen. The excitation wavelength was 440 nm, the excitation slit width was 4 nm, and the emission slit width was 2 nm. Each spectrum shown is the average of four spectra.

Q-band EPR of Photoaccumulated PS I Complexes

Photoaccumulation experiments were performed using a Bruker ER300E spectrometer and an ER 5106-QT resonator equipped with an opening for in-cavity illumination similar to that described in (Yang et al., 1998). Low temperatures were maintained with an ER4118CV liquid nitrogen cryostat and an ER4121 temperature controller. The microwave frequency was measured with a Hewlett-Packard 5352B frequency counter and the magnetic field was measured with a Bruker ER035M NMR gaussmeter. The pH of the sample was adjusted to 10.0 with 1.0 M glycine buffer, and sodium dithionite was added to a final concentration of 50 $\mu$M. After incubation for 10 min in the dark, the sample was placed into the resonator and the temperature was adjusted to
205 K. The sample was illuminated for 40 min with a 20 mW He-Ne laser operating at 630 nm. A dark background spectrum was subtracted from the photoaccumulated spectrum. EPR spectral simulations were carried out on a 466 MHz Power Macintosh G3 computer using a Windows 3.1 emulator (SoftWindows 3.0, Insignia Solutions, UK) and SimFonia software (Bruker Analytik GMBH, Germany).

*Time-Resolved EPR Spectroscopy at Multiple Frequencies*

Transient EPR spectra as well as pulsed EPR/ENDOR data were obtained using the same instrumentation described earlier (Zybailov et al., 2000; Bittl et al., 1997).

*Flash-Induced Absorbance Changes at 811 nm*

The transient absorbance changes were measured with a home-built, double-beam spectrophotometer described in (Vassiliev et al., 1997). The sample was prepared at a chlorophyll concentration of 7 µM in 50 mM Tris buffer, pH 8.3. Measurements were made at pH 8.3 in 50 mM Tris buffer in the presence of 2 mM sodium ascorbate and 5 µM 2,6-dichlorophenolindophenol. The samples were measured in a quartz cuvette with a 1-cm path for the measuring light. The excitation beam was provided by a frequency-doubled Nd-YAG laser (532 nm) with the flash energy attenuated to ca. 20 mJ using the Q-switch delay and neutral density filters. The measuring beam was provided by a 5 mW semiconductor laser operating at 811 nm. Kinetic analysis was performed using a non-linear regression algorithm in Igor Pro (WaveMetrics Inc., Lake Oswego, OR) (Vassiliev et al., 1997). All kinetic constants are reported as 1/e lifetimes (τ).
Room-temperature, transient EPR experiments were carried out using a modified Bruker ESP 200 equipped with a home-built broad-band amplifier (bandwidth >500 MHz) for direct detection experiments. Excitation was provided by a Continuum YAG/OPO laser system operating at 680 nm and 1 Hz. The EPR signals were digitized using a LeCroy LT322 500 MHz digital oscilloscope and transferred to a PC for storage and analysis. The samples were measured using a flat cell and a Bruker rectangular resonator fitted with a piece of rough-surfaced glass in front of the window to provide optimal illumination. The response time of the system in direct detection mode is limited by the bandwidth of the resonator and is estimated as ~50 ns, and the decay of the spin polarization limits the accessible time range to times shorter than a few microseconds. The same set-up can also be used with field modulation and lock-in detection. In this mode the response time is ~50 µs but the sensitivity is much higher, and the charge recombination can be monitored in the millisecond time range. In both modes of operation, full time/field data sets are collected and analyzed to determine the lifetimes of the species and their decay-associated spectra as described in detail (van der Est et al., 1994; Semenov et al., 2000).
REFERENCES


Carrington A, MacLachlan AD (1967) Introduction to Magnetic Resonance with Applications to Chemistry and Chemical Physics, Hamper and Row, New York


pathway mutants studied by time-resolved optical, EPR, and electromagnetic techniques. J Biol Chem 275: 23429-23438


Table 3.1: Doubling times of *Synechocystis* sp. PCC 6803 wild type and the *menG* mutant strains (hours).

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Photoautotrophic, 50 µE m⁻² s⁻¹ (n = 4)</td>
<td>9.8 ± 0.85</td>
</tr>
<tr>
<td>Photoautotrophic, 150 µE m⁻² s⁻¹ (n = 3)</td>
<td>8.1 ± 0.12</td>
</tr>
<tr>
<td>Photomixotrophic, 50 µE m⁻² s⁻¹ (n = 4)</td>
<td>7.9 ± 0.90</td>
</tr>
<tr>
<td>Photomixotrophic, 150 µE m⁻² s⁻¹ (n = 4)</td>
<td>5.6 ± 0.73</td>
</tr>
</tbody>
</table>
Table 3.2: Chlorophyll content in cells grown photoautotrophically at 32°C under various illumination conditions.

Chlorophylls were extracted from whole cells with methanol. The absorbance coefficient of 82 (µg/ml)^{-1} at 666 nm was used to calculate the chlorophyll content in 1 ml of the cultures with an optical density of 1.0 at 730 nm.

<table>
<thead>
<tr>
<th>Light intensity (µE m^{-2} s^{-1})</th>
<th>µg Chlorophyll / OD_{730nm} (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>50</td>
<td>3.55 ± 0.24</td>
</tr>
<tr>
<td>150</td>
<td>3.65 ± 0.21</td>
</tr>
<tr>
<td>300</td>
<td>2.48 ± 0.34</td>
</tr>
</tbody>
</table>
Table 3.3: Magnetic parameters of PhyQ ($A_1^-$) and 2-phytyl-1,4-NQ ($Q^-$) in the $A_1$ binding site.

<table>
<thead>
<tr>
<th></th>
<th>$g_{xx}$</th>
<th>$g_{yy}$</th>
<th>$g_{zz}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_1^-$</td>
<td>2.00622</td>
<td>2.00507</td>
<td>2.00218</td>
</tr>
<tr>
<td>$Q^-$</td>
<td>2.00633</td>
<td>2.00507</td>
<td>2.00218</td>
</tr>
</tbody>
</table>

Hyperfine coupling principal values (MHz)

<table>
<thead>
<tr>
<th></th>
<th>$A_{11}$</th>
<th>$A_{22}$</th>
<th>$A_{33}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_1^-$</td>
<td>12.8</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>$Q^-$</td>
<td>-15.5 ± 0.5</td>
<td>-11.8 ± 0.5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

$^a$ the principal axes of the $g$-tensors are shown in Fig 3.1. $^b$ Zech et al., (2000) *J. Phys. Chem.* B 104, 9728-9739 and references therein.
Fig 3.1: Relationship between the molecular structure of 2-phytyl-1,4-NQ and the axes of the electronic g-tensor.
The axes of the electronic g-tensor of 2-phytyl-1,4-NQ (indicated by arrows) are parallel to the molecular axes system.
Fig 3.2: Construction and verification of the menG mutant strain of *Synechocystis* sp. PCC 6803.

(A) Restriction map of the genomic region surrounding the menG gene in the wild type (top) and the menG mutant strain (bottom). The small arrows indicate the position of the PCR primers used to amplified the menG coding region. (B) Electrophoretic analysis of the DNA fragments amplified from the genomic DNA of wild type and the menG mutant strain through PCR analysis. (C) Southern-blot analysis of the genomic DNA isolated from wild type and menG mutant strain. Chromosomal DNA was digested using restriction enzymes BglII and HindIII. DNA fragment obtained by PCR from the wild type genome was used as probe, as shown in top panel.
Fig 3.3: Mass spectra recorded for pigments and quinone extracts from the PS I complexes isolated from the wild-type and menG mutant strains of Synechocystis sp. PCC 6803.

(A) Wild type, and (B) the menG mutant.
Fig 3.4: 77 K Fluorescence emission spectra from whole cells of *Synechocystis* sp. PCC 6803.

Chlorophyll fluorescence emission spectra were measured for the whole cells of wild type (solid lines) and the *menG* mutant strain (dotted lines) grown under three different light intensities, (A) 50 µE m\(^{-2}\) s\(^{-1}\), (B) 150 µE m\(^{-2}\) s\(^{-1}\), (C) 300 µE m\(^{-2}\) s\(^{-1}\). Each spectrum was recorded at the same cell density and presented as the average of four measurements.
Fig 3.5: SDS-PAGE analysis of PS I complexes isolated from the wild-type and *menG* mutant strains of *Synechocystis* sp PCC 6803. The equal amount of Chl *a* (5 µg) is loaded for each lane. Polypeptides were visualized after silver staining.
Fig 3.6: Photoaccumulated Q-band EPR spectra and simulations of PS I complexes from *Synechocystis* sp. PCC 6803.

Wild type (solid line), and the simulated spectrum (dotted line). B, *menG* mutant strain (solid line), and the simulated spectrum (dotted line). See text for details of the parameters of the simulation.
Fig 3.7: Transient spin-polarized EPR spectra (X-, Q- and W-) of the charge separated P700$^+$ Q$^-$ state in PS I trimers. Wild-type spectra (dashed lines) and the menG mutant (solid lines). The frequencies are from bottom to top: 9, 35, and 95 GHz.
Fig 3.8: Simulations of the Q-band spectrum of the menG mutant carried out for various angles between the largest principal axis $A_{11}$ and the $g_{xx}$ axis. Top: experimental spectrum as in Fig 3.4 (middle). Bottom: simulations as described and with the parameters specified in the text.
Fig 3.9: Spin-polarized EPR transients of wild type and menG mutant.

The traces a, b, c were collected using direct detection at the field positions indicated by the similarly labeled arrows in Fig 3.10. The traces on the left are for PS I complexes isolated from the menG mutant, and those on the right are those from the wild type.
Fig 3.10: Decay-associated transient EPR spectra at ambient temperature.

Spectra of PS I complexes the wild-type and menG mutant strains extracted from the full time/field datasets as described in detail in Van der Est et al., (1994) Biochemistry 33, 11789-11797. The solid curves correspond to the state $\text{P}^+ \text{Q}^-$ while the dashed curves are the spectra of $\text{P}^+ (\text{FeS})^-$. The arrows labeled a, b, c indicate the field positions corresponding to the transients shown in Fig 3.9.
Fig 3.11: Flash-induced absorbance changes at 812 nm in PS I complexes isolated from *Synechocystis* sp. PCC 6803. The experimental data are depicted as dots, and the computer-generated exponential fits are shown as solid lines, with the lifetimes of the phase indicated. Top: P700\(^{+}\) reduction kinetics in wild-type PS I complexes. Bottom: P700\(^{+}\) reduction kinetics in the menG mutant PS I complexes.
Fig 3.12: Steady-state rates of flavodoxin reduction in PS I complexes isolated from the wild-type and menG mutant strains.

Seven independent measurements were performed for PS I complexes from each strain at each light intensity. The open and filled diamonds indicate the average values obtained for the complexes for the wild-type and menG mutant strains, respectively. The solid and dashed lines indicate fitted curves derived from the Michaelis-Menten equation for the rates obtained for PS I complexes from the wild-type and menG mutant strains, respectively. Bars on the data points indicate the standard deviation.
Chapter 4

Physiological Characterization of Tocopherol Biosynthesis Mutants in the Cyanobacterium *Synechocystis* sp. PCC 6803: Demonstration of a Conditionally Lethal Phenotype in the Presence of Glucose at pH 7.0

Publications:

Zigang Cheng, Scott Sattler, Hiroshi Maeda, Yumiko Sakuragi, Donald A. Bryant, Dean DellaPenna (2003) Highly divergent methyltransferases catalyze a conserved reaction in tocopherol and plastoquinone synthesis in cyanobacteria and photosynthetic eukaryotes. *Plant Cell* 15: 2343-2356

Yumiko Sakuragi, Hiroshi Maeda, Dean DellaPenna and Donald A. Bryant, manuscript in preparation.
ABSTRACT

The phenotypes of three tocopherol mutant populations, slr1736, sll0418 and slr0089, previously selected in the presence of glucose in the cyanobacterium Synechocystis sp. PCC 6803, have been reexamined. When cultured on solid media containing glucose, the mutant populations showed extremely heterogeneous colony morphologies, which suggested that the strains were genotypically inhomogeneous. Newly isolated tocopherol mutant strains were unable to grow in the presence of glucose at pH values below 7.2. Therefore, it was concluded that “authentic” slr1736, sll0418, and slr0089 mutants are glucose-sensitive and are not able to grow in the presence of glucose. Further characterization of the “authentic” mutants revealed that the chlorophyll a, phylloquinone, and plastoquinone contents decreased when cells were grown in the presence of glucose, and Photosystem II (PS II) activity was completely lost after 24 h. However, levels of the D1 and PsbO proteins remained nearly constant, and the levels of the sodB transcripts (encoding the superoxide dismutase) were virtually identical between the wild-type and mutant strains before and after transfer to medium containing glucose. These results indicate that the loss of the D1 and PsbO proteins is not responsible for the loss of PS II activity and strongly support the conclusion that the glucose-induced phenotype is not directly associated with oxidative stress. The authentic mutants exhibited chlorosis when grown in the presence of glucose. The relative phycobiliprotein contents decreased to nearly one-third of the wild-type level, and the concomitant accumulation of the nblA (encoding a protein essential for the degradation of phycobilisomes) transcript was detected. These results indicate that the authentic mutants
experience macronutrient starvation when grown in the presence of glucose. Further
growth analyses revealed that the glucose-induced growth defects, PS II inactivation, and
the macronutrient starvation responses occur at pH below 7.2. At pH 7.0 in the presence
of glucose, the PS II activity was completely lost, and the growth ceased after 24 h. The
results further demonstrate that α-tocopherol plays an essential role for the growth and
survival of *Synechocystis* sp. PCC 6803 in the presence of glucose and suggest that α-
tocopherol plays a role in the regulation of macronutrient metabolism in cyanobacteria.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Chl a</td>
<td>chlorophyll a</td>
</tr>
<tr>
<td>DCMU</td>
<td>3-(3,4-dichlorophenyl)-1,1-dimethyl-urea</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-piperazine-N’-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HGA</td>
<td>homogentisic acid</td>
</tr>
<tr>
<td>HPP</td>
<td>4-hydroxyphenylpyruvate</td>
</tr>
<tr>
<td>HPPD</td>
<td>4-hydroxyphenylpyruvate dioxygenase</td>
</tr>
<tr>
<td>HPT</td>
<td>homogentisate phytoltransferase</td>
</tr>
<tr>
<td>MPBQ</td>
<td>2-methyl-6-phytyl-1,4-benzoquinol</td>
</tr>
<tr>
<td>MPBQ MT</td>
<td>2-methyl-6-phytyl-1,4-benzoquinol methyltransferase</td>
</tr>
<tr>
<td>PBP</td>
<td>phycobiliprotein</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PS II</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
</tbody>
</table>
α-Tocopherol (vitamin E) is a lipid-soluble, organic molecule that is synthesized only by oxygen-evolving phototrophs, including some cyanobacteria and all green algae and plants (Threlfall and Whistance, 1971; Collins and Jones, 1981). The conservation of α-tocopherol synthesis during the evolution of photosynthetic organisms suggests that the molecule performs one or more critical functions. In plants, α-tocopherol is synthesized and localized in the plastids (Soll et al., 1980, 1985), and it is particularly abundant in the thylakoid membranes (Fryer, 1992), in which it is thought to afford protection against various oxidative stresses (Munné-Bosch and Lenor, 2000). Because α-tocopherol is also an essential component of animal diets, most of our knowledge of tocopherol function has been obtained from studies in these systems. Studies in whole animals, animal cell cultures and artificial membranes have shown that tocopherols scavenge and quench various reactive oxygen species and lipid oxidation byproducts, which would otherwise propagate lipid peroxidation chain reactions in membranes (Kamal-Eldin and Appelqvist, 1996). In addition to these antioxidant functions, other “non-antioxidant” functions related to modulation of signaling and transcriptional regulation in mammals have also been reported (Chan et al., 2001; Azzi et al., 2002; Ricciarelli et al., 2002). In photosynthetic organisms, tocopherol functions have not yet been rigorously defined, but it is believed that they are likely to include some or all of the functions reported in animals, as well as other possible functions specific to photosynthetic organisms.
The biosynthetic pathway of $\alpha$-tocopherol is summarized in Fig 4.1. It was elucidated in plants in the mid-1980’s and during the past five years, most of the genes encoding the enzymes of this pathway have been identified in both plants and cyanobacteria (Threlfall and Whistance, 1971; d’Harlingue and Camara, 1985; Norris et al., 1998; Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Schledz et al., 2001; Porfirova et al., 2002; Savidge et al., 2002; Shintani et al., 2002; Dähnhardt et al., 2002; Collakova and DellaPenna, 2003; Koch et al., 2003; Cheng et al., 2003). Briefly, homogentisic acid (HGA) is the aromatic head-group precursor for all tocopherols, and HGA is produced from 4-hydroxyphenylpyruvate (HPP) by the enzyme HPP dioxygenase (Norris et al., 1998; Dähnhardt et al., 2002). The committed step in tocopherol synthesis is the condensation of HGA and phytol-pyrophosphate by the enzyme homogentisate phytoltransferase to produce 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) (Collakova and DellaPenna, 2001; Schledz et al., 2001; Savidge et al., 2002; Collakova and DellaPenna, 2003). The ring of MPBQ is methylated by MPBQ methyltransferase to yield 2,3-dimethyl-5-phytyl-1,4-benzoquinol (Soll et al., 1985; Shintani et al., 2002; Cheng et al., 2003), which is cyclized by tocopherol cyclase to yield $\gamma$-tocopherol (Soll et al., 1985; Porfirova et al., 2002; Sattler et al., 2003). Finally, a second ring methylation by $\gamma$-tocopherol methyltransferase yields $\alpha$-tocopherol (Soll et al., 1980; d’Harlingue and Camara, 1985; Shintani and DellaPenna, 1998, Koch et al., 2003). Genes encoding HPP dioxygenase (*slr0090*), homogentisate phytoltransferase (*slr1736*), MPBQ methyltransferase (*sll0418*) and $\gamma$-tocopherol methyltransferase (*slr0089*) have been identified in *Synechocystis sp.* PCC 6803 (Shintani and DellaPenna,
1998; Shintani et al., 2002; Collakova and DellaPenna, 2001; Dänghardt et al., 2002, Cheng et al., 2003). The remaining enzyme, tocopherol cyclase, has recently been shown to be the product of slr1737 in Synechocystis sp. PCC 6803 (Sattler et al., 2003).

In the cyanobacterium Synechocystis sp. PCC 6803, the tocopherol biosynthetic pathway has been studied genetically and homozygous mutants of the slr1736, slr0089, and sll0418 genes, encoding homogentisate phytlytransferase, MPBQ methyltransferase, and γ-tocopherol methyltransferase, respectively, have been obtained (Shintani and DellaPenna, 1998; Shintani et al., 2002; Cheng et al., 2003; Collakova and DellaPenna, 2001). As expected from the biosynthetic pathway (Fig 4.1), the slr0089 mutant accumulates only γ-tocopherol (Shintani and DellaPenna, 1998), the sll0418 mutant accumulates 30% of the wild-type level of α-tocopherol and a small amount of β-tocopherol (Cheng et al., 2003), and the slr1736 mutant does not accumulate any tocopherols (Collakova and DellaPenna, 2001). In light of the established antioxidant activity of α-tocopherol in animal systems, one would expect that the loss or reduction of α-tocopherol would lead to a detectable phenotypic difference between the wild-type and mutant strains. Intriguingly, however, the tocopherol-deficient slr1736 mutant grew similarly to the wild-type strain both in the absence and in the presence of glucose at a moderate light intensity (110 µE m\(^{-2}\) s\(^{-1}\)) (Collakova and DellaPenna, 2001). A limited comparative analysis of the photosynthetic activity of the wild-type and slr1736 mutant strains failed to reveal any differences. This led the authors to conclude that α-tocopherol is dispensable for the survival of Synechocystis sp. PCC6803 under the conditions tested (Collakova and DellaPenna, 2001).
In this study, it is demonstrated that the “authentic” tocopherol mutants are glucose-sensitive and that they cannot maintain growth in the presence of glucose at pH 7.2 or lower. Mutant populations obtained in previous studies probably contained mixtures of secondary suppressor mutations that accumulate during cultivation in the presence of glucose, although their exact nature has yet to be identified. The glucose-sensitive phenotype of the “authentic” mutants was characterized, and the data indicate that α-tocopherol is essential for the survival of *Synechocystis* sp. PCC 6803 in the presence of glucose at pH 7.2 or lower.
RESULTS

Extremely Heterogeneous Colony Morphologies in Tocopherol Mutants Previously Isolated in the Presence of Glucose

When maintained on solid media containing 5 mM glucose, three tocopherol mutants, *slr1736::aphII*, *sll0418::aphII*, and *slr0089::aphII* that had been isolated in previous studies in the presence of glucose, showed much larger variations in colony size and pigmentation than what is typically observed for the wild-type strain (Fig 4.2 E-H). The larger colonies exhibited more intense blue-green pigmentation, which is characteristic of wild-type colonies, while the smaller colonies appeared more chlorotic and were yellow-green in color. Generally such non-homogenous colony morphology is indicative of heterogeneous genotypes within a given population. However, PCR analysis of genomic DNA isolated from three mutant strains showed that the targeted, mutated loci were homozygous and that no corresponding wild-type allele could be detected in any of the three mutant strains. These results eliminate the possibility that the observed colony heterogeneity (Fig 4.2) was due to incomplete segregation of the mutant loci or to contamination of the cultures with wild-type cells. When the tocopherol mutants were maintained in the absence of glucose, more uniform colony morphologies were observed (Fig 4.2 A-D). Although the size of colonies showed somewhat larger variation as compared to wild type, all colonies showed intense blue-green pigmentation. Therefore it was concluded that the heterogeneity of colony morphologies observed for the tocopherol mutants is a specific response to growth in the presence of glucose.
The growth characteristics of two cell lines, one derived from a small colony and the other derived from a large colony of the \textit{slr0089} mutant selected in the presence of glucose, were analyzed in the absence and in the presence of glucose. In the absence of glucose, the growth behavior of both cell lines was virtually indistinguishable from that of wild type (Fig 4.3 A and B). Upon transfer to media containing glucose, however, the cell line derived from the small colony ceased growth within 24 h and did not recover during the time course of measurements (Fig 4.3 C). The cell line derived from the large colony grew somewhat more slowly than the wild-type strain, but it was able to maintain growth during the course of the experiment (Fig 4.3 D). The dramatic phenotypic difference between the two cell lines strongly suggested that they were genotypically distinct, and that the mutant populations previously obtained by selection in the presence of glucose (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Shintani et al., 2002) were genotypically inhomogeneous.

\textit{Tocopherol Mutants Are Glucose Intolerant}

“ Authentic” tocopherol mutant strains were constructed by transforming an immotile, glucose-tolerant wild-type strain of \textit{Synechocystis} sp. PCC 6803 with genomic DNAs isolated from the \textit{slr1736}, \textit{sll0418}, and \textit{slr0089} mutants selected in the presence of glucose. Because the presence of glucose was found to be unfavorable for some populations of these tocopherol mutants, kanamycin-resistant transformants were selected in the absence of glucose. Segregation of each mutant allele from the corresponding wild-type allele was tested by PCR analysis using specifically designed oligonucleotide primers (Fig 4.4). Products anticipated for the wild-type \textit{slr1736}, \textit{sll0418}, and \textit{slr0089}
genes were 1.3 kbp, 1.0 kbp, and 1.2 kbp, respectively. No such product was observed for any of the mutant strains; instead, a product ~1.3 kbp larger was detected in each mutant line. This difference in size corresponds to the \textit{aphII}-containing, kanamycin-resistance cartridge originally used for the insertional inactivation of each gene. It was therefore concluded that each mutant strain is homozygous and that the \textit{slr1736::aphII}, \textit{sll0418::aphII}, and \textit{slr0089::aphII} alleles in each mutant line segregated completely in the absence of glucose.

