ABSTRACT

Title of Document: NEW INSIGHT INTO ISOPRENOID BIOSYNTHESIS IN THE CYANOBACTERIUM *SYNECHOCYSTIS* SP. STRAIN PCC 6803

Kelly Ann Poliquin, Doctor of Philosophy, 2006

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In cyanobacteria, many compounds including chlorophylls, carotenoids, and quinones are synthesized from the isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These C_5 compounds are products of the well-studied methylerythritol phosphate (MEP) pathway found in cyanobacteria, plant plastids, and many bacteria. Previous studies suggest that isoprenoid biosynthesis via the MEP pathway in the cyanobacterium *Synechocystis* sp. strain PCC 6803 are more complex than those proposed for a model bacterium. Most notably, *in vitro* isoprenoid biosynthesis in *Synechocystis* is stimulated by compounds of the pentose phosphate cycle (PPC) and not by intermediates of the MEP pathway. Isoprenoid biosynthesis in *Synechocystis* was therefore further investigated by disrupting *sll1556*, a gene distantly related to type 2 IPP isomerase genes. This gene is not essential under optimal photosynthetic conditions (20 μmol photons/m^2/s). IPP isomerase activity could not be shown for the purified protein. Whereas *in vitro* PPC substrate stimulated isoprenoid biosynthesis could not be demonstrated in Δ*sll1556*

cell-free extracts, it was restorable upon addition of the recombinant Sll1556 protein. PPC-stimulated isoprenoid biosynthesis results in a progression of isoprenoid production (C_5 to C_{10} to C_{20}) *in vitro*, although PPC compounds were not found to serve as direct substrates. Isoprenoid synthesis activity was unaffected when LytB, the terminal enzyme of the MEP pathway responsible for the production of both IPP and DMAPP, was immunodepleted from the cell-free extract, suggesting LytB activity is not likely to contribute to the observed *in vitro* isoprenoid synthesis. The physiological importance of Sll1556 was revealed at high light (200 μmol photons/m^2/s). High light stress in the Δ*sll1556* mutant is evident by slower cell growth, a decrease in chlorophyll and carotenoid content, and in fewer thylakoids per cell. Myxoxanthophyll, but not zeaxanthin, increased in high light cells. The exact function of Sll1556 remains to be elucidated, but the combined results are consistent with a role in isoprenoid biosynthesis that is particularly important under high light stress. The mechanism by which Sll1556 is involved in PPC-stimulated isoprenoid synthesis is discussed, as are future areas of exploration for research.

NEW INSIGHT INTO ISOPRENOID BIOSYNTHESIS IN THE CYANOBACTERIUM *SYNECHOCYSTIS* SP. STRAIN PCC 6803

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Dissertation submitted to the Faculty of the Graduate School of the
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DEDICATION

This dissertation is dedicated to my family and friends, who mean more to me than anything. To my husband, John, whose love, support, and patience helped make this possible. To my parents, Bob and Jackie, who always stressed the importance of an education and were there every step of the way routing for me.

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CDP-ME	4-diphosphocytidyl-2*C*-methyl-D-erythritol
CDP-ME2P	4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate
CPM	counts per minute
DMAPP	dimethylallyl diphosphate
DMAOH	dimethylallyl alcohol
DTT	dithiothreitol
DXP	1-deoxyxylulose-5-phosphate
DXR	1-deoxyxylulose-5-phosphate reductoisomerase
DXS	1-deoxyxylulose-5-phosphate synthase
FOH	farnesol
FPP	farnesyl diphosphate
FR6P	fructose-6-phosphate
GA3P	glyceraldehyde-3-phosphate
GGOH	geranylgeraniol
GGPP	geranylgeranyl diphosphate
GOH	geraniol
GPP	geranyl diphosphate
GL6P	glucose-6-phosphate
HM-2B4PP	1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate
HMG-CoA	hydroxymethylglutaryl-CoA
IPI	IPP isomerase
IPP	isopentenyl diphosphate
ME-2,4cPP	2*C*-methyl-D-erythritol 2,4-cyclodiphosphate
MEP	2-*C*-methyl-D-erythritol-4-phosphate
MVA	mevalonic acid
PPC	pentose phosphate cycle
PYR	pyruvate
TAP tag	tandem affinity purification tag
RT-PCR	real-time polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
WT	wild type

CHAPTER 1

General Introduction

Functions of isoprenoids

Isoprenoids, also known as terpenes or terpenoids, are a diverse group of naturally synthesized compounds found in bacteria, fungi, animals, and plants (Kleinig, 1989). The more than 30,000 isoprenoids identified to date have a wide range of functions (Lange et al., 2000; Rodríguez-Concepción and Boronat, 2002). In bacteria, isoprenoids play a role in membrane stabilization in the form of hopanoids and bactoprenols (Rohmer et al., 1984; Rosa-Putra et al., 1998), as well as in the respiration chain as electron carriers such as ubiquinone and menaquinone (Gennis and Stewart, 1996). In animals, isoprenoid compounds include many hormones and sterols that contribute to membrane structure; dolichols involved in protein glycosylation; and ubiquinones in respiration chains (Rohmer, 1999; Goldstein and Brown, 1990).

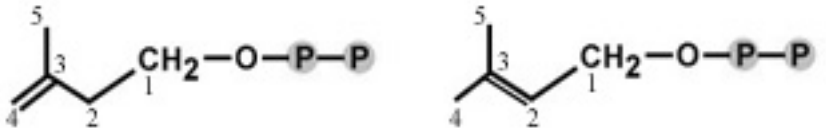
Plants contain the greatest variety of isoprenoids. In photosynthetic organisms, isoprenoids are essential components of the photosynthetic machinery such as carotenoids and the phytol tail of chlorophyll in the photosynthetic reaction centers and as light harvesting pigments with the former involved in photoprotection (Goodwin, 1980; Lawlor, 1987). Plant hormones involved in growth and development like gibberellic acid, abscisic acid, and cytokinin are derived from isoprenoids (Gray, 1987). Plants also use isoprenoids for defense against bacteria,

fungi, and herbivores in the form of phytoallexins and toxins, as well as for competition (Bach, 1995; Croteau et al., 2000). Finally, isoprenoids such as essential oils and pigments for coloration of flowers and fruits are important for attracting pollinators (Rohmer, 1999; Cunningham and Gantt, 2001). With such a wide array of functions, the elucidation of isoprenoid synthesis is of interest for many.

Classification of isoprenoids

Although diverse in function, all isoprenoids are derived from one or a combination of two 5-carbon (C_5) compounds, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Figure 1-1). Isoprenoids can be classified according to how many C_5 units they contain. The simplest isoprenoids are hemiterpenes, which have only five carbons. An example of a hemiterpene is isoprene, a volatile compound made directly from DMAPP that is greatly studied in higher plants because of its role in atmospheric chemistry (Sharkey and Yeh, 2001).

Generally, isoprenoids form by a head to tail addition of one IPP to an activated, allylic DMAPP. The number of IPP units added is determined by a family of enzymes called prenyl transferases (Tachibana, 1994). Addition of IPP to DMAPP results in a C_{10} compound called geranyl diphosphate (GPP), a monoterpene (Spurgeon and Porter, 1981). Examples of monoterpenes, which are often volatile compounds and considered secondary metabolites, include essential oils of plants such as geraniol (C_{10}) and a mixture of isomers (i.e. menthone) (Eisenreich et al., 1997; Rohmer, 1999). The condensation reaction of the allylic DMAPP with IPP to



A. IPP B. DMAPP

Figure 1-1. The 5-carbon building blocks of isoprenoids: A.) isopentenyl diphosphate (IPP) and B.) its isomer dimethylallyl diphosphate (DMAPP). The double bond between carbons 2 and 3 of DMAPP is allylic and allows DMAPP to react with additional molecules of IPP to form longer chained isoprenoids (Poulter and Rilling, 1981).

make a C_{10} compound makes the IPP molecule itself allylic, which then is able to accept another IPP unit (Gray, 1987). A C_{15} compound is called farnesyl diphosphate (FPP), which gives rise to a class of isoprenoids known as sesquiterpenes, also secondary metabolites. This group includes β-caryophyllene, a volatile compound emitted by some plants which attracts predators to ward off attacking herbivores (Owen and Peñuelas, 2005; Rasmann et al., 2005). A head to middle condensation of two FPP molecules results in the tripterpene squalene (C_{30}), which leads to sterols (Kleinig, 1989).

Geranylgeranyl diphosphate (GGPP), a C_{20} compound, can be produced by addition of IPP to FPP or by a condensation of two GPPs. The diterpene GGPP is a substrate for the production of gibberellins, which act as plant hormones, and the phytol side-chain of the photosynthetic pigment chlorophyll (Lange and Ghassemian, 2003). The head to head condensation of two GGPP molecules is the first committed step for the production of carotenoids, C_{40} tetraterpenes. Carotenoids are critical compounds that serve as light harvesting pigments and provide protection from excess light energy and oxygen radicals in photosynthetic organisms (Cunningham and Gantt, 1998). Isoprenoid compounds of C_{45} or greater are called polyterpenes. These include dolichols, rubber, and ubiquinone and plastoquinone, involved in electron transport in the mitochondria and chloroplast, respectively (Kesselmeier and Staudt, 1999; Bouvier et al., 2005). An overview of the different classes of isoprenoids is shown in Figure 1-2.

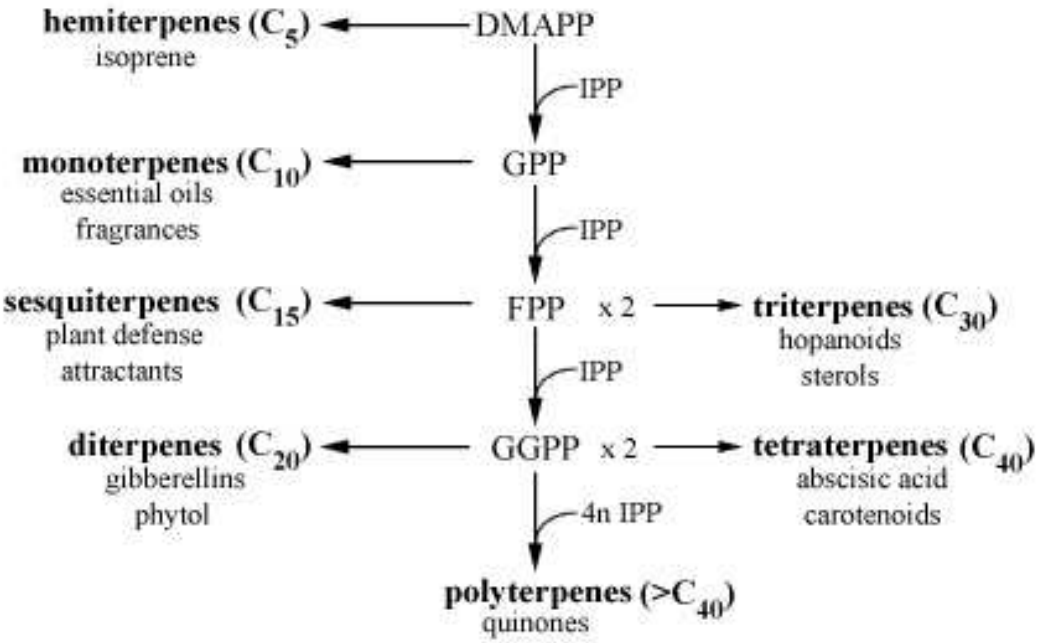


Figure 1-2. Classification of isoprenoids. Although numerous and diverse, isoprenoids can be classified according to the number of C_5 units they contain. Reviewed in Spurgeon and Porter (1981).

Biosynthesis of isoprenoids and discovery of a second pathway

Scientists first began studying isoprenoids in the 1800s and by the early 1900s it was found that their structural composition was based on 5-carbon units. It was not until the 1950s, however, that the mechanism of isoprenoid synthesis began to emerge through experiments studying sterol production in yeast and mammalian cells. Aided by the availability of isotopes that could be used to follow labeled substrates, it was discovered that the isoprenoids being study were derived from IPP via a pathway involving acetyl-CoA (reviewed in Spurgeon and Porter, 1981). Over time, many steps of the pathway were unraveled and it eventually became known as the mevalonate, or mevalonic acid (MVA), pathway (Figure 1-3). The MVA pathway begins with the conversion of acetyl-CoA into hydroxymethylglutaryl-CoA (HMG-CoA) through acetoacetyl-CoA. HMG-CoA is then converted into MVA by the enzyme HMG-CoA reductase, which requires NADPH. MVA is converted into IPP after two phosphorylations and a decarboxylation in the presence of ATP (Gray, 1987). IPP is then isomerized into DMAPP by the enzyme IPP isomerase (IPI) (Ramos-Valdivia et al., 1997).

Although the MVA pathway was thought to be universal for isoprenoid biosynthesis, studies using photosynthetic organisms were not consistent with this notion. One of the biggest oddities was that labeled MVA and acetate were not incorporated into monoterpenes, diterpenes, or carotenoids, but were found in sterols, sesquiterpenes, and triterpenes (Kleinig, 1989; Bach, 1995). Instead, it was found that the label from [^{14}C] carbon dioxide ended up in carotenoids from isolated chloroplasts of the green alga *Acetabularia* (Moore and Shephard, 1977) as well as

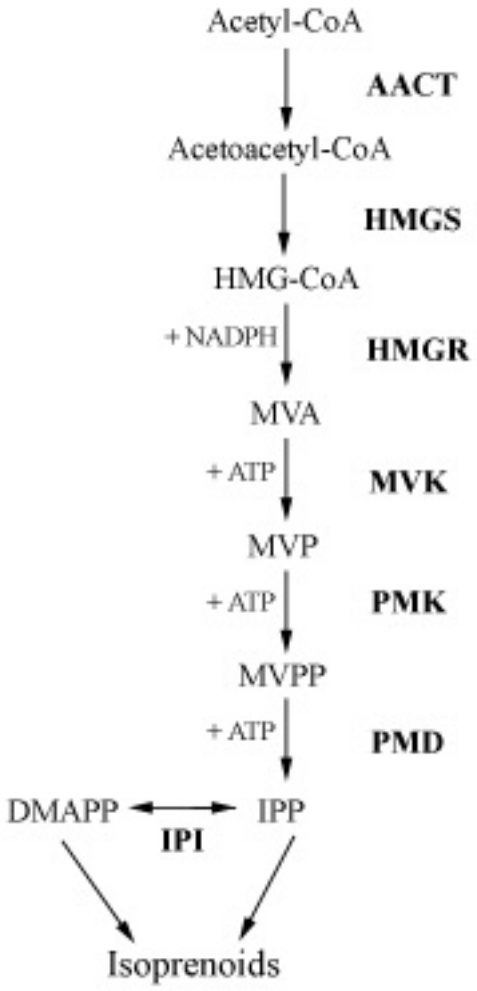


Figure 1-3. The mevalonic acid pathway in animals, fungi, the cytoplasm of plants, and archaebacteria (adapted from Rodríguez-Concepción and Boronat, 2002). HMG-CoA, hydroxymethylglutaryl-CoA; MVA, mevalonate; MVP, 5-phosphomevalonate; MVPP, 5-diphospho-mevalonate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate. Enzymes catalyzing the reactions are in bold. **AACT**, acetoacetyl-CoA thiolase; **HMGS**, HMG-CoA synthase; **HMGR**, HMG-CoA reductase; **MVK**, MVA kinase; **PMK**, MVP kinase; **PMD**, MVPP decarboxylase; **IPI**, IPP isomerase.

isolated spinach chloroplasts (Shulze-Siebert and Shultz, 1987). Additionally, mevinolin, an inhibitor of an enzyme of the MVA pathway, did not affect the synthesis of carotenoids and chlorophyll in plants, although it did inhibit production of sterols (Lichtenthaler, 1999). These discrepancies were thought to be the result of poor permeability of the chloroplast envelope to labeled substrates or the inhibitor. It was soon, discovered, however, that another means to isoprenoid biosynthesis existed.

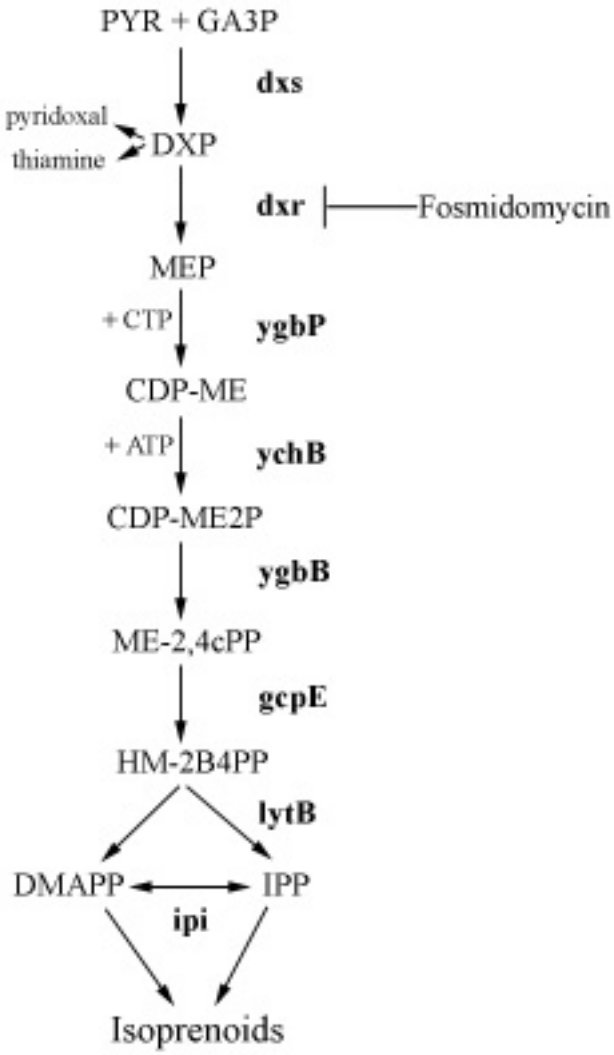
Rohmer and colleagues were the first to publish evidence supporting an alternate route for isoprenoid synthesis (Flesch and Rohmer, 1988). Using the eubacteria *Rhodopseudomonas* sp. and *Methylobacterium organophilum*, they followed the label from [^{13}C] acetate into hopanoids, bacterial isoprenoids that are components of cell membranes which are structurally similar to eukaryotic sterols. Surprisingly, the labeling pattern did not agree with what should have occurred if the hopanoids were being made by the MVA pathway. Instead, it suggested that the carbons from acetate were first going through the glyoxylate cycle and tricarboxylic acid cycle before being incorporated into isoprenoids (Flesch and Rohmer, 1988). Subsequent studies with the eubacteria *Zymomonas mobilis* and *Methylobacterium fujisawaense* using labeled [^{13}C] glucose revealed that the source of the carbons found in IPP were derived from pyruvate and a triose phosphate (Rohmer et al., 1993). The triose phosphate was ultimately identified as glyceraldehyde 3-phosphate (GA3P) using mutants of the eubacterium *Escherichia coli* lacking enzymes involved in triose phosphate metabolism and [^{13}C] labeled substrates (Rohmer et al., 1996). First termed the nonmevalonate or Rohmer pathway, it is now more commonly known as

either the deoxyxylulose 5-phosphate (DXP) pathway or the 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Eisenreich et al., 2001; Lichtenthaler, 1999) (Figure 1-4).

Numerous studies confirm the role of the MEP pathway in eubacterial isoprenoid synthesis (Rohmer, 1999). Evidence for the occurrence of this alternate pathway in a photosynthetic organism was first tested in the green alga *Scenedesmus obliquus* with methods similar to those used earlier in bacteria. It was found that this organism made all of the isoprenoids studied (sterols, ubiquinone, phytol, carotenoids, and plastoquinone) via the MEP pathway (Schwender et al., 1996). Similar results were also found for two other green alga, *Chlorella fusca* and *Chlamydomonas reinhardtii*, as well as the cyanobacterium *Synechocystis* PCC 6714 (Disch et al., 1998).

Much of the evidence that contradicted the longstanding theory of a single MVA-dependent route to isoprenoid synthesis, though, was from research with higher plants (Kleinig, 1989; Bach, 1995). Lichtenthaler et al. (1997) were the first to demonstrate that plants use both the MVA and the MEP pathways to produce their isoprenoids. Using [^{13}C]glucose for labeling studies in *Lemna gibba* (duckweed), *Daucus carota* (carrot), and *Hordeum vulgare* (barley), they determined that cytoplasmic sterols were made by the MVA pathway, while chloroplast localized carotenoids (β-carotene and lutein), chlorophyll, and plastoquinone were derived from the MEP pathway. Subsequent work from this and other groups have supported these results and it is now widely accepted that cytosolic isoprenoids in plants are made from the MVA pathway, while those produced in the chloroplast are made via

Figure 1-4. The 2-C-methyl-D-erythritol 4-phosphate pathway of Escherichia coli (modified from Rodríguez-Concepción and Boronat, 2002). PYR, pyruvate; GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-ME2P, 4-diphospho-cytidyl-2-C-methyl-D-erythritol 2-phosphate; ME-2,4cPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HM-2B4PP, 2-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate. The genes encoding the enzymes that catalyze the reactions are in bold: dxs, deoxyxylulose 5-phosphate synthase; dxr, deoxyxylulose 5-phosphate reductoisomerase. The MEP pathway inhibitor fosmidomycin is shown.


PYR + GA3P
dxs
pyridoxal
thiamine
DXP
dxr
Fosmidomycin
MEP
+ CTP
ygbP
CDP-ME
+ ATP
ychB
CDP-ME2P
ygbB
ME-2,4cPP
gcpE
HM-2B4PP
lytB
DMAPP
IPP
ipi
Isoprenoids

the MEP pathway (reviewed in Rohmer, 1999).