The newly isolated \textit{slr1736}, \textit{sll0418}, and \textit{slr0089} mutants produced uniform colonies and grew similarly to the wild type in the absence of glucose under moderate light intensity (< 50 \(\mu\)E m\(^{-2}\) s\(^{-1}\); Fig 4.5 A). In the presence of glucose, however, all three mutants showed severe growth defects and were incapable of maintaining growth after 24 h (Fig 4.5 B and C). This growth phenotype of the mutant strains selected in the absence of glucose is very similar to that observed for the cell line derived from the small colonies of the mutants selected in the presence of glucose (see Fig 4.3 C), but sharply contrasts with the growth behavior of the cell lines derived from the large colonies (Fig 4.3 D). These results clearly demonstrate that “authentic” \textit{slr1736}, \textit{sll0418}, and \textit{slr0089} mutants are glucose sensitive. The data also suggest that \(\alpha\)-tocopherol plays an essential role for the survival of \textit{Synechocystis} sp. PCC 6803 in the presence of glucose.

When cells from these “authentic” mutants grown in the presence of glucose for 72 h were transferred to fresh medium containing no glucose, no growth was detected even after 3 weeks of incubation. These results indicated that these “authentic” mutants lost viability during cultivation in the presence of
glucose for 72 h. The pH of the cultures grown in the presence of glucose typically shifted from the original value (pH 8.0) to a value in the range of 7.0 to 7.3. Preconditioned media prepared from cultures of the “authentic” mutants grown in the presence of glucose supported the normal growth of the wild-type cells. These results show that the glucose toxicity of the mutants is not the result of products excreted from the cells in the presence of glucose.

To test whether the growth light intensity was responsible for the observed failure of the “authentic” tocopherol mutants to grow in the presence of glucose, the “authentic” slr1736 mutant was grown in the presence of glucose at various light intensities. Under both weak (< 5 μE m⁻² s⁻¹) and strong (300 μE m⁻² s⁻¹) illumination conditions, however, the presence of glucose in the growth medium resulted in the death of the “authentic” slr1736 mutant (Fig 4.6 A and B). This indicates that light intensity is unlikely to be a contributing factor in the observed glucose toxicity of the tocopherol mutants. The effects of inhibitors of the electron transport chain on the glucose toxicity of the slr1736 mutant were also studied. Addition of a sublethal concentration of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl-urea) to the growth medium led to a small recovery of growth for the slr1736 mutant in the presence of glucose (Fig 4.6 C). However, increasing the DCMU concentration further did not improve the growth of the slr1736 mutant. Addition of potassium cyanide in various concentrations did not rescue the slr1736 mutant from the glucose toxicity (Fig 4.6 D).

The effects of other organic carbon sources were analyzed on the growth of the “authentic” slr1736 mutant. The presence of 5 mM pyruvate, succinate, and fumarate,
which are intermediates of the TCA cycle, did not cause growth defects in the *slr1736* mutant. (Fig 4.7 A-D). The presence of 15 mM glutamate did not affect the growth of the *slr1736* mutant (Fig 4.7 E). The presence of ornithine, however, resulted in growth defects similar to those caused by glucose (Fig 4.7 F). The results suggested that the toxicity induced by glucose is caused by one or more metabolic intermediate that accumulate during glucose and ornithine catabolism. Ornithine is an intermediate in the citrulline cycle, in which as much as 20% of CO₂ fixation can take place (Tabita, 1987). On the other hand, glucose is metabolized through the glycolytic pathway or oxidative pentose phosphate pathway. Intermediates of these pathways are also shared with the Calvin-Benson cycle (reductive pentose phosphate pathway), in which the major CO₂ fixation occurs (Tabita, 1987). The fact that both glucose and ornithine caused lethal effects on the tocopherol mutants implies that α-tocopherol is perhaps involved in carbon metabolism by coordinating inorganic and organic carbon metabolic balance in the cells.

*Chlorophyll* *a, Phylloquinone and Plastoquinone Content*

The complete loss of, or a decrease in, tocopherol content led to small changes in the pigmentation of the “authentic” tocopherol mutants grown in the absence of glucose. The chlorophyll *a* (Chl *a*), phylloquinone, and plastoquinone contents were approximately 10%, 10-20%, and 3-30% lower, respectively, in these tocopherol mutants than in the wild type (Fig 4.8). The *slr1736* mutant cells contained nearly the same amount of Chl *a* and quinones as wild type, while the *sll0418* and *slr0089* mutant cells contained somewhat lower amounts. These differences, however, did not seem to affect the photosynthetic electron transport activity; the rates oxygen evolution observed
for both whole-chain electron transport and the Photosystem II (PS II)-dependent electron transport for the three tocopherol mutants were very similar to, or perhaps somewhat higher than, those of wild type (Fig 4.9, see below).

When grown in the presence of glucose, however, the mutants exhibited more dramatic changes in the contents of Chl a, phylloquinone, and plastoquinone. The Chl a and phylloquinone contents were largely unchanged by 24 h after transfer from medium without glucose to medium containing glucose and decreased significantly after 48 to 72 h to as low as 50% of the corresponding wild-type level. Under these conditions, the Chl a as well as phylloquinone level of wild type was largely unaffected during the course of measurements (Fig 4.8 A and B). Because phylloquinone is associated exclusively with the Photosystem I complexes in cells, the parallel decreases in the Chl a and phylloquinone contents may imply a decrease in the Photosystem I content in the cells.

In contrast, following transfer from a medium without glucose to a medium containing glucose, the plastoquinone content gradually decreased in the wild-type strain (Fig 4.8 C); by 48 h, the plastoquinone content of wild type was only half of that found in the absence of glucose. An even more severe decrease was observed in the mutants, in which the plastoquinone content decreased to ~50% of the wild-type level by 24 h and as low as 15% by 72 h after transfer to medium containing glucose (Fig 4.8 C). Recent studies have shown that the redox state of the plastoquinone pool in the thylakoid membranes modulates the expression levels of photosynthetic genes and affects the cellular contents of components of the photosynthetic apparatus, including the phycobilisomes and Photosystem I (Alfonso et al., 2000; Li and Sherman, 2000). This
raises the question as to whether the dramatic decrease in the plastoquinone content of the “authentic” mutants could lead to a change in its redox state, which in turn could be responsible for the decrease in the phycobiliprotein content and PS II activity (see below), and ultimately cell death. The redox state of the plastoquinone pool can be modified by various growth conditions including the intensity of illumination applied during cell growth. Low light intensity (< 5 µE m\(^{-2}\) s\(^{-1}\)) typically causes reduction of the plastoquinone pool while high light intensity (300 µE m\(^{-2}\) s\(^{-1}\)) causes its oxidation (Cooley and Vermaas, 2001). However, incubation under either of these light conditions did not lead to recovery of growth in the “authentic” tocopherol mutants in the presence of glucose (Fig 4.6 A and B). Similarly, addition of various concentrations of potassium cyanide, which promotes reduction of the plastoquinone pool, did not alleviate the glucose sensitivity of these mutants (Fig 4.6 D), and addition of DCMU, which causes oxidation of the plastoquinone pool, did so only to a small extent (Fig 4.6 C). These combined results therefore suggest that the changes in the plastoquinone pool size, and its possibly altered redox state, are not the primary cause of the glucose toxicity observed for the authentic tocopherol mutants in the presence of glucose.

*Photosystem II Activity*

Given the significant reduction in the Chl a, phylloquinone, and plastoquinone contents in response to the presence of glucose, whole-chain and PS II-dependent electron transport was assessed in the mutants (Fig 4.9). In cells grown in the absence of glucose, the whole-chain and the PS II-dependent oxygen evolution rates of all three mutants were similar to, or even slightly higher, than that of the wild type. Likewise,
somewhat higher O₂-evolving activity (the whole-chain or the PS II-dependent) was detected for the wild type when grown in the presence of glucose. In contrast, no O₂-evolving activity (the whole-chain and the PS II-dependent) was detected for the tocopherol mutants when grown in the presence of glucose.

Loss of PS II activity is often seen when cells are exposed to oxidative stress caused by exposure to extremely high light, to low temperature, or to an environment containing a high salt concentration (Allakhverdiev et al., 1999; Hideg et al., 2000; see a review by Aro et al., 1993, and references therein), and this phenomenon is known as photoinhibition. Under such conditions, the D1 protein, a subunit that forms a part of the PS II core structure (Ferreira et al., 2004), rapidly degrades, and, as a result, the activity of PS II is lost. The possibility that the observed PS II inactivation is related to photoinhibition in the tocopherol mutant in response to glucose was tested by immunological assay. When cells were grown in the absence of glucose, the amount of immunologically detectable D1 protein was virtually indistinguishable between the wild-type and the mutant strains (Fig 4.10). After transfer to medium containing glucose, the amount of immunologically detectable D1 protein was only slightly reduced in the mutants compared to wild type, even though by 24 h a complete loss of PS II-mediated oxygen evolution activity was observed (Fig 4.9). PsbO, or the 33 kDa protein, is a subunit that forms a peripheral domain of PS II known as the oxygen-evolving complex (Ferreira et al., 2004). This complex is responsible for the catalysis of water splitting and the production of oxygen. The immunologically detectable PsbO protein was also largely unaffected in response to glucose in both wild-type and three tocopherol mutant strains.
These results demonstrate that the loss of PS II activity in the tocopherol mutants in the presence of glucose is not associated with structural damage that leads to a loss of the D1 protein and PsbO, and they suggest that oxidative stress is not the cause of the PS II inactivation. The expression of the *sodB* gene, encoding superoxide dismutase, which is known to accumulate several-fold in response to various reactive oxygen species (Ushimaru et al., 2002), was analyzed by RT-PCR (Fig 4.11). The amounts of the *sodB* transcript detected for the wild type and the *slr1736* mutant were very similar when cells were grown in the absence of glucose (Fig 4.11, shown at 0 h) and in the presence of glucose (Fig 4.11, at 4, 8, and 24 h). The combined results therefore strongly suggest that the loss of α-tocopherol does not cause oxidative stress in response to glucose and that the inactivation of PS II is unrelated to oxidative stress.

*Relative Phycobiliprotein Content*

The relative content of phycobiliproteins (PBPs), the primary protein components of the light-harvesting antennae known as phycobilisomes, was determined in response to the transfer of the wild type and the “authentic” tocopherol mutants to medium containing glucose (Fig 4.12 A). In the absence of glucose, all three tocopherol mutants contained 20-25% less PBP than the wild type. After 24 h of growth in the presence of glucose, the PBP content of wild-type cells decreased to ~60% of the level found in the absence of glucose, while the PBP content of the three tocopherol mutants decreased even more sharply to 18-26% of the level in their respective controls obtained for the cells grown in the absence of glucose. Time-course analyses revealed that the reduction of PBPs initiates by 4 h after transfer to medium containing glucose (Fig 4.12 B). In
comparing the decreased PBP levels of the wild type and the three tocopherol mutants during growth in the presence of glucose, it is obvious that the loss of PBPs is much more severe in the three mutants than in the wild type.

These data are consistent with the pale, chlorotic (yellow-green) coloration of the mutant colonies in the presence of glucose. The mutants had significantly lower absorption in the orange-red region of their visible spectra, and as a result were less blue-green in coloration than control cells. This bleaching, due to a decrease in the cellular content of PBPs, is well known to be associated with nutrient starvation, and can be caused by carbon (Miller and Holt, 1977), nitrogen (Allen and Smith, 1969; Foulds and Carr, 1977; Wood and Haselkorn, 1980; Yamanaka and Glazer, 1980; Stevens et al., 1981; Elmorjani and Herdman, 1987), phosphorous (Ihlenfeldt and Gibson, 1975), sulfur (Schmidt et al., 1982; Jensen and Rachlin, 1984; Warner et al., 1986), or iron limitation (Sherman and Sherman, 1983). The nblA gene, encoding a factor essential for the controlled degradation of phycobilisomes, is known to accumulate in Synechocystis sp. PCC 6803 cells in response to nitrogen deprivation (Richaud et al., 2001). RT-PCR analyses showed that, in the wild-type samples, the nblA transcript was barely detectable when cells were grown in the absence of glucose (Fig 4.11, shown at 0 h) and that a small induction occurred by 4 h after transfer to medium containing glucose. In the slr1736 mutant sample, the wild-type level of the nblA transcript was detected in the absence of glucose and a dramatic increase followed by 4 h after transfer to medium containing glucose. This correlates well with the observed behavior of PBP contents in the tocopherol mutants (Fig 4.12, also see above) and suggests that the slr1736 mutant is
experiencing nutrient limitation in response to glucose. Because the BHEPES medium used in culturing these mutants contains an appropriate mixture of all micronutrients and macronutrients to support the growth of *Synechocystis* sp. PCC 6803 (Stainer et al., 1971; see Materials and Methods), the observed macronutrient starvation response of the mutants in the presence of glucose is most likely to be due to an impairment in an essential biosynthetic or regulatory process, which involves carbon, nitrogen, and glucose.

*Involvement of CO₂ in the Glucose Toxicity*

The observed glucose-sensitive phenotype of the “authentic” tocopherol mutants resembled that of the *icfG* mutant reported in a previous study (Beuf et al., 1994). The *icfG* gene encodes a putative protein phosphatase similar to RsbU (anti anti-sigma factor B) in *Bacillus subtilis* (Price, 2000; Woodbury et al., 2004). This mutant showed a glucose sensitive-phenotype only at low CO₂ [air level, 0.035% (v/v)] levels. The presence of succinate, fumarate, pyruvate, and glutamate did not cause lethality at low CO₂, while the presence of ornithine caused a lethal effect. The striking behavioral resemblance between the *icfG* mutant and the “authentic” tocopherol mutants in their response to various carbon sources suggested a possible involvement of CO₂ in the glucose toxicity observed for the tocopherol mutants. Fig 4.13 shows the growth curves of wild type and the “authentic” *slr1736* mutant in the presence of glucose in air and 3 % (v/v) CO₂. The *slr1736* mutant grew similarly to the wild type in air even in the presence of glucose, and in fact, the growth of the two strains was virtually indistinguishable under these conditions. When cells were grown in the presence of glucose at 3% (v/v) CO₂,
however, the *slr1736* mutant exhibited severe growth defects as described above. Therefore, these results demonstrated that the glucose toxicity occurs only at high CO$_2$ levels. This led to the hypothesis that $\alpha$-tocopherol is involved in the global regulation of organic and inorganic carbon metabolism in *Synechocystis* sp. PCC 6803, possibly through a signal transduction cascade involving IcfG.

**Involvement of pH in Glucose Toxicity**

The observed dependence of the glucose toxicity on the CO$_2$ concentration was, however, questioned after discussion with Prof. Aaron Kaplan of the Hebrew University, Israel. In the presence of 3% (v/v) CO$_2$, the growth medium was found to acidify from the initial pH 8.0 to about pH 7 (see above). Acidification of cultures can be caused by a high concentration of CO$_2$, such as 3% (v/v), since the inorganic carbon (Ci) supplied into medium as dissolved CO$_2$ is rapidly hydrated and reaches equilibrium with HCO$_3^-$.

Thus, the possibility that the high-CO$_2$ requirement of the glucose toxicity was actually because of medium acidification was tested directly. The wild-type and the “authentic” tocopherol mutants were grown at various pH values at 1% CO$_2$ (v/v) in medium BHEPES40, which is a modified BHEPES medium supplemented with 40 mM HEPES for higher pH buffering capacity (see Materials and Methods). After culturing for 2 days in this medium at 1% (v/v) CO$_2$, the pH remained near the original value within a deviation of $\leq$ 0.05 pH unit. In the absence of glucose, both the wild type and the tocopherol mutant strains grew well at all pH values tested between 6.8 and 8.0, and no significant difference was observed among the four strains (Fig 4.14). This establishes that the loss of $\alpha$-tocopherol does not affect the growth rates of the cells at all pH values.
between 6.8 and 8.0 in the absence of glucose. When the cells were transferred to media containing glucose, the wild type grew faster as compared to the growth in the absence of glucose (Fig 4.14), which confirms that the glucose is taken up and metabolized in the wild-type cells. The tocopherol mutants grew similarly to wild type at pH 8.0 and 7.6 (Fig 4.14). However, they grew very poorly or not at all at pH 7.2, 7.0, and 6.8. In comparing the pH-independent growth phenotype in the absence of glucose and the pH-dependent growth in the presence of glucose, it is obvious that the observed glucose toxicity of the tocopherol mutants occurs at pH 7.2 or lower. Therefore, the glucose-sensitive phenotype characterized in detail in the earlier section of this chapter is also due to the lowering of the pH caused by the acidification of the growth media during growth in the presence of 3% (v/v) CO₂.

Fig 4.15 shows that growth curves of the wild-type and the “authentic” slr1736 mutant cultures at various pH values in air and 1% (v/v) CO₂. The slr1736 mutant grew similarly to wild type at pH 8.0 and 7.6 both in air and 1% (v/v) CO₂ (Fig 4.15 A and B). At pH 7.2, the slr1736 mutant grew slightly slower than wild type during the initial 20 h and grew even more slowly after 20 h (Fig 4.15 C). At pH 7.0 and 6.8, the mutant grew substantially slower than wild type during the first 20 h and then stopped growing after 20 h (Fig 5.2 D and E). The growth behavior of the slr1736 mutant was virtually indistinguishable either when grown with air or with 1% (v/v) CO₂ (Fig 4.15 A-E). These results demonstrate it is the pH of the medium and not the extracellular CO₂ concentration that causes the glucose-sensitive growth phenotype of the slr1736 mutant.
It is noteworthy that the pH-dependent glucose toxicity occurs after 20 h, which suggests that the cells were accumulating a toxic substance during the initial 20 h. 3-\( O \)-methyl-glucose is known to be transported into the cells through the glucose transporter; however, it is not phosphorylated and therefore is not metabolized further (Beuf et al., 1994). Both the wild type and the \( slr1736 \) mutant grew well in the presence of this glucose analogue in the medium at pH 7.0 (Fig 4.16, top). The growth rates were very similar to those in the absence of glucose (based on visual inspection). The fact that the \( slr1736 \) mutant was able to grow similarly to wild type in the presence of this glucose analog suggests that the cause of the glucose toxicity is not the presence of glucose \textit{per se} that accumulates inside the cells. The presence of 5 mM pyruvate, succinate, or fumarate in growth medium at pH 7.0 caused the tocopherol mutant to grow slightly slower yet very similarly to wild type (Fig 4.16, top). These results strongly suggest that the causative agent for the glucose toxicity at pH 7.0 in the tocopherol mutants is likely to be metabolites accumulated either during glucose metabolism through the glycolytic pathway or the pentose phosphate pathway. The glucose toxicity was observed when cells were grown at RT (ca. 25 °C). The growth experiments described so far were conducted under constant pH values; in other words, the cells were grown at pH 7.0 in the absence of glucose before being transfer to medium containing glucose at pH 7.0 in order to assess the effect of glucose on the growth of these cells. Likewise, the cells were grown at pH 8.0 in the absence of glucose before transferred to medium containing glucose at pH 8.0. Fig 4.16 (bottom) shows a similar, yet somewhat different experiment. In this experiment, the cells were initially grown at pH 8.0 in the absence of glucose and then were transferred to medium containing glucose at pH 7.0. The purpose of this
experiment was to test whether the incubation at pH 7.0 (in the absence of glucose) “sensitizes” the tocopherol mutant cells to glucose or its by-product. The results showed that the tocopherol mutants were unable to grow after transfer from medium at pH 8.0 (containing no glucose) to medium containing glucose at pH 7.0. This suggested that the pH value of the medium before transfer to medium containing glucose has nothing to do with the observed glucose toxicity in the tocopherol mutant cells.

*Photosynthetic Activity*

Fig 4.17 shows the activities of PS II measured for cells grown at various pH values in the absence and the presence of glucose. In the absence of glucose, PS II activities of the *slr1736* cells (filled squares) were higher those that of wild type (filled circles) at all pH values. Although the reason for this higher activity for the *slr1736* mutant is not known, this result is reproducible, as can be also seen in Fig 4.9. In the presence of glucose, the PS II activities of the wild-type cells were higher than those in the absence of glucose at all pH values (open circles). On the contrary, no PS II activity was detectable for the *slr1736* mutant at pH 6.8 and 7.0. Higher activity was observed as the pH value was increased, and at pH 8.0 the PS II activity was virtually indistinguishable from that of wild type. Therefore, the inactivation of the PS II is also dependent on the presence of glucose, the pH of the surrounding environment, and the absence of tocopherols.

*The PBP contents*

When the cells were grown at pH 7.0 in the absence of glucose and with 1% CO₂ (v/v), the PBP contents of the wild type and the “authentic” tocopherol mutants were
virtually indistinguishable (Fig 4.18A). When cells were grown in the presence of glucose at pH 7.0, however, the PBP contents in the tocopherol mutants decreased to about one-third of the wild type level (Fig 4.18A). This is consistent with the results described in Fig 4.12, and demonstrates that the tocopherol mutants are experiencing macronutrient starvation in response to glucose at pH 7. The effect of pH on the PBP content was analyzed for the wild-type and the slr1736 mutant cells grown in the presence of glucose (Fig 4.18B). At all pH values tested, the PBP content in the wild-type cells remained constant. The PBP content in the slr1736 mutant cells was significantly lower, less than 40% of the wild-type level at pH 7.0 and below, which confirms the reproducibility of the pH-dependent macronutrient starvation response in the tocopherol mutant as described above. As the pH of the medium increased, higher PBP contents were detected. At pH 8.0, the PBP content of the slr1736 mutant was 70% of the wild-type level. The observed reduction of the PBP content over a wide range of pH values strongly indicates that the presence of glucose alone induces a macronutrient starvation response in the tocopherol mutants and that the macronutrient starvation response is severely exacerbated at pH values below 7.2.
DISCUSSION

In this study, it was determined that the previously reported *Synechocystis* sp. PCC 6803 mutants of genes *slr1736*, *sll0418*, and *slr0089*, encoding homogentisate phytlyltransferase, MPBQ methyltransferase and γ-tocopherol methyltransferase, respectively, are probably genotypically heterogeneous mixtures of secondary suppressor mutants. These mutants with defects in tocopherol biosynthesis were originally selected and maintained in the presence of glucose, and although the targeted genes were completely disrupted, a variety of colony growth morphologies have now been observed, which is consistent with genetic heterogeneity. When these mutant lines were reconstructed by a second round of transformation, followed by selection and maintenance in the absence of glucose, the resulting mutant lines (here denoted as “authentic” mutants to distinguish them from the original mutants previously selected in the presence of glucose) were also found to be completely disrupted at each respective locus, but the observed colony morphology and growth characteristics were now uniform. It was found that the “authentic” mutants are highly sensitive to the presence of glucose at pH values less than 7.2, and that the growth of each mutant strain ceased within 24 h following transfer to medium containing glucose under those conditions. These results clearly show that α-tocopherol is conditionally essential for the survival of *Synechocystis* sp. PCC 6803 in the presence of glucose. It is likely that the selection and culturing of the original transformants in the presence of glucose caused the selection of secondary suppressor mutations that permitted survival under these otherwise suboptimal or lethal conditions. Although the exact nature of the suppressor mutations has yet to be
determined, one can propose a few possibilities that enable the glucose-sensitive cells to become glucose tolerant: inactivation of the glucose transporter, alteration of flux through the glycolytic and/or oxidative pentose phosphate pathways, or the alteration of the regulatory pathway(s) that controls glucose metabolism (Beuf et al., 1994, Hihara et al., 1997, also see below).

The glucose toxic phenotype was found to be dependent on the pH of medium and occurs at pH ~7.2 or lower. PS II inactivation and the reduction of PBP contents were also found to be pH-dependent when glucose is present in the medium and α-tocopherol synthesis is interrupted. Similar pH-dependent growth defects and reduction of photosynthetic activities were observed for the cotA mutant (Katoh et al., 1996). CotA is involved in light-induced proton extrusion, and its absence has been shown to lead to severe growth defects at pH 7.0 or lower. Therefore, it is possible that the glucose toxicity observed for the “authentic” tocopherol mutants are related to defects in proton extrusion. However, a simple comparison between the cotA mutant and the tocopherol mutants is not appropriate, because of the significant phenotypic differences between them. The cotA mutant shows pH-dependent growth defects in the absence of glucose, whereas the tocopherol mutant does not under the same conditions. Growth rate of the cotA mutant is slower than wild type (60-70% of the wild-type level) at pH 8.0 in the absence of glucose, whereas the tocopherol mutants grow similarly to wild type under the same conditions (visual inspection). Therefore, the pH-dependent glucose toxicity of the tocopherol mutants seems to be very complex and involves one or more factors that are associated with the pH homeostasis of the cells. Further studies are required to resolve
the molecular mechanism of the pH-dependent glucose toxicity in the tocopherol mutants.

Trebst and co-workers reported that the herbicide-mediated loss of α-tocopherol led to the complete loss of PS II activity with concomitant degradation of the D1 protein following high light treatment in the green alga *Chlamydomonas reinhardtii* (Trebst et al., 2002). This was certainly not the case in the tocopherol mutants of *Synechocystis* sp. PCC 6803, as the level of the D1 protein remained similar to the wild type even when the PS II activity was completely lost. The growth light intensity (50 µE m⁻² s⁻¹) used in this study was less than one half of the saturating intensity, and this value seems unlikely to promote a photoinhibitory oxidative stress. In fact, the glucose toxicity observed for these mutants is light-independent, since it occurs over a wide range of light intensity, from < 5 to 300 µE m⁻² s⁻¹, as described in the Results. Given that the transcript level of the *sodB* gene is largely unaffected in the mutants under these lethal conditions, it is highly unlikely that the inactivation of PS II and the death of the cells are directly associated with an oxidative stress. Furthermore, the deleterious effects of glucose on the *slr1736*, *sll0418*, and *slr0089* mutants were virtually indistinguishable despite the varying compositions and contents of tocopherols in each mutant. Should α-tocopherol function solely as an antioxidant, an increasing susceptibility to glucose with decreasing tocopherol content would reasonably be expected among the various tocopherol mutants, but this is not observed. Therefore, it appears most likely that α-tocopherol plays a role other than, or at least in addition to, that as a bulk antioxidant in the survival of *Synechocystis* sp. PCC 6803 in the presence of glucose. Taking all of these considerations...
together, it was concluded that the observed metabolic perturbation, PS II inactivation, and cell death of the tocopherol mutants grown in the presence of glucose at pH values below 7.2 are unlikely to be directly associated with the antioxidant activity of α-tocopherol in *Synechocystis* sp. PCC 6803. Moreover, it appears most likely that α-tocopherol plays a role other than as a bulk antioxidant in the survival of *Synechocystis* sp. PCC 6803 in the presence of glucose.

PBPs are the major constituent of the light harvesting antennae in cyanobacteria, and they can constitute nearly half of the total soluble protein (Grossman et al., 1994). When cyanobacteria are subjected to macronutrient deprivation conditions for carbon, nitrogen, phosphorus, sulfur, and iron, the synthesis of PBPs ceases and their active degradation begins (Allen and Smith, 1969; Ihlenfeldt and Gibson, 1975; Foulds and Carr, 1977; Yamanaka and Glazer, 1980; Wood and Haselkorn 1980; Stevens et al., 1981; Schmidt et al., 1982; Sherman and Sherman, 1983; Jensen and Rachlin, 1984; Elmorjani and Herdman, 1987). It is generally believed that the degradation of these proteins provides amino acids for energy production and maintenance protein synthesis, while reducing the potential for photooxidative stress due to light stress at PS II. Therefore, it is highly likely that the observed, dramatic decrease in the PBP contents in the “authentic” tocopherol mutants during growth in the presence of glucose represents a response to a perceived state of nutritional deprivation. Supporting this interpretation is the observation that transcription of the *nbla* gene, known to accumulate in response to nitrogen deprivation (Richaud et al., 2001), also accumulated in the tocopherol-deficient mutant in response to glucose. The combined results hence suggest that the complete loss
of, or a substantial decrease of, α-tocopherol perturbs metabolic processes related to nitrogen, carbon, and sulfur, and that this disruption ultimately leads to phycobilisome degradation and cell death.

Recent studies in mammalian systems have demonstrated the involvement of α-tocopherol in modulating the production of signaling molecules and pathways (Chan et al., 2001; Azzi et al., 2002; Ricciarelli et al., 2002). For example, α-tocopherol has been shown to bind to phospholipase A₂ specifically at the substrate-binding pocket and act as a competitive inhibitor, thereby decreasing the release of arachidonic acid for eicosanoid synthesis (Chandra et al., 2002). α-Tocopherol has also been suggested to modulate the phosphorylation state of protein kinase Cα in rat smooth-muscle cells, possibly via phosphorylation of protein phosphatase 2A (Ricciarelli et al., 1998). α-Tocopherol is also directly involved in transcriptional regulation in animals, including the expression of the genes encoding liver collagen αI, α-tocopherol transfer protein, and α-tropomyosin collagenase (Yamaguchi et al., 2001; Azzi et al., 2002). Therefore, it seems possible that, in addition to acting as a bulk lipid-soluble antioxidant, α-tocopherol could also play a regulatory role in Synechocystis sp. PCC 6803, possibly in the regulation of metabolic processes such as those involved in carbon and nitrogen metabolism.

Two glucose-tolerant mutants reported previously appear to be relevant to the observations reported here. The pmgA (sll1986) and icfG (slr1680) loci have been shown to be determinants for glucose sensitivity (Hihara and Ikeuchi, 1997; Beuf et al., 1994).
Inactivation of the \textit{pmgA} locus has been shown to result in a glucose-sensitive phenotype and enhanced photoautotrophic growth capability (Hihara and Ikeuchi, 1997). Inactivation of the \textit{icfG} locus, on the other hand, results in a glucose-sensitive phenotype under low CO$_2$ conditions (Beuf et al., 1994). The “authentic” tocopherol mutants, whose phenotypes are characterized in this chapter, are glucose-sensitive both under high and low CO$_2$ environments but only at pH values less than 7.2. The amino acid sequences deduced from the \textit{pmgA} and \textit{icfG} coding regions show weak similarity to RsbT/W (a serine/threonine protein kinase) and RsbU/X (a protein phosphatase and a negative regulator of sigma factor B) in \textit{Bacillus subtilis}. These proteins form a signal transduction network with other Rsb proteins and regulate the activity of sigma factor B through phosphorylation/dephosphorylation interactions upon perception of environmental stress (Price 2000; Woodbury et al., 2004). Homologous protein sequences of other Rsb proteins are also found in the \textit{Synechocystis} sp. PCC 6803 genome: Slr2031 (RsbU/X), Ssr1600 (RsbS/V), Slr1856 (RsbS/V), and Slr1861 (RsbT/W). It is therefore plausible that an Rsb-like pathway is present in this organism and may participate in the regulation of glucose metabolism. In fact, studies have shown that recombinant Slr1861 phosphorylates Slr1856 and IcfG dephosphorylates the phosphorylated Slr1856 (Shi et al., 1999). Whether the role of $\alpha$-tocopherol is related to this hypothetical pathway is an open question and is a subject of continuing investigation.
SUMMARY

The tocopherol mutants (slr1736, sll0418, and slr0089), which had been previously isolated in the presence of glucose, were shown to be phenotypically and genotypically heterogeneous populations. Newly isolated “authentic” tocopherol mutants were glucose-sensitive and were not able to grow in the presence of glucose after 24 h. This glucose toxicity was shown to occur when the mutants were grown at pH values below 7.2. Under these conditions, the PS II activities were significantly reduced or not detected at all in the tocopherol mutants.
MATERIALS AND METHODS

Growth Conditions and Strains

The slr1736, sll0418, and slr0089 mutants were generated by targeted insertion with the aphII gene, which confers kanamycin resistance, as described previously (Shintani and DellaPenna, 1998; Collakova and DellaPenna, 2001; Shintani et al., 2002). The wild-type strain used for transformation and further analysis was a glucose-tolerant strain of Synechocystis sp. PCC 6803 (Williams, 1988). Medium BHEPES, pH 8.0, was used for selection, maintenance, and growth measurements of wild type and mutants. This medium is prepared by supplementing BG-11 medium (Stainer et al., 1971) with 4.6 mM HEPES [4-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)]-KOH and 18 mg L⁻¹ ferric ammonium citrate. Medium BEHEPS40, modified medium BEHEPS after supplementing with 40 mM HEPES for higher buffer strength, was used for pH-dependent or pH-controlled growth analyses. Wild-type cells were maintained on solid B-HEPES medium containing 1.5 % (w/v) agar and 5 mM glucose, and the “authentic” tocopherol mutants were maintained on solid medium BHEPES containing 1.5 % (w/v) agar, 50 µg kanamycin ml⁻¹, and importantly, no glucose. Prior to all growth measurements and preparations for subsequent analyses, cells were transferred from solid to liquid medium BHEPES and allowed to grow exponentially for several generations in the absence of glucose. For determination of growth characteristics, late-exponential phase cultures were diluted in fresh liquid medium BHEPES to an OD₇₃₀ nm of approximately 0.05 cm⁻¹. The diluted cultures were grown at 32 °C with continuous
bubbling with air containing 1% or 3% (v/v) CO₂. Growth was monitored by measuring the optical density at 730 nm. For growth in the presence of glucose, the medium was supplemented with 5 mM glucose. The growth light intensity was 50 µE m⁻² s⁻¹, unless otherwise noted. For growth measurements in the presence of inhibitors of the electron transport chain, the following concentrations were used: 0.03 µM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl-urea); 2, 20, or 200 µM potassium cyanide. Pre-conditioned medium was prepared from a culture of an authentic tocopherol mutant that had been grown in the presence of glucose for 72 h. The cells were removed by centrifugation at 8000 × g, and the supernatant was filtered through a sterile 0.22 µm pore-size membrane. The filtrate was then mixed with five-fold strength medium BHEPES in a four-to-one ratio (v/v) to achieve a normal, one-fold-strength medium.

Isolation of Tocopherol Mutants

*Synechocystis sp.* PCC 6803 wild type was transformed with genomic DNA extracted from the *slr1736*, *sll0418*, and *slr0089* mutants selected in the presence of glucose as previously described (Williams, 1988). Segregation of mutant alleles from the wild type allele was carried out in the absence of glucose and in the presence of 50 µg kanamycin ml⁻¹. Oligonucleotide primers used for PCR analysis were as follows; *slr1736* forward primer (5’-GGCTTCTCCTACCCGGAATTCTACTTCTCCTG-3’), *slr1736* reverse primer (5’-GCTTTCTAAGTGTACATCTAGACTCCGCCA-3’), *sll0418* forward primer (5’-ATGCCCGAGTATTTGCTTCTGCC-3’), *sll0418* reverse primer (5’-GCACTGCTTTGAACATACCGAAG-3’), *slr0089* forward primer (5’-GCTTTCTCCTACCCGGAATTCTACTTCTCCTG-3’), *slr0089* reverse primer (5’-GCACTGCTTTGAACATACCGAAG-3’).
TCTACCGGAAATTGCCAACTACCA-3’), and slr0089 reverse primer (5’-CCTAGGAGATTGTGGACTCAA-3’).

**Oxygen Evolution and Consumption Measurements**

Cells grown in the absence and in the presence of glucose for 24 h were harvested by centrifugation at 8,000 × g at room temperature and resuspended in a 25-mM HEPES buffer, pH 7.0, to obtain an optical density of 1.0 cm⁻¹ at 730 nm. For whole-chain oxygen evolution and consumption measurements, the cell suspensions were incubated in the dark in the presence of 10 mM NaHCO₃ for at least 15 min prior to measurements. For the Photosystem II (PS II)-dependent oxygen evolution measurements, 1 mM 1,4-benzoquinone and 0.8 mM K₃Fe(CN)₆ were added instead of NaHCO₃ and measurements were performed immediately. The excitation light intensity was approximately 3 mE m⁻² s⁻¹. The oxygen concentration was measured polarographically with a Clark-type electrode as previously described (Sakamoto and Bryant, 1998).

**Quantification of Pigments and Quinones by High Performance Liquid Chromatography**

Chl a in whole cells was extracted with methanol, and its molar content per unit optical density at 730 nm was determined using the molar absorption coefficient 82 L g⁻¹ cm⁻¹ (MacKinney, 1941). For quinone analyses, cells were harvested by centrifugation at 8,000 × g, at 4 °C for 6 min, washed in 1 ml of 50 mM Tris-HCl buffer, pH 8.0, and centrifuged at 18,000 × g for 1 min to obtain tight cell pellets. Pigments and quinones were extracted by ultrasonication in 400 µl of cold acetone-methanol (7:2, v/v) in the
High performance liquid chromatography (HPLC) analysis was performed with an Agilent 1100 system equipped with a diode array detector (Agilent Technologies, Wilmington, DE, USA). One hundred microliters of extract was mixed with 10 µl of 1 M ammonium acetate and injected onto a NovaPak C18 column (3.9 × 300 mm, 4 µm packing, Waters, Milford, MA). Analytes were eluted by a linear gradient program consisting of solvent A (100% methanol) and solvent B (100% isopropanol) using the following protocol: [100% A] for 10 min, [100% A] to [20% A : 80% B] in 20 min, [20% A: 80% B] for 5 min, and [20% A : 80% B] to [100% A] for 5 min. The flow rate was 0.75 ml min⁻¹. The Chl a, phylloquinone, and plastoquinone contents of each sample were determined based on integrated peak areas and their molar absorption coefficients, which are 17.4 mM⁻¹ cm⁻¹ at 618 nm, 18.9 mM⁻¹ cm⁻¹ at 270 nm (Dunphy and Brodie, 1971), and 15.2 mM⁻¹ cm⁻¹ at 254 nm (Crane and Dilley, 1963), respectively. The absorption coefficient of Chl a at 618 nm was calculated from the ratio of the absorption peaks at 618 nm and 666 nm in methanol-isopropanol (6:4, v/v) and from its absorption coefficient at 666 nm in methanol (MacKinney, 1941). The quinone content per unit optical density at 730 nm was calculated by multiplying the molar ratio of quinone to Chl a by the molar Chl a content per optical density at 730 nm.

**Estimation of Relative Content of Phycobiliproteins**

Cells were harvested by centrifugation at 8000 × g for 6 min, and pellets were resuspended in the 25 mM HEPES buffer, pH 7.0, to obtain 2 ml of the cell suspensions with an optical density of 0.5 cm⁻¹ at 730 nm. One milliliter of this suspension was heated
at 100 °C for 1 min. A modification was made in the method previously described (Zhao and Brand, 1989) for estimation of the relative PBP content. The absorbance at 635 nm and 730 nm were recorded for unheated and heated samples, and the data were then inserted into the following equation: the relative PBP content = \((\Delta OD_{635nm} - \Delta OD_{730nm})/OD_{730nm\text{-unheated}}\), where \(\Delta\) indicates OD_{unheated sample} minus OD_{heated sample}

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting of the D1 protein**

Cells were grown in the absence of glucose to mid-exponential phase or in the presence of glucose for 24 h, and harvested by centrifugation at 8,000 \(\times\) g at 4 °C for 6 min. The resulting cell pellets were immediately frozen in liquid N\(_2\) and stored at –80 °C until being used. Frozen cells were thawed on ice and resuspended in the 25 mM HEPES buffer, pH 7.0, at an optical density of 100 cm\(^{-1}\) at 730 nm. An equal volume of glass beads was added to the cell suspension, which was allowed to stand on ice for 5 min. Using a home-built bead-beater, the samples were vigorously shaken 4 times for 30 s at 4 °C with 30 s intervals on ice. An aliquot (10 µl) of each sample was mixed with an equal volume of loading buffer, and the mixture was incubated at 65 °C for 20 min, and applied onto a discontinuous SDS-polyacrylamide gel with 10% (w/v) acrylamide in the separating gel as described (Schägger and van Jagow, 1987). Electrophoresis was carried out at 50 V for 12 h. The antibodies raised against the amino acids 234-242 of the D1 protein of *Synechocystis* sp. PCC 6803 were a kind gift from Prof. Eva-Mari Aro of the University of Turku, Finland. The antibodies raised against the PsbO protein of *Synechocystis* sp. PCC 6803 were a kind gift from Prof. Robert Burnap of the Oklahoma
State University, USA. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Proteins were detected by immunoblotting by using an enhanced chemiluminescence reagents and analysis system (Amersham Biosciences, Piscataway, NJ) according to manufacturer’s specifications.

Isolation of total RNA and RT-PCR analyses

Total RNA was isolated from cells using the Mini-to-Midi RNA isolation kit (Invitrogen Corp., Carlsbad, CA) followed by an RNase-free DNase treatment, and repurification by using the same kit. The RNAs obtained were adjusted to a final concentration of 50 ng mL\(^{-1}\) in RNase free double distilled water and store at -80°C until being used. RT-PCR reactions were carried out by using the One-Step RT-PCR kit (Qiagen Inc., Valencia, CA) for detecting the *rnpB* transcript. The reaction volume was 25 µL, and 4 ng µL\(^{-1}\) of the total RNAs was used. For detecting the *nblA* and *sodB* transcripts, 400 ng of total RNA was first converted to cDNA by the RT reaction using M-MLV reverse transcriptase (Promega, Madison, WI). The reaction volume was 20 µL for each sample. The resulting cDNA was diluted with 80 µL of TE buffer, and stored at -20 °C until being used. Two microliters of cDNA stock were used for PCR (final volume, 25 µL) using Hot-Start Taq polymerase (Qiagen Inc., Valencia, CA). RNasin (Promega, Madison, WI), an RNase inhibitor, was present in one-step RT-PCR reactions and during the cDNA synthesis.
ACKNOWLEDGEMENTS

The authors thank Prof. Eva-Mari Aro (University of Turku) for kindly providing the D1 antibodies, Prof. Robert Burnap (Oklahoma State University) for kindly providing the PsbO antibodies, and Prof. Aaron Kaplan, Hebrew University for discussion, critical comments, and suggestions.
REFERENCES


Fig 4.1: Biosynthetic pathway of α-tocopherol in *Synechocystis* sp. PCC 6803.
HPPD, 4-hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytlytransferase; MPBQ MT, 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase; TC, tocopherol cyclase; γ-TMT, γ-tocopherol methyltransferase.
Fig 4.2: Colony morphologies of three tocopherol mutants previously isolated in the presence of glucose.
Cells were grown in the absence of glucose (A-D) and in the presence of glucose (E-H). Wild type (A, E), the slr1736 mutant (A, E), the sll0418 mutant (C, G), and the slr0089 mutant (D, H), on solid BHEPES medium.
Fig 4.3: Growth curves of wild type and cell lines derived from small (A, C) and large colonies (B, D) of tocopherol mutants selected in the presence of glucose (see Fig 4.2) at 32 °C, 50 µE m⁻² s⁻¹ with 3% (v/v) CO₂.

The data shown for each strain are averages of at least three independent cultures, and standard error bars are shown. Growth in the absence of glucose (A, B) and in the presence of glucose (C, D). (A, C) Wild type (filled circles) and a cell line derived from a small colony slr0089 mutant selected in the presence of glucose (open circles) (see Fig 4.2). (B, D) Wild type (filled circles) and cell lines derived from large colonies the slr0089 (open circles), sll0418 (open triangles), and slr1736 mutants (open squares) selected in the presence of glucose (see Fig 4.2).
Fig 4.4: PCR analysis of the genomic DNAs extracted from the newly isolated tocopherol mutants selected in the presence of glucose. (A) The slr1736 mutant, (B) the sll0418 mutant, and (C) the slr0089 mutant. Lane 1 and lane 2 in each panel show PCR products amplified from the wild-type and mutant genomic DNA templates, respectively. The DNA fragments amplified from the mutant templates are 1.3-kb longer than those from the wild-type template because of the insertion of the aphII cassette encoding resistance to kanamycin.
Fig 4.5: Growth curves of wild type and the “authentic” tocopherol mutants (A) in the absence and (B, C) in the presence of glucose at 32 °C, 50 µE m⁻² s⁻¹, 3% (v/v) CO₂.

Closed circles indicate the wild-type strain; open squares, triangles, and circles indicate the authentic slr1736, sll0418, and slr0089 mutant strains, respectively. The data shown for each strain are averages of three independent cultures, and standard error bars are shown.
Fig 4.6: Growth curves of wild type and the “authentic” slr1736 mutant in the presence of glucose at 32 °C, 3% (v/v) CO₂.

Growth (A) at 300 μE m⁻² s⁻¹, (B) at < 5 μM m⁻² s⁻¹, (C) in the presence of 0.03 μM DCMU at 50 μE m⁻² s⁻¹, and (D) in the presence of potassium cyanide (2 μM and 20 μM) at 50 μE m⁻² s⁻¹. Closed circles and open squares indicate the wild-type and the “authentic” slr1736 mutant strains, respectively. Data shown for each strain are averages of three independent cultures, and standard error bars are shown. Dotted lines in (D) and (E) indicate the presence of DCMU and potassium cyanide, respectively.
Fig 4.7: Growth analyses of wild type and the “authentic” slr1736 mutant of *Synechocystis* sp. PCC 6803 at 32 °C, 50 µE m⁻² s⁻¹, 3% CO₂ (v/v), in the presence various carbon sources. (A) 5 mM glucose, (B) 5 mM succinate, (C) 5 mM pyruvate, (D) 5 mM fumarate, (E) 15 mM glutamate, and (F) 15 mM ornithine. Filled circles and open squares indicate the wild-type and the *slr1736* mutant strains, respectively. Data shown here average of three independent measurements, and standard error bars are shown. In panel F, the tube on the left is the wild-type strain grown in the presence of 15 mM ornithine, while the tube on the right is the *slr1736* mutant grown under the same conditions.
Figure 4.8: Chlorophyll $a$ (Chl $a$) contents, and quinone contents in wild type and the “authentic” tocopherol mutants grown in the absence and in the presence of glucose.

(A) Chl $a$ content, (B) phylloquinone (PhyQ) content, and (c) plastoquinone (PQ) content. The data shown are averages of three determinations, and standard error bars are shown. Cultures grown in the absence of glucose (shows at 0 h) were transferred to media containing glucose for 24, 48, and 72 h. Wild type, the $slr0089$, the $sll0418$, and the $slr1736$ mutants are shown in black, diagonally slashed, checked, and open columns, respectively.
Fig 4.9: Oxygen-evolution activities of the whole-chain and the Photosystem II-dependent electron transport chains in wild type and the “authentic” tocopherol mutants of *Synechocystis* sp. PCC 6803.