A recent field of study to emerge from this is whether there is cross-talk between the two pathways or an exchange of intermediates across the chloroplast envelope. Two groups have reported data supporting the transport of IPP and GPP (C_{10}) from the chloroplast to the cytosol (Bick and Lange, 2003; Laule et al., 2003). Nagata et al. (2002) provided evidence for exchange in the opposite direction, stating that MVA could partially restore plastid isoprenoid levels in an *Arabidopsis thaliana* mutant lacking a necessary enzyme of the MEP pathway. This was further supported by the work of Hemmerlin et al. (2003), who used mevinolin, the MVA pathway inhibitor, and fosmidomycin, the MEP pathway inhibitor, to show that the label from an intermediate of each pathway could be incorporated into plastidic and cytosolic isoprenoids, respectively. Recent data acquired from *Nicotiana benthamiana* (tobacco) leaves impaired in isoprenoid synthesis via the MEP pathway do not agree with these findings, however. A cytosolic contribution of isoprenoid intermediates did not rescue the loss of chlorophyll and carotenoids that resulted from this impairment (Page et al., 2004). The interrelatedness of the two pathways in plants requires considerable future work for its elucidation.

From the many studies on isoprenoid biosynthesis, it has been determined that the MEP pathway is present in most eubacteria, cyanobacteria, and plant plastids while the MVA pathway occurs in archaebacteria, animals, fungi, and the cytosol of plants (Boucher and Doolittle, 2000). It is probable that eukaryotes acquired their genes for isoprenoid synthesis from prokaryotes (MVA pathway from archaea, MEP pathway from eubacteria). Only eukaryotes with plastids possess genes for the MEP

pathway, most likely from the cyanobacterial ancestor of plastids (Lange et al., 2000). Although most organisms fit into the accepted classification of having the MEP or MVA pathway, the distribution of isoprenoid biosynthetic pathways is not without exceptions. Some green algae use only the MEP pathway (Schwender et al., 1996; Disch et al., 1998), while *Euglena gracilis*, another chloroplast-containing organism, possesses only the MVA pathway. Some bacteria have only the MVA pathway, such as *Staphylococcus aureus*, while others, like *Streptomyces* sp. strain CL190, use both pathways to make their isoprenoids at different stages of development (Kuzuyama and Seto, 2003). A few bacteria operate on one pathway, yet contain an incomplete portion of the other, as is the case in *Bacillus subtilis* (Boucher and Doolittle, 2000). These discrepancies in distribution are thought to be the result of lateral gene transfer (Boucher and Doolittle, 2000).

Elucidation of the steps of the MEP pathay

The MEP pathway has been well studied in the gram-negative eubacterium *Escherichia coli* and many of the steps have been determined (Figure 1-4). The pathway begins with the condensation of pyruvate (PYR) and glyceraldehyde 3-phosphate (GA3P) to form 1-deoxy-D-xylulose 5-phosphate (DXP) (Schwarz, 1994). The enzyme catalyzing this step was determined to be DXP synthase (*dxs*) (Sprenger et al., 1997; Lois et al., 1998). DXP was found to be important in other cell processes besides isoprenoid production, serving as the precursor to thiamine (B_1) and pyridoxal (B_6) (Sprenger et al., 1997). The second step of the pathway is the rearrangement and reduction of DXP to MEP (Rohmer, 1996). Using a genetic approach, Kuzuyama et

al. (1998) and Takahashi et al. (1998) determined the enzyme responsible was DXP reductoisomerase (*dxr*). In addition, it was discovered that this enzyme is inhibited by fosmidomycin (3-[*N*-formyl-*N*-hydroxyamino] propylphosphonic acid), a compound structurally similar to MEP (Kuzuyama et al., 1998; Zeidler et al., 1998). Labeling experiments and NMR analysis of products revealed that the next step is the CTP-dependent conversion of MEP to 4-diphosphocytidyl-2*C*-methyl-D-erythritol (CDP-ME). Database searches yielded a potential gene, *ygbP*, that might be involved and activity assays of the purified recombinant protein confirmed this (Rohdich et al., 1999). Subsequently, Lüttgen et al. (2000) determined that *ychB* catalyzes the ATP-dependent phosphorylation of CDP-ME to 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate (CDP-ME2P) by searching for genes found in eubacteria and plants, but not in animals, fungi, or archaebacteria (which have only the MVA pathway) and testing the recombinant protein for activity.

The final enzymes of the pathway, encoded by *ygbB*, *gcpE*, and *lytB*, were found similarly through database searching. The product of *ygbB* was shown to be involved in the next step of the pathway by converting CDP-ME2P to 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (ME-2,4cPP) (Herz et al., 2000). Cunningham et al. (2000) predicted roles for both *gcpE* and *lytB* in the pathway based on their pattern of occurrence with other MEP pathway enzymes. *gcpE* from *E. coli* was later shown to encode an enzyme that catalyzes the conversion of ME-2,4cPP to 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (HM-2B4PP) (Hecht et al., 2001; Hintz et al., 2001). The recombinant enzyme from the hypothermophilic bacterium *Thermus thermophilus* (Kollas et al., 2002) and from *E. coli* (Seemann et al., 2002) was

purified and active only after reconstitution of its iron-sulfur cluster under reducing, anaerobic conditions. Recently, ferredoxin was shown to be the potential electron donor for GcpE in the cyanobacterium *Thermosynechococcus elongatus* BP-1 (Okada et al., 2005).

The final step of the MEP pathway involves *lytB*. Early studies indicated that LytB acts at a point before the production of IPP and DMAPP (Cunningham et al., 2000). Experiments in *E. coli* using labeled intermediates of the MEP pathway indicated that two different routes to IPP and DMAPP from a common intermediate downstream of MEP existed (Charon et al., 2000). It was later shown that purified LytB from *E. coli* and from the thermophilic eubacterium *Aquifex aeolicus* catalyzed the conversion of HM-2B4PP to both IPP and DMAPP when also assayed in oxygen-free conditions in the presence of a reductant (Adam et al., 2002; Altincicek et al., 2002; Rohdich et al., 2002, Rhodich et al., 2003). Like GcpE, LytB was recently shown to interact with and have activity in the presence of ferredoxin in the malaria parasite *Plasmodium falciparum*, indicating that it may be a necessary redox partner (Rohrich et al., 2005).

Isoprenoid biosynthesis in the cyanobacterium *Synechocystis* strain PCC 6803

Cyanobacteria are generally photosynthetic autotrophs (using only light as an energy source). Because they are prokaryotic, they contain no chloroplast to conduct photosynthesis. Instead, they have intracellular membranes resembling the thylakoid membranes of chloroplasts that contain the photosynthetic machinery including photosystem II, photosystem I, and the pigments chlorophyll a, phycocyanin,

allophycocyanin, and carotenoids (Moore et al., 1998). It is thought that cyanobacteria are the progenitors of chloroplasts in algae and plants (Delwiche et al., 1995). Because cyanobacteria require isoprenoids for photosynthesis (carotenoids, phytol tail of chlorophyll), electron transport (quinones), and membrane stability (hopanoids), they are an interesting model to study isoprenoid biosynthesis in a photosynthetic system.

The genome of the freshwater cyanobacterium *Synechocystis* sp. strain PCC 6803 (Figure 1-5) has been completely sequenced (Kaneko et al., 1996) and contains homologs for the MEP pathway genes *dxs*, *dxr*, *ygbB*, *ygbP*, *ychB*, *gcpE*, and *lytB*. No homologs for enzymes of the MVA pathway are present (Cunningham et al., 2000). While most of the genes of the MEP pathway were initially discovered in *E. coli*, *lytB* was first found to be involved in *Synechocystis* PCC 6803. Cunningham et al. (2000) showed that an insertion in the promoter region of *lytB* resulted in slow growing yellow/green colonies that eventually bleached, while an insertion in the coding region was lethal. Cells were rescued if supplemented with the alcohol analogs of IPP and DMAPP. Overexpression of *Synechocystis* PCC 6803 *lytB* in an *E. coli* strain engineered to produce the pink carotenoid lycopene resulted in an increased production of the pigment. Interestingly, when *lytB* (from *Adonis aestivalis*) and *dxs* (from marigold) were overexpressed in the same lycopene producing strain of *E. coli*, a greater accumulation of lycopene occurred then with either *lytB* or *dxs* alone, while an overexpression of *lytB* and the IPP isomerase gene

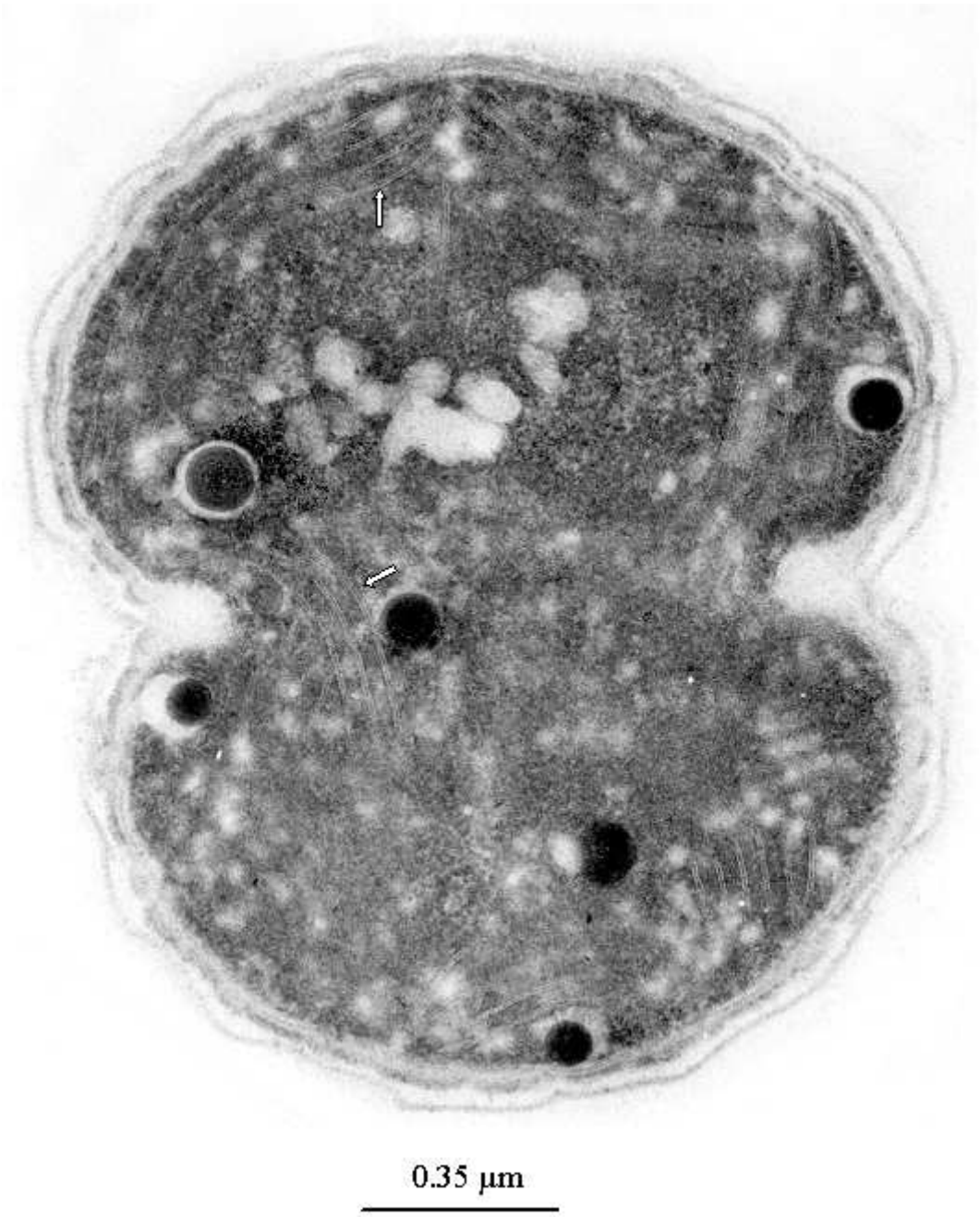


Figure 1-5. Transmission electron micrograph of a wild type *Synechocystis* strain PCC 6803 cell undergoing division. Thylakoid membranes, which contain the photosynthetic machinery, are indicated by the arrows. Scale, 0.35 µm.

(*ipi*; from *Arabidopsis*) resulted in no increased lycopene production. From these studies, it was concluded that the product of *lytB* plays a role in isoprenoid synthesis, acting at or before the branching point in the MEP pathway. Soon after it was discovered that the catalytic action of bacterial LytB occurs in the main trunk of the pathway and that it converts HM-2B4PP to both IPP and DMAPP in a 5:1 ratio, respectively (Altincicek et al., 2001; Hintz et al., 2001; McAteer et al., 2001; Adam et al., 2002; Altincicek et al., 2002; Rohdich et al., 2002).

Further studies of isoprenoid biosynthesis via the MEP pathway in *Synechocystis* PCC 6803 in the laboratory of E. Gantt led to the discovery of a number of differences when compared to *E. coli*. The first was the apparent lack of a homolog for IPP isomerase, the enzyme that interconverts IPP and DMAPP (Cunningham et al., 2000). Using *Synechocystis* PCC 6803, Ershov et al. (2000) demonstrated a lack of IPP isomerase activity using similar assay conditions as for *E. coli*. In cell-free extracts, radiolabeled IPP could only be incorporated into isoprenoids if DMAPP was added as a primer. The same was true for extracts of another cyanobacterium, *Synechococcus* sp. PCC 7942. These results suggested that DMAPP is being made independently of IPP and not from an interconversion by an IPP isomerase in these organisms.

Interestingly, Hahn et al. (1999) reported that IPP isomerase in *E. coli* is not essential for normal isoprenoid synthesis. Rodríguez-Concepción et al. (2000) further demonstrated that the product of the *E. coli ipi* gene was active and the only enzyme in that organism that could interconvert IPP and DMAPP. IPP isomerase does seem to be required for normal isoprenoid biosynthesis in plant chloroplasts. Using virus-

induced gene silencing, Page et al. (2004) posttranscriptionally silenced expression of the tobacco IPP isomerase in leaves and found that chlorophyll and carotenoid levels were each reduced by about 80% in infected tissue. These results suggest that although the MEP pathway is capable of making both IPP and DMAPP, IPP isomerase is necessary for optimal isoprenoid production in tobacco chloroplasts, perhaps through balancing the ratio of IPP to DMAPP (Page et al., 2004).

In addition to the lack of IPP isomerase, Ershov et al. (2002) reported other ways in which isoprenoid synthesis in *Synechocystis* PCC 6803 differed from that described in *E. coli*. Fosmidomycin, a potent inhibitor of the MEP pathway enzyme DXR, had no effect on *Synechocystis* PCC 6803 cells grown under photoautotrophic conditions or on isoprenoid production *in vitro*. Furthermore, addition of the MEP pathway substrates pyruvate and deoxyxylulose 5-phosphate (DXP) to cell-free extracts of *Synechocystis* PCC 6803 did not enhance isoprenoid production, as measured by the incorporation of radiolabeled IPP. Instead, compounds of the pentose phosphate cycle, such as fructose 6-phosphate, glucose 6-phosphate, and erythrose 4-phosphate stimulated isoprenoid biosynthesis. It was hypothesized that these phosphorylated compounds may enter into the MEP pathway prior to the reaction involving LytB to produce IPP and DMAPP (Ershov et al., 2002). Under certain growth conditions, utilization of alternate substrates for isoprenoid production (in the form of photosynthetic metabolites) might prove beneficial for the organism.

During investigations into the MEP pathway in *Synechocystis* PCC 6803, Kaneda et al. (2001) discovered a gene within a cluster of other MVA pathway genes in the bacterium *Streptomyces* sp. strain CL190 (Kaneda et al., 2001).

Characterization of the purified protein led to the discovery that it catalyzed the conversion of IPP to DMAPP. Furthermore, it was shown to complement the disrupted IPP isomerase gene in *E. coli*. Because this enzyme required NADPH and FMN as cofactors and had no sequence similarity to known IPP isomerases, it was classified as a type 2 IPP isomerase. All other known IPP isomerases were grouped as type 1, based on their lack of a need for cofactors. The *Streptomyces* sp. strain CL190 type 2 IPP isomerase gene has homology to others in archaebacteria and some eubacteria, including *Synechocystis* PCC 6803. *Synechocystis* PCC 6803 open reading frame sll1556 has 44.9% similarity and 35.1% identity to the *Streptomyces* sp. strain CL190 type 2 IPP isomerase gene (Kaneda et al., 2001).

Objectives of this disseratation

Although isoprenoid biosynthesis has been well studied, there is still much that is not known. Recent work done in our laboratory (Ershov et al., 2000 and 2002) has uncovered significant differences in regard to isoprenoid synthesis in the cyanobacterium *Synechocystis* PCC 6803 from what is expected of a typical MEP pathway operating organism. The focus of this dissertation is to further investigate isoprenoid production in *Synechocystis* PCC 6803. Chapter 2 aims to characterize a *Synechocystis* PCC 6803 mutant with a loss of function insertion in *sll1556,* a gene that has been annotated as a type 2 IPP isomerase, in hopes of better understanding its role in isoprenoid biosynthesis in this organism. Chapter 3 explores how the isoprenoid products of pentose phosphate cycle substrate stimulated isoprenoid synthesis. Chapter 4 examines the effect of the *sll1556* mutation on isoprenoid

synthesis and its importance under environmental stress. Chapter 5 summarizes this work and discusses areas for future research.

CHAPTER 2

Inactivation of *sll1556* in *Synechocystis* PCC 6803 Impairs Isoprenoid Biosynthesis from Pentose Phosphate Cycle Substrates *In Vitro*

Summary

In cyanobacteria many compounds, including chlorophylls, carotenoids, and hopanoids are synthesized from the isoprenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Isoprenoid biosynthesis in extracts of the cyanobacterium *Synechocystis* PCC 6803 grown under photosynthetic conditions, stimulated by pentose phosphate cycle substrates, does not appear to require methylerythritol phosphate pathway intermediates. The *sll1556* gene, distantly related to type 2 IPP isomerase genes, was disrupted by insertion of a Kan^r cassette. The mutant was fully viable under photosynthetic conditions although impaired in the utilization of pentose phosphate cycle substrates. Compared to the parental strain the Δ*sll1556* mutant (a) is deficient in isoprenoid biosynthesis *in vitro* with substrates including glyceraldehyde 3-phosphate, fructose 6-phosphate, and glucose 6-phosphate, (b) has smaller cells (diam. *ca*.13% less), (c) has fewer thylakoids (*ca*. 30% less), and (d) has a more extensive fibrous outer wall layer. Isoprenoid biosynthesis is restored with pentose phosphate cycle substrates plus the recombinant Sll1556 protein in the Δ*sll1556* supernatant fraction. IPP isomerase activity could not be demonstrated for the Sll1556 protein, and the exact function of the protein remains to be elucidated. The reduction of thylakoid area and the effect on

outer wall layer components is consistent with an impairment of isoprenoid biosynthesis in the mutant, possibly via hopanoid biosynthesis. Our findings are consistent with an alternate metabolic shunt for biosynthesis of isoprenoids. This chapter has been published in the Journal of Bacteriology (Poliquin et al., 2004).

Introduction

Isoprenoids are required for many cell processes including photosynthesis, membrane stability, electron transport, and the cellular production of carotenoids, rubber, and fragrances. More than 30,000 isoprenoid compounds have been described. Synthesis of the essential 5-carbon building blocks of isoprenoids, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), can be attributed to one of two pathways. The mevalonic acid (MVA) pathway, the sole pathway in animals and in many bacteria, also occurs in the cytoplasm of plant cells where it is responsible for synthesis of sterols and ubiquinones (Rodríguez-Concepción and Boronat, 2002; Rohmer, 1999). The 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway occurs in *Escherichia coli* and many other bacteria as well as in cyanobacteria. It is also present in chloroplasts where it provides substrates for the synthesis of carotenoids, chlorophylls, and quinones. This pathway is believed important for cyanobacterial photosynthetic pigment biosynthesis including carotenoids and the phytolation of chlorophyll and in hopanoid synthesis of bacterial and cyanobacterial cell walls and membranes (Jürgens et al., 1992; Rohmer, 1999; Simonin et al., 1996). Most of the MEP pathway genes have been functionally verified in the gram negative heterotrophic bacterium *Escherichia coli* (reviewed in

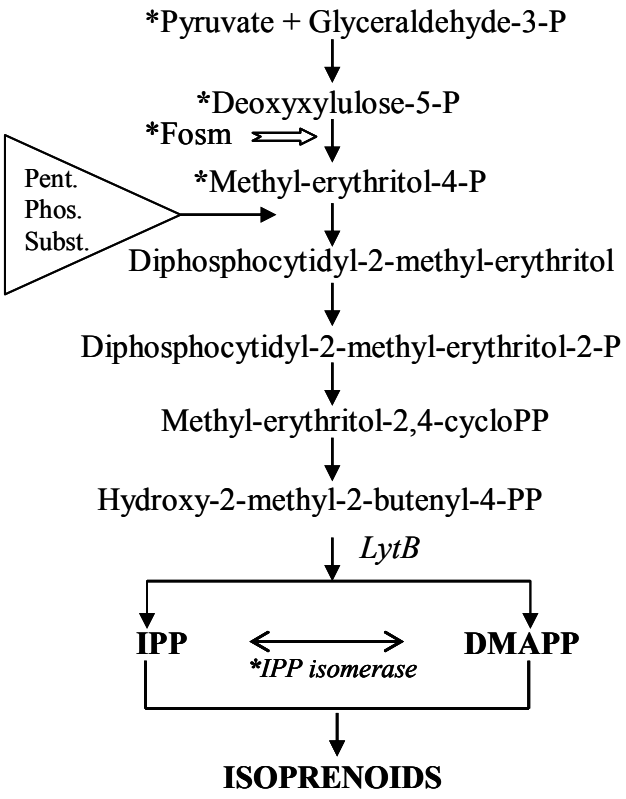
Eisenreich et al., 2001; Kuzuyama and Seto, 2003; Rodríguez-Concepción and Boronat, 2002; Rohmer, 1999), and this bacterium has thus become the standard for defining the MEP pathway in other organisms. The MEP pathway is typically represented as a linear sequence of reactions commencing with pyruvate (PYR) and glyceraldehyde 3-phosphate (GA3P) as substrates with a condensation to 1-deoxy-D-xylulose 5-phosphate (DXP) leading to the synthesis of IPP and DMAPP, and is usually assumed to involve an IPP isomerase for IPP and DMAPP interconversion (Eisenreich et al., 2001; Kuzuyama and Seto, 2003; Rodríguez-Concepción and Boronat, 2002; Rohmer, 1999).