O₂-evolution activities of whole-chain electron transport (H₂O → HCO₃⁻) were measured for cells grown in the absence of glucose (black columns) and in the absence of glucose (diagonally slashed columns). O₂-evolution activities of PSII-dependent electron transport (H₂O → 1,4-BQ/K₃Fe(CN)₆) were measured for cells grown in the absence of glucose (checked columns) and in the presence of glucose (open columns). Data shown here average of five independent measurements, and standard error bars are shown. The cells were grown for 24 h under the designated conditions before measurements. N.D., not detectable.
Fig 4.10: Immunoblotting analysis for the D1 and PsbO proteins in whole cells of wild type and three “authentic” tocopherol mutants grown in the absence of glucose (Photoautotrophic) and in the presence of glucose (Photomixotrophic) at 32 °C, 50 µE m⁻² s⁻¹, with 3% (v/v) CO₂.

Proteins from equal amount of cells (10 µL of cell suspension with OD₇₃₀ nm =100) was loaded in each lane. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Proteins were detected by immunoblotting as described in the Materials and Methods.
**Fig 4.11:** Time-dependent RT-PCR analysis of the *sodB* and *nblA* transcripts in wild type and the “authentic” *slr1736* mutant of *Synechocystis* sp. PCC 6803

The cells were initially grown in the absence of glucose (shown at 0 h) and transferred to media containing 5mM glucose and grown for 4, 8, 24 h at 3% CO₂ (v/v), 32°C, 50 µE m⁻² s⁻¹. *rnpB*, encoding the catalytic RNA subunit of RNase P, was used as control.
Fig 4.12: Relative phycobiliprotein contents in wild type and the “authentic” tocopherol mutants of *Synechocystis* sp. PCC 6803 grown at 3% (v/v) CO₂, 32°C, 50 µM E m⁻² s⁻¹.  
(A) Cells grown in the absence of glucose (black columns) were transferred to glucose-containing BHEPES media and grown for 24 h (diagonally slashed columns). (B) Time-course analyses in wild type (closed circles) and the *slr1736* mutant (open squares) grown in the absence of glucose (shown at 0 h), and transferred to medium containing glucose and grown for 4, 8, and 24 h. The values represent the ratio relative to wild type grown in the absence of glucose. Data shown here are the average of six independent measurements, and standard error bars are shown.
Fig 4.13: Growth curves of wild type and the “authentic” \textit{slr1736} mutant in the presence of glucose at 3\% (v/v) CO$_2$ and air.
Closed circles and open squares indicate the wild-type and the \textit{slr1736} mutant strains, respectively. Solid and dotted lines indicated the presence of 3\% (v/v) CO$_2$ and air in medium, respectively. Cells were grown at 32°C, 50 \mu E m$^{-2}$ s$^{-1}$. 
Fig 4.14: Cultures of wild type and the “authentic” tocopherol mutants of Synechocystis sp. PCC 6803 grown at various pH for 2 days at 1% (v/v) CO₂, 32°C, 50 µE m⁻² s⁻¹.

Cells were grown in the absence (above) or in the presence (below) of glucose in medium BHEPES40 at pH 6.8, 7.0, 7.2, 7.6, and 8.0.
Fig 4.15: Growth curves of wild type and the “authentic” slr1736 mutant of *Synechocystis* sp. PCC 6803 at various pH values in the presence of glucose in air and 1% (v/v) CO₂.

Cells were grown in BHEPES40 medium at (A) pH 8, (B) pH 7.6, (C) pH 7.2, (D) pH 7.0, (E) pH 6.8, in 1% (v/v) CO₂ (circles) and air (squares). Wild type and the “authentic” slr1736 mutant strains are indicated with filled symbols and open symbols, respectively. Growth analyses were carried out at 50 µE m⁻² s⁻¹, 32°C.
Fig 4.16: Cultures of wild type and the “authentic” slr1736 mutant of *Synechocystis* sp. PCC 6803 grown at various conditions for 48 h at pH 7. (Top) Growth in the presence of 5 mM pyruvate, fumarate, succinate, and 3-O-methyl-glucose at pH 7. (Bottom) Growth in the presence of 5 mM glucose at pH 7 with 5 mM NaCl (left), at room temperature (middle), and cultures pre-conditioned at pH 8. Growth analyses were carried out at 50 µE m⁻² s⁻¹, 32°C, 1% (v/v) CO₂ using medium BHEPES40.
Fig 4.17: PS II-dependent O₂-evolution activities of wild type and the “authentic” slr1736 mutant grown in the absence (closed symbols) and in the presence (open symbols) of glucose. Wild type and the slr1736 mutant are indicated by circles and squares, respectively. Cells were grown in medium BHEPES40 at the designated pH. O₂ evolution was measured for 2 mL of cell suspension (OD
730nm = 1.0) in 50 mM HEPES buffer, pH 7.0, using 1mM 2,6-dimethyl-1,4-benzoquinone as the electron acceptor. Excitation light intensity was ca. 3 mE m⁻² s⁻¹. Temperature of the measurement chamber was maintained at 30 °C. A Clark-type electrode equipped with a magnetic stirrer was used for the assay.
Fig 4.18: Phycobiliprotein contents in wild type and the tocopherol mutants of *Synechocystis* sp. PCC 6803 grown at 1% (v/v) CO₂, 32°C, 50 μE m⁻² s⁻¹.

(A) Wild type, and the *slr1736*, *sll0418*, and *slr0089* mutants were grown for 24 h at pH 7 in the absence (black columns) and in the presence (open columns) of glucose. (B) Wild type and the *slr1736* mutant were grown for 24 h at various pH values in the presence of glucose.
Chapter 5

Transcriptional Regulation of Metabolic and Sigma Factor Genes in the Vitamin E-Deficient Mutant of *Synechocystis* sp. PCC 6803

Publication:

Yumiko Sakuragi, Dean DellaPenna, Donald A. Bryant, manuscript in preparation
ABSTRACT

Transcriptional regulation of metabolic genes and sigma factor genes was studied in the vitamin E (α-tocopherol)-deficient mutant (slr1736) of *Synechocystis* sp. PCC 6803. When glucose was present in growth medium at pH 8.0, the transcripts of nblA (a factor essential for the controlled degradation of PBP), sbpA (the periplasmic sulfate binding protein), and sigB, sigC, sigD, sigE, sigH, sigI (alternative sigma factors) were shown to accumulate in the tocopherol-less mutant cells. These results indicate that the α-tocopherol mutant responds to the presence of glucose as though nitrogen, carbon, and sulfur were limiting in the environment. When glucose is present in the growth medium at pH 7.0, the tocopherol mutant ceased growth after 20 h. Under these conditions, the amounts of the sigA (the principal sigma factor), rbcL (the RuBisCO large subunit), and ccmK1 and ccmL (carboxysome shell proteins) transcripts diminished after 4 h. Because the amounts of these transcripts remained unaltered when cells were grown in the absence of glucose or in the presence of glucose pH 8.0, it was concluded that the pH-dependent, glucose-induced misregulation of the sigA and rbcL are largely responsible for the glucose-induced lethality of the tocopherol mutants under these conditions. Thin-section electron micrographs revealed the absence of carboxysomes in the tocopherol mutant grown in the presence of glucose at pH 7.0. The transcription of slr2031 (a putative protein phosphatase that shows sequence similarity with RsbU and that is essential under sulfur- and nitrogen-limiting conditions) and ndhF3, ndhR, sbtA (inorganic carbon uptake mechanisms) were shown to be constitutively down-regulated under all conditions tested in the tocopherol mutant. This result demonstrates that α-
tocopherol is involved in the transcriptional regulation of these metabolic genes in *Synechocystis* sp. PCC 6803. Thin-section electron micrographs of the tocopherol-less mutant showed that a large number of glycogen granules accumulated in the cells when grown even in the absence of glucose. The targeted interruption of the *pmgA* gene (a putative anti-sigma factor homologous to RsbW) resulted in a similar pH-dependent, glucose-induced lethal phenotype. Based on these results, it is concluded that α-tocopherol acts as a global regulatory molecule in carbon, sulfur, and nitrogen metabolism in *Synechocystis* sp. PCC 6803.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>CCM</td>
<td>inorganic carbon concentrating mechanisms</td>
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<tr>
<td>DCMU</td>
<td>3-(3,4-dichlorophenyl)-1,1-dimethyl-urea</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-piperazine-N’-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PBP</td>
<td>phycobiliprotein</td>
</tr>
<tr>
<td>PS II</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RuBisCO</td>
<td>ribulose-1,5-bisphosphate carboxylase/oxygenase</td>
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α-Tocopherol is synthesized only in oxygenic phototrophs including cyanobacteria, green algae and higher plants (Threlfall and Whistance 1971; Collins and Jones, 1981). Given that α-tocopherol constitutes an essential component of our diet, most of our knowledge concerning the roles and functions of this molecule has been obtained from studies in animal systems. Based on results gathered in animals, animal cell cultures, and artificial membranes, it has been shown that tocopherols function as antioxidants and scavenge or quench various reactive oxygen species and lipid oxidation byproducts that would otherwise propagate lipid peroxidation chain reactions in membranes (Kamal-Eldin and Appelqvist, 1996). This antioxidant role of α-tocopherol has now been demonstrated in eukaryotic phototrophs owing to studies in the past several years. The herbicide-mediated interruption of the α-tocopherol biosynthesis pathway has been shown to render PS II more susceptible to oxidative stress under extremely high light illumination in the green alga Chlamydomonas reinhardtii (Trebst et al., 2002). The presence of α-tocopherol has been shown to suppress the propagation of lipid peroxidation during seedling germination in Arabidopsis thaliana (Sattler et al., 2004). Thus, there is little doubt that α-tocopherol functions as a bulk antioxidant in the eukaryotic phototrophs as well as in animals. The antioxidant role of α-tocopherol is also being investigated in the cyanobacterium Synechocystis sp. PCC 6803, and the results obtained so far support an antioxidant role of α-tocopherol in this organism (H. Maeda, Y. Sakuragi, D. A. Bryant, and D. DellaPenna, personal communication)
Perhaps the most novel and exciting functions of α-tocopherol have been uncovered by various research groups during the last few years. In addition to the antioxidant functions, other “non-antioxidant” functions related to modulation of signaling and transcriptional regulation in mammals have also been reported (Chan et al., 2001; Azzi et al., 2002; Ricciarelli et al., 2002). For example, α-tocopherol has been shown to bind to phospholipase A₂ specifically at the substrate-binding pocket and to act as a competitive inhibitor, thereby decreasing the release of arachidonic acid for eicosanoid synthesis (Chandra et al., 2002). α-Tocopherol is also directly involved in transcriptional regulation in animals, including the expression of the genes encoding liver collagen αI, α-tocopherol transfer protein, and α-tropomyosin collagenase (Yamaguchi et al., 2001; Azzi et al., 2002). The presence of α-tocopherol resulted in a modulation of the phosphorylation state of protein kinase Cα in rat smooth-muscle cells, possibly via phosphorylation of protein phosphatase 2A (Ricciarelli et al., 1998). None of these non-antioxidant functions of α-tocopherol has yet been experimentally demonstrated in oxygenic phototrophs.

In the previous studies described in Chapter 4, the role(s) of α-tocopherol in cyanobacteria was investigated by using three mutants, *slr1736, sll0418*, and *slr0089*, deficient in enzymes involved in the α-tocopherol biosynthetic pathway (see Fig 4.1). The tocopherol mutants are able to grow similarly to wild type at pH values between 6.8 and 8.0 in the absence of glucose (control conditions), under which conditions the PS II activities and the phycobiliprotein (PBP) contents were comparable to those of wild type. After transfer to a medium containing glucose at pH 8.0, the tocopherol mutants also
grew similarly to wild type and the PS II activity remained unaltered, although the PBP content decreased by ~30 % (permissive conditions). After transfer to medium containing glucose at pH 7.0, however, the tocopherol mutants ceased growth by 24 h, and the PS II activity was completely lost. Under these conditions the PBP content was reduced by ≥ 60 % (lethal conditions). The involvement of oxidative stress in the PS II inactivation was largely ruled out, because the levels of the D1 protein and the sodB transcript were comparable between wild type and the tocopherol mutants under these conditions. Based on the observed reduction in the PBP content and the accumulation of the nblA transcript, which is known to accumulate in response to nutrient limitation (Collier and Grossman, 1994; Richaud et al., 2001), it was suggested that the tocopherol mutants are experiencing macronutrient starvation. This led to the hypothesis that α-tocopherol plays a role as a regulatory molecule in macronutrient metabolism in Synechocystis sp. PCC 6803.

In this chapter, a study of the transcriptional regulation of metabolic genes and sigma factor genes in a tocopherol-deficient mutant (slr1736) is described. Of 39 genes initially analyzed, the amounts of the transcripts of 10 genes were shown to respond either to the presence of glucose alone or to the presence of glucose and pH in the medium, whereas the amounts of the transcripts of 4 genes were shown to be constitutively down regulated upon loss of α-tocopherol. Based on these observations, it is concluded that α-tocopherol is involved in transcriptional regulation of some key metabolic genes and plays a role in the global coordination of macronutrient metabolism in Synechocystis sp. PCC 6803.
RESULTS

Transcription of nblA and sbpA

Given the significant reduction of the PBPs in response to glucose and lowered pH (see Chapter 4), the nblA transcript was analyzed in wild type and slr1736 mutant cells grown at pH 7.0 and pH 8.0 (Fig 5.1). In the absence of glucose, the wild-type strain barely accumulated detectable nblA transcripts both at pH 7.0 and pH 8.0, and only a small increase followed in response to glucose in the growth medium. This appeared somewhat contradictory to the observed behavior of the PBP content in the wild type, in which the PBP content increased by 20% after transfer to medium containing glucose when cells were grown at pH 7.0 (see Chapter 4, Fig 4.18). Furthermore, the medium used for culturing these cells contains a growth-rate saturating concentration of nitrate (ca. 18 mM) (Stainer 1971; see Materials and Methods). Thus, it is inconceivable that these cells were actually limited by the availability of nitrogen. The presence of glucose in the medium might lead to an increase in the intracellular carbon pool and, as a result, facilitate the faster growth of this organism. These “carbon-saturated”, fast-dividing cells perhaps are not capable of metabolizing nitrogen at an equivalent rate, and thus respond as though they are limited by nitrogen in their environment.

The slr1736 mutant accumulated a higher amount of nblA transcript in the absence of glucose as compared to the wild-type control (Fig 5.1, shown at 0 h), which suggests that the slr1736 mutant is “perceiving” nitrogen limitation even in the absence of glucose. In response to glucose, the amount of the nblA transcripts increased dramatically in the slr1736 mutant after 4 h when cells were grown in medium at pH 7.0.
(Fig 5.1). Similar although more moderate increases were observed when cells were grown in the presence of glucose at pH 8.0. These results indicate that the slr1736 mutant is “perceiving” a severe nitrogen limitation at pH 7.0 and moderate nitrogen limitation at pH 8.0. This is consistent with the dramatic reduction of PBP contents at these pHs (ca. 40% of the wild-type level at pH 7.0 and ca. 70% of the wild-type level at pH 8.0).

The sbpA transcript, encoding the periplasmic sulfate binding protein, is known to accumulate in response to sulfate limitation (Laudenbach et al., 1991). No detectable sbpA transcripts were present in wild-type cells grown at pH 7.0 and pH 8.0 in the absence or presence of glucose (Fig 5.1). In the slr1736 mutant cells grown at pH 7.0, a small yet discernible amount of the sbpA transcripts was detected in the absence of glucose, and these transcripts dramatically increased by 8 h after transfer to medium containing glucose. When the slr1736 mutant cells were grown at pH 8.0, no sbpA transcripts were detectable in the absence of glucose, and a gradual increase was observed in 24 h after transfer to a medium containing glucose. These results indicate that the slr1736 mutant is “perceiving” sulfur limitation even in the absence of glucose at pH 7.0, and the response is exacerbated after transfer to a medium containing glucose. It is noteworthy that the amounts of the sbpA and nblA transcripts accumulated at pH 7.0 and 8.0 coincided well with the degree of reduction of the PBP contents at these pHs in the presence of glucose (See Chapter 4, Fig 4.18, and Fig 5.1). The combined results hence demonstrate that the reduction of the PBP content is associated with “perceived” nitrogen and sulfur limitation in the slr1736 mutant.
The \textit{cpcA} transcripts, encoding the phycocyanin $\alpha$-subunit, which is a subunit of the most abundant PBPs, were found to decrease gradually in the tocopherol mutant in response to glucose when the \textit{slr1736} mutant cells were grown at pH 7.0. At pH 8.0 its level remained largely unaltered both in wild type and the \textit{slr1736} mutant before and after addition of glucose. Therefore, it was concluded that the mechanism of the reduction of the PBP content at pH 7.0 and 8.0 in the \textit{slr1736} mutant grown in the presence of glucose are different. At pH 8.0, the reduction of the PBP content primarily involves active degradation of the PBP proteins, while at pH 7.0 it involves both the active degradation, as characterized by the accumulation of the \textit{nblA} transcript, and the transcriptional down regulation of the genes encoding PBPs, which further supports the conclusion that the cells are perceiving nutrient limitation and responding to it (Grossman et al., 1994; also see references therein).

\textit{Transcription of Alternative Sigma Factor Genes}

The \textit{Synechocystis} sp. PCC 6803 genome encodes eight $\sigma^{70}$-type alternative sigma factors (SigB through I) in addition to the principal sigma factor (SigA), and they play roles as transcription factors that are activated in response to various kinds of stresses (Caslake et al., 1997; Gruber and Bryant, 1998; Huchauf et al., 2000; Muro-Pastor et al., 2001; Imamura et al., 2003a, b). Fig 5.2 shows the results of the RT-PCR analysis of \textit{sigB}, \textit{sigC}, \textit{sigD}, \textit{sigE}, \textit{sigH}, and \textit{sigI} in wild type and the \textit{slr1736} mutant in the absence of glucose and in the presence of glucose. SigB has been suggested to be involved in a variety of stress responses, including dark response (Imamura et al., 2003a) and heat-shock response (Huchauf et al., 2000) in \textit{Synechocystis} sp. PCC 6803, and carbon and
nitrogen starvation responses in *Synechococcus* sp. PCC 7002 (Caslake et al., 1997). When cells were grown at pH 7.0, the amount of the *sigB* transcripts was largely unaltered in wild type before and after addition of glucose to the medium (Fig 5.2). In the *slr1736* mutant, the amount of the *sigB* transcript was somewhat higher in the absence of glucose as compared to the wild-type control, followed by a large increase by 4 h after transfer to medium containing glucose. A similar response was observed for the *slr1736* mutant grown at pH 8.0. Therefore the amount of the *sigB* transcripts responded to glucose alone and was not influenced by the pH of the medium. Similar accumulation patterns of transcripts were also observed for *sigC*, *sigH*, and *sigI*. SigH is involved in the heat-shock response (Hackauf et al., 2000), while SigC, which is related to SigE in *Synechococcus* sp. PCC 7002, is likely to be involved in the post-exponential phase of growth (Gruber and Bryant, 1998). The role of SigI has not yet been elucidated. The results suggest that the *slr1736* mutant is experiencing a mixture to stresses in response of glucose.

Transcription of *sigE* is under the control of NtcA, a global nitrogen regulator, which binds to its characteristic binding site located in the upstream flanking sequence of the *sigE* transcription initiation site and induces transcription of *sigE* in response to nitrogen deprivation (Muro-Pastor et al., 2001). Interestingly, the amount of the *sigE* transcripts increased in response to glucose in wild type both at pH 7.0 and 8.0 (Fig 5.2). This is consistent with the pattern of the accumulation observed for the *nblA* transcripts (Fig 5.1, see above). Although the reason for this glucose-induced nitrogen limitation response has not yet been defined, it is possible that this is due to an altered intracellular
carbon-nitrogen balance caused by increased carbon flux induced by the glucose metabolism (see also above). In the slr1736 mutant, the amount of the sigE transcripts increased more dramatically than for the wild type in response to glucose. This glucose-induced accumulation of the sigE transcripts occurred both at pH 7.0 and pH 8.0, which is consistent with the results obtained for the nblA transcripts (Fig 5.1, see above) and which indicates that the slr1736 mutant is “perceiving” a nitrogen limitation in response to glucose.

In contrast to sigE, the amount of the sigD transcripts was largely unaffected by the presence of glucose in wild type (Fig 5.2). In the tocopherol mutant, the amount of the sigD transcript was lower than that of wild type in the absence of glucose and increased by several-fold in response to glucose (by visual inspection). SigD is highly expressed during exposure to high light (Imamura et al., 2003a,b). It has been suggested that this light-dependent behavior of sigD expression is regulated by the redox state of PS II (Imamura et al., 2003a). Because the growth conditions used in this study employ continuous illumination of the cultures with a moderate light intensity (50 µE m⁻² s⁻¹), and because the PS II activity is nearly at the wild-type level for the slr1736 mutant in the absence of glucose at pH 7.0 and 8.0 or in the presence of glucose at pH 8.0, the observed alteration of the sigD transcription can not be explained simply by the previously suggested light-responsive role of SigD.

Taking these results together, it was firmly concluded that the transcription of these alternative sigma factor genes is modulated in the slr1736 mutant in response to glucose and that pH plays little role in the glucose-induced response of the transcription
of the alternative sigma factors. This indicates that \(\alpha\)-tocopherol plays a role in regulating the stress response related at least to carbon, nitrogen and sulfur metabolism in

*Synechocystis* sp. PCC 6803.

**Transcription of sigA and Ci Genes**

The *sigA* gene encodes the principal sigma factor, which is responsible for the transcription of genes encoding proteins essential for growth and reproduction in eubacteria including cyanobacteria (Gruber and Bryant, 1997; Caslake and Bryant, 1996). The amount of the *sigA* transcripts was analyzed for wild type and the *slr1736* mutant grown at pH 7.0 and 8.0 (Fig 5.3). At pH 7.0, the *sigA* transcripts in wild type remained constant after addition of glucose to medium, which is consistent with the fact that the wild-type cells grow normally under these conditions. The *sigA* transcript level in the *slr1736* mutant at pH 7.0 was virtually identical to that for the wild type in the absence of glucose, which is also consistent with the fact that the *slr1736* mutant grows similarly to wild type in the absence of glucose at pH 7.0. The level of the *sigA* transcripts remained unaltered for 4 h when the *slr1736* mutant was transferred to medium containing glucose at pH 7.0. However, after 8 h the *sigA* transcript level significantly diminished. Such a dramatic reduction in the amount of the *sigA* transcript is likely to cause a reduction of the content of SigA available for transcription of house-keeping genes, which would in turn severely impair the growth capacity of the cells. Indeed, the tocopherol mutants are unable to grow in the presence of glucose at pH 7.0 (see Chapter 4, Fig 4.14 and Fig 4.15). In contrast, when the *slr1736* mutant was grown in the presence of glucose at pH 8.0, the amount of the *sigA* transcript was virtually unaltered and was very similar to that
in the wild type control. This is consistent with the fact that the *slr1736* mutant grew similarly to wild type under these conditions (see Chapter 4, Fig 4.14 and Fig 4.15). The direct correlation between the amounts of the *sigA* transcripts and the growth behavior of the *slr1736* mutant in the absence and in the presence of glucose at pH 7.0 and 8.0 strongly indicate that the pH-dependent glucose toxicity is caused by the pH-dependent, glucose-induced alteration of the *sigA* transcript level in the *slr1736* mutant.