Work in our lab has concentrated on the photosynthetic prokaryote *Synechocystis* PCC 6803, which possesses homologs of all the essential genes for the MEP pathway (Kaneko et al., 1996). These recent studies (Ershov et al., 2000; Ershov et al., 2002) provided evidence that isoprenoid biosynthesis in cells grown photoautotrophically exhibits some major differences from the pathway predicted from *E. coli* as summarized in Figure 2-1. This cyanobacterium does not utilize the predicted MEP pathway substrates PYR and DXP *in vitro*. Instead it utilizes products of photosynthesis as substrates. In addition it was shown that isoprenoid biosynthesis in cultures of *Synechocystis* PCC 6803 was not affected *in vivo* or *in vitro* by fosmidomycin (Ershov et al., 2002), the inhibitor of the key MEP enzyme deoxyxylulose phosphate reductoisomerase. Collectively, these results strongly suggest that alternate substrate paths are used in isoprenoid biosynthesis. In accordance with the data, Ershov et al. (2002) proposed that in this cyanobacterium the linear MEP pathway as defined for *E. coli* is not the primary pathway by which

isoprenoids are synthesized under photosynthetic conditions, but rather that products of the pentose phosphate cycle serve as substrates, and it was hypothesized that they could subsequently enter the MEP pathway downstream of MEP (Figure 2-1, and in Fig. 6 of Ershov et al., 2002). Cunningham et al. (2000) previously found that LytB, which is near the branch point of IPP and DMAPP synthesis, is essential for the survival of *Synechocystis* PCC 6803 and therefore is likely required for the formation of IPP and/or DMAPP. It is generally expected that an IPP isomerase is involved in DMAPP production from IPP (Koyama and Ogura, 1999; Kuzuyama and Seto, 2003; Ramos-Valdivia et al., 1997). However, in previous work with *Synechocystis* PCC 6803, our lab was unable to demonstrate IPP isomerase activity consonant with a lack of type 1 IPP isomerase gene (Kaneko et al., 1996) and it was suggested that DMAPP and IPP are separately generated (Ershov et al., 2000; Ershov et al., 2002) which had been independently also suggested for plant cell cultures (Arigoni et al., 1999). It should be noted that a possible homolog of a type 2 IPP isomerase in *Synechocystis*, similar to the *Streptomyces* type 2 IPP isomerase (Kaneda et al., 2001), was suggested from sequence identity (32%) from the *sll1556* open reading frame (ORF). To test this possibility the knock-out mutant Δ*sll1556* was created and the recombinant protein from this ORF was examined for IPP isomerase activity.

In the current work we report that Sll1556 is an enzyme that does not have IPP isomerase activity, and that the non-lethal *Synechocystis* PCC 6803 mutant was impaired in the utilization of pentose phosphate cycle substrates for isoprenoid biosynthesis *in vitro*. The impairment is correlated with a significant reduction of thylakoid membranes in the mutant and an increase in outer wall components. We

Figure 2-1. The isoprenoid biosynthetic MEP pathway as determined for Escherichia coli beginning with pyruvate plus glyceraldehyde 3-P leading to the formation of methylerythritol 4-P from deoxyxylulose 5-P and through subsequent steps via the LytB enzyme to IPP and DMAPP (Cunningham et al., 2000; Kuzuyama and Seto, 2003; Rodriguez-Concepcion et al., 2000; Rodriguez-Concepcion and Boronat, 2002; Rohdich et al., 2003; Rohmer, 1999; Wolff et al., 2003). Fosmidomycin (white arrow) inhibits the synthesis of methylerythritol 4-P and blocks the growth of the bacterium. Asterisks (*) denote differences in Synechocystis PCC 6803 where pyruvate, deoxyxylulose 5-P, and methylerythritol 4-P do not serve as substrates in vitro; where IPP isomerase activity has not been observed; and where fosmidomycin does not inhibit isoprenoid biosynthesis or growth in the cyanobacterium (Ershov et al., 2002); and the triangle indicates where pentose phosphate cycle substrates may possibly enter downstream of methylerythritol 4-P.


*Pyruvate + Glyceraldehyde-3-P
*Deoxyxylulose-5-P
*Fosm
*Methyl-erythritol-4-P
Pent.
Phos.
Subst.
Diphosphocytidyl-2-methyl-erythritol
Diphosphocytidyl-2-methyl-erythritol-2-P
Methyl-erythritol-2,4-cycloPP
Hydroxy-2-methyl-2-butenyl-4-PP
LytB
IPP
*IPP isomerase
DMAPP
ISOPRENOIDS

suggest that DMAPP and IPP biosynthesis do not involve interconversion by an IPP isomerase (of either type 1 or type 2), and that more than one metabolic path leads to isoprenoid biosynthesis.

Materials and Methods

Cell culture and fractionation

Liquid cultures of the glucose-tolerant strain of *Synechocystis* PCC 6803 (obtained from Wim Vermaas, Arizona State University) were routinely grown at ca. 30ºC in continuous light (15-20 µmol photons/m2/s) with continuous shaking and slow bubbling of 5% CO_2 in air. The parental strain (WT) of *Synechocystis*, referred throughout the manuscript as WT, is the widely used glucose tolerant strain (29). The culture medium BG-11 was supplemented with 5 mM potassium-TES (N-tris (hydroxymethyl) methyl-2-aminoethane-sulfonic acid, pH 8.3).

For *in vitro* assays *Synechocystis* PCC 6803 cells were harvested in the log phase of growth. Cells pelleted by centrifugation were quickly rinsed in 100 mM HEPES/KOH (pH 7.7) and 1 mM DTT, treated with lysozyme (10 mg/ml, 60 min, 37ºC) rinsed in the same buffer, and broken in a French pressure cell (20,000 psi, 4ºC). The supernatant fraction (3-5.5 milligram protein per ml), after centrifugation at 60,000 x *g* for 1 h, was stored at -80ºC for use within less than 3 weeks. An alternate improvement in the procedure was to prepare the 60,000 x *g* supernatant directly from pelleted frozen cells (-80ºC) (0.7 ml), previously rinsed in 100 mM HEPES/1 mM DTT (pH 7.7), and broken (for 30 s with intermittent cooling, 4 times) in buffer with a Mini-Bead beater (Biospec, Products Inc. Bartlesville, OK). Also,

the reaction mixture was pre-incubated with unlabeled IPP for 20 min (37ºC) before addition of ^{14}C-IPP and further incubation and assayed from 0-60 min. The preincubation served to deplete any residual DMAPP that might be present in the cells extracts. It should be noted that the results were essentially the same as with the previous procedure (Ershov et al., 2002), except that variation in activity with time of stored supernatant was eliminated, as was a small stimulation from residual MEP previously observed (as in Figure 3 of Ershov et al., 2002).

Isoprenoid biosynthesis with radiolabeled IPP incorporation

The incorporation of [^{14}C]IPP into the acid labile fraction of a petroleum ether extract (Ershov et al., 2002) was used as an indirect assay for DMAPP synthesis in extracts of *Synechocystis* PCC 6803 cells. The reaction mixture was composed of the cell-free supernatant fraction (60,000 x *g*) with 100 mM HEPES/KOH (pH 7.7), 5 mM glutathione, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 500 μM ATP, 250 μM CTP, 100 μM thiamine-PP, 10 μM coenzyme B_{12} (5'-deoxyadenosyl-cobalamine), 1 mM NADPH, 500 μM NADP, and 1 mM FAD. Each incubation was carried out in a total volume of 1 ml with a final concentration of 8.5 μM [1-^{14}C]IPP (Amersham) [and 8.25x10^5 dpm/ml] at 37ºC. Following a 20 min pre-incubation with 3.0 μM IPP, [^{14}C]IPP and compounds tested were added individually, or in combination: DMAPP - dimethylallyl diphosphate (2 μM), DXP – 1-deoxy-D-xylulose 5-phosphate (500 μM) (Echelon Research Labs. Inc., Salt Lake City, UT), GA3P – glyceraldehyde 3-phosphate (1 mM), FR6P – fructose 6-phosphate (500 μM), GL6P – glucose 6 phosphate (500 μM), MEP - 2-*C*-methyl-D-erythritol 4-phosphate (500 μM) (Echelon

Research Labs. Inc.), and PYR – pyruvate (500 μM). Aliquots of 0.2 ml (0.2 to 0.46 mg protein) were assayed for radioactivity.

The incorporation of [^{14}C]IPP into allylic prenols was verified by extraction into petroleum ether (b.p. 55-110ºC) after hydrolysis (0.5 N HCl, 37ºC, 20 min) as in Ershov et al. (2000, 2002), and 1ml of each extracted sample was counted in 10 ml ScintiSafe Econo 2 cocktail (Fisher Scientific). Verification that [^{14}C]IPP was incorporated into isoprenoids had been previously established by reversed-phase column chromatography (Ershov et al., 2000). Each 0.5 ml sample of the petroleum ether extract was applied to a silica gel 60, RP-18 (EM Industries) column (24 x 1 cm), previously equilibrated in, and then eluted with 100% acetonitrile. The following alcohols served as calibration standards: isopentenyl alcohol (C_5), geraniol (C_{10}), linalool (C_{10}), farnesol (C_{15}), nerolidol (C_{15}), and geranyl geraniol (C_{20}) (Aldrich). Controls for phosphatase activity showed that the petroleum ether extract had very few counts without acid hydrolysis, indicating little or no phosphatase activity. Furthermore, the ^{14}C incorporation into isoprenoids was time course dependent where longer chains, i.e. > C_{10} became predominant with increased time of incubation (data not shown).

Cloning and disruption of *Synechocystis* sp. PCC 6803 gene *sll1556*

The nucleotide sequence of *Synechocystis* sp. PCC6803 open reading frame *sll1556* (Kaneko et al., 1996) was obtained at Cyanobase (http://www.kazusa.or.jp/cyanobase/). Oligonucleotide primers sll1556N (gagaggatccatggatagcacccccaccgtaag; the initiation codon is underlined) and

sll1556C (tcgtcaaccagagcaaaatgtc) were designed, with the assistance of the program Primer3 (Rozen and Skaletsky, 2000) to amplify the entire ORF and introduce *Nco*I and *BamH*I sites at the N terminus. Genomic DNA was extracted from *Synechocystis* as earlier described (Williams, 1988). The PCR was performed using an MJ Research (Waltham, MA) PTC-150-25 MiniCycler with heated lid. The Advantage-HF2 PCR kit (Clontech Laboratories, Inc.) was used in a reaction volume of 50 µl in 100 µl thin-walled tubes. After an initial denaturation for 1 min at 94°C, amplification was for 30 cycles at 94/60/68°C for 10/60/150 s with a final extension for 10 min at 68°C. The amplified *sll1556* PCR product was purified by extraction with phenol/chloroform, precipitated with sodium acetate and ethanol, washed with 70% ethanol, dried, and resuspended in TE buffer (Sambrook et al., 1989). After digestion with *BamH*I, a 1.1 kB fragment containing *sll1556* was purified by gel electrophoresis (1% agarose), recovered using the Geneclean kit (Bio 101, Inc., Carlsbad, CA), and cloned in frame in the *Bgl*II and *Pvu*II sites of plasmid vector pBAD/His-B (Invitrogen) to give plasmid pBAD/His-sll1556. The *sll1556* insert was sequenced to confirm the reading frame and the absence of mutations introduced by PCR.

A kanamycin-resistance gene, originally obtained from *Tn*903, was excised as a 1.3 kB *Eco*RV-*Eco*RV fragment and cloned into the *Msc*I site within *sll1556* (Figure 2-2) to give plasmid pBAD/His-sll1556Kan. This plasmid was linearized by digestion with *Eco*RI (a site present in the multiple cloning site of the vector), and then extracted with phenol/chloroform, precipitated and resuspended in TE as described above. This linearized plasmid preparation was used to transform

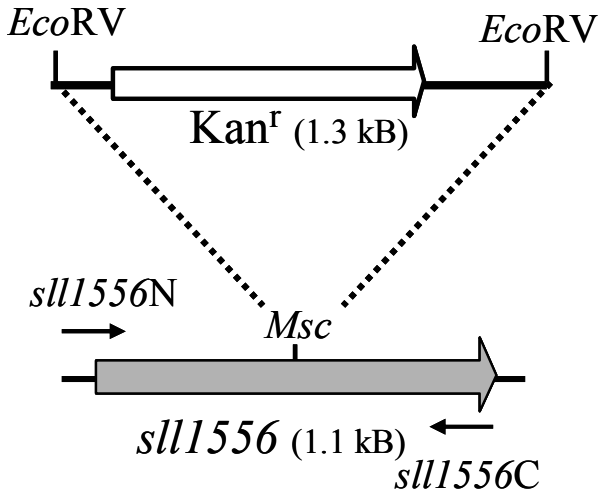
Synechocystis PCC 6803, essentially as described by Williams (1988). Segregation of the *sll1556*::Kan^r (*Δsll1556*) mutant was confirmed by PCR (Figure 2-2) using the primers and reaction conditions described above.

Expression and purification of proteins

E. coli strain Rosetta (Novagen) containing plasmid pBAD/His-sll1556 (see above) was grown in LB medium supplemented with ampicilllin (150 μg/ml) at 30°C at 300 cycles/min shaking until the culture reached an A_{600} of approximately 0.5. At this point 20% arabinose was added to give a final concentration of 0.2%. After 16 h of further growth at 20°C, the culture flask was chilled in ice water and cells were harvested at 500 x *g* for 10 min at 4°C. Well-drained cell pellets were frozen at -80°C. The recombinant protein was also produced using growth at 30°C with 3 h induction in Top10 cells (Invitrogen).

For production of recombinant *Streptomyces* type 2 IPP isomerase, conditions were the same except that IPTG (2mM final concentration) was used as the inducer, with cells grown at 20°C for 16 h before harvest. Cells contained the plasmid pQCLD41 (Kaneda et al., 2001).

Recombinant proteins were purified after cell breakage by sonication, and eluted from a Ni-NTA column with 100 mM imidazole Tris-HCl pH 8.0. Alternatively, cells were extracted with the B-PER® bacterial protein extraction reagent (Pierce Biotechnology, Co.) and the recombinant protein was purified on Ni-NTA mini columns (Qiagen, Chatsworth, CA) by elution with buffered 125 mM imidazole (50 mM phosphate, 300 mM NaCl, 1 mM DTT, pH 8.0). In Figure 2-3 are



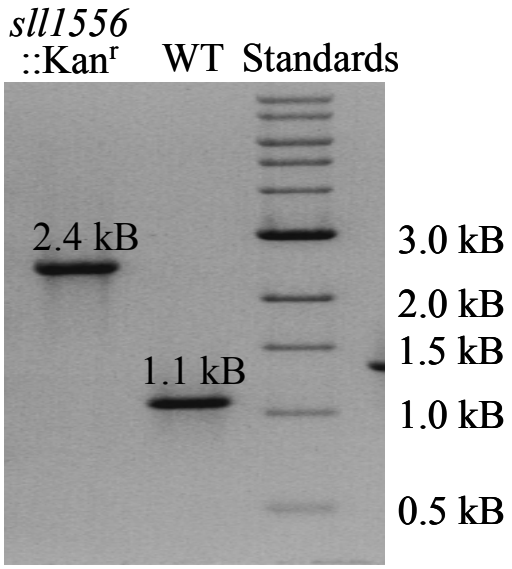


Figure 2-2. Upper: Schematic illustration of *Synechocystis* PCC 6803 genomic DNA encompassing gene sll1556 (gray arrow). A kanamycin-resistance gene (Kan^r) (white arrow) was inserted in the *Msc*I site to inactivate the gene. Lower: agarose gel electrophoresis of PCR products obtained using genomic DNA from the wild type strain (WT) and mutant *sll1556*::Kan^r indicates complete segregation of the mutant (*i.e.* all gene copies have been inactivated).

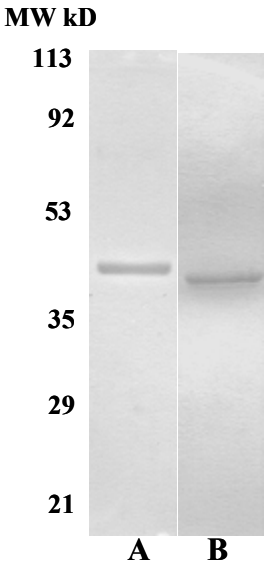


Figure 2-3. SDS-PAGE of His-tagged recombinant proteins purified on Ni-NTA columns and stained with Coomassie blue**:** (A) Sll1556 protein (ca. 41.6 kD) of *Synechocystis* strain PCC 6803, and (B) type 2 IPP isomerase (37 kD) of *Streptomyces* sp. strain CL190 (B). Molecular weight range as indicated on the left.

shown the purified proteins on SDS-PAGE after Coomassie blue staining. The size estimates of 41.6 kDa for Sll1556 of *Synechocystis* PCC 6803 and of 37 kDa for the type 2 IPP isomerase of *Streptomyces* sp. strain CL190, using molecular weight markers (Low Range) from Bio-Rad Labs., are consistent with the expected molecular weights (including the His-tag).

Enzymatic activity tests

(i) IPP isomerase assay. For the type 2 IPP isomerase assay the procedure developed by Kaneda et al. (2001) was used. Assays were conducted with FAD plus and minus NADPH, or with FMN plus and minus NADPH. Thus, the requirement for FAD (or FMN) and NADPH was confirmed. Briefly, the activity was measured by [^{14}C]IPP incorporation into the petroleum either layer, dependent on the acid lability of DMAPP as above. Each reaction at 37ºC (0.5 ml final volume) contained 8.5 μM [^{14}C]IPP, 100 mM HEPES (pH 7.7), 2 mM DTT, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM NADPH, 1 mM FAD (or FMN) and recombinant protein (over a range of 0.1-50 μg/0.5ml). The standard assay developed for the type 1 IPP isomerase was done according to Spurgeon et al. (1984) to ascertain activity of the recombinant proteins with and without cell supernatants. The results with Sll1556 were negative, consistent with earlier results on cell extracts (Ershov et al., 2000; Ershov et al., 2002).

(ii) Glycolate oxidase. The Sll1556 protein was tested for glycolate oxidase activity by two separate methods. The first was according to Zelitch (1955) involving the loss of color at 620 nm of 2,6-dichlorophenylindophenol (DCPIP). The oxidase

assay in 1.0 ml (final volume) contained 30 mM potassium phosphate buffer (pH 8.0), 0.003% DCPIP, +/- 5 mM FMN, 42 mM imidazole, 30 mM potassium cyanide, and Sll1556 protein (ca. 3µg). After a 20 min. incubation the validity of the glycolate oxidase assay was verified by addition of spinach glycolate oxidase (E.C.1.1.2.15) (ca. 5 ug). Phenylhydrazone formation was used for the second assay by measuring the increased optical density at 324 nm. Reaction conditions were as above, except that 3 mM phenylhydrazine replaced DCPIP and cyanide, and the assay validity was verified by addition of glyoxylic acid.

(iii) Lactate dehydrogenase. The Sll1556 protein was tested for lactate dehydrogenase activity by the decrease of optical density at 340 nm by modifying the procedure of Kornberg (1955). A 1.0 ml incubation mixture contained 30 mM Tris-HCl, 5 mM $MgCl_2$, with or without 5 mM FMN, 50 mM pyruvic acid, 1 mM NADH (or NADPH) and 1 mM DTT and Sll1556 protein (ca. 3µg). Lactate dehydrogenase in NADH (rabbit muscle E.C. 1.1.1.27) was added to validate the incubation conditions.

Protein concentrations were determined as in Ershov et al. (2002) by the bicinchoninic acid assay, and the Micro BCA protein assay (Pierce Biotechnology, Co.) with bovine serum albumin as standard. Except where otherwise indicated, the chemicals used in this study were purchased from Sigma Chemical Co.

Electron microscopy

Cells from the log phase of growth, rinsed in 0.25 M phosphate buffer (pH 7.0), were fixed in 2.0% phosphate-buffered glutaraldehyde (2 h) and rinsed several

times in buffer. The secondary fixation in 1% OsO_4 (2 h) was also followed by several phosphate buffer rinses. For transmission electron microscopy cells were dehydrated in ethanol and propylene oxide before embeddement in Epon. Sections were stained with 1% uranyl acetate and lead citrate and examined in a Zeiss EM10 CA.

For scanning electron microscopy cells were fixed in glutaraldehyde as above and were then collected on a Nucleopore (0.5 μm) filter, dehydrated in ethanol (75%-100%) for critical point drying and shadowed with gold-palladium in a Denton Vacuum DCP-1 apparatus for examination with a Hitachi S-4700 scanning electron microscope.