Accompanying the reduction of the amounts of the *sigA* transcript, the amounts of the transcripts of carboxysome-related genes (*rbcL*, *ccmK1*, *ccmL*), a carbon-concentrating mechanism (CCM) gene (*ndhF4*), and *sigG* were also decreased in a pH- and glucose-dependent manner in the *slr1736* mutant (Fig 5.3). *rbcL* encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which is responsible for the fixation of CO$_2$ and is essential for the survival of the cells, because this gene cannot be completely inactivated in cyanobacteria (Pierce et al., 1989). *ccmK1* and *ccmL* encode carboxysome shell proteins, which form the outer shell of carboxysomes and encapsulate RuBisCO within the carboxysomal structure (Price et al., 1993). *ndhF4* encodes a subunit of a multi-subunit complex that is a low-affinity constitutive CO$_2$ uptake transporter (Shibata et al., 2001). The reduction in the levels of the carboxysome transcripts most likely leads to the reduction in the amount of carboxysomes in the cells; as a result, the ability to fix CO$_2$ would be severely impaired (Price et al., 1993).
Intracellular Structures

Thin-section electron micrographs of wild-type and slr1736 mutant cells grown at pH 7.0 in the absence and the presence of glucose were recorded. Fig 5.4 shows the electron micrographs of wild type grown in the absence (Fig 5.4A) and in the presence of glucose (Fig 5.4B). In the wild-type cells grown in the absence of glucose, the space between the thylakoid membranes was filled with electron-opaque material, which most likely represents the protein-rich phycobilisomes (Fig 5.9A). In the cytoplasm, electron-opaque polyhedral bodies are visible, which are characteristic of carboxysomes. Slightly larger electron-transparent bodies with round or oval shapes are most likely due to poly-β-hydroxybutyrate (Stainer, 1988). In the wild-type cells grown in the presence of glucose (Fig 5.4B), on the other hand, the intermembrane space is filled with small electron-transparent objects with irregular shapes, which are characteristic of glycogen granules. Glycogen granules serve as general carbohydrate reserves (Stainer, 1998). The cellular content of glycogen accounts for 10-20 % of the dry cell weight when cells are growing photoautotrophically and exponentially, whereas it accounts for up to 60 % of dry cell weight when cells are subjected to nitrogen-limiting conditions (Stainer, 1988; Smith 1982). That few glycogen granules are observed in the wild-type cells grown in the absence of glucose is consistent with the fact that these cells grow in a balanced manner and optimally under these conditions. The observed accumulation of glycogen in the wild-type cells grown in the presence of glucose most likely reflects a carbon-saturated, or nitrogen-limited metabolic state of the cells.
Fig 5.5A shows an electron micrograph of an slr1736-mutant cell grown in the absence of glucose at pH 7.0. The intracellular structures, such as the presence of thylakoid membranes, carboxysomes, and poly-β-hydroxybutyrate, are very similar to those of the wild-type control grown under the same conditions (Fig 5.4 A). However, in the slr1736 mutant, the intermembrane space was filled with glycogen granules. This clearly contrasts with the situation in the wild-type cells, in which only a small number of glycogen granules was detectable (Fig 5.4A). Rather, this feature of the slr1736 mutant cell resembles that of the wild-type cell grown in the presence of glucose (Fig 5.4B). This suggests that the slr1736 mutant accumulates an unusually large amount of carbohydrate even in the absence of glucose. Therefore, the slr1736 mutant cells seem either to be experiencing nitrogen limitation, or to be performing CO₂ fixation or glycogen synthesis much more efficiently than the wild-type cells. The former is not likely to be the case as the mutant cells grow similarly to wild type under these conditions. Therefore, it is most likely that the mutant is overproducing photosynthate. In either case, it is obvious that the “metabolic state” of the slr1736 mutant is different from that of the wild type even in the absence of glucose.

When the slr1736 mutant was grown in the presence of glucose, the appearance of the intracellular structures changed dramatically (Fig 5.5B). The intermembrane space is no longer electron-opaque, which is consistent with the significant reduction in the PBP content. No carboxysomes were observed in the majority of the cells, which supports the results that the transcripts of the carboxysome genes are substantially reduced in the slr1736 mutant under these conditions. The number of layers formed by the thylakoid
membranes, however, does not seem to be affected significantly. Interestingly, new oval structures with an electron-opaque appearance are visible between the thylakoid membranes; these structures were absent in wild type grown in the absence and in the presence of glucose and in the slr1736 mutant grown in the absence of glucose.

**Constitutive Alteration of the CCM Genes and slr2031 Expression**

Given the alteration of the “metabolic state” in the slr1736 in the absence of glucose, further transcription analyses were carried out in order to understand the involvement of α-tocopherol in macronutrient metabolism. Of 39 genes analyzed for their transcript level, 4 genes (ndhF3, ndhR, sbtA, slr2031) showed constitutive down-regulation both in the presence and in the absence of glucose (Fig 5.6). ndhF3, ndhR, and sbtA encode components of high-affinity CO2 uptake transporters (Klughammer et al., 1999; Shibata et al., 2001, 2002), a repressor of the ndhF3 operon, and the sodium-dependent bicarbonate transporter, respectively, and all participate in inorganic carbon concentrating mechanisms (CCM) under limited inorganic carbon environments. The transcripts of these genes were detected in wild type both in the absence and the presence of glucose at pH 7.0 and 8.0. On the contrary, the transcript levels for these genes were significantly reduced in the slr1736 mutant both in the presence and in the absence of glucose both at pH 7.0 and 8.0. Thus, the constitutive alteration of these CCM genes is directly linked to the loss of α-tocopherol.

These CCM genes are known to be upregulated in response to low inorganic carbon environments, and are hardly detectable under high CO2 conditions as analyzed by Northern-blot hybridization (Figge et al., 2001). This raises a question as to whether
the detection of the CCM genes in this study [at 1% (v/v) CO₂] is due to an artifact such as contamination with genomic DNA during the RNA isolations. The RNA preparation is carried out through three steps, including isolation, RNase-free DNase treatment, and finally purification; thus, it is unlikely that genomic DNA is present in the preparation. Indeed, PCR analyses on the same RNA templates used for RT-PCR analyses using primers targeted to the *rnpB* gene resulted in no product formation, which confirms that the RNA preparations are free of genomic DNA contamination. Currently, we do not have an explanation other than attributing the discrepancy to a difference in the sensitivity of the detection methods. Typically one-step RT-PCR analyses using the Qiagen One-Step RT-PCR kit (Qiagen inc., Valencia, CA) requires 50 pg µL⁻¹ to 5 ng µL⁻¹ of total RNA in order to detect the target transcripts, whereas typical Northern-blot hybridization reactions require several micrograms of RNA. Indeed, when more sensitive methods were employed, other groups have also detected the presence of the *ndhF3* and *sbtA* transcripts in RNA preparations obtained from cells grown at high CO₂ level [3-5% (v/v) CO₂] (Shibata et al., 2002; McGinn et al., 2003).

*slr2031* encodes a putative protein phosphatase (similar to RsbU) involved in the regulation of SigB in *B. subtilis*. In wild-type cells, the amount of *slr2031* transcripts were low yet detectable in the absence of glucose, and transcript levels substantially increased by 8 h in response to glucose both at pH 7.0 and pH 8.0 (Fig 5.6). In the *slr1736* mutant, the *slr2031* transcript is hardly detectable in the absence of glucose, and only a slight induction was observed in response to glucose at pH 7.0. At pH 8.0, the *slr2031* transcript behaved similarly at pH 7.0, although a moderate increase was
observed in the presence glucose by 24 h. In *Synechocystis* sp. PCC 6803, Slr2031 has been shown to be somehow involved in sulfur and nitrogen metabolism, and its absence is deleterious under sulfur- and nitrogen-limiting conditions (Huchauf et al., 2000). An independent study has shown that the presence of Slr0231 is also essential when the *cotA* gene is inactivated (Katoh et al., 1995). CotA has been suggested to be involved in light-induced proton extrusion and CO₂ transport in *Synechocystis* sp. PCC 6803 (Katoh et al., 1996a,b). Thus, it is possible that Slr2031 is involved in the transcriptional regulation of genes that are involved in regulating processes including carbon, nitrogen, and sulfur metabolism. The fact that the expression of *slr2031*, as well as *ndhF3*, *ndhR*, and *sbtA*, is constitutively down-regulated in the absence of α-tocopherol demonstrates that α-tocopherol plays a role in the transcriptional regulation of metabolic genes in *Synechocystis* sp. PCC 6803.

Is the significant reduction of *slr2031* transcription directly related to the cause of the pH-dependent glucose toxicity of the tocopherol mutant? In order to answer this question, an *slr2031* disruption mutant was generated by insertion of the *aadA* gene cassette, conferring spectinomycin resistance, into the unique *MfeI* site of the *slr2031* coding region. Complete segregation of the mutant allele from the wild-type allele was demonstrated by PCR analysis (Fig 5.7). Using the primers designed to target and amplify a 0.9-kb N-terminal portion of the *slr2031* loci resulted in the amplification of a 0.9-kb DNA fragment when the genomic DNA isolated from wild type was used as the template. No 0.9-kb product was detectable when the genomic DNA isolated from the *slr2031*::*addA* transformant was used as the template. Instead, a product of 2 kb was
detected. The size difference between the two products corresponds to the size of the addA gene cassette, which demonstrates that the slr2031::addA transformant is homozygous and free from the slr2031 wild-type allele.

A preliminary growth analysis was carried out at pH 7.0 and pH 8.0. The slr2031 mutant (slr2031::aadA) was able to grow both at pH 7.0 and pH 8.0 in the absence of glucose. When the cells were transferred to medium containing glucose, the slr2031 mutant was able to grow similarly to wild type both at pH 7.0 and pH 8.0 (Fig 5.8 A and B). An slr1736 slr2031 double mutant behaved similarly to the slr1736 mutant at both pHs in the presence of glucose (Fig 5.8 A and B). Therefore, it was concluded that the constitutive down-regulation of slr2031 transcription is not the direct cause of the pH-dependent glucose toxicity observed for the tocopherol mutant.

As mentioned in Chapter 4, the pmgA mutant of Synechocystis sp. PCC 6803 is unable to grow in the presence of glucose, which indicates that PmgA plays a role in the regulation of photomixotrophic growth (growth on CO₂ and glucose as the carbon source) (Hihara and Ikeuchi, 1997). To test whether the role of PmgA is related to the role of α-tocopherol, a pmgA inactivation mutant was constructed by inserting the accC1 gene cassette, conferring gentamicin resistance, into the unique SpeI site within the pmgA coding region. The complete segregation of the mutant allele from that of wild type was demonstrated by PCR analysis (Fig 5.7). PCR using the primers designed to target and amplify a 0.8-kbp of the pmgA locus resulted in the amplification of a 0.8-kbp fragment when genomic DNA isolated from wild-type cells was used as the template. No 0.8-kbp product was detectable for the PCR product when genomic DNA isolated from the
pmgA::aacC1 transformant was used as the template. Instead, a product of 1.9 kb was detected. The size difference between the two products corresponds the size of the aacC1 gene cassette, which demonstrates that the pmgA::aacC1 transformant is homozygous and free from the pmgA wild-type allele.

A preliminary growth analysis was carried out in the absence of glucose and in the presence of glucose at pH 7.0 and pH 8.0. The results showed that the pmgA mutant grew similarly to wild type in the absence of glucose at pH 7.0 and at pH 8.0 (by visual inspection). In the presence of glucose, the pmgA mutant grew similarly to wild type in medium at pH 8.0; however, its growth was found to be severely impaired in the presence of glucose at pH 7.0. This apparent similarity in the growth behavior between the tocopherol and pmgA mutants in response to glucose and pH suggests that α-tocopherol and PmgA play roles in the same regulatory pathway. PmgA shows similarity to RsbW/RsbT, protein serine/threonine kinase, in B. subtilis (Price CW, 2000; Woodbury et al., 2004). RsbW functions as an anti-SigB factor, while RsbT functions as an activator of RsbU, which in turns activates SigB in the signal transduction cascade that regulates the activity SigB. They act together with other protein phosphatases and kinases including RbsU, an Slr2031 homologue (Price CW, 2000; Woodbury et al., 2004). More than 6 Rsb-like proteins were found to be conserved in the genome of Synechocystis sp. PCC 6803, and PmgA, Slr2031, and Ssr1600 are also found to be present in the genomes of Thermosynechococcus elongatus BP-1, Nostoc sp. PCC 7120, Nostoc punctiforme, Trichodesmium erythraeum, Synechococcus sp. PCC 7002, Prochlorococcus marinus sp. MED4, P. marinus sp. MIT9313, P. marinus sp. SS120, and Synechococcus sp.
WH8102. Thus, it is highly plausible that a pathway similar to the Rsb pathway in *B. subtilis* is present in cyanobacteria and regulates the balance between carbon, nitrogen and sulfur metabolism.
DISCUSSION

The potential involvement of α-tocopherol in macronutrient metabolism was investigated. RT-PCR analyses performed on the cells grown at pH 7.0 and 8.0 in the absence and in the presence of glucose demonstrated that the transcripts of the alternative sigma factors accumulate in the slr1736 mutant in response to glucose, which indicated that the slr1736 mutant is experiencing a variety of stresses when grown in the presence of glucose (Caslake et al., 1997; Gruber and Bryant, 1998; Huchauf et al., 2000; Muro-Pastor et al., 2001; Imamura et al., 2003a,b). The nblA and sigE transcripts, known to accumulate in response to nitrogen limitation (Mulo-Pastor, 2001; Richaud 2001), also accumulated in response to glucose in the slr1736 mutant. The sbpA transcript, known to accumulate in response to sulfur limitation (Laudenbach and Grossman, 1992), also accumulated in response to glucose in the slr1736 mutant. In combination with the results obtained for the PBP content as described in Chapter 4, these results demonstrate that slr1736 mutant is experiencing macronutrient starvation when grown in the presence of glucose, and that α-tocopherol is somehow influencing carbon, nitrogen, and sulfur metabolism in Synechocystis sp. PCC 6803.

When cells were grown at pH 7.0 in the presence of glucose, the level of transcripts of sigA, ndhF4, cpcA, and carboxysome genes (rbcL, ccmK1, ccmL) decreased substantially. These genes were constitutively expressed, and their products play housekeeping roles for the survival of cyanobacteria. Thus, it is possible that the reduction of the ndhF4, and cpcA, and carboxysome transcripts is caused by the reduction of the cellular content of SigA (Pierce et al., 1989; Caslake and Bryant, 1996; Gruber and
Because the functions of SigA and RuBisCO are absolutely indispensable for the survival of cyanobacteria, the observed pH-dependent, glucose-induced transcriptional misregulation of \textit{sigA} and \textit{rbcL} is a likely cause of the cell death of the tocopherol mutant in the presence of glucose in medium at pH 7.0.

The mechanism of the pH-dependent, glucose-induced misregulation of \textit{sigA} transcription has yet to be determined. A study has suggested that \textit{sigA} transcription is regulated by the activity of the photosynthetic electron transport chain, and in the presence of DCMU or in the dark the amount of \textit{sigA} transcripts is rapidly lowered (Tuominen et al., 2003). Interestingly, the activity of PS II, and thus the activity of the photosynthetic electron transport chain, is reduced in a pH-dependent manner in the tocopherol mutant in response to glucose, and PS II activity was completely lost at pH 7.0 (see Chapter 4, Fig 4.17). The PS II content in the tocopherol mutant under these conditions appeared to be largely unaltered based on the contents of the D1 and PsbO proteins (see Chapter 4, Fig 4.10). Thus, the inactivation of PS II is thought to be related either to the loss of electron transfer cofactors or to the generation of metabolites that inhibit the activity of PS II. Decades of studies and recent X-ray crystallographic structures of the PS II complexes from the cyanobacterium \textit{Synechococcus elongatus} have confirmed the presence of one bicarbonate molecule in the vicinity of the non-heme iron located between the two bound plastoquinone molecules (Q\textsubscript{A} and Q\textsubscript{B}) (Blubaugh and Govindjee, 1986; Petrouleas et al., 1994; Ferreira et al., 2004, see also reviews by Blubaugh and Govindjee, 1988; Govindjee and van Rensen, 1993; van Rensen et al., 1999, and references therein ). It has been demonstrated that this bicarbonate molecule is
displaced *in vitro* by other molecules such as formate, NO, glycolate, glyoxylate, and oxalate (Petrouleas et al., 1994). Glycolate is a metabolite generated by the oxygenase activity of RuBisCO, which occurs when the intracellular CO₂ concentration is substantially reduced. Although, there is no evidence suggesting a significant reduction in the intracellular Ci pool in the tocopherol mutant, this possibility needs to be examined nevertheless.

Perhaps the most exciting outcome of the RT-PCR analyses is the discovery that the expression of *slr2031* and CCM genes (*ndhF3, ndhR, sbtA*) is constitutively down-regulated in the tocopherol mutant in the presence and absence of glucose both at pH 7.0 and 8.0. This direct effect of the loss of α-tocopherol on gene expression is a strong indication that α-tocopherol is involved in transcriptional regulation in cyanobacteria. Because the function of these products are directly related to macronutrient metabolism, this further leads to the conclusion that α-tocopherol is involved in the regulation of the macronutrient metabolism as a transcriptional regulator in cyanobacteria. It is noteworthy that, in mammalian systems, it has been demonstrated that α-tocopherol is directly involved in transcriptional regulation of the genes encoding liver collagen α1, α-tocopherol transfer protein, and α-tropomyosin collagenase (Yamaguchi et al., 2001; Azzi et al., 2002). The mechanisms of these transcriptional regulation processes are not completely understood; however, it is a reasonable assumption that it involves a modulation of a signal transduction cascade that regulates the expression of these genes. Indeed, α-tocopherol has been suggested to modulate the cell signaling pathway by modulation of the phosphorylation state of protein kinase Cα in rat smooth-muscle cells,
possibly via phosphorylation of protein phosphatase 2A (Ricciarelli et al., 1998). In light of the observed involvement of α-tocopherol in cell signaling in the animal system, it is intriguing to recognize the phenotypic similarity between the tocopherol mutant and the pmgA mutant in *Synechocystis* sp. PCC 6803. As mentioned earlier, the pmgA gene has been demonstrated to be a determinant of glucose tolerance in this organism (Hiahara and Ikeuchi, 1997), and its deduced amino acid sequence shows similarity to a serine/threonine protein kinase, which has been characterized as the anti-sigma factor (RsbW) or the activator for the anti-RsbW (RsbT) in the regulatory pathway of SigB in *B. subtilis* (Price, 2000; Woodbury et al., 2004). Thus, it is highly plausible that α-tocopherol plays a regulatory role through this Rsb-like pathway in *Synechocystis* sp. PCC 6803 as summarized in Fig 5.9. This is the first time, to the best of my knowledge, that the involvement of α-tocopherol in transcriptional regulation has been experimentally demonstrated in the oxygenic phototrophs. This newly discovered role of α-tocopherol in *Synechocystis* sp. PCC 6803 thus strongly indicates that α-tocopherol plays a regulatory role in addition to a role as a bulk antioxidant in some cyanobacteria.

The next question is whether such a regulatory role is evolutionarily conserved among green algae and plants. Recent studies have shown that a mutant in maize, originally isolated because of its deficiency in sucrose transport through plasmodesmata, is also deficient in tocopherol cyclase (Porfirova et al., 2002; Sattler et al., 2003). This suggests that α-tocopherol plays a role in sugar metabolism in maize. It is therefore highly plausible that α-tocopherol has similar regulatory functions in macronutrient metabolism in plants.
SUMMARY

It was shown that a α-tocopherol mutant responds to the presence of glucose as though nitrogen and sulfur were limiting in the environment. This conclusion is based on the accumulation of stress-responsive gene transcripts. When glucose is present in growth medium at pH 7.0, the amounts of the sigA and rbcL transcripts diminished after 4 h. Because the amount of these transcripts remained unaltered when cells were grown in the absence of glucose or in the presence of glucose pH 8.0, it was concluded that the pH-dependent glucose-induced misregulations of the sigA and rbcL are largely responsible for the glucose-induced lethality of the tocopherol mutants under these conditions. The transcription of metabolic genes (slr2031, ndhF3, ndhR, and sbtA) is shown to be constitutively down regulated under all conditions tested in the tocopherol mutant. This result demonstrates that α-tocopherol is involved in transcriptional regulation of these metabolic genes in Synechocystis sp. PCC 6803. Thin-section electron micrographs of the tocopherol mutant showed that large amounts of glycogen granules accumulated in the cells. The targeted interruption of the pmgA gene (a putative anti sigma factor homologous to RsbW) resulted in the similar pH-dependent glucose-induced lethal phenotype. Based on theses results, it was concluded that α-tocopherol is involved as a regulatory molecule in the global regulation of carbon, sulfur, and nitrogen metabolism in Synechocystis sp. PCC 6803.
MATERIALS AND METHODS

Strains and Growth Conditions

The wild-type strain used for transformation and further analyses was a glucose-tolerant strain of *Synechocystis sp.* PCC 6803 (Williams 1988). Medium BHEPES, pH 8.0, was used for maintenance of the wild type and mutant strains. This medium is prepared by supplementing BG-11 medium (Stainer et al., 1971) with 4.6 mM of HEPES [4-(2-hydroxyethyl)piperazine-N’-(2-ethnesulfonic acid)]-KOH and 18 mg L⁻¹ ferric ammonium citrate. Medium BHEPES40 is prepared by supplementing the medium BHEPES with 40 mM HEPES. Prior to all growth measurements and the preparation of cells for subsequent analyses, cells were transferred from solid to liquid medium BHEPES and allowed to grow exponentially for several generations in the absence of glucose. For the determination of growth characteristics, late-exponential phase cultures were diluted in fresh liquid medium BHEPES40, pH 7.0 or 8.0, to an OD₇₅₀ nm of approximately 0.05 cm⁻¹. The diluted cultures were grown at 32 °C with continuous bubbling with air containing 1% (v/v) CO₂. For growth in the presence of glucose, the medium was supplemented with 5 mM glucose. The light intensity was 50 µE m⁻² s⁻¹, unless otherwise noted. For isolation of RNA, late-exponential phase cultures were diluted in fresh liquid medium BHEPES40 to an OD₇₅₀ nm of approximately 0.3 cm⁻¹ in a total volume of 80 mL. The diluted cultures were grown under the same conditions as described above.
Generation of the slr2031 and the pmgA Mutants

The 5’-terminal portion of the slr2031 locus was amplified by PCR using primers designed to target this genomic region [slr2031-F, 5’-AGGAGTTGGTGCTAAGCTTTACCAGGAACAAA-3’, including an engineered HindIII cleavage site (underlined); slr2031-R, 5’-TGACAAAGCGATGGGAATTC-3’, including an engineered EcoRI cleavage site (underlined)]. The pmgA locus was amplified by PCR using primers designed to target this genomic region [pmgA-F, 5’-TTCTCTGTGCCGAAGCTTATG-3’, including an engineered HindIII cleavage site (underlined); pmgA-R, 5’-CACCATGGTGCCGAATTCAGGC-3’, including an engineered EcoRI cleavage (underlined)]. The amplified DNA fragments were digested with HindIII and EcoRI and ligated with pUC19 obtained after digestion of pUC19 with HindIII and EcoRI. An EcoRI fragment of pSRA2, containing the aadA gene conferring spectinomycin resistance, was inserted into the unique MfeI site within the 5’ slr2031 coding region. An XbaI fragment of pMS266, containing the accC1 gene conferring gentamicin resistance, was inserted into the unique SpeI site within the pmgA coding region. The resulting constructs were linearized after digestion with EcoRI and used to transform wild-type Synechocystis sp. PCC 6803 cells. Selection of transformants was carried out on solid medium BHEPES, pH 8.0, in the presence of 50 µg mL⁻¹ spectinomycin or in the presence of 20 µg mL⁻¹ gentamicin at 27 °C, under a moderate light intensity (~50 µE m⁻² s⁻¹). PCR analyses were carried out using the same sets of primers used for the cloning.
Isolation of Total RNA and RT-PCR Analyses

Total RNA was isolated from cells using the Mini-to-Midi RNA isolation kit (Invitrogen corp., Carlsbad, CA) followed by an RNase-free DNase treatment and repurification by using the same kit. The final concentration of the purified RNA preparations was 50 ng mL\(^{-1}\) in RNase free double-distilled water; the RNA samples were stored at -80°\(^{\circ}\)C until being used. RT-PCR reactions were carried out by using the One-Step RT-PCR kit (Qiagen Inc., Valencia, CA) for detecting the \(rnpB, spbA, sigC, sigE, sigI, ndhF4, ndhF3, ndhR, sbtA\) and \(slr2031\) transcripts. The reaction volume was 25 µL, and 4 ng µL\(^{-1}\) of the RNA preparation was used for each reaction. For detecting the other transcripts, 400 ng of total RNA was first converted to cDNA by a reverse transcription reaction using M-MLV reverse transcriptase (Promega, Madison, WI). The reaction volume was 20 µL for each sample. The resulting cDNA was diluted with 80 µL of TE buffer, and stored at –20 °C until being used. Two microliters of cDNA stock were used for each PCR reaction (final volume, 25 µL) using Hot-Start Taq polymerase (Qiagen Inc., Valencia, CA). RNasin (Promega, Madison, WI), an RNase inhibitor, was present in the one-step RT-PCR reactions and during the cDNA synthesis.