Multiple sequence alignment and construction of neighbor-joining tree

The predicted amino acid sequences of bacterial type 2 IPP isomerases and polypeptides of undetermined function related to these enzymes were aligned using the program Clustal X (Thompson et al., 1997) with the Blosum series weight matrix. All parameters were set at their default values. A Neighbor-Joining tree (Saitou and Nei, 1987) was constructed from the alignment, with gaps excluded from the analysis and correction made for multiple substitutions (Kimura , 1980). One thousand bootstrap trials were carried out with a random number generator seed of 111. The tree was displayed using the program NJplot (Perrière and Gouy, 1996).

Results

Sll1556 does not exhibit IPP isomerase activity *in vitro*

The gene *sll1556* of *Synechocystis* PCC 6803 was identified as a probable type 2 IPP isomerase based on a 32% a.a. sequence identity with a homologous gene of *Streptomyces* (Kaneda et al., 2001). In fact, Kaneda et al. (2001) were the first to identify the FMN- or FAD- plus NADPH requiring novel type 2 IPP isomerase, that lacks homology with the well characterized IPP isomerase (type 1) (Hahn et al., 1999; Ramos-Valdivia et al., 1997; Spurgeon et al., 1984). The type 2 IPP isomerase activity has also been shown in *Bacillus subitilis* (Steinbacher et al., 2003) and in *Staphylococcus aureus* (Kaneda et al., 2001) (Table 2-1). In several protein databases the ORF *sll1556* is annotated as IPP isomerase type 2, or as FMN-dependent lactate dehydrogenase. Genes related to *sll1556* of *Synechocystis* PCC 6803 are also found in a number of other cyanobacteria. However, notably absent are predicted homologs in three strains of *Prochlorococcus marinus* (SS 120, MED 4, MIT 9313) as well as in *Synechococcus* sp. WH 8102 and *Gloeobacter violaceus* PCC 742 (Table 2-1). To our knowledge, activity for neither IPP isomerase (type 2) nor lactate dehydrogenase has been functionally demonstrated for Sll1556 nor for a related protein in any of the cyanobacteria listed in Table 2-1. This prompted us to first assay the IPP isomerase activity of the expressed Sll1556 recombinant protein of *Synechocystis* PCC 6803, and to examine the consequences of inactivating this gene.

IPP isomerase activity could not be demonstrated (Figure 2-4) with the recombinant Sll1556 protein purified on Ni-NTA columns (Figure 2-3) under the conditions used for the homologous *Streptomyces* gene product. Yet, as seen in

Organism	Type 1	Type 2	Source reference or database[a]
Organisms with IPP isomerase function demonstrated:			
Escherichia coli	+		Hahn et al., 1999
Bacillus subtilis		+	Steinbacher et al., 2003
Streptomyces sp. CL190		+	Kaneda et al., 2001
Staphylococcus aureus		+	Kaneda et al., 2001
Cyanobacteria with genes related to type 2:			
Anabaena (Nostoc) PCC 7120		gi:17133727 (all4519)	Kazusa DNA Research Inst., Japan
Nostoc punctiforme PCC 73102		gi:23128293	DOE Joint Genome Institute, USA
Synechocystis PCC 6803		gi:2829616 (sll1556)	Kazusa DNA Research Inst., Japan
Synechococcus elongatus PCC 7942		*Draft genome prediction*	*DOE Joint Genome Institute, USA*
Thermosynechococcus elongatus ***BP-1***		***gi:22295127 (tll1403)***	***Kazusa DNA Research Inst., Japan***
Trichodesmium erythraeum BP-1		gi:23041526	DOE Joint Genome Institute, USA
Cyanobacteria lacking type 2-related genes:			
Gloeobacter violaceus PCC 742			Kazusa DNA Research Inst., Japan
Prochlorococcus marinus SS 120			Genoscope, France
Prochlorococcus marinus MED 4			DOE Joint Genome Institute, USA
Prochlorococcus marinus MIT 9313			DOE Joint Genome Institute, USA
Synechococcus sp. strain WH 8102			

Table 2-1. Demonstrated IPP isomerase function in bacteria and genes (by gene bank identification numbers) related to IPP isomerase in cyanobacteria

[a]Kazusa DNA Research Institute, Kazusa, Japan; DOE Joint Genome Institute, Walnut Creek, Calif.; Genescope, Evry, France. USA, United States of America

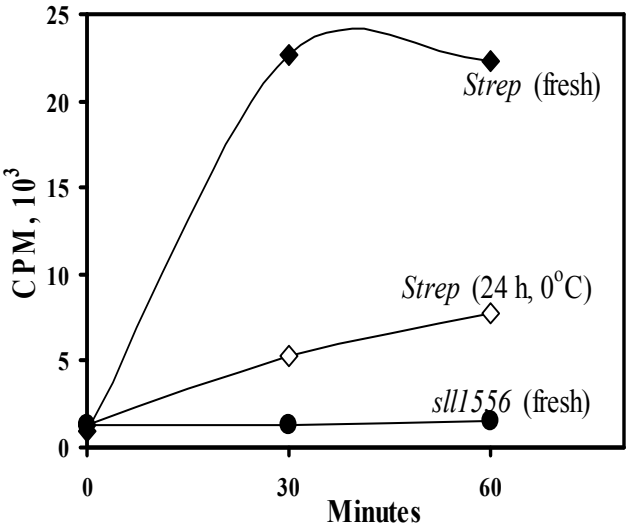


Figure 2-4. [^{14}C]IPP conversion to DMAPP (as in Methods) by IPP isomerase type 2 (of *Streptomyces* sp. strain CL190), but not by the Sll1556 protein (of *Synechocystis* strain PCC 6803). Freshly purified recombinant Sll1556 protein of *Synechocystis* strain PCC 6803 (1.0 µg/500 µl) (●). *Streptomyces* sp.: freshly purified enzyme (0.57 µg/500 µl) (♦); and stored 24 h, 0ºC (1.14 µg/500 µl) (◊). The Sll1556 protein lacks IPP isomerase activity, whereas freshly purified *Streptomyces* sp. strain CL190 protein is clearly active and this activity declined upon storage. Incubation mixture: 8.5 µM [^{14}C]IPP, 100 mM HEPES, 2 mM DTT, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM NADPH, 1 mM FAD, pH 7.7 (37ºC).

Figure 2-4, IPP isomerase activity was obtained with the recombinant protein of *Streptomyces* consistent with the previous report (Kaneda et al., 2001). These results confirm the initial observation that the type 2 isomerase requires FAD (or FMN) and NADPH (data not shown). The recombinant enzyme of *Streptomyces* was very active initially but declined over time at 0ºC (Figure 2-4), and its activity was totally abolished by freezing with or without 50% glycerol. Numerous alternate purification conditions were also tried in isolating the protein including breakage of *E. coli* cells by sonication, decreasing or omitting DTT, Tris HCl pH 7.9 buffer substitution, FMN presence throughout, and varying the protein concentration, but in no case was IPP isomerase activity found for the *sll1556* gene product. It was concluded that if this gene product is an IPP isomerase it must require conditions as yet unknown, or that the protein has a different function.

Pentose phosphate cycle substrate stimulation of isoprenoid biosynthesis is impaired in the Δ*sll1556* mutant

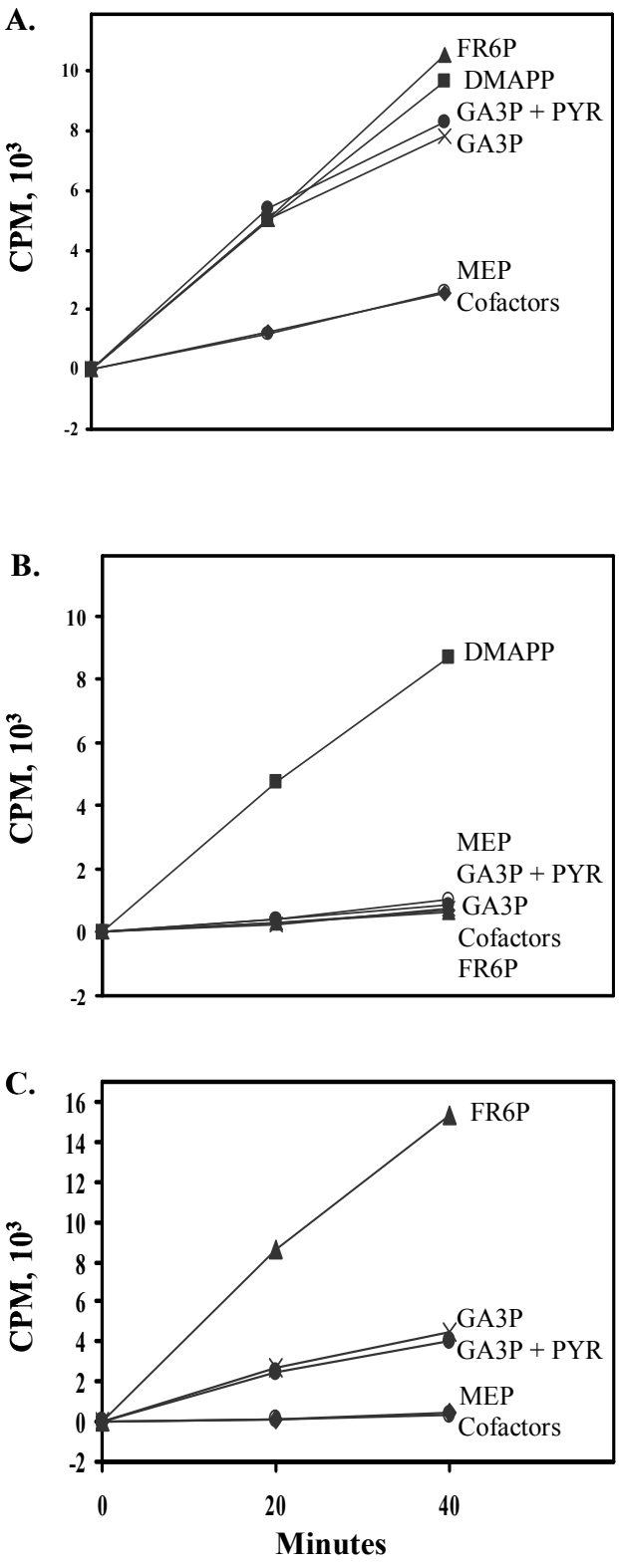
If the *sll1556* gene product is involved in isoprenoid biosynthesis then this should be reflected in the mutant where the gene has been inactivated (as in Figure 2-2). Isoprenoid biosynthesis, requiring both DMAPP and IPP for formation of compounds of C_{10} and greater, was measured *in vitro* for WT and Δ*sll1556* as in Ershov et al. (2002). Basically, the [^{14}C]IPP incorporated in the cell supernatant extract (60,000 x *g*) is retrieved in the petroleum ether fraction (after acid hydrolysis). Analysis by reverse phase chromatography verified that the labeled isoprenoids were compounds of C_{10} and greater (Ershov et al., 2000) (data not shown). This

incorporation into isoprenoids larger than C_5, together with the lack of IPP isomerase activity, indicates DMAPP production. Furthermore, stimulation of isoprenoid biosynthesis was obtained by the addition of exogenous DMAPP with both WT and Δ*sll1556* mutant supernatantt (Figure 2-5A and B, respectively). Hence, we equate [^{14}C]IPP uptake with DMAPP production under our *in vitro* conditions. Also, in WT supernatant [^{14}C]IPP stimulation was observed with pentose phosphate cycle intermediates (Figure 2-5A.), in addition to stimulation by DMAPP. Typical MEP pathway intermediates such as MEP, PYR, and DXP (not shown) did not stimulate [^{14}C]IPP incorporation above that obtained with the incubation cofactor mixture alone in WT. Pentose phosphate cycle intermediates (such as GL6P, FR6P) which may feed into the MEP pathway downstream (or possibly at alternate sites), were again active as had been previously shown in WT (Ershov et al., 2002).

However, with the Δ*sll1556* mutant there was no stimulation of isoprenoid synthesis with FR6P, GA3P, MEP, or GL6P (not shown), nor by the cofactor mixture alone (Figure 2-5B.) indicating a lack of DMAPP production from these substrates. These results clearly imply that the missing factor, probably an enzyme, is required for incorporation of these pentose phosphate cycle components into the formation of a pool of isoprenoids.

The stimulation of pentose phosphate cycle substrates could be restored upon addition of the Sll1556 protein to the mutant Δ*sll1556* supernatant as seen in Figure 2-5C. The inferred production of DMAPP, as indicated by ^{14}C-IPP incorporation, occurred with GA3P, FR6P and GL6P (not shown) but not with MEP or the Sll1556 protein alone. In fact, the restoration of activity with FR6P, under the same

Figure 2-5. [^{14}C]IPP incorporation into the isoprenoid fraction of the supernatant of wild type is stimulated by FR6P and GA3P, indicating DMAPP production (A.). In the supernant of the *sll1556* -mutant the same substrates failed to stimulate [^{14}C]IPP incorporation (B.). Reconstitution of activity by addition of Sll1556 protein, 0.15 μg/ml final concentration in reaction mixture (C.). FR6P (500 μM) (▲), DMAPP (2 μM) (■), GA3P (1 mM) (**X**), GA3P (1 mM) plus PYR (500 μM) (●), MEP (500 μM) (○), and incubation cofactor mixture (♦) (500 μM ATP, 250 μM CTP, 100 μM thiamine PP, 1 mM NADPH, 500 μM NADP, 1mM FAD, 5 mM glutathione, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 10 μM coenzyme B_{12}, 100 mM HEPES/KOH pH 7.7) incubated at 37ºC. Incorporation with radiolabeled [^{14}C]IPP (8.5 μM) followed pre-incubation with IPP (3 μM) (Materials and Methods).


A.
CPM, 10³
10
8
6
4
2
0
-2
FR6P
DMAPP
GA3P + PYR
GA3P
MEP
Cofactors
B.
CPM, 10³
10
8
6
4
2
0
-2
DMAPP
MEP
GA3P + PYR
GA3P
Cofactors
FR6P
C.
CPM, 10³
16
14
12
10
8
6
4
2
0
-2
FR6P
GA3P
GA3P + PYR
MEP
Cofactors
0
20
40
Minutes

experimental conditions was equivalent to, or greater, than that obtained with the WT (with addition of 2.0 μM DMAPP/0.5 mM FR6P). The Sll1556 protein is likely an enzyme as judged by the increased activity upon higher Sll1556 concentrations (not shown), and because heating of the protein (2 min,100ºC) destroyed the activity. The nature of this apparent enzyme was subsequently further investigated.

Other suggested Sll1556 enzyme functions

There is little doubt that the Sll1556 protein has activity as evident from the reconstitution experiments (Figure 2-5C), which suggests that the protein influences another route to DMAPP production and isoprenoid biosynthesis. A BlastP search suggests that the *sll1556* gene product is functionally similar to FMN dependent alpha-hydroxy acid dehydrogenases, as well as IPP isomerase (type 2). Glycolate oxidase and lactate dehydrogenase are suggested albeit with rather low similarities. Hence, their substrate utilization was assayed by standard procedures (see Materials and Methods) with enzymes of known function to verify the assay conditions. Glycolate oxidase activity could not be confirmed, nor could activity for lactate dehydrogenase. At this point the functional identity of Sll1556 remains to be determined.

The Δ*sll1556* mutation affects membrane development

A comparison of the cell morphology of WT *Synechocystis* PCC 6803 and the Δ*sll1556* mutant was made using cultures in the log phase of growth and of similar cell density (Table 2-2). Cells of the WT, when grown at moderate light condition

Table 2-2. Comparison of *Synechocystis* PCC 6803 wild type and Δ*sll1556* mutant

	WT	Δ*sll1556* mutant
Cell diameter (μm):	1.75 ± 0.1 (SD)	1.53 ± 0.1 (SD)
No. of thylakoids/section	9-11	5-8
Outer wall:	Smooth	More fibrous
IPP isomerase:	Deficient	Deficient
Isoprenoid production *in vitro*:	Pentose phosphate cycle substrate stimulation	No pentose phosphate cycle substrate stimulation

(20 μmol/m^2s) are typically coccoid in shape except during division when the daughter cells are oblong (Figure 2-6). In scanning electron micrographs the cell diameter is 1.53 ± 0.12 μm for mutant cells, and 1.75 ± 0.11 μm for WT cells. The outer wall layer of the mutant is considerably more fibrous than that of the WT. These fibrous extensions may be elongated stretches of outer wall layers. They are more coarse than fimbriae (Vaara and Vaara, 1988), but could be related to some of the coarser pili observed in *Synechocystis* PCC 6803 (Bhaya et al., 2000; Yoshimura et al., 2002). Negatively stained images of whole mounts of fixed cells did not reveal pili on either WT or mutants which does not rule out their absence. Thylakoid membranes in sectioned cells (Figure 2-7) tended to be peripherally arranged but could also fill the cell center. Also common were discontinuations of thylakoid membranes near the cell wall, suggestive of recent cell divisions. In the mutant there were fewer thylakoids (ca. 6.8) per central cell section than in WT (ca. 9.4). Mutant and WT cultures had about the same growth rate at 20 μmol photons/m^2/s, suggesting that the lesser thylakoid content in the mutant was not a limiting factor.

Discussion

That the Sll1556 protein is not an IPP isomerase (type 2) is not proven by the inability to demonstrate such an activity *in vitro*, although the activity of the *Streptomyces* homolog was confirmed (Figure 2-4). With an amino acid identity of only 32% it must be considered that the function of Sll1556 may not be that of an IPP isomerase. A BlastP analysis together with searches of databases of cyanobacterial genomes revealed a number of gene sequences related to *sll1556* as a putative IPP isomerase

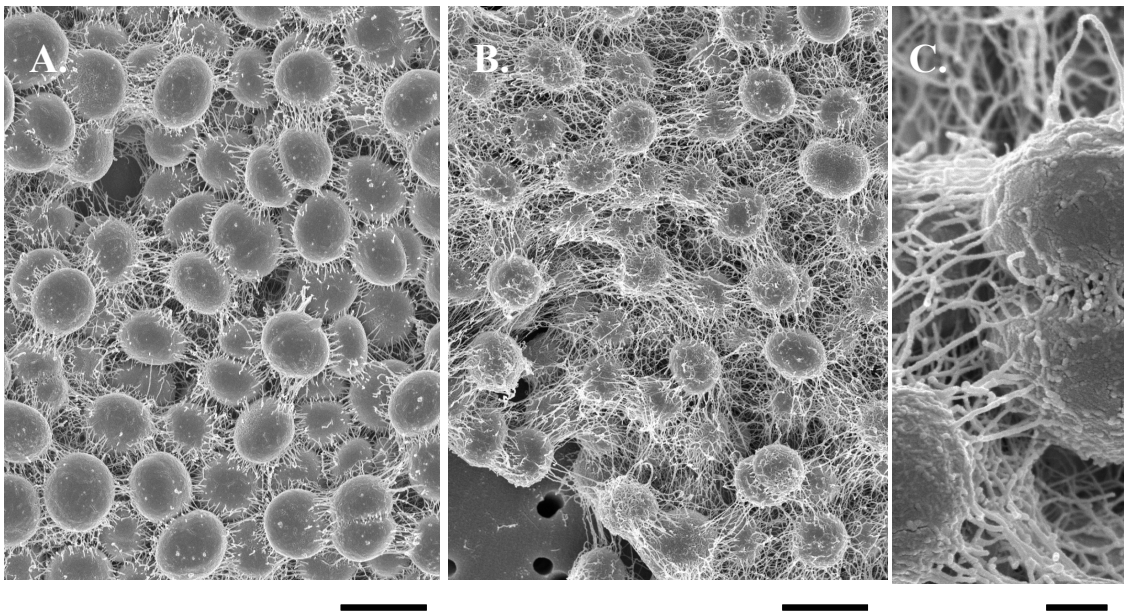


Figure 2-6. Scanning electron micrographs of *Synechocystis* PCC 6803 wild type (A.) and Δ*sll1556* mutant cells (B.) from log phase cultures (as is evident by several new daughter cells that are kidney-shaped). The wild type cells have a relatively smoother outer surface, and the mutant cell surfaces have greater fibrous extensions shown at greater magnification in C. Scale bar: A. & B. 2 μm, C. 0.15 μm.

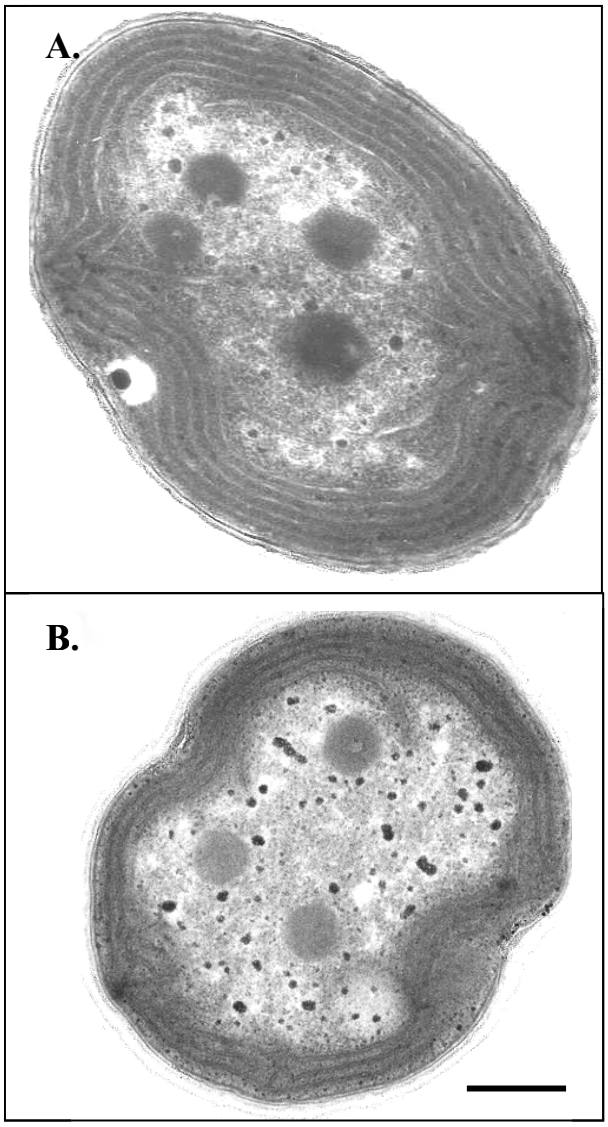


Figure 2-7. Transmission electron micrographs of a *Synechocystis* PCC 6803 wild type cell (A.) and a Δ*sll1556* mutant cell (B.). Thylakoid density in Δ*sll1556* mutant cells was typically less than in wild type cells. Scale bar: A. & B. 0.3 µm

type 2 (Table 2-1), but as noted above a functional confirmation for purified gene products is lacking. Activities suggested from gene sequence annotations, such as glycolate oxidase and FMN-dependent lactate dehydrogenase were also not obtained. Sequence comparisons with Sll1556 indicate that homologous proteins similar to that in *Synechocystis PCC* 6803 are present in five other cyanobacteria: *Anabaena (Nostoc)* PCC, *Nostoc punctiforme* PCC 73102, *Synechococcus elongatus* PCC 7942, *Thermosynechococcus elongates* BP-1, and *Trichodesmium erythraeum* BP-1. For the species listed in Table 2-1 and shown in the tree (Figure 2-8) the *sll1556* related gene similarities are ca. 30% between bacteria and cyanobacteria, and ca. 50% among cyanobacteria. Many of these were designated as IPP isomerases in databases, but the functional annotation can only be considered valid for the three bacterial species where type 2 IPP isomerase has been functionally verified (Kaneda et al., 2001; Steinbacher et al., 2003). Even though a meaningful relatedness is suggested from the tree (Figure 2-8), any functional designation for the Sll1556 protein and its probable cyanobacterial homologs (ca. 50% similarity) is at this time premature.

A requirement for an IPP isomerase would only apply if an organism had only one pathway by which IPP and DMAPP are produced, as implied by a linear pathway as assumed for *E. coli* (Figure 2-1). Yet, even in *E. coli* it has been shown that an IPP isomerase is not essential (Hahn et al., 1999). In addition, Kuzuyama and Seto (2003) further reported that a number of bacteria do not posses genes for either type of IPP isomerase, thus indicating considerable diversity.

There is little doubt that the *sll1556* gene product affects isoprenoid biosynthesis *in vitro*. Since the Δ*sll1556* mutant was impaired in inferred DMAPP

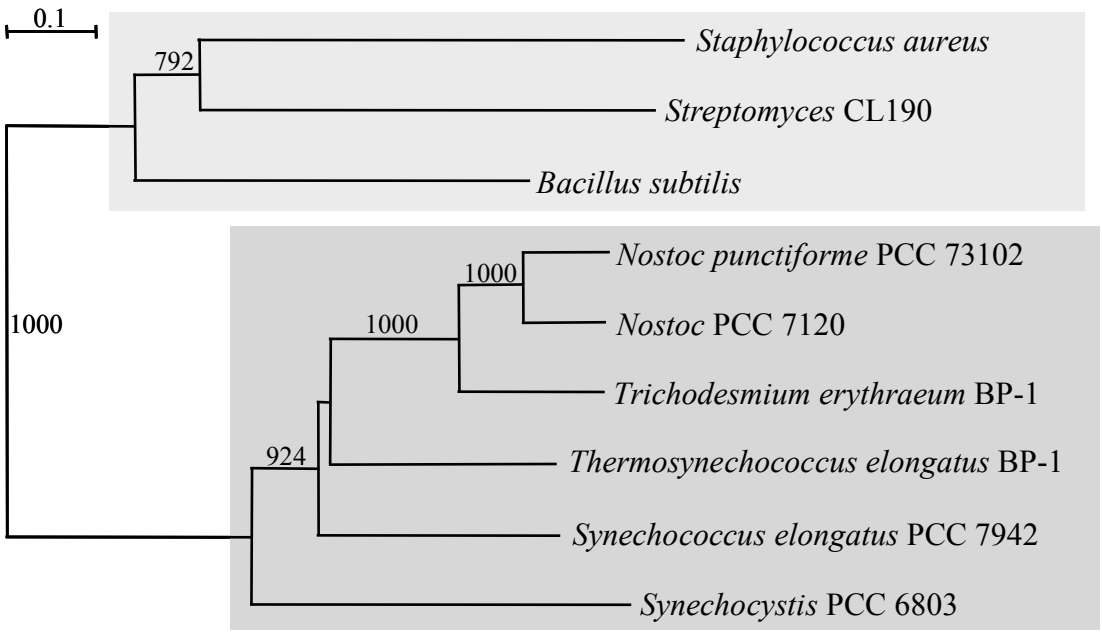


Figure 2-8. Neighbor-joining tree of the known bacterial type 2 IPP isomerases and related polypeptides encoded in cyanobacterial genomes. Type 2 IPP isomerase has been functionally determined only for the top group. Bootstrap values greater than 50% (for 1000 trials) are indicated. See Table 2-1 for accession numbers of the polypeptides.

production by lack of stimulation by FR6P and other pentose phosphate cycle substrates (Figure 2-5B), it can be assumed that it was deficient in an alternate path of isoprenoid production. The rather significant membrane reduction in the mutant implies that this alternate path could be very significant under certain conditions. These results require further exploration of pentose phosphate cycle substrates under varied *in vivo* and *in vitro* conditions to clarify the nature and role of the Sll1556 protein.

The analysis of the wild type *Synechocystis* PCC 6803 strain and the Δ*sll1556* mutant, as summarized in Table 2-2, reveals some notable physiological and morphological phenotypical characteristics. It is well known that hopanoids are present in the membranes (thylakoids, cell membranes, and wall layers) of cyanobacteria (Jürgens et al., 1992; Simonin et al., 1996). Hence, a reduction in thylakoid synthesis in the mutant (Figure 2-7) is readily attributable to reduced isoprenoid biosynthesis, since isoprenoid precursors are essential building blocks of carotenoids, chlorophylls and certain quinones required for construction and assembly of the photosynthetic apparatus. Modifications in normal isoprenoid biosynthesis are also consistent with alterations of the outer wall layers (glycocalyx or pili?), as suggested by the greater fibrous appearance of the outer surface in the mutant (Figure 2-6B & C). It is likely that these combined effects result from a deficiency, but not absence, of isoprenoid biosynthesis.

Under normal photosynthetic conditions the Sll1556 protein is dispensable: the growth of the mutant culture in mineral medium did not differ significantly from that of the WT (at the same light intensity). It is likely that the cells rely more heavily

on an alternate metabolic path in which the Sll1556 protein does not play a critical role. Differences in metabolite utilization can depend on the organism's environment. For example, recently Yang et al. (2002) analyzed the fluxes of central metabolites by following the products from ^{13}C-glucose in *Synechocystis* PCC 6803 under different conditions. They found significant differences of the flow through the pentose phosphate pathway and through the glycolytic path between cells grown heterotrophically vs. photomixotrophically.

This brings us back to the presumption of a linear isoprenoid production pathway via MEP in *Synechocystis* PCC 6803. For *E. coli* there is clear support for the proposed MEP pathway (Kuzuyama and Seto, 2003; Rodríguez-Concepción et al., 2000; Rodríguez-Concepción and Boronat, 2002; Rohdich et al., 2003; Rohmer, 1999; Wolff et al., 2003) (Figure 2-1) for isoprenoid biosynthesis as mentioned in the Introduction. Both organisms have essential genes for the MEP pathway. However, as noted above for *Synechocystis,* the MEP pathway is not the only pathway of isoprenoid biosynthesis under photosynthetic growth conditions. It was previously shown that pentose phosphate cycle substrates lead to isoprenoid biosynthesis (Ershov et al., 2002), which was confirmed here. Furthermore, the current results with the Δ*sll1556* mutant show that in addition to the pentose phosphate cycle substrates there may yet be another path to DMAPP biosynthesis, and perhaps also to IPP synthesis. Although we fully recognize the essential nature of the MEP pathway for the survival of *Synechocystis* PCC 6803, previous and current results lead to the suggestion of alternate paths.

These studies on the *sll1556* mutant have provided further evidence to suggest that isoprenoid biosynthesis in this photosynthetic cyanobacterium is more complex than predicted from *E. coli*. It appears that biosynthesis of isoprenoids in this organism is not linear, but involves more than one pool of substrates and probably at least one alternate path to DMAPP biosynthesis. An alternate route to DMAPP production from pentose phosphate cycle substrates can be metabolically advantageous for a photosynthetic organism at optimal growth conditions where an increased supply of isoprenoids would enhance thylakoid and cell wall synthesis.

CHAPTER 3

In vitro Pentose Phosphate Cycle Substrate Stimulation of C_5-C_{20} Isoprenoid Biosynthesis in *Synechocystis* PCC 6803

Summary

In cyanobacteria and chloroplasts the 2-*C*-methyl-*D*-erythritol-4-phosphate (MEP) pathway is the primary path by which isoprenoid precursors are synthesized, with LytB as the final enzyme in the pathway leading to IPP and DMAPP. In the cyanobacterium *Synechocystis* PCC 6803, we show with [^{14}C]IPP a progression of isoprenoid production (C_5 to C_{10} to C_{20}) *in vitro* that is stimulated by pentose phosphate cycle (PPC) compounds, although radiolabeled glucose 6-phosphate, glyceraldehyde 3-phosphate, or fructose 6-phosphate do not directly serve as substrates. This isoprenoid synthesis activity was unimpaired when LytB was immunodepleted from the supernatant; therefore, LytB catalysis is not likely to contribute to the observed *in vitro* isoprenoid synthesis. This chapter has been prepared for submission to the journal FEBS Letters.

Introduction

Isoprenoids constitute a diverse class of natural compounds found in all organisms. In plants, algae, and cyanobacteria, isoprenoids are involved in photosynthesis, electron transport, and membrane structure (Lichtenthaler, 1999). Although quite varied in their functions and structures, all isoprenoids are derived

from one or a combination of two five-carbon compounds (C_5), isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Isoprenoids are grouped according to the number of C_5 units they contain. Chain elongation generally occurs through a head to tail condensation of IPP to DMAPP, as is the case in C_{10}, C_{15}, and C_{20} production, which are catalyzed by enzymes called prenyltransferases (Koyama, 1999).

In plant chloroplasts, cyanobacteria, and many eubacteria, the C_5 building blocks are synthesized by the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Rohmer, 1999). In photosynthetic organisms, the MEP pathway is largely responsible for the production of the IPP and DMAPP used to synthesize carotenoids, the phytol side chain of chlorophyll, plastoquinone, and numerous mono- and di-terpenes (Rohmer, 1999; Eisenreich et al., 2004; Bouvier et al., 2005). Much of the MEP pathway has been elucidated using the bacterium *Escherichia coli* as a model organism (reviewed in Rodriguez-Concepcion and Boronat, 2002). In the MEP pathway, pyruvate and glyceraldehyde 3-phosphate condense to form 1-deoxy-D-xylulose 5-phosphate (DXP), which through a linear sequence of reactions is eventually converted to both IPP and DMAPP through the action of LytB, an essential enzyme that catalyzes the terminal step of the pathway. An IPP isomerase further interconverts IPP and DMAPP (Kuzuyama and Seto, 2003; Barkley et al. 2004a). In *E. coli*, however, this enzyme was found not to be essential (Hahn et al., 1999).

Synechocystis strain PCC 6803 is a photosynthetic cyanobacterium that possesses homologs of all of the genes of the MEP pathway (Kaneko et al., 1996).

However, previous work from our laboratory has yielded results contrary to a simple linear pathway to IPP and DMAPP. Fosmidomycin, a potent inhibitor of MEP synthesis (Kuzuyama et al., 1998; Zeidler et al., 1998), has no affect on the viability or growth rate of *Synechocystis* strain PCC 6803 cells grown photoautotrophically and does not affect isoprenoid synthesis *in vitro* (Ershov et al., 2002). Also, additions of pathway substrates and intermediates such as pyruvate and DXP have not yielded any stimulation of isoprenoid production *in vitro* (Ershov et al., 2002). In contrast, phosphorylated photosynthetic metabolites of the pentose phosphate cycle (PPC) greatly stimulate isoprenoid biosynthesis *in vitro*. Based on these observations, we hypothesized that PPC compounds served as alternate substrates for IPP and/or DMAPP synthesis, perhaps feeding into the MEP pathway downstream of MEP (Ershov et al., 2002; Poliquin et al., 2004).

Here, we examine the progression of PPC-stimulated isoprenoid biosynthesis *in vitro*, and show that depletion of LytB, the terminal MEP pathway enzyme, has no significant effect on isoprenoid production under the conditions employed. These results provide further insight into PPC substrate stimulated isoprenoid synthesis in *Synechocystis* and can provide an approach useful for studies on the regulation of IPP/DMAPP metabolic adjustments or in controlling prenyltransferase activities *in vitro*.

Materials and methods

Cell culture and fractionation

Synechocystis strain PCC 6803 (obtained from Wim Vermaas, Arizona State University) was grown in liquid cultures in continuous light (20-25 μmol/m^2/s) with shaking and slow bubbling with 5% CO2 in air at 30ºC. The culture medium used for this glucose-tolerant strain was BG-11 supplemented with 5 mM potassium-TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 8.3]. All chemicals used in this study were purchased from Sigma Chemical Co. unless otherwise noted.

In vitro assays were conducted with *Synechocystis* strain PCC 6803 cells harvested in the log phase of growth. Cells were pelleted by centrifugation, rinsed with 100 mM HEPES/KOH-1 mM dithiothreitol (DTT) (pH 7.7), and frozen at -80°C in 1 L or 0.7 mL aliquots. Cells were thawed on ice and broken (4 x 30 s with intermittent cooling) using a Mini-Bead Beater (Biospec Products Inc., Bartlesville, Okla.). The breakate was diluted approximately 5-fold and centrifuged 1 h at 60,000 x g at 4°C. The resulting supernatant (3 to 5 mg protein/mL) was kept on ice for immediate use.

Incorporation of radiolabeled IPP as a measure of isoprenoid synthesis

[^{14}C]IPP incorporation into compounds extractable with petroleum ether after acid hydrolysis (i.e. allylic diphosphates) (as in Poliquin et al., 2004) was used as an indirect assay of DMAPP synthesis in cell-free extracts of *Synechocystis* strain PCC 6803. The reaction mixture contained the 60,000 x g supernatant (1.3 to 1.8 mg

protein/ml) with 100 mM HEPES/KOH-1 mM DTT (pH 7.7), 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM glutathione, 1 mM FAD, 1 mM NADPH, 500 μM NADP, 500 μM ATP, 250 μM CTP, 100 μM thiamine-PP, and 10 μM coenzyme B_{12}. Each reaction, in a final volume of 1 mL, was preincubated with 3 μM IPP for 20 min at 37°C to deplete endogenous DMAPP. Following this, 8.5 μM [1-^{14}C]IPP (American Radiolabeled Chemicals, Inc) (8.25×10^5 dpm/ml) was added along with 500 μM fructose 6-phosphate (FR6P) when indicated. Aliquots of 0.2 mL (containing 0.25 to 0.36 mg protein) were removed at various times after addition of [^{14}C]IPP.

The incorporation of [^{14}C]IPP into the isoprenoid fraction was measured after acid hydrolysis (0.5 N HCl, 20 min, 37°C) and extraction into petroleum ether (boiling point, 35 to 60°C) as in Poliquin et al. (2004). Each sample was counted in 10 mL ScintiSafe Econo 2 cocktail (Fisher Scientific).

Reverse-phase column chromatography was used to analyze radiolabeled compounds in the petroleum ether fraction. An aliquot of 0.8 mL of a 3 mL petroleum ether extract obtained from a typical [^{14}C]IPP incorporation assay as described above was applied to a column (Pharmacia; 1.8 cm × 26 cm) of silica gel 60, RP-18 (EM Industries) previously equilibrated and then eluted with either 100% acetonitrile or acetonitrile:H_2O 20:5 at a flow rate of about 7.8 mL/h. Fractions of 1.3 ml were collected and counted in 10 mL ScintiSafe Econo 2 cocktail. The column was calibrated with the following standards: dimethylallyl alcohol (C_5), geraniol (C_{10}), farnesol (C_{15}), and geranylgeraniol (C_{20}). Standard elution profiles were monitored by the absorbance at 196 nm.

Alternatively, thin layer chromatography was used to identify radiolabeled isoprenoids. Petroleum ether extracts were evaporated to near dryness and then spotted onto silica gel RP-18 glass plates (5 x 20cm) (EM Industries). The above standards were spotted onto the same plate and the plates were developed with 25:2 acetonitrile/water. The adsorbant was scraped from sections of the plate and analyzed for radioactivity, which was then compared to the migration of the standards, the positions of which were ascertained by staining with iodine vapor.

LytB tandem affinity tagging

The *slr038* (*lytB*) gene of *Synechocystis* PCC 6803 was modified so as to produce a polypeptide with an epitope tag appended to the C terminus. Plasmid pBS1539 (http://www-db.embl-heidelberg.de/jss/servlet/de.embl.bk.wwwTools.GroupLeftEMBL/ExternalInfo/seraphin/TAP.html; Rigaut et al., 1999), containing the TAP (tandem affinity purification) tag, was obtained from Bertrand Séraphin of the EMBL in Heidelberg (now at the CNRS, Centre de Génétique Moléculaire, Avenue de la Terrasse - Bât. 24, 91198 GIF-SUR-YVETTE Cedex, FRANCE). The URA3 selectable marker (used for selection in yeast) was removed from pBS1539 (by digestion of the plasmid with *Sma* I and *Xho* I) and replaced with a kanamycin resistance gene [excised from plasmid pBR329K (Chamovitz *et al.,* 1990) using *Pst* I] after blunting both the vector and insert with mung bean nuclease. The resulting plasmid is referred to as p1539Kan. Constructs containing the *Synechocystis* PCC6803 *slr0348* genes, followed by and fused to the TAP tag, followed by the kanamycin resistance gene, and then followed

by *Synechocystis* genomic DNA sequence immediately downstream of *slr0348*, was created by overlap PCR (essentially as in Murphy *et al.,* 2000) using the primers listed in Table 3-1. The TAP tag and Kan^R gene were amplified from pBS1539Kan using the primers TAP-KanN and TAP-KanC. The *slr0348* gene was amplified from genomic DNA of *Synechocystis* PCC 6803 using the primers 6803lytBNdn and 6803lytBNup. The 6803lytBNup primer included sequence at the 5' end that was complementary to the TAP tag (see Table 3-1). Nucleotide sequences immediately downstream of *slr0348* was amplified using the primers 6803lytBCdn 6803lytBCup, with 6803lytBCdn containing 5' sequence complementary to sequence downstream of the Kan^R gene in the PCR product produced from p1539Kan.

PCR was performed with an MJ Research (Waltham, Mass.) PTC-150-25 MiniCycler with a heated lid. The Advantage KlenTaq polymerase mix (Clontech Laboratories, Inc., Palo Alto, Calif.) was used with a reaction volume of 50 µl in 100-µl thin-wall tubes. All PCR reactions were carried out using a high fidelity DNA polymerase (HF-2 from BD Biosciences Clontech). The primary PCR reactions contained about 0.5 µg of *Synechocystis* genomic DNA or 0.1 µg of plasmid (p1539Kan) DNA, with primers at a final concentration of 0.4 µM. Genomic DNA was prepared from cells of *Synechocystis* PCC 6803 as previously described (Williams, 1988). An initial denaturation at 94°C for 1 min was followed by 5 cycles of 94, 64, and 68°C for 10, 60, and 120 s, respectively, and then 25 more cycles with the annealing temperature reduced to 60°C. PCR products of the expected size were purified by electrophoresis in a 1% (wt/vol) agarose gel, and recovered in a final volume of 50 µL TE using the Geneclean kit (Bio 101, Inc., Carlsbad, Calif.). A

Table 3-1. Oligonucleotide primers used for epitope tagging of LytB

Primer	*Sequence
TAP-KanN	tccatggaaaagagaagatggaa
Tap-KanC	agcgtaatgctctgccagtgtta
6803lytBNdn	ggaaatgcagctactcaacgac
6803lytBNup	ttccatcttctctttccatggatcccgcaatttctaggacgggtt
6803lytBCdn	taacactggcagagcattacgctaggcctggctgttgagcatgag
6803lytBCup	cgctcttaaacatcgccataac

*Underlined bases introduce overhangs complementary to the product of the PCR reaction using primers TAP-KanN and TAP-KanC.

secondary PCR reaction was then carried out using 4 µl of each of the primary reaction products and the "outer" primers (6803lytBNdn and 6803lytBCup) at 1/5 the normal concentration (final concentration of 0.08 µM rather tham 0.4 µM). An initial denaturation at 94°C for 1 min was followed by 5 cycles of 94, 64, and 68°C for 10, 60, and 270 s, respectively, and then 22 more cycles with the annealing temperature reduced to 60°C. A PCR product of the expected size (3.1 kB) was purified by electrophoresis in a 1% (wt/vol) agarose gel, recovered using the Geneclean kit, precipitated with ethanol to concentrate and sterilize it, and then used to transform *Synechocystis* PCC 6803 as earlier described (Cunningham *et al.,* 2000), except that the concentrated cultures were grown for 24 h after addition of the transforming DNA and spread directly on BG-11 agar plates supplemented with 10 µg of kanamycin sulfate per ml. Colonies appearing on these plates were streaked onto plates with 25 µM kanamycin, and colonies arising on these plates were streaked, in turn, on plates containing 50 µM kanamycin.