Transmission Electron Microscopy

Cells grown in the absence or in the presence of glucose for 24 h were harvested and immediately fixed in a 2.5 % glutaraldehyde solution prepared in a 0.1 M cacodylate buffer, pH 7.4, over night at 4°C. The fixed cells were washed in the 0.1 M cacodylate buffer for three times at room temperature, and subjected to the second fixation in a 1% osmium tetroxide solution prepared in the 0.1 M cacodylate buffer for 1.5 h at room
temperature. The cells were washed twice in the 0.1 M cacodylate buffer and then once in double distilled water. After incubation in a 2 % uranyl acetate solution for 2 h at room temperature, the cells were washed in the following concentration of ethanol: 50% (v/v), 70% (v/v), 90% (v/v), 95% (v/v) ethanol in water followed by two washes in 100% (v/v) ethanol. Final washes were performed three times in 100% ethanol (EM grade) and then three times in acetone at room temperature before infiltration. The washed cells were incubated in the presence of the following concentration of the resin Spurr (Spurr 1969) over night at room temperature: 50% (v/v) and 72 % (v/v) in acetone and twice in 100% (v/v) Spurr. The samples were then polymerized at 60°C over night. Thin sections (ca. 50-60 nm thickness) were stained with 2 % (v/v) uranyl acetate before examination under a JEM 1200 EXII transmission electron microscope (Joel USA Inc., Peabody, MA, USA).
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Fig 5.1: Time-course RT-PCR analysis of the *cpcA*, *nblA*, and *sbpA* transcripts in wild type and *slr1736* mutant grown at pH 7.0 and pH 8.0. Cell were grown in the absence of glucose (shown at 0 h) and in the presence of glucose for 4, 8, and 24 h, at 1% (v/v) CO₂, 32 °C at 50 µE m⁻² s⁻¹.
Fig 5.2: Time-course RT-PCR analysis of alternative sigma factor transcripts in wild type and slr1736 mutant grown at pH 7.0 and pH 8.0.
Cell were grown in the absence of glucose (shown at 0 h) and in the presence of glucose for 4, 8, and 24 h, at 1% (v/v) CO₂, 32 °C at 50 µE m⁻² s⁻¹.
Fig 5.3: Time-course RT-PCR analysis of transcripts for *sigA*, *sigG* and genes involved in Ci uptake and fixation in wild type and *slr1736* mutant grown at pH 7.0 and pH 8.0.

Cell were grown in the absence of glucose (shown at 0 h) and in the presence of glucose for 4, 8, and 24 h, at 1% CO$_2$ (v/v), 32 °C at 50 µE m$^{-2}$ s$^{-1}$. 
Fig 5.4. Thin-section electron micrographs of the wild-type cells grown (A) in the absence and (B) in the presence of glucose for 24 h
Cells were grown in medium BHEPES40, pH 7.0, 1% (v/v) CO₂, 32 °C, 50 µE m⁻² s⁻¹, at 32 °C. Cells in the mid-exponential growth phase (A) were transferred to medium containing glucose and allowed to grow for 24 h (B).
Fig 5.5: Thin-section micrographs of the slr1736 mutants grown (A) in the absence and (B) in the presence of glucose for 24 h.

Arrows in (B) show the novel, proteinaceous structure (see text). Cells were grown in medium BHEPES40, pH 7.0, 1% (v/v) CO₂, 32 °C, 50 µE m⁻² s⁻¹, at 32 °C. Cells in the mid-exponential growth phase (A) were transferred to medium containing glucose and allowed to grow for 24 h (B).
Fig 5.6: Time-course RT-PCR analyses of the *slr2031* and various CCM gene transcripts in wild type and *slr1736* mutant grown at pH 7.0 and pH 8.0, 1% (v/v) CO₂, 32 °C at 50 µE m⁻² s⁻¹. Cell were grown in the absence of glucose (shown at 0 h) and in the presence of glucose for 4, 8, and 24 h.

<table>
<thead>
<tr>
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<th>pH 8.0</th>
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<td><em>slr1736</em></td>
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<tr>
<td>24</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
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*sigB, sigC, sigD, sigE, sigH, sigI, mpB*
Fig 5.7: PCR analysis of the genomic DNAs extracted from the *slr2031::aadA* and *pmgA::accC1* mutants.

Reactions were carried out using (A) the primer set slr2031-F and slr2031-R, and (B) the primer set pmgA-F and pmgA-R (see Materials and Methods). Left and right lanes in each panel shows PCR products amplified from the wild type (WT) and mutant genomic DNAs.
Fig 5.8: Cultures of wild type, *slr1736, slr2031, and pmgA* mutants of *Synechocystis* sp. PCC 6803 grown in the presence of glucose (A) at pH 8.0 and (B) at pH 7.0. Cell were grown at 1% (v/v) CO₂, 32 °C at 50 µE m⁻² s⁻¹.
Fig 5.9: Summary and a model of the non-antioxidant role of $\alpha$-tocopherol in *Synechocystis* sp. PCC 6803

A Rsb-like regulatory pathway, which involves PmgA, appears to exist in *Synechocystis* sp. PCC 6803 and possibly in other cyanobacteria (see text). In this model, $\alpha$-tocopherol either directly or indirectly interacts with the Rsb-like pathway, possibly via the function of PmgA. Through such interaction, it is hypothesized that $\alpha$-tocopherol affects the transcriptional regulation of some metabolic genes, which may be required for photomixotrophic growth of this organism.
Chapter 6

Comparative Genome Analyses and Genetic Studies of the Plastoquinone Biosynthesis Pathway in Cyanobacteria

Publication:

Yumiko Sakuragi and Donald A. Bryant, manuscript in preparation
ABSTRACT

The biosynthetic pathway of plastoquinone-9 (PQ-9) was studied by means of comparative genome analyses in 14 cyanobacteria. The presence of genes homologous to \textit{ubiA}, \textit{ubiC}, \textit{ubiD}, \textit{ubiE}, \textit{ubiH}, and \textit{ubiX} involved in ubiquinone-8 (UQ) biosynthesis in \textit{Escherichia coli} was detected in cyanobacterial genomes, which suggests that PQ-9 biosynthesis in cyanobacteria occurs from 4-hydroxybenzoate through a pathway similar to that for the UQ biosynthesis in proteobacteria. Attempted insertional inactivation of the \textit{ubiA}, \textit{ubiX}, and \textit{ubiH} homologs in \textit{Synechocystis} sp. PCC 6803 resulted in an incomplete segregation of the alleles, indicating that the products of these genes are essential for the survival of this cyanobacterium. Of the several homologs of \textit{ubiE} genes found in the cyanobacterial genomes, only two are conserved among 14 species. However, inactivation mutagenesis of these two conserved \textit{ubiE} homologs, \textit{sll1653} and \textit{sll0418}, as well as others, including \textit{sll0829}, \textit{slr1039}, \textit{sll0487}, \textit{slr0407}, and \textit{slr1618} had little effect on the cellular PQ-9 content. These results therefore demonstrate that these UbiE homologs are probably not involved in PQ-9 biosynthesis in cyanobacteria. After exhaustive database searching, the presence of \textit{ubiC} homologs, which are closely related to the plastid \textit{ycf21} gene, was detected in many cyanobacterial genomes. Taking these observations together, a hypothetical PQ-9 biosynthetic pathway in cyanobacteria is proposed.
<table>
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<th>Description</th>
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<td>plastoquinone-9</td>
</tr>
<tr>
<td>UQ-8</td>
<td>ubiquinone-8</td>
</tr>
<tr>
<td>MSBQ</td>
<td>2-methyl-6-solanyl-1,4-benzoquinone</td>
</tr>
<tr>
<td>MPBQ</td>
<td>2-methyl-6-phytyl-1,4-benzoquinone</td>
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INTRODUCTION

Plastoquinone (PQ-9, 2-3-dimethyl-5-solanyl-1,4-benzoquinone) is an isoprenoid quinone that is only synthesized in oxygenic phototrophs including cyanobacteria, algae, and higher plants (Threlfall and Whistance, 1971; Collins and Jones, 1981). It serves as a one-electron carrier (QA) as well as a two-electron carrier (QB) within Photosystem II and stabilizes the charge separation between P680⁺ and QB⁻ upon photoexcitation (Barry et al., 1994; Diner and Babcock, 1994; Bricker and Ghanotakis, 1996; Britt, 1996). PQ-9 also shuttles electrons from NADH dehydrogenase to cytochrome b₆f complex, therefore serving as an essential membrane-associated, lipid-soluble electron carrier both in photosynthetic and respiratory electron transport chains.

Studies of PQ-9 biosynthesis have been carried out since the 1960’s in higher plants by using isotopic tracers and direct enzyme activity assays (see a review by Threlfall and Whistance, 1970) as well as by genetic methods (Collakova and DellaPenna, 2001; Shintani et al., 2002; Cheng et al., 2003). The combined results have shown that PQ is synthesized from homogentisate after two enzymatic reactions: condensation of solanyl diphosphate and homogentisate by a solanyl transferase resulting in 2-methyl-6-solanyl-1,4-benzoquinone (MSBQ), and a methylation reaction at the C₃ position of the benzoquinoid ring system by MSBQ methyltransferase. This MSBQ methyltransferase is also responsible for methylation of 2-methyl-6-phytanyl-1,4-benzoquinone (MPBQ) in α-tocopherol biosynthesis, and therefore it is a bifunctional enzyme in higher plants (Shintani et al., 2002; Cheng et al., 2003). Given the frequent similarity of biochemical pathways in plants and cyanobacteria, it might be assumed that
the same pathway functions in cyanobacteria. However, recent studies (Dänhardt et al., 2002; Cheng et al., 2003) showed that neither the synthesis of homogentisate nor the presence of MPBQ methyltransferase activity is required for PQ-9 biosynthesis in the cyanobacterium *Synechocystis* sp. PCC 6803. These results suggested that PQ-9 biosynthesis in cyanobacteria is different from that in higher plants and that the PQ-9 biosynthetic pathways convergently evolved in cyanobacteria and higher plants (Cheng et al., 2003).

How is PQ synthesized in cyanobacteria? A preliminary inspection of the whole genome sequence of *Synechocystis* sp. PCC 6803 has shown the presence of ORFs that show sequence similarity to enzymes responsible for ubiquinone-8 (UQ-8, 2,3-dimethoxy-5-methyl-6-octaprenyl-1,4-benzoquinone) biosynthesis. UQ-8 and PQ-9 are both members of the alkyl-substituted benzoquinone family and contain a 1,4-benzoquinoid ring with a polyprenyl side chain (45 carbon units for PQ-9 and 40 carbon units for UQ-8) at the C₆ position of the benzoquinoid ring system. UQ-9 biosynthesis occurs as follows (Fig 6.1, see a review by Meganathan, 2001, and references therein). The first committed step of the pathway is the aromatization of chorismate to 4-hydroxybenzoate (4-HBA), which is catalyzed by chorismate-pyruvate lyase (UbiC). Prenylation by 4-HBA octaprenyltransferase (UbiA) and decarboxylation by 3-octaprenyl-4-HBA decarboxylase (UbiD and UbiX) results in the synthesis of 2-octaprenyl phenol, which is converted to UQ after three hydroxylation reactions that alternate with three methylation reactions. A hydroxyl group is first introduced by 2-octaprenyl-phenol monooxygenase (UbiB) (at the position *ortho* to the C₁ OH), which is
subsequently methylated by an $O$-methyltransferase (UbiG) resulting in 2-methoxy-6-octaprenyl phenol. Secondly a hydroxyl group is introduced by a monooxygenase (UbiH) at the position $para$ to the C$_1$ hydroxyl group, resulting in the synthesis of 2-methoxy-6-octaprenyl-1,4-benzoquinol. UbiE, a $C$-methyltransferase, delivers a methyl group to a position $ortho$ to the C$_6$ prenyl side chain, resulting in the synthesis of 2-methoxy-5-methyl-6-octaprenyl-1,4-benzoquinol. Further hydroxylation by a monooxygenase (UbiF) and methylation by UbiG at the C$_3$ position of the ring system results in UQ-8. In the structure of PQ-9, the two methoxy groups are replaced by two methyl groups (see Fig 6.1), and no additional methyl group is present at the ring position $ortho$ to the prenyl side chain. Therefore, it is possible that the PQ-9 biosynthesis occurs by a pathway that is similar to the UQ-8 biosynthesis pathway in proteobacteria, at least in that part of the pathway that leads to the formation of the prenyl benzoquinone.

To investigate the PQ-9 biosynthesis pathway in cyanobacteria, comparative genome analyses using 14 cyanobacterial genome sequences were performed in combination with a reverse genetic approach. The results suggest that the PQ-9 biosynthesis occurs by a pathway similar to that for the UQ-8 pathway in proteobacteria.
RESULTS

Comparative Genome Analyses

As of April 26, 2004, largely completed genomic sequences of 14 cyanobacteria are available: eight complete sequences exist for Synechocystis sp. PCC 6803 (Kaneko et al., 1996), Nostoc sp. PCC 7120 (Kaneko et al., 2001), Thermosynechococcus elongatus BP-1 (Nakamura et al., 2002a,b), Gloeobacter violaceus (Nakamura et al., 2003), Prochlorococcus marinus MED4 and Prochlorococcus marinus MIT9313 (Rocap et al., 2003), Prochlorococcus marinus SS120 (Dufresne et al., 2003), Synechococcus sp. WH8102 (Palenik et al., 2003); and incomplete sequences exist for Synechococcus sp. PCC 7002 (J. Marquardt, T. Li, J. Zhao, and D. A. Bryant, unpublished), Nostoc punctiforme (Gene Bank accession number: NZ_AAAY00000000), Trichodesmium erythraeum (Gene Bank accession number: NZ_AABK00000000), Synechococcus elongatus PCC 7942 (Gene Bank accession number: NZ_AADZ01000001), Anabaena variabilis ATCC 29413 (Gene Bank accession number: NZ_AAEA01000001), and Crocosphaera watsonii WH8502 (Gene Bank accession number: NZ_AADV01000004). Database searches using the E. coli UbiA, UbiB, UbiC, UbiD, UbiE, UbiF, UbiG, UbiH, and UbiX protein sequences against these genomes revealed the presence of homologs for UbiA, UbiD, UbiX, UbiE, and UbiH. Similarities between the sequences of E. coli Ubi proteins and the cyanobacterial homologs were about 50% for UbiA (Table 6.1), 30-50% for UbiH (Table 6.2), 60% for UbiD (Table 6.3), and 50% for UbiX (Table 6.4). Multiple UbiE/UbiG homologues were detected, and the number varied depending on the species (Table 6.5). The observed conservation of these Ubi proteins among
cyanobacteria and between cyanobacteria and *E. coli* suggests that the part of the UQ-8 biosynthesis pathway involving prenylation, decarboxylation and the introduction of a hydroxyl group is probably functioning in cyanobacteria. The only exception to this is *P. marinus* MED4, in which no UbiD and UbiX homologs were detected. Reverse-phase HPLC analyses of the whole cell extracts confirmed that *P. marinus* MED4 synthesizes about two molecules of PQ-9 per 100 chlorophyll *a* molecules. Similar value was obtained for the whole cell extracts from *Synechocystis* sp. PCC 6803. Therefore, it is safely concluded that *P. marinus* MED4 synthesizes PQ-9 and it is suggested that the carboxylation reaction is probably catalyzed by yet unknown enzymes.

UQ-8 biosynthesis in *E. coli* involves eight enzymes and two of these were absent in cyanobacteria: UbiB, and UbiF. The absence of UbiB and UbiF, the monooxygenases that introduce hydroxyl groups into ring system (which are subsequently methylated by UbiG), is consistent with the absence of the methoxy substituents in PQ-9. In combination with the results described above, it was hypothesized that biosynthesis of PQ-9 in cyanobacteria occurs as shown in Fig. 6.2. In this pathway, 4-hydroxybenzoate is converted by UbiA to 2-solanyl-4-hydroxybenzoate, which is decarboxylated by UbiD/UbiX giving rise to 2-solanyl phenol. It is proposed that 2-solanyl phenol is first hydroxylated by UbiH, followed by two methylation reactions performed by one or more of the UbiE homologs, although it is entirely possible that one or both of the methylation reactions occurs before hydroxylation, as is the case for O-methylation by UbiG, which precedes hydroxylation by UbiH during UQ-8 synthesis (see Meganathan, 2001, and Fig 6.1).
UbiC, chorismate lyase, is responsible for the first committed step in the pathway (Fig 6.1). The *ubiC* gene in some β-proteobacteria and γ-proteobacteria, including *Neisseria meningitidis* Z2491, *Pseudomonas aeruginosa* PA01, *Salmonella enterica* subsp. enterica serovar Typhi, *Shigella flexneri* str. 301 setotype 2a, *Yersinia pestis* C092 and *E. coli*, is located immediately upstream of the *ubiA* gene based on genome analyses performed on these organisms. In cyanobacteria, no *ubiC* homolog was found in the vicinity of the *ubiA* gene, and no obvious *ubiC* homologs were found in their genomes. These observations appeared to indicate that homologs of this gene were absent in the cyanobacteria, and that synthesis of 4-hydroxybenzoate occurs through an alternative pathway (see discussion). After exhaustive database searching, however, ORFs that showed very weak similarity to the UbiC sequences of *E. coli* (< 30%) were detected (Table 6.6). These ORFs were highly conserved among cyanobacteria (50–80% similarity) and showed amino acid sequence similarity to Ycf21 proteins, which are also found in the plastid genomes of eukaryotic phototrophs including *Porphyra purpurea* and *Cyanophora paradoxa*. Fig 6.3 shows an alignment of sequences of the UbiC protein in *E. coli* and the Yfc21 homologs in the cyanobacteria and eukaryotic phototrophs. In the alignment, a glutamate residue at the position 155 (UbiC) was found to be conserved among the Ycf21 homologs in both the cyanobacteria and eukaryotic phototrophs. This residue constitutes a portion of the catalytic site and is hydrogen-bonded to the hydroxyl oxygen of 4-hydroxybenzoate as previously shown in the 1.4-Å resolution X-ray crystal structure of the UbiC protein (Gallagher et al., 2001). An arginine residue at the position 79, which has also been shown to constitute a portion of the active site, was conserved among many cyanobacteria, although not all. Therefore, it is possible that the Ycf21
homologs catalyze the conversion of chorismate to 4-hydroxybenzoate in the PQ-9 biosynthesis in cyanobacteria.

*Mutagenesis of ubiA, ubiX, and ubiH*

An attempt was made to inactivate the ORFs *slr0926* (*ubiA*), *slr1099* (*ubiX*), and *slr1300* (*ubiH*) in *Synechocystis* sp. PCC 6803 by insertion of the *aphII* gene conferring kanamycin resistance. These attempts, however, failed to lead to homozygous mutants and resulted in incomplete segregation of the alleles under photoautotrophic conditions even under selective conditions in the presence of 50 µg mL⁻¹ kanamycin (data not shown). Addition of glucose to growth medium, which provides electrons to the electron transport chains through NADH (thereby bypassing Photosystem II), likewise did not lead to complete segregation. Addition of various quinoid compounds to the growth medium either to inhibit the Photosystem II activity (atrazine) or to compensate the loss of PQ-9 (2,6-dimethyl-1,4-benzoquinone, PhyQ, UQ-0, UQ-1, and UQ-2) did not lead to complete segregation (data not shown). Merodiploid mutants were unstable both under photoautotrophic and photomixotrophic conditions under illumination at a normal light intensity (20 - 50 µE m⁻² s⁻¹), as no further growth was observed after several transfers on solid media. These results indicates that the *ubiA*, *ubiX*, and *ubiH* genes are indispensable for the survival of *Synechocystis* sp. PCC 6803, which is consistent with the anticipated essential role of plastoquinone in cyanobacteria.
Phylogenetic Reconstruction with Ubi proteins of Cyanobacteria and Proteobacteria

Evolutionary relationships between the Ubi homologues of cyanobacteria and proteobacteria (*E. coli*, *Rhodopseudomonas palustris*, *Rhodospirillum rubrum* and *Rhodobacter sphaeroides*) were analyzed by constructing phylogenetic trees based on deduced amino acid sequences and by comparing the results to the phylogenetic tree based on 16S ribosomal RNA (rRNA) (Fig 6.4). 16S rRNA is universally present and highly conserved among prokaryotes and thus is often used as a molecular marker to reflect the evolutionary clock (Woese, 1987). Phylogenetic trees based on the nucleotide sequence of 16S rRNA showed that the cyanobacteria and proteobacteria formed independent groups, which is consistent with previous results (Woese, 1987). Within the cyanobacteria, two major clades were observed: clade A consisted of *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002, *Nostoc* sp. PCC 7120, *N. punctiforme*, and *T. erythraeum*, and clade B consisted of *Synechococcus* sp. WH8102 and the three *Prochlorococcus* species (Fig 6.4). Phylogenetic trees based on the amino acid sequences of UbiA, UbiD, and UbiX showed similar branching orders and topologies to the tree for 16S rRNA. The trees were supported by high bootstrap values (Fig 6.4), suggesting that the *ubiA*, *ubiD*, and *ubiX* genes have evolved through vertical heritage from the last common ancestor of both cyanobacteria and the proteobacteria. The topology of the tree based on the UbiH sequences appeared different, however. Within the group formed by all cyanobacteria, the proteobacteria formed a cluster with clade A, which may indicate the lateral transfer of the *ubiH* gene between these two groups of organisms. However, the bootstrap value assigned to the branch connecting a node where cyanobacteria in clade A and clade B bifurcate and a node where the proteobacteria deviate from the
cyanobacteria in clade A is only 52, which only poorly supports the likeliness of this branching order. Alternatively, it is possible that the out-group used to construct the tree (Mlr2092, a putative UbiH homologue in *Mesorhizobium loti*) may be distorting the topology and thereby gives rise to an artifact. This happens if the sequence of the out-group is more closely related to the cyanobacterial sequences than to the proteobacterial sequences. As shown in Table 6.2, the amino acid sequence of Mlr2092 that was used as an out-group is equally distantly related to any of the UbiH sequences analyzed. The replacement of Mlr2092 by another protein sequence, NahG (salicylate hydroxylase in *Pseudomonas putida*) or Bsu1050 (a putative monooxygenase in *Bacillus subtilis*), did not alter the topology of the tree. Therefore, it is possible that the proteobacteria form a group within the cyanobacteria and a lateral exchange of *ubiH* took place between proteobacteria and the cyanobacteria in clade A. This evolutionarily close relationship of UbiH homologs between the two groups of organisms provides further support for the involvement of these proteins in the PQ-9 biosynthesis in cyanobacteria.