Replacement of *slr0348* with a TAP-tagged gene, and the segregation of the gene replacement, was confirmed by PCR using the "outer" primer pair. Production of the TAP-tagged Slr0348 polypeptide was ascertained by immunoblotting using an antiserum specific for the TAP tag. Note: The TAP tag adds 184 amino acids and 20,624 to the molecular weight of the Slr0348 polypeptide.

Preparation of LytB depleted cell-free extracts

The method of sample preparation and depletion of TAP-tagged LytB from *Synechocystis* cells was modified from Rigaut et al. (1999). Frozen pellets obtained from 1 L cultures of LytB TAP-tagged *Synechocystis* cultures harvested in the log

phase of growth were thawed and broken in 100 mM HEPES/KOH (pH 7.7), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, and Roche Molecular Biochemicals Complete, Mini, EDTA-free protease inhibitor cocktail using a Bead Beater (Biospec Products Inc., Bartlesville, Okla.) (5 x 20 s with intermittent cooling). The breakate was centrifuged for 1 h at 60,000 x g at 4°C. The supernatant was diluted 1:1 with the above buffer and 5 mL (2.1 to 2.6 mg protein/ml) was applied to a column (BioRad poly-prep 10 mL chromatography column) containing 200 µL IgG Sepharose™ 6 Fast Flow resin (Amersham Pharmacia Biotech AB) that had been equilibrated with 100 mM HEPES/KOH (pH 7.7). The column was rotated for 2 h at 4°C, after which the flow-through was collected.

Depletion of TAP-tagged LytB from *Synechocystis* extracts was verified by immunoblot. Protein concentration of samples before and after depletion was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology Co.). Denaturing SDS-PAGE was used to separate proteins with a 4% polyacrylamide stacking gel and 12% polyacrylamide resolving gel containing 0.1% SDS as described by Sambrook et al. (1989). Proteins were transferred to an Immobilon transfer membrane (Millipore) for 1 h at 100 V according to Peluso and Rosenberg (1987) in a Bio-Rad transblot apparatus. Detection of epitope-tagged protein was based on the methodology of Walke et al. (2001). The membrane was blocked with 5% (w/v) dry milk in PBS-0.1% Tween, followed by incubation with a 1:2000 dilution of peroxidase anti-peroxidase (PAP; Sigma) in buffer containing 1% (w/v) dry milk in PBS-1% Tween. After 4 washes with PBS-1% Tween, the blot was

developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Co.) and exposed on film (X-OMAT LS, Kodak) for 30 sec to 15 min.

Results and Discussion

PPC-stimulation results in the synthesis of new isoprenoids

Our previous studies of isoprenoid production in *Synechocystis* cell-free extracts relied on the incorporation of [^{14}C]IPP into allylic compounds (Ershov et al. 2000). The incorporation of radiolabel was greatly enhanced by addition of various phosphorylated PPC compounds but not by non-phosphorylated compounds, with glucose 6-phosphate and fructose 6-phosphate giving the greatest stimulation (Ershov et al., 2002). We had assumed earlier that the PPC compound stimulation was likely due to an enhanced DMAPP production (Poliquin et al., 2004). In order to identify the isoprenoids synthesized as a result of PPC stimulation, [^{14}C]IPP incorporation was performed in the presence of FR6P (Figure 3-1). Radioactive products present in petroleum ether extracts of acid hydrolyzed reaction mixtures were analyzed after 5 min, 15 min, 30 min, and 60 min of incubation (arrows, Figure 3-1) by reverse-phase C_{18} silica-gel column chromatography.

In Figure 3-2 is seen a typical elution profile for extracts taken after incubation with FR6P for 5 min and 60 min. DMAOH, the C_5 alcohol of DMAPP, was the major product detected at the earliest time point (5 min). It eluted in fractions ca. 24 and 30. The first peak corresponded exactly with a co-eluting peak of a standard of DMAOH (non acid-hydrolyzed). The second peak, here referred to as

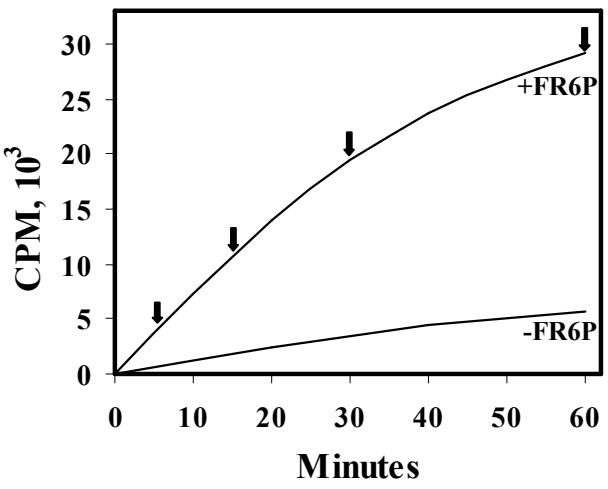


Figure 3-1. *In vitro* [^{14}C]IPP incorporation into *Synechocystis* supernatant when stimulated by 500 μM fructose 6-phosphate (+FR6P) with incorporation much lower with no addition of fructose 6-phosphate (-FR6P). The reaction mixture contained 60,000 x g supernatant with 100 mM HEPES/KOH-1 mM DTT (pH 7.7), 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM glutathione, 1 mM FAD, 1 mM NADPH, 500 μM NADP, 500 μM ATP, 250 μM CTP, 100 μM thiamine-PP, and 10 μM coenzyme B_{12}. Arrows denote times when samples were taken for analysis of isoprenoid products (as in Materials and Methods).

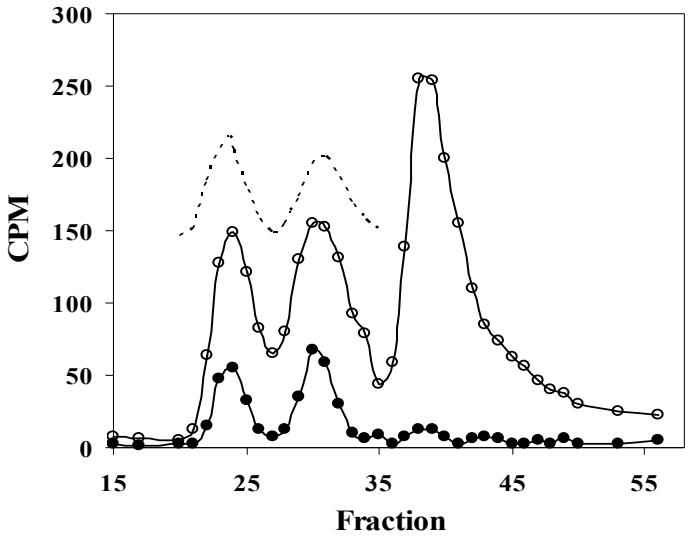


Figure 3-2. Elution of the [^{14}C]-allylic fraction on a reverse-phase silica-gel column from supernatant incubations of 5 min (●) and 60 min (○) shows increasing isoprenoid length over time. Superimposed is the [^{3}H]DMAOH elution (·····) and that of an isomer [^{3}H]DMAOH* after acid hydrolysis of [^{3}H]DMAPP. Column eluted with 100% acetonitrile.

DMAOH*, routinely appeared upon acid hydrolysis of DMAPP and [^{3}H]DMAPP. In attempting to identify the DMAOH* compound we compared its retention time with methyl-3-buten-2-ol, a compound Fisher et al. (2001) had reported to be a product of DMAPP after acid hydrolysis. In our system, the elution time for methyl-3-buten-2-ol did not correspond with that of DMAOH*. We combined the counts of radioactivity in the peaks corresponding to DMAOH and DMAOH* and consider this combination to represent the DMAPP formed from [^{14}C]IPP in the cell-free reaction mixture (as in Figure 3-3). Moreover, the DMAOH represented by these 2 peaks were likely considerably greater than observed here since DMAOH is highly volatile and the loss was not well controlled during the column fractionation. By 60 min incubation time the predominant radioactivity is found in fractions 38-39. This elution time corresponds to that of the C_{20} isoprenoid alcohol geranylgeraniol (GGOH). Similar elution profiles were obtained using alternate PPC compounds other than FR6P, including GL6P, ribulose 5-phosphate and erythrose 4-phosphate (data not shown). In reaction mixtures that lacked PPC compounds the recoverable radioactivity was too low for reliable analysis (Figure 3-1). It should be noted that with PPC-stimulation up to ca. 50% of the radiolabel was in the C_5-C_{20} fractions by 60 min. While our analysis focused on isoprenoids of up to C_{20} in length, radioactivity was also found in larger, unidentified compounds as well, perhaps squalene (C_{30}) or carotenoids (C_{40}), that were recovered in a 1:1 butanol/acetonitrile wash of the column after the C_5-C_{20} compounds had been eluted. These compounds were not further analyzed.

To better ascertain if intermediate size isoprenoids (C_{10}, C_{15}) were being

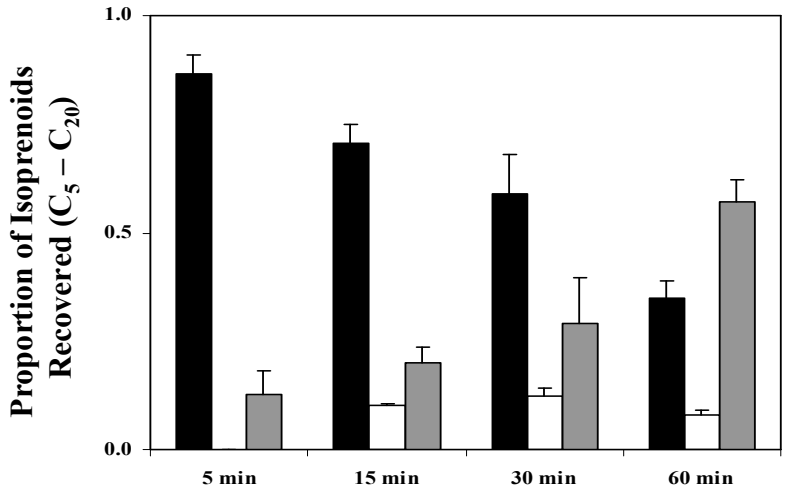


Figure 3-3. Progressive changes of [^{14}C] incorporated into C_5 (DMAOH + DMAOH*) (black), C_{10} (white) and C_{20} (gray) isoprenoids synthesized over time, with fructose 6-phosphate addition and incubation conditions as in Figure 3-1. Shown are averages (± S.D.) of two (5 min, 30 min) or three (15 min, 60 min) experiments.

produced, a weaker mobile phase (4:1 acetonitrile/H_2O) was used to elute the reverse phase column. The increased resolution revealed a small peak that corresponded with the C_{10} geraniol (GOH) (data not shown), but no label was observed in fractions coinciding with the C_{15} compound farnesol. The proportion of GOH remained relatively small over a time course for 5-60 min incubation. Analysis of the proportion of label in C_5, C_{10}, and C_{20} compounds after a 5 min, 15 min, 30 min, and 60 min incubation with FR6P supports the notion of a progression of synthesis (Figure 3-3). The proportion of C_5 isoprenoids gradually decreased over time and the amount of C_{20} isoprenoids steadily increased. PPC substrate stimulation of [^{14}C]IPP incorporation into isoprenoids of C_5 and greater over time indicates that there is new synthesis. Presumably, elongation of prior (non-radioactive) isoprenoids further suggests that one or more prenyltransferases are active in the cell-free extract.

It was surprising that we could not reliably detect the C_{15} isoprenoid alcohol farnesol (FOH) at any point in the time course by column chromatography because it is generally assumed that FOH is within the 5 carbon linear progression to longer chained isoprenoids. With thin layer chromatography a very small amount of radioactivity was observed to co-migrate with a C_{15} FOH standard. The relatively low level of radioactivity detected for both GOH and FOH, whereas GGOH was increasingly labeled over time, is congruent with prenyltransferases leading primarily to C_{20} compounds. In fact, within the sequenced genome of *Synechocystis* PCC 6803 (Kaneko et al., 1996), no homologs to C_{10} or C_{15} prenyltransferases exist, whereas two genes have been predicted to be GGPP synthases. One was later shown to have activity as a nonaprenyl diphosphate (C_{45}) synthase (Okada et al., 1997). GPP and

FPP may be produced through the enzymatic activity of an as yet unidentified prenyltransferase, or more likely, that the prenyltransferase(s) responsible for GPP and FPP synthesis can act on multiple isoprenoid substrates to make various chain lengths. For example, a novel prenyltransferase was found in the thermophilic cyanobacterium *Synechococcus elongatus* by Ohto et al. (1999) where the recombinant enzyme catalyzed reactions leading to the production of C_{15}, C_{20}, C_{25}, C_{30}, and C_{35} isoprenoids using C_5-C_{15} isoprenoids as substrates.

Although isoprenoid chain length is largely determined by prenyltransferase structure (Tarshis et al., 1996), the relative amounts of IPP and DMAPP within the cell may also affect product synthesis. The isoprenoid compound most predominant in Arabidopsis chloroplasts incubated with [^{14}C]IPP was identified as C_{20}, suggesting a rapid turnover of substrates to make larger isoprenoids (Bouvier et al., 2000). Upon increasing the concentration of DMAPP, however, C_{10} (GPP) compounds accumulated. Considering C_{20} compounds serve as substrates for the synthesis of phytol, carotenoids, and plastoquinone in photosynthetic organisms (Rohmer, 1999; Bouvier et al., 2005), it is not surprising that it would be the major isoprenoid that accumulates.

Are PPC compounds substrates for isoprenoid biosynthesis in vitro?

The question remains as to how substrates of the PPC affect the synthesis of isoprenoids, specifically whether they serve as substrates for DMAPP synthesis or if they otherwise somehow stimulate the incorporation of [^{14}C]IPP. Under our standard assay conditions three radiolabeled compounds were separately added to the

Synechocystis cell-free extract: [2-^{14}C] and [6-^{14}C]glucose 6-phosphate, [U-^{14}C]glyceraldehyde 3-phosphate, and [U-^{14}C]FR6P. Considerable amounts of radioactivity were recovered in the petroleum ether fraction in each case. However, when aliquots were analyzed by reverse-phase liquid chromatography as in Figure 3-2, no significant isoprenoid labeling was observed in the fractions corresponding to the C_5-C_{20} isoprenoids. In any case, it appears that the stimulation of [^{14}C]IPP incorporation by the PPC compounds is indirect.

LytB does not contribute to PPC-stimulated isoprenoid biosynthesis

LytB is an essential enzyme of the MEP pathway, first identified as such by Cunningham et al. (2000) in *Synechocystis* PCC 6803. It was inferred that LytB acted at or beyond the point at which the pathway branches to form IPP and DMAPP and its importance was demonstrated by the inability to construct a viable loss of function mutant. Subsequently, it was shown in *E. coli* that LytB catalyzed the conversion of hydroxy-2-methyl-2-butenyl 4-diphosphate into both IPP and DMAPP in a 5:1 ratio, respectively (Rohdich et al., 2002; Adam et al., 2002). Further studies with purified recombinant LytB demonstrated the requirement for reducing agents for activity, as well as reconstitution of a dioxygen sensitive [4Fe-4S] cluster (Rohdich et al., 2003; Wolff et al., 2003; Gräwert et al., 2004). More recently, LytB from *Plasmodium falciparum* was shown to form a complex with and have activity in the presence of ferredoxin and ferredoxin-NADP(+) reductase, suggesting that ferredoxin may be the redox partner necessary for LytB catalysis (Röhrich et al., 2005). Notwithstanding the recognized susceptibility to O_2 of the 4Fe-4S cluster enzyme in *Plasmodium*, it is

possible that in an oxygen producing photosynthetic cyanobacterium LytB may remain active in a cell-free supernatant.

To determine whether LytB activity is involved in PPC substrate stimulated isoprenoid synthesis, we epitope-tagged (according to Rigaut et al., 1999) LytB of *Synechocystis* in order to assay cell-free supernatants with and without LytB. The growth of this mutant was unimpaired, indicating that LytB retained function despite the C-terminal epitope tag for the enzyme *in vivo*. Affinity purification effectively depleted the cell supernatant extract (60,000 × g) as ascertained by SDS-PAGE and Western blot analysis (Figure 3-4). While TAP-tagged LytB is present within 'pre-depletion' cell-free extracts, it could not be detected after the immunodepletion (Figure 3-4B).

Using cell-free extracts before and after LytB depletion, PPC substrate stimulated isoprenoid biosynthesis using FR6P was measured. Regardless of whether LytB protein was present in the cell supernatant extract, PPC substrates stimulated [^{14}C]IPP incorporation into the isoprenoid fraction (Figure 3-4A). Furthermore, the amount of [^{14}C]IPP incorporation was comparable for both extracts. The lack of participation by LytB in PPC substrate stimulated isoprenoid production in our assay system supports the reported requirement for anaerobic, reducing conditions for the activity of this enzyme (Rohdich et al., 2003; Wolff et al., 2003; Gräwert et al., 2004, Röhrich et al., 2005). Previously we reported our speculation that PPC compounds stimulated isoprenoid production by entering the MEP pathway downstream of MEP in *Synechocystis* (Ershov et al., 2002, Poliquin et al., 2004). Our most recent results, however, indicate LytB catalysis is not required for PPC substrate stimulation of

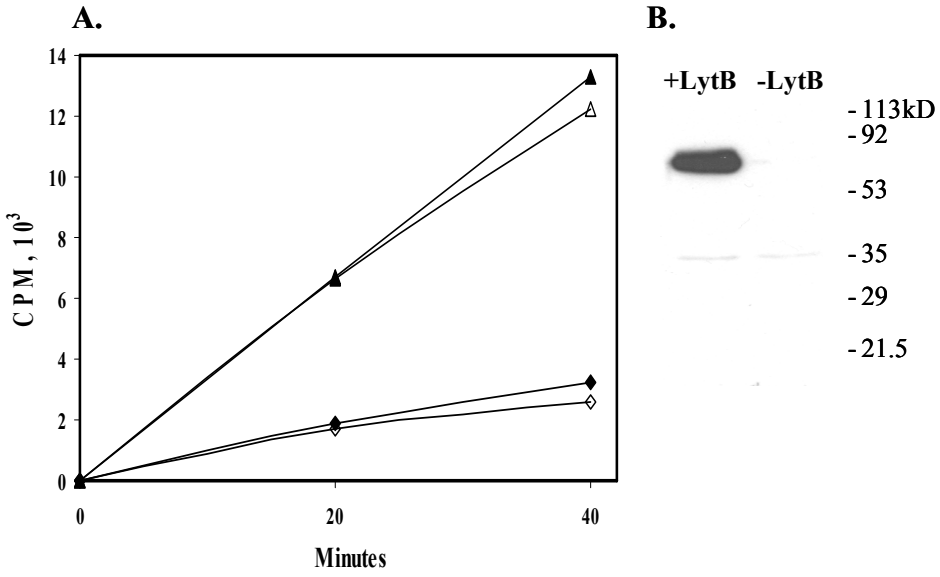


Figure 3-4. A. Isoprenoid production as indicated by [^{14}C]IPP incorporation into cell-free supernatants with fructose 6-phosphate stimulation plus cofactors containing LytB (▲) and depleted of LytB (Δ). Supernatant plus cofactors with LytB (♦) and after LytB depletion (◊). B. Immunoblots of supernatant from TAP-tagged LytB cells before (+) and after LytB depletion (-). The 66 kD region is larger than what is predicted for LytB because it also contains the epitope tag.

isoprenoid biosynthesis in our *in vitro* system.

Conclusions

These result of this study show that *Synechocystis* PPC-stimulated isoprenoid biosynthesis *in vitro* leads to the production of C_5, C_{10}, and C_{20} compounds, indicating the presence of an active prenyltransferase(s) within this organism. Moreover, we report that LytB catalysis is not part of the *in vitro* PPC-stimulated synthesis of these isoprenoid compounds. This methodological approach can help in elucidating isoprenoid production in *Synechocystis*, which may not be as straightforward as has been generalized for MEP pathway-containing organisms.

CHAPTER 4

Functional Importance of *Synechocystis* PCC 6803 Sll1556 Under High Light Stress

Summary

In this chapter, the Δ*sll1556* mutant was further characterized in an effort to better understand its role in isoprenoid biosynthesis in *Synechocystis* PCC 6803. Under high light stress, Δ*sll1556* cells had a significant impairment in growth and were out-competed by WT cells in a mixed culture. Consistent with these results, Sll1556 protein expression was greater in cells under high light than under non-stress conditions. Furthermore, Δ*sll1556* cells grown under high light had altered cell structure and a reduction in chlorophyll and carotenoids, as did cells grown under non-stress conditions. It is suggested that the Sll1556 protein is indeed important for *Synechocystis* PCC 6803, especially under high light stress. Its possible role in isoprenoid biosynthesis is discussed.

Introduction

The photosynthetic machinery of cyanobacteria and plants has evolved into efficient light harvesting apparatuses that are well-equipped to respond to changes in the environment. These pigment-protein complexes, also known as photosystems, are found within the thylakoid membranes and utilize light energy for the photochemical reactions of photosynthesis (Barry et al., 1994). Three types of pigments are

associated with the photosynthetic apparatus in cyanobacteria, each with a characteristic absorption spectra to maximize light harvesting: phycobilins, chlorophyll, and carotenoids (Lawlor, 1987). Phycobilins, which are assembled into protein complexes called phycobilisomes, are found only in cyanobacteria and red algae and serve as the major light harvesting antenna pigments, transferring light energy to the photosystem reaction centers (Gantt, 1980). Chlorophyll serves as light harvesting antenna pigments, with the exception of the reaction center P_{680} and P_{700} chlorophyll, which are involved in driving electron transport to the photochemical reactions (Hillier and Babcock, 2001; Vavilin et al., 2005). Finally, carotenoids are found within the photosynthetic apparatus. They may act as antenna pigments that transfer light energy to the reaction centers, but can also have an additional function in protection against excess light energy in the form of excited triplet chlorophyll and reactive oxygen species (Cogdell and Frank, 1987; Britton, 1995; Demmig-Adams et al., 1996). This protective role is important, since such reactive molecules can oxidize and damage the proteins and pigments of the photosynthetic apparatus, as well as membrane lipids (Niyogi, 1999). Their importance is reflected by the fact that some plant herbicides, such as norflurazon, are so effective because they inhibit carotenoid synthesis (Sandmann and Böger, 1989). In cyanobacteria, protection from harmful, excess light energy generally comes from the carotenoids β-carotene, echinenone, zeaxanthin, and myxoxanthophyll (Goodwin, 1980).

Carotenoids and the phytol tail of chlorophyll are part of a diverse group of compounds called isoprenoids. In cyanobacteria, including *Synechocystis* strain sp. PCC 6803, both are synthesized by the 2*C*-methyl-D-erythritol 4-phosphate (MEP)

pathway (reviewed in Rohmer, 1999). Although many of the steps of the MEP pathway have been determined using the eubacterium *Escherichia coli*, *Synechocystis* PCC 6803 has proven an excellent model in which to study isoprenoid synthesis because of its completely sequenced genome (Kaneko et al., 1996) and ease of genetic manipulation. Moreover, as a cyanobacterium it is useful for studying isoprenoid biosynthesis in a photosynthetic system.

Isoprenoid production via the MEP pathway in *Synechocystis* may not be as simple as that which has been described for other organisms. Work in our laboratory has discovered that compounds from the pentose phosphate cycle (PPC) stimulate isoprenoid biosynthesis *in vitro*, and that this stimulation requires the Sll1556 protein (Ershov et al., 2002; Poliquin et al., 2004). Sll1556 was first annotated as a type 2 IPP isomerase, an enzyme that interconverts IPP and DMAPP (Kaneda et al., 2001). This new class of IPP isomerase differs in that it requires NADPH and FMN for activity. The first type 2 IPP isomerase was discovered in *Streptomyces* sp. strain CL190 by Kaneda et al. (2001). Homologs are found in many archaebacteria and eubacteria, including the *sll1556* gene of *Synechocystis* PCC 6803 which has 32% sequence identity with the *Streptomyces* type 2 IPP isomerase. Poliquin et al. (2004), however, could not demonstrate type 2 IPP isomerase activity for purified recombinant Sll1556 under similar assay conditions. Furthermore, disruption of the gene revealed that *sll1556* is not essential for *Synechocystis* PCC 6803 and growth under photoautotrophic conditions was not impaired (20 μmol photons/m^2/s of light at 30ºC) (Poliquin et al., 2004). Morphologically, there were only small differences between WT and Δ*sll1556* cells. One notable difference was about a 30% reduction

of thylakoid membranes in the mutant, which suggested a function for Sll1556 protein in isoprenoid biosynthesis (Poliquin et al., 2004).

To gain further insight into the function of Sll1556, the effect of light and temperature stress on Δ*sll1556* cells was investigated. Although no difference was seen between WT and Δ*sll1556* cells grown at low temperatures, an obvious impairment in the mutant was observed under high light. Compared to WT cells, Δ*sll1556* cells had a reduced rate of growth, altered cell structure, and a decrease in chlorophyll and carotenoid content, suggesting an important function for the Sll1556 protein under high light stress.

Materials and Methods

Culture and growth conditions

A glucose tolerant strain of *Synechocystis* strain PCC 6803 (Wim Vermaas, Arizona State University) was grown in liquid cultures of BG-11 media supplemented with 5 mM potassium-TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 8.3] (Williams, 1988). This strain is referred throughout as wild type (WT), while one with a disruption in the *sll1556* gene is referred to as Δ*sll1556* (Poliquin et al., 2004). For routine experiments, cells were grown in 1 liter of medium in 2.5 L Fernbach flasks at 30ºC under 20 µmol photons/m^2/s or 200 µmol photons/m^2/s cool white fluorescent lamps with continuous shaking and bubbling with 5% CO_2 in air. Cells were harvested in early to mid log phase of growth and stored, if necessary, as pellets at -80ºC. For rapid growth assessment, WT and Δ*sll1556* cultures were grown in 250 ml side-arm flasks in 20 ml of the same media inoculated with cells to a final

optical density of 0.05-0.1 at 730 nm. Except where otherwise indicated, the chemicals used in this study were purchased from Sigma Chemical Co.

Epitope-tagged Sll1556 expression

The *sll1556* gene in *Synechocystis* PCC 6803 was modified so as to produce a polypeptide with an epitope tag appended to the C terminus. Plasmid pBS1539, containing the TAP tag (http://www-db.embl-heidelberg.de/jss/servlet/de.embl.bk.wwwTools.GroupLeftEMBL/ExternalInfo/seraphin/TAP.html; Rigaut et al., 1999), was obtained from Bertrand Séraphin of the EMBL in Heidelberg (now at the CNRS, Centre de Génétique Moléculaire, Avenue de la Terrasse - Bât. 24, 91198 GIF-SUR-YVETTE Cedex, FRANCE). The URA3 selectable marker (used for selection in yeast) was removed from pBS1539 (by digestion of the plasmid with *Sma* I and *Xho* I) and replaced with a kanamycin resistance gene [excised from plasmid pBR329K (Chamovitz et al., 1990) using *Pst* I] after blunting both vector and insert with mung bean nuclease. The resulting plasmid is referred to as p1539Kan. A construct containing the *Synechocystis* PCC 6803 *sll1556* gene, followed by and fused to the TAP tag, followed by the kanamycin resistance gene, and then followed by *Synechocystis* genomic DNA sequence immediately downstream of *sll1556*, was created by overlap PCR (as in Murphy et al., 2000) using the primers listed in Table 4-1. The TAP tag and Kan^R gene were amplified from pBS1539Kan using the primers TAP-KanN and TAP-KanC. *sll1556* was amplified from genomic DNA of *Synechocystis* PCC 6803 using the primers 6803sll1556Ndn and 6803sll1556Nup. The latter primer included sequence at the 5' end that was complementary to the TAP tag. Sequence immediately downstream of

Table 4-1. Oligonucleotide primers used for epitope tagging of Sll1556

Primer	*Sequence
TAP-KanN	Tccatggaaaagagaagatggaa
Tap-KanC	Agcgtaatgctctgccagtgtta
6803sll1556Ndn	Cccttggttgatcagcagtatg
6803sll1556Nup	Ttccatcttctcttttccatggaaggtttagttaacctttgtcccgattg
6803sll1556Cdn	Taacactggcagagcattacgctaagggacattttgctctggttg
6803sll1556Cup	Ggggacgtttatgggacagttg

*Underlined bases introduce overhangs complementary to the product of the PCR reaction using primers TAP-KanN and TAP-KanC.

sll1556 was amplified using the primers 6803sll1556Cdn and 6803sll1556Cup, with the former primer containing 5' sequence complementary to sequence downstream of the Kan^R gene in the PCR product produced from p1539Kan. The 3 PCR products were purified by agarose gel electrophoresis and then combined. A secondary PCR reaction was then carried out using only the outer primers (6803sll1556Ndn and 6803sll1556Cup). All PCR reactions were carried out using a high fidelity DNA polymerase (HF-2 from BD Biosciences Clontech) and a minimum number of amplification cycles were employed in order to minimize introduction of mutations by the PCR process. The product of the second PCR reaction was purified by agarose gel electrophoresis, precipitated with ethanol to concentrate and sterilize it, and then used to transform *Synechocystis* PCC 6803 as earlier described (Cunningham et al., 2000). Replacement of s*ll1556* with a TAP-tagged gene was confirmed by PCR. Production of the TAP-tagged Sll1556 protein was ascertained by immunoblotting using an antiserum specific for the TAP tag (below).

Sll1556-TAP cultures were grown in 20 ml BG-11 media in 250 ml side-arm flasks (0.1-0.2 initial optical density at 730 nm) under 20 µmol photons/m^2/s or 200 µmol photons/m^2/s as above. Based on the optical density at 660 nm (chlorophyll absorption), equal amounts of cells were harvested in early to mid log phase of growth, pelleted, and broken with glass beads and vortexing in 30 µl 2X SDS loading buffer . Samples were boiled 5 min, centrifuged at top speed for 10 min, and run on denaturing SDS-PAGE (4% polyacrylamide stacking gel, 12% polyacrylamide resolving gel containing 0.1% SDS) as described by Sambrook et al. (1989). Proteins were transferred to an Immobilon transfer membrane (Millipore) for 1 h at 100 V

according to Peluso and Rosenberg (1987) in a Bio-Rad transblot apparatus. TAP-tagged Sll1556 was detected by a procedure based on Walke et al. (2001). The membrane was blocked with 5% (w/v) dry milk in PBS-0.1% Tween, followed by incubation with a 1:2000 dilution of peroxidase anti-peroxidase (PAP; Sigma) in buffer containing 1% (w/v) dry milk in PBS-1% Tween. After 4 washes with PBS-1% Tween, the blot was developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Co.) and exposed on film (X-OMAT LS, Kodak) for 10 min.

Competitive growth experiments and quantitative RT-PCR

Competitive growth experiments were conducted using a WT control strain with a kanamycin resistance gene inserted into a neutral site (WT_{Kan}) (Cunningham and Gantt, unpublished) and Δ*sll1556* cells. The WT_{Kan} strain was used to allow growth under similar conditions in kanamycin-containing media. Equal amounts of each cell culture (based on the optical density at 660 nm) were combined and added to BG-11 media and allowed to acclimate for 2 days under 20 μmol photons/m^2/s. Such a culture was diluted to an optical density of 0.15-0.45 at 660 nm with BG-11 media to a final volume of 20 ml in 250 ml Erlenmyer flasks, always with two replicates. Growth was allowed to proceed at 20 μmol photons/m^2/s or 200 μmol photons/m^2/s, and at days 0, 2, 4, and 6, equal amounts of cell culture were removed and the cells were pelleted and frozen at -20ºC.

Genomic DNA was extracted from the thawed pelleted cells of *Synechocystis* as described in Williams (1988) and used to perform real time polymerase chain reaction (RT-PCR). This method of PCR measures the relative amount of a particular

sequence of DNA that is present in a sample based on the fluorescence of a dye when it binds to double-stranded DNA. In this experiment, RT-PCR was used to quantify the amount of WT_{Kan} or Δ*sll1556* DNA present in the mixed culture, an indication of the proportion of each cell strain within the culture. Oligonucleotide primers were designed to amplify either WT_{Kan} or Δ*sll1556* DNA, as well as a sequence from both WT_{Kan} and Δ*sll1556* DNA to verify the results of the PCR reaction with the individual primers (Table 4-2). The PCR reaction contained 5 μl of the extracted DNA (diluted 1:250), 0.24 μM of the appropriate primers, and SYBR Green PCR Master mix prepared according to the manufacturer's recommendations (Applied Biosystems) in a final volume of 30 μl. Additional reactions were set up using serial dilutions of a sample of DNA in order to construct a relative standard curve. Each PCR reaction was done in triplicate. DNA samples analyzed were from 3 independent growth experiments (3 each for 20 μmol/m^2/s and 200 μmol/m^2/s light), with each experiment done in replicates of two.

The PCR was performed in an ABI Prism® 7700 Sequence Detector System. The program consisted of 1 cycle of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative amounts of WT_{Kan} or Δ*sll1556* DNA were calculated using a standard curve constructed from the log of the concentration of the standard versus the threshold cycle of amplification (C_t; the PCR cycle at which a significant level of fluorescence is detected) (Applied Biosystems User Manual). The results obtained from the RT-PCR reaction with each individual primer for samples taken on Day 0 were normalized to 50%, based on the assumption that equal amounts of each culture were initially mixed together. The total DNA

Table 4-2. Oligonucleotide primers used for RT-PCR of *Synechocystis* WT_{Kan} and Δ*sll1556* DNA.

Primer	*Sequence
TAPtagRTPCRN (WT_{Kan})	Cagcagccaaccgcttta
TAPtagRTPCRC (WT_{Kan})	Ggttttgagctcaccctgaa
sll1556RTPCRN	Tggaagtgccggtaattgtc
sll1556RTPCRC	Tggctcataacacccttgt
RTPCRgenN	Cccaccgtaagtccgatcat
RTPCRgenC	Ccagagggtcagtcccaaatc

within a sample was calculated by adding the results obtained from RT-PCR with each individual primer. The percentage of WT_{Kan} and Δ*sll1556* DNA within the sample could then be determined. These values were confirmed by comparing the results of RT-PCR with each individual primer to those obtained using a primer that amplified a sequence from both strains.

Chlorophyll and carotenoid determination

Synechocystis PCC 6803 cells were pelleted from 5 ml of a culture in log phase. Pigments were extracted with 95% dimethylformamide for 15 minutes in the dark. After a 10 minute centrifugation, the optical density of the resulting extract at 461 nm and 664 nm was measured. Carotenoid and chlorophyll determinations were made based on their extinction coefficients and their absorption maxima (Cunningham, unpublished). Carotenoid content (μg/ml) was ascertained using the formula $A_{461} - (0.0465 \times A_{664}) \times 4$. Chlorophyll concentration (μg/ml) was calculated by the equation $A_{664} \times 11.92$. Carotenoid and chlorophyll concentrations were expressed on a per cell basis, which was determined using a Spencer Bright-Line hemacytometer and Olympus BHTU light microscope.

Pigment analysis

For chlorophyll and carotenoid pigment analysis, 10 ml aliquots of a culture in the log phase of growth were pelleted and frozen at -80ºC. Pigments were extracted from the thawed pellets with 400 μl acetone:methanol (1:1) in dim light. After a brief centrifugation, the extract was removed to a new tube and 400 μl of ethyl acetate was added to the pellet. After another brief centrifugation, the second extraction was

added to the first, along with 600 µl H_2O. After multiple inversions, the mixture was centrifuged for 5 minutes. The upper phase was transferred to a new tube and centrifuged for another 10 minutes to clarify the extract for immediate analysis.

HPLC was performed on a Hewlett-Packard HP 1100 Series HPLC system with diode array detector (Agilent Technologies, Palo Alto, CA, USA). The column (4.6 mm × 25 cm) contained Spherisorb ODS2 5 µm (Supelco, Inc., Belefonte, PA, USA). 20 µl of sample was loaded onto the column and pigments were eluted with a gradient of 10-60% ethyl acetate in acetonitrile:H_2O:triethylamine (9:1:0.01) over 35 minutes at a flow rate of 1 ml/min. Pigment identification was confirmed by comparing the retention times with those of known standards.

Electron microscopy

Transmission electron microscopy was essentially performed as in Poliquin et al. (2004). Cells from cultures in log phase of growth were pelleted by centrifugation and fixed in 2% phosphate-buffered glutaraldehyde for 30 minutes. The cells were washed three times with H_2O, post-fixed in 1% OsO_4 for 60 minutes, with three subsequent water rinses. Fixed cells were taken up in melted 3% agar and small blocks were dehydrated in an ethanol to propylene oxide series before embedment in Epon. Sections were stained with 1% uranyl acetate and lead citrate and examined in a Zeiss EM10 CA microscope.

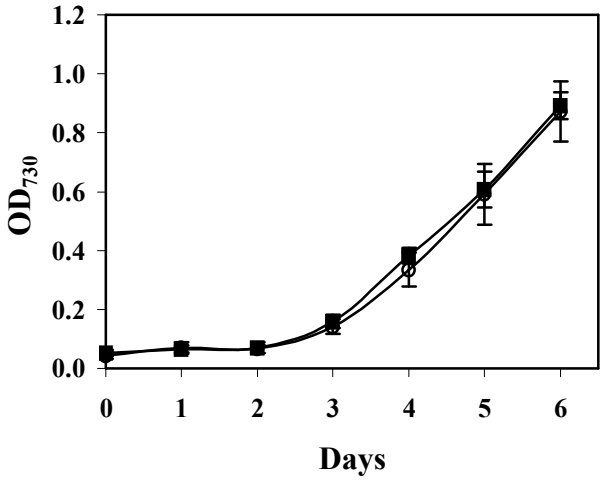
Results

Growth under environmental stress

To increase our understanding of isoprenoid biosynthesis in *Synechocystis* PCC 6803, we previously disrupted *sll1556* (Poliquin et al., 2004), a gene first annotated as a type 2 IPP isomerase based on sequence similarity to the *Streptomyces* sp. strain CL190 enzyme (Kaneda et al., 2001). Cells of the Δ*sll1556* mutant were viable, suggesting a nonessential function for this protein. Type 2 IPP isomerase activity could not be demonstrated for the purified recombinant protein, however (Poliquin et al., 2004). In an effort to elucidate the function of this protein, the effect of environmental stresses on Δ*sll1556* cells was tested. A change in the physiology or morphology of the mutant when subjected to a stress could indicate an important role for Sll1556 under certain growth conditions.

Growth of the Δ*sll1556* cells was first compared to WT cells at 20 μmol photons/m^2/s irradiance. This light intensity is within the reported range of compensation points (when photosynthesis and respiration are in balance) for aquatic phycobilisome-containing photosynthetic organisms (Gantt, 1990) and is not considered a stress. In comparison, a typical compensation level for sun plants is 1000-2000 μmol photons/m^2/s and for shade plants 100-500 μmol photons/m^2/s (Harvey, 1979). At 20 μmol photons/m^2/s, Δ*sll1556* growth did not measurably differ from that of WT (Figure 4-1A), as had been previously noted (Poliquin et al., 2004). High light is known to be a common stress for photosynthetic organisms and may inhibit growth. In the case of *Synechocystis*, high light stress responses have been reported to occur at 160 μmol photons/m^2/s (Satoh, 1998). Thus, it was decided

A.



B.

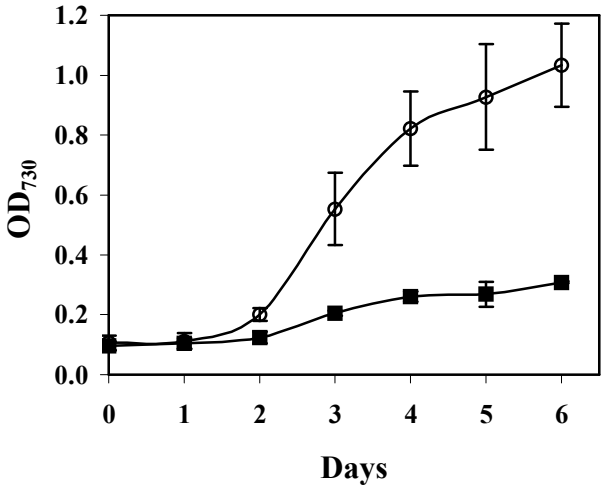


Figure 4-1. Growth of *Synechocystis* PCC 6803 Δ*sll1556* cells is impaired under high light stress. WT (○) and Δ*sll1556* (▪) cells were grown in side-arm flasks in air under continuous light at 20 μmol photons/m^2/s (A) and 200 μmol photons/m^2/s (B). While growth was comparable for the two strains under 20 μmol photons/m^2/s (A), the mutant had a reduced rate of growth under high light stress (B). Growth was monitored by the change in optical density at 730 nm. Results are the average of two independent experiments. Standard deviations are indicated.

to compare growth of Δ*sll1556* and WT cells under 200 μmol photons/m^2/s. At this light intensity there was a noticeable lag as compared to WT cultures (Figure 4-1B). After 6 days of growth, the WT culture had an optical density of over three times that of the Δ*sll1556* culture. The lag in growth clearly indicates an impairment in cells lacking the Sll1556 protein.

Synechocystis is a mesophilic cyanobacterium with optimal growth at 30ºC, and Inoue et al. (2001) had noted growth effects at 25ºC and complete inhibition at 15ºC. Therefore, growth of Δ*sll1556* and WT cells at 15ºC and 20ºC was tested under our light conditions. We found no growth at 15ºC, supporting the previous observations. Although cultures grew at 20º, the difference in growth between WT and Δ*sll1556* cells was not further reduced than at 30ºC. Apparently, lower temperatures do not serve as a stress by which WT and Δ*sll1556* cells can be differentiated.

Growth competition of WT and Δ*sll1556*

With the observed difference in growth of WT and Δ*sll1556* cultures under high light stress (Figure 4-1B), it was of interest to ascertain if in a mixed culture WT cells were at a competitive advantage. A competition experiment was conducted in which WT_{Kan} and Δ*sll1556* cells were grown together in the same flask under 20 μmol photons/m^2/s or 200 μmol photons/m^2/s. RT-PCR was performed (as described in Materials and Methods) using the DNA extracted from the mixed cultures to determine the proportion of WT_{Kan} and Δ*sll1556* DNA present within the samples. Under 20 μmol photons/m^2/s, the percentage of WT_{Kan} and Δ*sll1556* DNA within the mixed culture remained essentially equal, with minor fluctuations over the course of 6

days (Figure 4-2A). This indicates comparable growth between the two strains under non-stress conditions, in agreement with earlier growth measurements (Figure 4-1A). In contrast, differences in the amount of DNA for each strain were seen when the cultures were grown under 200 μmol photons/m^2/s (Figure 4-2B). After 2 days, the relative amount of Δ*sll1556* DNA decreased as compared to WT_{Kan} DNA, signifying that more WT_{Kan} cells were present within the mixed culture. By 6 days of growth, only ca. 18% of the total DNA was from Δ*sll1556* cells. This increase in the amount of WT_{Kan} cells suggests that under 200 μmol photons/m^2/s, the Sll1556 protein provides WT_{Kan} cells a competitive advantage over Δ*sll1556* cells.