**UbiE**

Database search of the genomes of cyanobacteria showed the presence of multiple UbiE homologs (Table 6.5, Fig 6.5). There are two ORFs that are conserved in all 14 cyanobacteria: these are homologs of Sll1653 and Sll0418 of *Synechocystis* sp. PCC 6803. Note that the incomplete genome sequence of *N. punctiforme* only contained a homolog of Slr0089, which encodes γ-tocopherol methyltransferase. Whether a homolog of Sll0418 is present in this organism is yet to be determined. Homologs of Sll0829 and Sll0487 are conserved in all cyanobacteria except *P. marinus* MED4 and *P. marinus*
SS120. Slr1618 and Slr0407 seem to be specific to *Synechocystis* sp. PCC 6803 and *C. watsonii*, or *Synechocystis* sp. PCC 6803, *A. variabilis*, and *G. violaceus*, respectively (Table 6.5). Amino acid sequences of all these homologs contain the conserved S-adenosyl-L-methionine binding motif (data not shown). UbiE in *E. coli* is a bifunctional enzyme and is responsible for the C-methylation reactions in both UQ-8 and menaquinone biosyntheses (Lee et al., 1997). Phylogenetic analyses conducted on the UbiE homologues of cyanobacteria showed that Sll1653 forms a cluster with the *E. coli* UbiE (Fig 6.5). Furthermore, as described in Chapter 2 and Chapter 3 (also see Sakuragi et al., 2002), this ORF is responsible for transferring a methyl group to 2-prenyl-1,4-naphthoquinone in phylloquinone and menaquinone-4 biosyntheses in cyanobacteria. Therefore, it was expected that Sll1653 would be responsible for at least one of the methylation reactions in PQ-9 synthesis. Whole-cell extracts from the sll1653 mutant were analyzed by reverse-phase HPLC. PQ-9 eluted at 36 min using the current analytical system (see “Materials and Methods”, see also Chapter 2). Whereas the chemically synthesized 2-methyl-6-solanyl-1,4-benzoquinone (a kind gift from Prof. DellaPenna in the Michigan State University, see Shintani et al., 2000), a predicted intermediate in the PQ-9 synthesis, eluted at 33 min as shown in Fig 6.6A. The HPLC profile of the whole-cell solvent extracts from the sll1653 mutant showed a peak at 35.5 min. The absorption spectrum of the component eluting at this retention time showed a maximum at 256 nm (Fig 6.6B, solid line), which is characteristic of PQ-9 (Dunphy and Brodie, 1971) and is a few nanometers red shifted as compared to the absorption maximum observed for the MPBQ standard (Fig 6.6B, dotted line). The results therefore demonstrate that PQ-9 is present in the sll1653 mutant cells. The contents of PQ-9 were analyzed based on the
integrated peak area in the HPLC chromatograms and were expressed as relative to the cellular contents of chlorophyll a (Fig 6.7). The results showed that the PQ-9 content in the sll1653 mutant is comparable to that in wild type. Therefore, it was concluded that Sll1653 is not involved in the PQ-9 biosynthesis or that a redundant activity conferred by another methyltransferase(s) is present.

An in vitro study has previously demonstrated that recombinant Sll0418, MPBQ methyltransferase, can catalyze methylation of MSBQ and lead to the synthesis of PQ-9 (Shintani et al., 2002). Because this ORF is also highly conserved among the cyanobacteria (Table 6.5, Fig 6.5), it was predicted that Sll0418 is either responsible for transferring of one or two of the methyl groups in the PQ-9 biosynthesis or that it provides a redundant activity with Sll1653. Targeted inactivation and complete segregation of the mutated allele was performed and confirmed by PCR (see Chapter 3). The sll0418 mutant was, however, capable of synthesizing the wild-type level of PQ-9 as shown by HPLC analysis on the whole-cell lipid extracts (Fig 6.7).

Double and triple mutants of the UbiE homologs were constructed in Synechocystis sp. PCC 6803 in further attempt to identify methyltransferase(s) involved in the PQ-9 biosynthesis. Combinations of mutated loci are as follows: double mutations were constructed for sll1653 and sll0418, sll1653 and slr0407, sll1653 and sll0829, sll0418 and sll0829, sll0418 and slr0407, and sll0418 and slr0089; and triple mutations were constructed for of sll1653, sll0418 and sll0829; and sll1653, slr0407, and sll0829. Antibiotic resistance cartridges were used to insertionally inactivate these genes (Fig 6.8). The full segregation of the mutated alleles from the respective wild-type alleles was
analyzed by PCR as shown in Fig 6.9. PCR using the designed primers (Table 6.7) that are targeted to the *sll0829, sll01653, sll0418, slr0407, slr0089, slr1618* resulted in products of 0.84 kbp, 0.3 kbp, 0.92 kbp, 0.94 kbp, 1.2 kbp, and 1.8 kbp, respectively (Fig 6.9, lane 1), which are expected based on the restriction maps as shown in Fig 6.8. No product with corresponding wild-type size was detected for the mutants (Fig 6.9, lanes 2); instead products with larger sizes were detected in all cases. The difference between the mutants and the wild-type corresponds either to the size of antibiotic resistance cartridges or to the size of antibiotic resistance cartridges minus the deleted regions (Fig 6.8). Therefore, it was concluded that all the mutants are free of respective wild-type alleles and were homozygous in the allele for which the drug cartridges had been introduced.

Reverse-phase HPLC analyses on whole-cell lipid extracts showed that none of the double and triple mutants showed a significant difference from the wild type in the amount of PQ-9 accumulated in the cells (Fig 6.7). The fact that the *sll1653 sll0418* double mutant did not affect the PQ-9 content strongly indicates that Sll1653 and Sll0418, despite their conservation among all the cyanobacteria and their predicted functioning in PhyQ/MQ synthesis (or the demonstration of function *in vitro* PQ-9 synthesis), do not play major roles in the PQ-9 biosynthesis. This is further supported by the construction of the *sll1635 sll0418* double mutant, which also synthesized a wild-type level of PQ-9 (Fig 6.7). The possibility that another UbiE homolog is providing an additional activity to Sll01653 and/or Sll0418 was tested by analyzing the PQ-9 contents in the *sll0418 slr1618, sll0418 slr0407, sll0418 slr0829, sll1653 sll0829, and sll1653 slr0407* double mutants by reverse-phase HPLC. The results showed that all of the double
mutants contained essentially the wild-type level of PQ-9, which indicates that Slr0829, Slr0407, and Slr1618 are not involved in the PQ-9 biosynthesis. This is further supported by the result that the two triple mutants, sll1653 sll0418 sll0829 and sll1653 slr0407 sll0829, contained a wild-type level of the PQ-9 in the whole-cell solvent extracts. Participation of Slr0089 in the PQ synthesis was also ruled out as the sll0418 slr0089 double mutant synthesized the wild-type level of PQ-9. Slr1039 and Slr0487 are two hypothetical proteins with an $S$-adenosyl-L-methionine binding motif, and they are well conserved among most of the cyanobacteria (Fig 6.5, Table 6.5). Targeted inactivation of these ORFs, however, did not affect the PQ-9 biosynthesis. In combination, these results strongly suggest that the UbiE homologs do not play a major role in the PQ-9 biosynthesis in *Synechocystis* sp. PCC 6803.
DISCUSSION

Database searches of the whole genome sequences of 14 cyanobacteria (8 complete and 6 incomplete) revealed the presence of the UbiA, UbiC, UbiD, UbiE, UbiH, and UbiX homologs, which are proteins involved in the UQ-8 biosynthetic pathway in proteobacteria. Attempted insertional inactivation of the \textit{ubiA}, \textit{ubiH}, and \textit{ubiX} genes in \textit{Synechocystis} sp. PCC 6803 resulted in the generation of unstable merodiploids, which indicates that these genes are essential for the survival of this organism and which is consistent with the presumed essential role of PQ-9 in cyanobacteria. Based on these findings, it is proposed that the PQ biosynthesis occurs in cyanobacteria as shown in Fig 6.2. In this proposal, UbiA catalyzes the condensation of 4-hydroxybenzoate and solanyldiphosphate resulting in 3-solanyl-4-hydroxybenzoate, which is decarboxylated by UbiD/UbiX resulting in 2-solanyl phenol. Hydroxylation by UbiH is expected to take place at the C\textsubscript{4} position of the ring system, resulting in 2-solanyl-1,4-benzoquinol. Whether the two methylation reactions at the C\textsubscript{2} and C\textsubscript{3} position take place before or after the hydroxylation is an open question. These methylation reactions, however, are not catalyzed by the UbiE homologs (Sll1653, Sll0418, Sll0829, Slr0407, Sll0487, Sll1618 and Slr1039) in \textit{Synechocystis} sp. PCC 6803 as demonstrated by insertional mutagenesis and subsequent HPLC analyses. Phylogenetic analyses have indicated that the presence of the UbiA, UbiD, and UbiX homologs in cyanobacteria are the result of vertical heritage from the common ancestor of cyanobacteria and proteobacteria, suggesting a possible common evolutionary origin of the UQ-8 and PQ-9 biosyntheses. UbiE (Sll1653) is probably inherited from the common ancestor and evolved in cyanobacteria.
to be specific to the methylation reaction in the PhyQ/MQ biosynthesis, while another methyltransferase(s) has been recruited to function for the PQ-9 biosynthesis.

The methyltransferase(s) that function in the PQ-9 biosynthesis is/are still a mystery. The fact that mutants of 7 UbiE homologs contained wild-type levels of PQ-9 suggests i) that a SAM (S-adenosyl-L-methionine)-dependent methyltransferase(s) that is only very distantly related to UbiE is responsible for these reactions, or ii) that a SAM-independent methyltransferase(s) is responsible for the reactions. Of the few SAM-independent methyltransferases detected in the *Synechocystis* sp. PCC 6803 genome, none is conserved among cyanobacteria except for *slr0212*, which is annotated as encoding the methionine synthase and uses 5-methyltetrahydrofolate as the methyl donor ([EC 2.1.1.13], see http://www.chem.qmul.ac.uk/iubmb/enzyme/EC2/1/1/13.html). This ORF is, however, highly conserved among other bacteria and eukaryotes and is present in the genome as a single copy. Therefore, this gene product most likely functions as methionine synthase and is unlikely to be involved in the PQ biosynthesis. When the genome was searched for SAM-binding proteins, ORFs *sll1407*, *slr1115*, *slr0303*, *slr1071*, *sll1693*, *slr1815* were detected. Of these, *slr0303* is the only ORF that is conserved among all the cyanobacteria. The possible involvement of this hypothetical protein in PQ-9 biosynthesis needs to be tested.

The presence of conserved Ycf21 homologs in cyanobacteria, which are weakly similar to UbiC in *E. coli*, suggested that 4-hydroxybenzoate derives from chorismate by the action of chorismate-lyase activity possibly conferred by these Ycf21 homologs. This was thought to be a highly plausible hypothesis because catalytic residues of the UbiC
protein are largely conserved among the Ycf21 homologs in cyanobacteria. However, the poor amino acid sequence similarity between the Ycf21 homologs and UbiC proteins leaves a possibility that an alternative pathway exists that leads to the synthesis of 4-hydroxybenzoate from a compound other than chorismate. Genome analyses have revealed that many organisms that synthesize UQ lack UbiC, including higher plants, animals, and even some proteobacteria such as *Agrobacterium tumefaciens*, *Brucella melitensis*, *Rhodopseudomonas palustris*, *Chromobacterium violaceum*, and *Xanthomonas axonopodis*. There are three pathways that are known to lead to the synthesis of the intermediate 4-hydroxybenzoate in these organisms: i) the UbiC-dependent chorismate pathway in *E. coli* and some β- and γ-proteobacteria; ii) the 4-hydroxyphenyllactate pathway in rat (see review by Meganathan, 2001, and references therein); and iii) the aromatic amino acid pathway in higher plants (see a review by Threlfall and Whistance, 1971, and references therein). In UQ biosynthesis, 4-hydroxybenzoate is synthesized from the aromatic amino acid tyrosine or phenylalanine via *p*-coumarate (Loscher and Heide, 1994). It has also been reported that the cyanobacterium *Anacystis nidulans* converts the aromatic amino acid tyrosine to 4-hydroxybenzoate, and that this conversion may be carried out by thylakoid-bound enzyme complexes consisting of tyrosine ammonia-lyase and 4-hydroxybenzoate synthase (Löffelhardt, 1976). Therefore, it is plausible that a similar aromatic amino acid pathway may exist in other cyanobacteria and could provide 4-hydroxybenzoate for PQ-9 biosynthesis as proposed in Fig 6.10.
Lastly, the fact that the sll0418 mutation did not affect the cellular PQ content in *Synechocystis* sp. PCC 6803 indicates that Sll0418 is only involved in α-tocopherol biosynthesis. However, this ORF is conserved in nearly all cyanobacteria whose genome sequence is so far available, including those that are predicted not to be capable of α-tocopherol synthesis (*Synechococcus* sp. WH 8102 and three *Prochlorococcus* species, see Chapter 5). What is the function of the Sll0418 homologs in these cyanobacteria? Is it possible that the Sll0418 homologs are involved in PQ biosynthesis in these cyanobacteria, given that the recombinant Sll0418 can catalyze the methylation of MSBQ and generate PQ-9. Genetic analyses of the role of Sll0418 needs to be performed in these other organisms.
SUMMARY

The PQ-9 biosynthesis pathway was studied by means of comparative genome analyses in 14 cyanobacteria. The presence of UbiA, UbiD, UbiE, UbiH, and UbiX homologs, which are required for UQ-8 biosynthesis in *E. coli*, was detected in the cyanobacteria. The presence of Ycf21 homologs, which show weak similarity to UbiC, was detected after exhaustive database searching in cyanobacteria. The results suggest that PQ-9 synthesis occurs similarly to UQ-8 biosynthesis pathway. Targeted inactivation of six UbiE homologs (methyltransferases), however, did not affect PQ-9 biosynthesis in *Synechocystis* sp. PCC 6803. This suggested that enzymes that are not evolutionarily related to UbiE in *E. coli* catalyze the methylation reactions in PQ-9 biosynthesis in cyanobacteria.
MATERIALS AND METHODS

Growth Conditions

The wild-type strain used for transformation and further analysis was a glucose-tolerant strain of *Synechocystis sp*. PCC 6803 (Williams, 1988). Medium BHEPES, pH 8.0, was used for selection, maintenance, and growth measurements of wild type and mutants. This medium is prepared by supplementing BG-11 medium (Stainer et al., 1971) with 4.6 mM of HEPES [4-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)]-KOH and 18 mg L^{-1} ferric ammonium citrate. The wild-type and *ubiA*, *ubiH*, and *ubiX* mutant strains were grown on solid medium BHEPES containing the following additions: 5 mM glucose alone; 5 mM glucose and various concentrations (5 µM, 50 µM, 500 µM, 1 mM, and 10 mM) of ubiquinone-0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone), ubiquinone-1 (2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone), ubiquinone-2 (2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone), or phylloquinone; 5 mM glucose and 5 µM atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine); or 5 mM glucose, 5 µM ubiquinone-2, and 20 µM 2,6-di-<i>O</i>-methyl-<i>ß</i>-cyclodextrin. The cells on these solid media were grown at room temperature at moderate light intensity (ca. 50 µE m^{-2} s^{-1}) or at low light intensity (ca. 5 µE m^{-2} s^{-1}).

Constructions of Single and Double Methyltransferase Mutants

DNA fragments containing the *sll0829*, *sll0418*, *slr0407*, *slr1618*, *slr1039*, and *slr0089* genes were amplified from isolated genomic DNA by PCR using a set of forward and reverse primers (see Table 6.7 for sequences and Fig 6.8 for positions). The
constructions of the insertional inactivation of these alleles are described in Table 6.8.

The \textit{aacC1} gene, conferring gentamicin resistance, derived from the pMS266 plasmid (Becker et al., 1995) and the \textit{aadA} gene, conferring spectinomycin resistance, derived from the pSRA2 plasmid (Frigaard et al., 2004) were inserted into the selected site within these gene coding regions (see Table 6.8 and Fig 6.8). The resulting p829Gm and p1039Sp plasmids were linearized and used to transform the wild-type \textit{Synechocystis} sp. PCC 6803 to obtain the homozygous \textit{sll0829::accC1} and \textit{slr1039::aadA} mutants.

Genomic DNA isolated from the \textit{sll0418::aphII} mutant (Chapter 4) and the \textit{sll1653::aphII} mutant (Chapter 3) were used to transform the homozygous \textit{sll0829::accC1} mutant to generate the \textit{sll0418::aphII sll0829::accC1} and \textit{sll1653::aphII sll0829::accC1} double mutants. The linearized p418Sp and p407Sp were used to transform the homozygous \textit{sll1653::aphII sll0829::accC1} double mutant to generate the \textit{sll1653::aphII sll0418::aadA sll0829::accC1} and \textit{sll1653::aphII slr0407::aadA sll0829::accC1} triple mutants. The linearized p418Sp was used to transform the homozygous \textit{sll1653::aphII} mutant to generate the \textit{sll0418::aadA sll1653::aphII} double mutant. The linearized p1618Sp, p407Sp, and p89Gm were used to transform the homozygous \textit{sll0418::aphII} mutant to generate the \textit{sll0418::aadA 1618::aadA}, \textit{sll0418::aphII slr0407::aadA}, and \textit{sll0418::aphII slr0089::accC1} double mutants. A homozygous \textit{sll0487::aphII} mutant was constructed in a previous study (W. M. Schluchter, G. Shen, and D. A. Bryant, unpublished results). Complete segregation of the each mutated alleles from the corresponding wild-type allele was confirmed by PCR. Primer used for the PCR analyses are summarized in Table 6.7.
Quinone and Chlorophyll Analysis

Cells were harvested from photoautotrophically grown cultures and recovered in a tight pellet. Pigments and quinones were extracted with 400 µL of cold acetone:methanol (7:2 v/v) after brief ultrasonication. After centrifugation and filtration through a PTFE filter membrane with a 0.2-µm pore size (Whatman International Ltd., Maldstone, UK), the extract in the organic phase were directly injected into an Agilent Technology 1100 series HPLC system (Agilent Technology, Palo Alto, CA, USA) equipped with SUPELCO (Sigma-Aldrich Corp., St. Louis, MO, USA) Discovery® C18 column (25 cm x 4.6 mm, 5 µm). Analyses were carried out using the following protocol: 80%A/20%B from 0 to 10 min, a linear change to 20%A/80%B in 40 min, and 20%A/80%B for 5 min, where solvent A and solvent B are 100% methanol and 100% isopropanol, respectively. The flow rate was 0.75 ml min⁻¹. Detection of eluates was performed with a diode array detector (Agilent 1100 series). The chlorophyll a, phylloquinone, and plastoquinone contents of each sample were determined based on integrated peak areas and their molar absorption coefficients, which are 17.4 mM⁻¹ cm⁻¹ at 618 nm, 18.9 mM⁻¹ cm⁻¹ at 270 nm (Dunphy and Brodie, 1971), and 15.2 mM⁻¹ cm⁻¹ at 254 nm (Crane and Dilley, 1963), respectively. The absorption coefficient of chlorophyll a at 618 nm was calculated from the ratio of the absorption peaks at 618 nm and 666 nm in methanol-isopropanol (6:4, v/v) and from its absorption coefficient at 666 nm in methanol (MacKinney, 1941).
REFERENCES


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* Incomplete genome sequences.
Table 6.2: Amino acid sequence similarities between UbiH homologs in 14 cyanobacteria and *E. coli* (expressed as %)

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Table 6.3: Amino acid sequence similarities between UbiD homologs in 14 cyanobacteria and *E. coli* (expressed as %)

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## Table 6.4: Amino acid sequence similarities between UbiX homologs in 14 cyanobacteria and *E. coli* (expressed as %)

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<td>-</td>
<td>78</td>
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<tr>
<td><em>G. violaceus</em> 7421</td>
<td>Gll0529</td>
<td>78</td>
<td>77</td>
<td>80</td>
<td>80</td>
<td>-</td>
<td>78</td>
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<td>68</td>
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<tr>
<td><em>E. coli</em></td>
<td>UbiX</td>
<td>52</td>
<td>52</td>
<td>50</td>
<td>52</td>
<td>-</td>
<td>54</td>
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<td>-</td>
<td>50</td>
<td>55</td>
<td>52</td>
<td>100</td>
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6803, *Synechocystis* sp. PCC 6803; 7002, *Synechococcus* sp. PCC 7002; Nost, *Nostoc* sp. PCC 7120; Npun, *Nostoc punctiforme*; Tery, *Trichodesmium erythraeum*; Telon, *Tetraselmis* sp. PCC 8102, *Synechococcus* sp. WH8102; Selo, *Synechococcus* elongatus PCC 7942; Avar, *Anabaena variabilis* ATCC 29413; Cwat, *Crocosphaera watsonii* WH8501; *Prochlorococcus marinus* MED4; MIT, *Prochlorococcus marinus* MIT9313; S120, *Prochlorococcus marinus* SS120; Gloe, *Gloeobacter violaceus* PCC 7421; Eco, *E. coli*. Pairwise alignments were generated with BLOSUM30 using ClustalW. * Incomplete genome sequences. The N-terminal 107 and 62 residues were truncated in the tery2980 and Avar334101 sequences were 107 and 62 residues as compared to the Slr1099 sequence.
Table 6.5: List of the UbiE homologs in 14 cyanobacteria

<table>
<thead>
<tr>
<th></th>
<th>Sll1653</th>
<th>Sll0418</th>
<th>Sll0829</th>
<th>Slr0407</th>
<th>Slr1618</th>
<th>Slr1039</th>
<th>Sll0487</th>
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<tbody>
<tr>
<td>Synechocystis 6803</td>
<td>89-c70244</td>
<td>80-162268</td>
<td>89-46731</td>
<td>-</td>
<td>-</td>
<td>aAAD26590</td>
<td>aAAC14722</td>
</tr>
<tr>
<td>Synechococcus 7002 *</td>
<td>Alr5252</td>
<td>All2121</td>
<td>All3750</td>
<td>-</td>
<td>-</td>
<td>All3016</td>
<td>All0012</td>
</tr>
<tr>
<td>N. punctiforme *</td>
<td>Npun3758</td>
<td>-</td>
<td>Npun0871</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Npun5885</td>
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<tr>
<td>T. erythraeum *</td>
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<td>Tery1223</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>T. elongatus BP-1</td>
<td>Tll2373</td>
<td>Tll11726</td>
<td>Tll1604</td>
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<tr>
<td>Synechococcus 8102</td>
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<td>Synw2141</td>
<td>Synw1749</td>
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<td>Synw1148</td>
<td>Symw0487</td>
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<tr>
<td>S. elongatus 7942*</td>
<td>Selo20383</td>
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<td>Selo232101</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Selo213301</td>
</tr>
<tr>
<td>A. variabilis 29413*</td>
<td>Avar287101</td>
<td>Avar184601</td>
<td>Avar395201</td>
<td>Avar025741</td>
<td>-</td>
<td>Avar020095</td>
<td>-</td>
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<tr>
<td>C. watsonii 8501*</td>
<td>Cwat179201</td>
<td>Cwat194101</td>
<td>Cwat026375</td>
<td>-</td>
<td>Cwat020748</td>
<td>-</td>
<td>Cwat023036</td>
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<tr>
<td>P. marinus MED4</td>
<td>Pmm0431</td>
<td>Pmm1505</td>
<td>-</td>
<td>-</td>
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<tr>
<td>P. marinus MIT</td>
<td>Pmt0276</td>
<td>Pmt1785</td>
<td>Pmt1242</td>
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<td>Pmt0792</td>
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<td>P. marinus SSI120</td>
<td>Pro0427</td>
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<td>-</td>
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<td>-</td>
<td>Pro0816</td>
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<tr>
<td>G. violaceus 7421</td>
<td>Gll0127</td>
<td>Gllr3039</td>
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<td>-</td>
<td>Gllr3970</td>
<td>Gll1180</td>
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</tbody>
</table>

*Incomplete genome sequences. Locus tags were used to identify the proteins. For proteins in Synechococcus sp. PCC 7002, proteins are designated with a contig number and translation initiation site separated with a hyphen, or  * Gene Bank accession numbers are provided if available.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Locus tag</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Synechocystis</em> sp. PCC 6803</td>
<td>Sll1797</td>
</tr>
<tr>
<td><em>Crocosphaera watsonii</em> WH 8501</td>
<td>Cwat020398</td>
</tr>
<tr>
<td><em>Nostoc</em> sp. PCC 7120</td>
<td>All0938</td>
</tr>
<tr>
<td><em>Nostoc punctiforme</em></td>
<td>Npun5760</td>
</tr>
<tr>
<td><em>Anabaena variabilis</em> ATCC 29413</td>
<td>Avar303301</td>
</tr>
<tr>
<td><em>Trichodesmium erythraeum</em></td>
<td>Tery3876</td>
</tr>
<tr>
<td><em>Thermosynechococcus elongatus</em></td>
<td>Tll1562</td>
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<tr>
<td><em>Synechococcus elongatus</em> sp. PCC 7942</td>
<td>Selo236201</td>
</tr>
<tr>
<td><em>Synechococcus</em> sp. WH8102</td>
<td>Synw2384</td>
</tr>
<tr>
<td><em>Prochlorococcus marinus</em> sp. MIT9313</td>
<td>Pmt2179</td>
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<tr>
<td><em>Prochlorococcus marinus</em> sp. MED4</td>
<td>Pmm1676</td>
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<tr>
<td><em>Prochlorococcus marinus</em> sp. SS120</td>
<td>Pro1837</td>
</tr>
<tr>
<td><em>Porphyra purpurea</em> (red algae)</td>
<td>PopuCp195</td>
</tr>
<tr>
<td><em>Cyanophora paradoxa</em> (a photoautotrophic protist)</td>
<td>CypaCp147</td>
</tr>
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</table>
### Table 6.7: Sequences of primers used for PCR analyses

Lower case letters indicate heterologous nucleotides that are introduced in order to engineer restriction enzyme cleavage sites, which are indicated with underlines.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>sll0829-F</td>
<td>5'-GGTCCGCCAAGATTTAGCAGTAAG-3’</td>
</tr>
<tr>
<td>sll0829-R</td>
<td>5'-AGATTGGGACAGTAATCGAAGGC-3’</td>
</tr>
<tr>
<td>sll0418-F1</td>
<td>5'-'CGACTGAGGAAACGGTTGAAATTCCCGCACC-3’ (EcoRI cleavage site).</td>
</tr>
<tr>
<td></td>
<td>Used for cloning the sll0418 coding region</td>
</tr>
<tr>
<td>sll0418-R1</td>
<td>5'-CTTACGTGGCAATTAAAGCTTGAGTGCGT-3’</td>
</tr>
<tr>
<td></td>
<td>Used for cloning the sll0418 coding region</td>
</tr>
<tr>
<td>sll0418-F2</td>
<td>5'-ATGCCCGAGTATTTGCTTGCC-3’</td>
</tr>
<tr>
<td></td>
<td>Used for verification of segregation</td>
</tr>
<tr>
<td>sll0418-R2</td>
<td>5'-GCACTGCTTGGAAACATACCGAAG-3’</td>
</tr>
<tr>
<td></td>
<td>Used for verification of segregation</td>
</tr>
<tr>
<td>slr0089-F</td>
<td>5'-TCTACCGGAAATTTGCAACTACCA-3’</td>
</tr>
<tr>
<td>slr0089-R</td>
<td>5'-CCTAGGAGATTTGGGACTTCAA</td>
</tr>
<tr>
<td>slr0407-F</td>
<td>5'-CTCTGGAAACATTTGAAAGCTTTATCCG-3’ (HindIII cleavage site)</td>
</tr>
<tr>
<td>slr0407-R</td>
<td>5'-TACGTTACTTATCCCGGCAATTCCCTCT-3’ (EcoRI cleavage site)</td>
</tr>
<tr>
<td>slr1618-F</td>
<td>5'-TCTATTCTGGATATGCTGGCACA-3’</td>
</tr>
<tr>
<td>slr1618-R</td>
<td>5'-TTGTTTGGCGCTTGCTTGC-3’</td>
</tr>
<tr>
<td>slr1039-F</td>
<td>5'-GGGTGTATTACAATAACAGG-3’</td>
</tr>
<tr>
<td>slr1039-R</td>
<td>5'-ACTGGTGGGAGGTGGTGCC-3’</td>
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</table>
Table 6.8: Insertional inactivation constructs of the methyltransferase mutants in *Synechocystis* sp. PCC 6803.

*Hind*III (blunt) indicates that the 3’-overhand generated after digestion with *Hind*III is filled by Klenow to yield a blunt end.

<table>
<thead>
<tr>
<th>Target allele (Construct)</th>
<th>Restriction digestions</th>
<th>Site of insertion in pUC19</th>
<th>Insertion of drug cartridge</th>
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<tr>
<td><em>sll</em>0829 (p829Gm)</td>
<td><em>Hind</em>III, <em>Hinc</em>II</td>
<td><em>Hind</em>III, <em>Hinc</em>II</td>
<td><em>aacC1</em> between the <em>Bst</em>XI sites</td>
</tr>
<tr>
<td><em>sll</em>0418 (p418Sp)</td>
<td><em>Eco</em>RI, <em>Hind</em>III</td>
<td><em>Eco</em>RI, <em>Hind</em>III</td>
<td><em>aadA</em> at the unique <em>Sma</em>I site</td>
</tr>
<tr>
<td><em>slr</em>1618 (p1618Sp)</td>
<td><em>Hind</em>III, <em>Hinc</em>II</td>
<td><em>Hind</em>III, <em>Hinc</em>II</td>
<td><em>aadA</em> at the unique <em>Nhe</em>I site</td>
</tr>
<tr>
<td><em>slr</em>0089 (p89Gm)</td>
<td><em>Hind</em>II (blunt), <em>Kpn</em>I</td>
<td><em>Hind</em>II (blunt), <em>Kpn</em>I</td>
<td><em>aacC1</em> between the two <em>Pst</em>I sites</td>
</tr>
</tbody>
</table>
Fig 6.1 UQ biosynthesis pathway in *E. coli*
Fig 6.2. Hypothetical PQ-9 biosynthesis pathway in cyanobacteria
The question mark indicates the possibility that an alternative route exists for 4-hydroxybenzoate synthesis.
**Fig 6.3:** An alignment of the Ycf21/UbiC homologs in cyanobacteria and eukaryotic phototrophs.

Two catalytic residues (arginine at the position 76 and glutamate at position 155) of the UbiC protein are shown by black arrows.
Fig 6.4: Phylogenetic analyses based on nucleotide sequences of 16S rRNA and amino acid sequences of Ubi proteins in cyanobacteria and proteobacteria. Multiple alignments were generated with BLOSUM30 using ClustalW. Phylogenetic reconstruction was carried out using the Neighbor Joining method using PAUP* 4.0 beta. Bootstrap values were obtained after 1000 times repetitions. Ctep, Chlorobium tepidum; Cpar, Cyanophora paradoxa; Cwat, Crocosphaera watsonii WH 8501; Ecol, E. coli; Gvio, G. violaceus PCC 7421; Nostoc, Nostoc sp. PCC 7120; Npun, N. punctiforme; PMM, P. marinus MED4; PMT, P. marinus MIT 9313; S122, P. marinus SS120; Ppur, Porphyra purpurea; Rpal, Rps. palustris; Rrub, Rs. rubrum; Rsphe, Rb. sphaeroides; S6803, Synechocystis sp. PCC 6803; S7002, Synechococcus sp. PCC 7002; Synw, Synechococcus sp. WH 8102; Selon, Synechococcus elongatus PCC 7942; Tery, T. erythraeum; Telon, T. elongatus. Aeropyrum pernix K1 (locus tag: Ape1674), C. tepidum (locus tag: CT1511), Bacillus halodurance (locus tag: Bh3930), and Mesorhizobium loti (locus tag: Mlr2092) were used as out-groups.
Fig 6.5: Phylogenetic trees based on amino acid sequences of UbiE homologs in cyanobacteria.

Multiple alignments were generated with BLOSUM30 using ClustalW. This phylogenetic tree was constructed by the Neighbor Joining method using PAUP* 4.0 beta. Locus tags (Prefix followed by a 4-digit number) are used to indicate organisms and sequence identity (summarized in Table 6.6). Sll or Slr, *Synechocystis* sp. PCC 6803; All or Alr, *Nostoc* sp. PCC 7120; Npun, *N. punctiforme*; Tery, *T. erythraeum*; Tll or Tlr, *T. elongatus*; Glr or Gill, *G. violaceus*; Pmm, *P. marinus* MED4; Pmt, *P. marinus* MIT9313; Pro, *P. marinus* SS120; Synw, *Synechococcus* sp. WH8102.
Fig 6.6: HPLC analysis of the *sll1653* mutant of *Synechocystis* sp. PCC 6803 and the 2-phytyl-1,4-benzoquinone (MPBQ) standard.

(A) Chromatograms of the whole-cell extract of the *sll1653* mutant of *Synechocystis* sp. PCC 6803 (solid line) and the MPBQ standard (dotted line). (B) Absorption spectra of PQ-9 eluting at 35.5 min in the *sll1653* mutant sample (dotted line) and of MPBQ eluting at 33 min.
Fig 6.7: PQ-9 content in whole cells of the wild-type and methyltransferase mutant strains of *Synechocystis* sp. PCC 6803
An error bar for the wild-type strain represents the standard error derived from 6 determinations. At least two independent samples were analyzed for each mutant and, although somewhat varied, similar results were obtained.
Fig 6.8: Restriction maps of coding regions and their flanking sequences for the UbiE homologs in *Synechocystis* sp. PCC 6803.

Primers used for PCR for both cloning and PCR verifications are shown by black arrows. F1 and R1 primers were used for cloning the *sll0418* coding region, while F2 and R2 primers were used for verification of segregation.
Fig 6.9: PCR analyses of genomic DNA isolated from the single, double, and triple methyltransferase (UbiE homolog) mutants of *Synechocystis* sp. PCC 6803. Primers used for the analyses are shown by black arrows in Fig 6.5. The thin arrows indicate the relationship between parental strains and mutant strains. Identities of mutants are shown above the black lines and the primers used for the analyses are shown below. PCR reactions carried out using genomic DNA isolated from mutants are shown in lane 2 and reactions carried out using genomic DNA isolated from wild type are shown in lane 1 for comparison.
Fig 6.10: Aromatic amino acid biosynthesis and an alternative proposal for 4-hydroxybenzoate synthesis in cyanobacteria.
CM, chorismate mutase; PD, prephenate dehydratase; PPAT, prephenate amino transferase; ADH, arogenenate dehydrogenase; TAL, tyrosine amino-lyase; PAT, phenylalanine aminotransferase; PAL, phenylalanine amino-lyase; CH, chorismate hydratase. The pathways leading to tyrosine and phenylalanine biosynthesis in cyanobacteria are as previously described (Bonner et al., 1995).
Appendix

A.1. Amino acid sequence alignments of phylloquinone (Men) and plastoquinone (Ubi) biosynthetic enzymes.

A.1.1. MenA homologs in cyanobacteria and other organisms
A.1.2. MenB homologs in cyanobacteria and other organisms
A.1.3. MenD homologs in cyanobacteria and other organisms
A.1.4. UbiA homologs in cyanobacteria and other organisms
A.1.5. UbiD homologs in cyanobacteria and other organisms
A.1.6. UbiH homologs in cyanobacteria and other organisms
A.1.7. UbiX homologs in cyanobacteria and other organisms
A.1.8. UbiE homologs in Synechocystis sp. PCC 6803
A.1.9. Sll1653 homologs in cyanobacteria
A.1.10. Sll0418 homologs in cyanobacteria
A.1.11. Sll0829 homologs in cyanobacteria
A.1.12. Sll0487 homologs in cyanobacteria
A.1.13. Slr1039 homologs in cyanobacteria

A.2. Photosystem I (PS I) complexes


A.2.2. Illustration of genetically engineered PS I in cyanobacteria
A.1.1. An amino acid sequence alignment of MenA homologs in cyanobacteria and other organisms

The multiple alignment was generated by BLOSUM30 using Clustal W. The locus tags used to indicate organisms are summarized in Table 2.1, except for the following: CymeCp095 in *Cyanidiochyzon merolae* and OJ1217B09.18 in *Oryza sativa*. AF2036, a hypothetical protein in the archaeon *Archeoglobus fulgidus* DSM4304, was used as an out-group in phylogenetic construction as shown in Fig 2.3.
A.1.2. An amino acid sequence alignment of MenB homologs in cyanobacteria and other organisms

The multiple alignment was generated by BLOSUM30 using Clustal W. The locus tags used to indicate organisms are summarized in Table 2.1, except for the following: Cymecp092 in Cyanidiochyzon merolae and P0671D0118 in Oryza sativa. AF0435, a hypothetical protein in the archaeon Archeoglobus fulgidus DSM4304, was used as an out-group in phylogenetic construction as shown in Fig 2.3.
A.1.3. An amino acid sequence alignment of MenD homologs in cyanobacteria and other organisms
The multiple alignment was generated by BLOSUM30 using Clustal W. The locus tags used to indicate organisms are summarized in Table 2.1, except for the following: CymeCp094 in Cyanidiochyzon merolae. ML2270, a MenD homolog in Mycobacterium leprae, was used as an out-group in phylogenetic construction as shown in Fig 2.3.
A.1.4. An amino acid sequence alignment of UbiA homologs in cyanobacteria and other organisms

A.1.5. An amino acid sequence alignment of UbiD homologs in cyanobacteria and other organisms
A.1.6. An amino acid sequence alignment of UbiH homologs in cyanobacteria and other organisms
A.1.7. An amino acid sequence alignment of UbiX homologs in cyanobacteria and other organisms

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession</th>
<th>Type</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechocystis</td>
<td>sp. PCC 7421</td>
<td>Rpal</td>
<td>10-20</td>
</tr>
<tr>
<td>Synechococcus</td>
<td>sp. PCC 7002</td>
<td>Npun</td>
<td>30-40</td>
</tr>
<tr>
<td>Nostoc</td>
<td>sp. PCC 7120</td>
<td>A7120</td>
<td>50-60</td>
</tr>
<tr>
<td>Aeropyrum</td>
<td>pernix</td>
<td>S8102</td>
<td>70-80</td>
</tr>
<tr>
<td>R. sphaeroides</td>
<td>sp. PCC 6301</td>
<td>Telon</td>
<td>90-100</td>
</tr>
<tr>
<td>P. marinus</td>
<td>MIT9313</td>
<td>PmarMIT9313</td>
<td>110-120</td>
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<td>P. marinus</td>
<td>SS122</td>
<td>PmarSS122</td>
<td>130-140</td>
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<tr>
<td>G. violaceus</td>
<td>PCC7421</td>
<td>Gvio</td>
<td>150-160</td>
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<tr>
<td>R. palustris</td>
<td>sp. PCC 7131</td>
<td>Rpal</td>
<td>170-180</td>
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<td>R. sphaeroides</td>
<td>sp. PCC 6301</td>
<td>Rpal</td>
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<tr>
<td>E. coli</td>
<td>K1</td>
<td>Apex647</td>
<td>210-220</td>
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</table>

The multiple alignment was generated by BLOSUM30 using Clustal W. S6803, Synechocystis sp. PCC 6803; S7002, Synechococcus sp. PCC 7002; A7120, Nostoc sp. PCC 7120; Npun, N. punctiforme; Telon, T. elongatus BP-1; S8120, Synechococcus sp. WH8102; PmarMIT9313, P. marinus MIT9313; PmarSS122, P. marinus SS120; Gvio, G. violaceus PCC7421; Rpal, R. palustris; Rsphe, R. sphaeroides; Ecol, E. coli; Apex647, Aeropyrum pernix K1.
A.1.8. An amino acid sequence alignment of UbiE homologs in *Synechocystis* sp. PCC 6803

The multiple alignment was generated by BLOSUM30 using Clustal W. Sequences with “Sll” and “Slr” prefixes indicate the UbiE homologs of *Synechocystis* sp. PCC 6803. UbiE indicate the *E. coli* sequence.
A.1.9. An amino acid sequence alignment of Sll1653 homologs in cyanobacteria

The multiple alignment was generated by BLOSUM30 using Clustal W. Locus tags are used to indicate each organism (see also Table 6.5): Sll1653, *Synechocystis* sp. PCC 6803; Ahr5252, *Nostoc* sp. PCC 7120; Npun3758, *N. punctiforme*; Tery3321, *T. erythraeum*; Til2373, *T. elongatus* BP-1; Synv1674, *Synechococcus* sp. WH8102; Pmm0431, *P. marinus* MED4; Pmt0276, *P. marinus* MIT9313; Pro0427, *P. marinus* SS120; Gll0127, *G. violaceus* PCC7421. 89-c70244 indicates a tentative locus in *Synechococcus* sp. PCC 7002.
A.1.10. An amino acid sequence alignment of SI0418 homologs in cyanobacteria
The multiple alignment was generated by BLOSUM30 using Clustal W. Locus tags are used to indicate each organism (see also Table 6.5): Sll0418 and Slr0089, *Synechocystis* sp. PCC 6803; All2121, *Nostoc* sp. PCC 7120; Npun3758, *N. punctiforme*; Tery4209, *T. erythraeum*; Tll1726, *T. elongatus* BP-1; Synw2141, *Synechococcus* sp. WH8102; Pmm1505, *P. marinus* MED4; Pmt1785, *P. marinus* MIT9313; Pro1661, *P. marinus* SS120; Gll3039, *G. violaceus* PCC7421. 80a16268 indicates a tentative locus in *Synechococcus* sp. PCC 7002.
A.1.11. An amino acid sequence alignment of Sll0829 homologs in cyanobacteria

The multiple alignment was generated by BLOSUM30 using Clustal W. Locus tags are used to indicate each organism (see also Table 6.5): Sll0829, *Synechocystis* sp. PCC 6803; All3750, *Nostoc* sp. PCC 7120; Npun0871, *N. punctiforme*; Tery1223, *T. erythraeum*; Tll1604, *T. elongatus* BP-1; Synw1749, *Synechococcus* sp. WH8102; Pnt1242, *P. marinus* MIT9313; Gll1483, *G. violaceus* PCC7421. 89a46731 indicates a tentative locus in *Synechococcus* sp. PCC 7002.
A.1.12. An amino acid sequence alignment of Sll0487 homologs in cyanobacteria

The multiple alignment was generated by BLOSUM30 using Clustal W. Locus tags are used to indicate each organism (see also Table 6.5): Sll0487, Synechocystis sp. PCC 6803; All0012, Nostoc sp. PCC 7120; Npun5885, N. punctiforme; Tery3855, T. erythraeum; Tlr1948, T. elongatus BP-1; Synw0487, Synechococcus sp. WH8102; P. marinus MIT1474; Grl1180, G. violaceus PCC7421.
A.1.13. An amino acid sequence alignment of Slr1039 homologs in cyanobacteria

The multiple alignment was generated by BLOSUM30 using Clustal W. Locus tags are used to indicate each organism (see also Table 6.5): Slr1039, Synechocystis sp. PCC 6803; All3016, Nostoc sp. PCC 7120; Tlr1953, T. elongatus BP-1; Synw1148, Synechococcus sp. WH8102; Pmt0662, P. marinus MED4; Pmt0792, P. marinus MIT9313; Pro0816, P. marinus SS120; Glr3970, G. violaceus PCC7421. 91a179267 indicates a tentative locus in Synechococcus sp. PCC 7002.
A.2.1. Polypeptide composition of isolated PS I complexes isolated from the wild-type, *menG*, *menF*, *rubA*, *rubA menG*, and *rubA menB* mutants of *Synechococcus* sp. PCC 7002

M and T indicate monomeric and trimeric PS I complexes isolated from the respective wild type (WT) and mutant strains. SDS-PAGE gel containing 16% acrylamide and 6 M urea were used to resolve the polypeptides (electrophoresis at 80 mV for 12 h at room temperature). Small blue, yellow, and red arrows indicate polypeptide bands that are not detectable in the wild-type PS I trimeric forms. The polypeptide bands were visualized after silver staining.
Presence of all the polypeptides was confirmed in the PS I complexes isolated from \textit{menF} and \textit{menG} mutants. Immunoblotting analysis showed that PsaA and PsaB polypeptides were present in the PS I complexes isolated from the \textit{rubA menB} and \textit{rubA menB} double mutants, while PsaC, PsaD, and PsaE were absent. The absence of the latter three polypeptides is expected as a result of the inactivation of the \textit{rubA} gene, as previously reported [Shen G, Antonkine ML, van der Est A, Vassiliev IR, Brettel K, Bittl R, Zech SG, Zhao J, Stehlik D, Bryant DA, Golbeck JH (2002) J Biol Chem 277: 20355-20366]. In the \textit{rubA menB} mutant, no PsaL was detectable when the isolated PSI complexes were analyzed, while it was detectable when thylakoid membranes was analyzed. This suggests that PsaL was lost during the preparation of PS I complexes. The absence of PsaL is also visible in the SDS-PAGE gel (a band immediately below PsaF is missing). It has been reported that PsaL is required for the formation of trimeric forms of PS I complexes [Chitnis VP and Chitnis PR (1993) FEBS Lett 336: 330-334]. Indeed, after the first sucrose-density gradient centrifugation, most of the chlorophyll (an thus, PS I complexes) was collected in the PS I monomeric fraction from the \textit{rubA menB} mutant.
A.2.2. Illustration of genetically engineered PS I in cyanobacteria.

Strain names are shown below the respective PS I complexes. WT, wild-type strains of *Synechocystis* sp. PCC 6803 (containing PhyQ) and *Synechococcus* sp. PCC 7002 (containing MQ-4); DMQ, demethylmenaquinone-4; DphQ, demethylphyloquinone; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinone; PQ, plastoquinone-9; AQ, 9,10-anthraquinone. The PS I core is shown in green, while the PsAC subunit is shown in blue. P700 and A0 are indicated by purple circles, A1 is indicated by red diamonds, and [4Fe-4S] (Fx, Fa, Fb) clusters are indicated by yellow squares. All the PS I complexes shown here have been generated, except for the DMPBQ-containing complexes, which are expected to be produced in a menF cyclase (in α-tocopherol biosynthetic pathway) double mutant that is yet to be constructed. For constructions of the mutants, see Chapter 2 and 3. The *rubA menG* mutant in *Synechococcus* sp. PCC 7002 was generated by transforming the *rubA* mutant with the menG:accC1 construct (see the construction of the *rubA menB* double mutant described in Chapter 2). The PS I complex devoid of quinone and [4Fe-4S] clusters has been recently generated by treating the PS I complexes isolated from the *rubA menB* mutant with AQ followed by extensive washing (Y. Sakuragi, B. Zybaiov, G. Shen, R. Balasubramanian, B.A. Diner, I. Karygina, Y. Pushkar, D. Stehlik, D.A. Bryant, and J.H. Golbeck, unpublished).
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