Epitope-tagged Sll1556 expression

The impairment in growth of Δ*sll1556* cells under 200 μmol photons/m^2/s suggests that the Sll1556 protein is important for *Synechocystis* PCC 6803 success under high light. To obtain further confirmation of this, epitope-tagged Sll1556 expression in *Synechocystis* cells grown under 20 μmol photons/m^2/s and 200 μmol photons/m^2/s was investigated. When compared on an equal chlorophyll basis, epitope-tagged Sll1556 expression was significantly greater in cells grown under high light as compared to those grown under non-stress conditions (Figure 4-3). This indicates that Sll1556 is up regulated under high light and, in agreement with the results of the earlier growth experiments, suggests an increased role for this protein under high light stress.

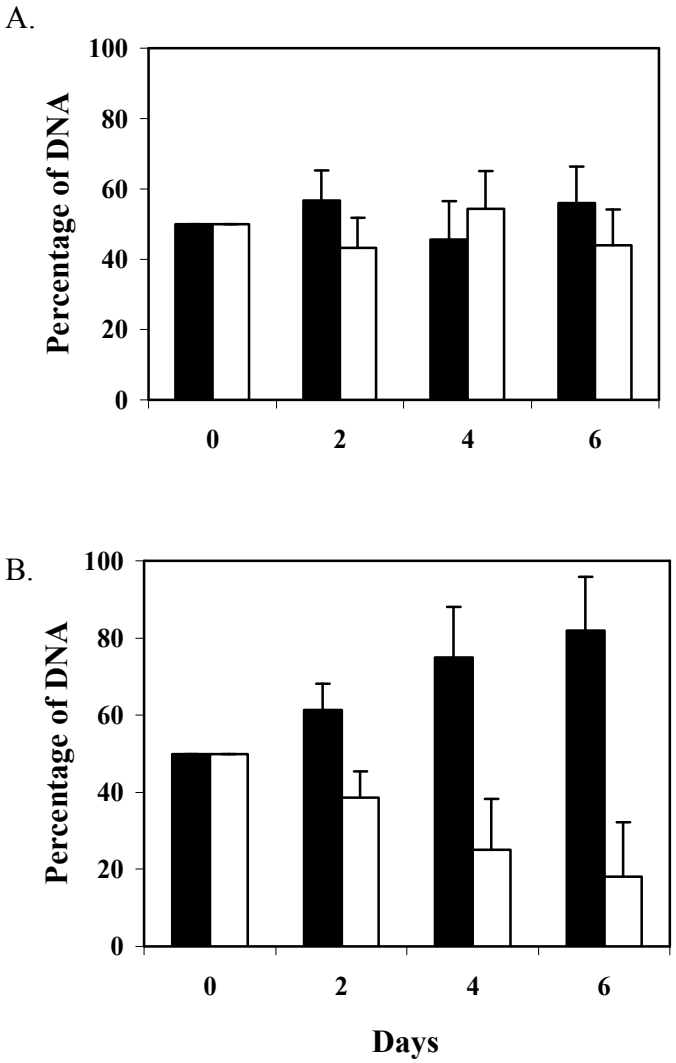


Figure 4-2. *Synechocystis* PCC 6803 WT_{Kan} cells have a competitive advantage over Δ*sll1556* cells under high light stress. Quantitative RT-PCR was performed on DNA extracted from mixed cultures of WT_{Kan} and Δ*sll1556* cells grown under 20 μmol photons/m^2/s (A) or 200 μmol photons/m^2/s (B) to determine the proportion of WT_{Kan} DNA (filled bars) and Δ*sll1556* DNA (open bars) within the culture (as described in Materials and Methods). Results are expressed as the percentage of DNA extracted and are the average of three independent experiments, each with two replicates. Standard deviations are indicated.

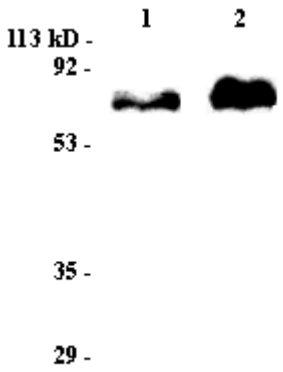


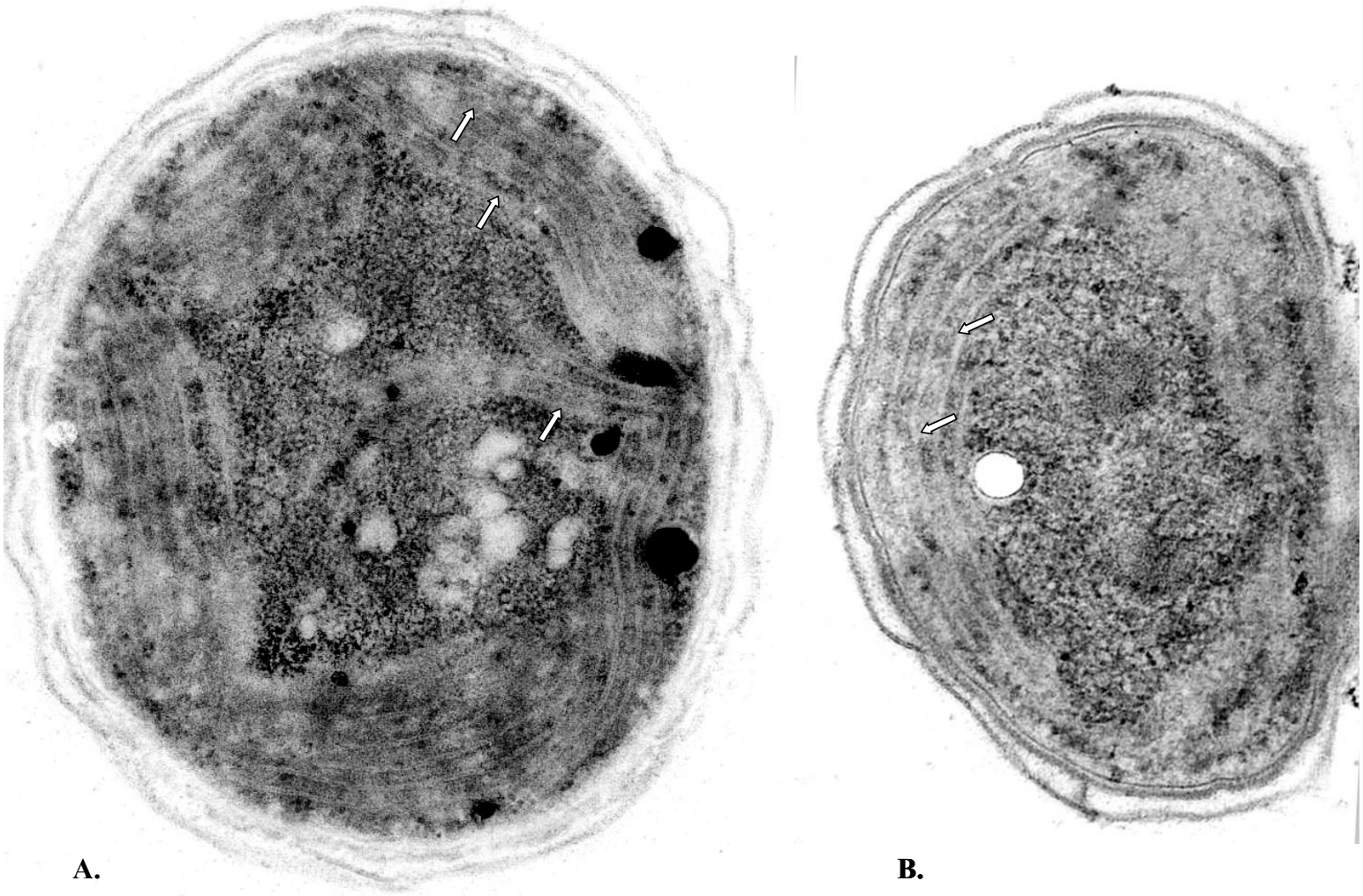
Figure 4-3. Expression of Sll1556 is greater in cells grown under high light than in those grown under non-stress conditions. *Synechocystis* cells containing epitope-tagged Sll1556 (58.1 kD) were grown under 20 µmol photons/m^2/s (lane 1) or 200 µmol photons/m^2/s (lane 2) and analyzed by SDS-PAGE and Western blot with a probe that detects a portion of the epitope tag. Samples were loaded on an equal chlorophyll basis. Molecular weight markers are indicated on the left.

Ultrastructure of *Synechocystis* cells grown under light stress

That Sll1556 is a protein that is involved in isoprenoid biosynthesis in *Synechocystis* PCC 6803 has been shown (Poliquin et al., 2004). It was previously reported that when grown under 20 μmol photons/m^2/s, Δ*sll1556* cells had morphological differences when compared to WT cells (Figure 4-4, as in Poliquin et al., 2004), even though growth was comparable between the two strains (Figures 4-1A and 4-2A). Δ*sll1556* cells were found to have a slightly reduced diameter (ca. 13% smaller) and fewer thylakoid membranes (ca. 30% less). Furthermore, mutant cells had a significant increase in the amount of fibrous extensions from the outer cell wall layer (glycocalyx; see Figure 4-6 for structural terminology), although sectioned cells revealed no differences in the structure or composition of this layer between the two strains. The effect of the mutation on the morphology of the cell, however, does not appear to be a detriment to its viability at this lower light intensity.

Because of the impairment in Δ*sll1556* growth under high light stress, it was of interest to examine the structural phenotype of both the mutant and WT strains grown under 200 μmol photons/m^2/s. Growth at this light intensity resulted in considerable pleiomorphy for both strains. While overall features were characteristic of each strain, cells with varying phenotypes (either more or less severe) were occasionally seen. This variability may explain the greater standard deviation found in WT cell growth under high light (Figure 4-1B). Such irregularities in morphology within the same cell strain do not seem to be uncommon for this organism, though. Mohamed et al. (2005) recently reported similar findings after examining the cell

Figure 4-4. Transmission electron micrograph of a *Synechocystis* PCC 6803 A) WT and B) Δ*sll1556* cell grown under 20 μmol photons/m^2/s. Cells were harvested in log phase of growth. WT cells are typically larger and have more thylakoid membranes (indicated by arrows) than Δ*sll1556* cells. Scale, 0.4μm.


A.
B.
0.4 µm

structure of *Synechocystis* PCC 6803 mutants impaired in carotenoid biosynthesis, possibly suggesting that some of the cells may be acclimating in response to the mutation or more probably that the cell development and division are not exactly synchronized. In fact, about 20-30% of WT and Δ*sll1556* cells observed were dividing

Under 200 μmol photons/m^2/s, both WT and Δ*sll1556* cells had fewer thylakoid membranes as compared to those grown under non-stress conditions, with Δ*sll1556* cells typically having even less (Figure 4-5). Furthermore, the thylakoid membranes of Δ*sll1556* cells appeared to be less electron dense, and tended toward greater luminal extensions than those found in WT cells. The most noticeable difference between WT and mutant cells was a significant enlargement of the inner periplasmic layer (Figures 4-5B, 4-6). The periplasmic layers are generally regarded as conduits for solutes and compounds for macromolecular formations. The inner periplasmic layer borders the plasma membrane on the cytoplasmic side and the peptidoglycan later on the outer side (Figure 4-6) (Gantt, 1994). In spite of the enlargement of the inner periplasmic layer, the other layers appear normal. In sections of both WT and Δ*sll1556* cells, the outer membrane and glycocalyx appear unimpaired, and neither the width nor density of the peptidoglycan layer appears to be reduced. This is similar to what was found for cells of both strains grown under non-stress conditions (Figure 4-4). A lack of electron density in the extended periplasmic layer might suggest cell plasmolysis, or what is more likely an accumulation of components resulting from impaired transport.

Figure 4-5. Transmission electron micrograph of a *Synechocystis* PCC 6803 A) WT and B) Δ*sll1556* cell grown under 200 μmol photons/m^2/s. Cells were harvested in log phase of growth. Δ*sll1556* cells generally had fewer thylakoid membranes (indicated by arrows) than WT and had a characteristic extended inner periplasmic layer (as indicated by bracket). Scale, 0.3μm.

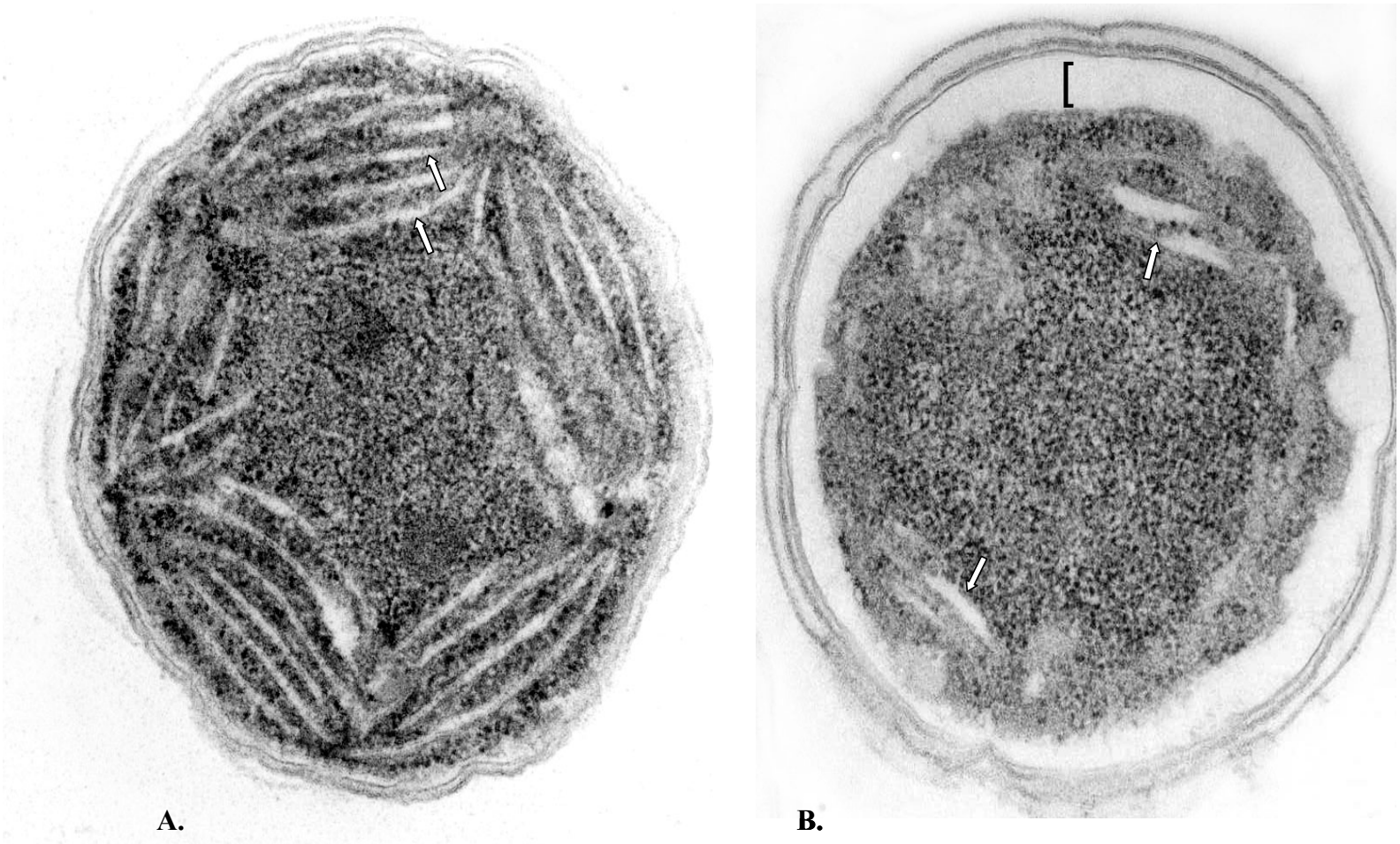
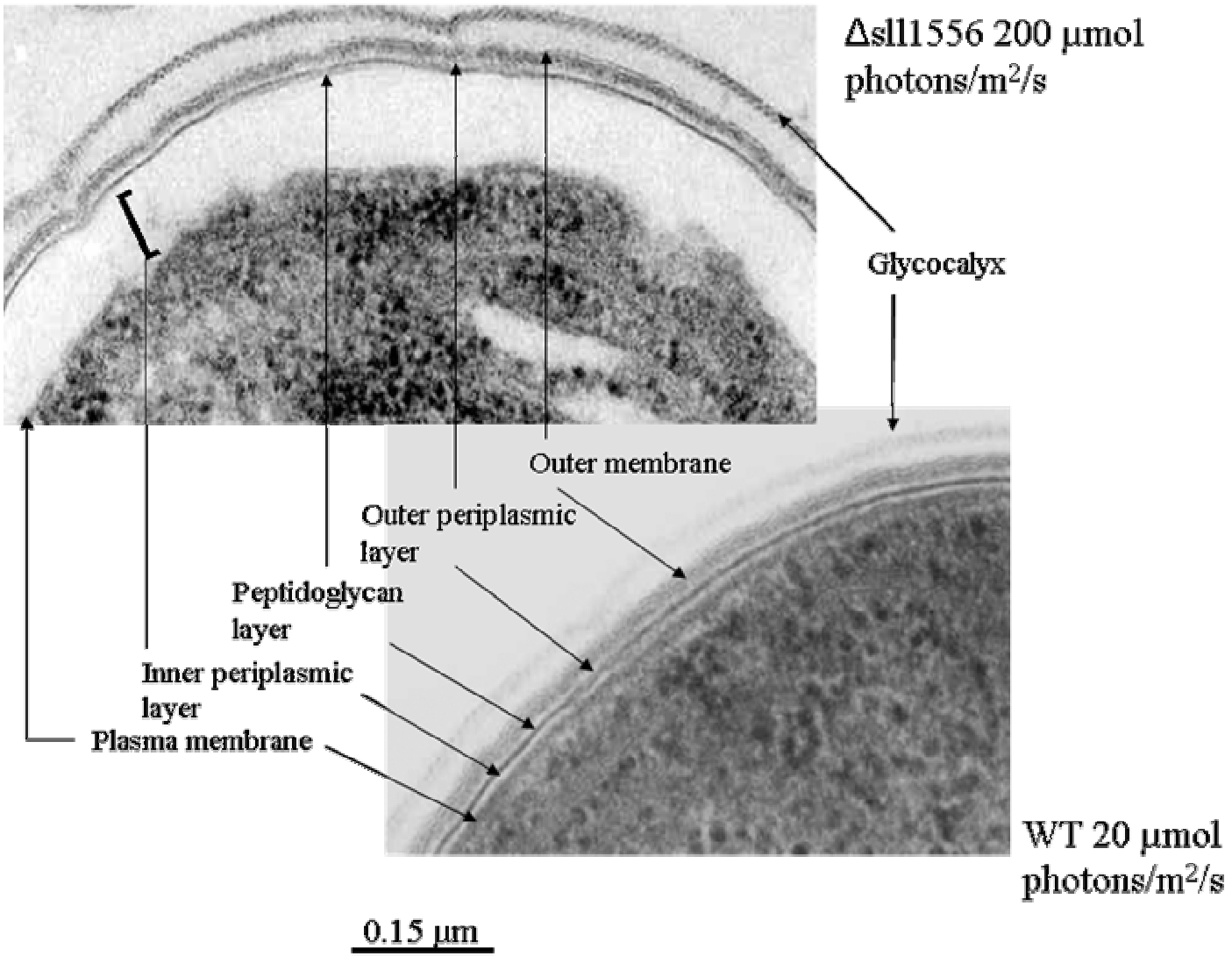

A.
B.
0.3 µm

Figure 4-6. Transmission electron micrograph of the enlarged outer wall layers of a *Synechocystis* PCC 6803 WT and Δ*sll1556* cell (as noted). Δ*sll1556* cells typically exhibited an extended inner periplasmic space (bracket) when grown under 200 μmol photons/m^2/s that is not apparent in WT cells grown under similar conditions. A WT cell grown under 20 μmol photons/m^2/s is pictured to aid in the identification of the various wall layers. Scale, 0.15 μm.


Δsll1556 200 µmol photons/m²/s
Glycocalyx
Outer membrane
Outer periplasmic layer
Peptidoglycan layer
Inner periplasmic layer
Plasma membrane
WT 20 µmol photons/m²/s
0.15 µm

Pigment analysis of Δ*sll1556* cells

With the observed impairment in growth and altered cell structure of the Δ*sll1556* mutant, it was of interest to possibly correlate the isoprenoid-dependent pigments (carotenoids and chlorophyll). Carotenoids and the phytol tail of chlorophyll are both products of the MEP pathway in photosynthetic organisms (Eisenreich et al., 1998; Rohmer, 1999). These pigments are part of the photosynthetic apparatus and are involved in the absorption and transmission of light energy for photosynthesis (Lawlor, 1987). Carotenoids have an additional function as photoprotectants against excited triplet chlorophyll and oxygen radicals which result from excess light energy (Britton, 1995; Demmig-Adams et al., 1996; Frank and Cogdell, 1996). Because of their importance, a reduction in chlorophyll or carotenoid synthesis could be a detriment to the cell.

Chlorophyll and carotenoid concentrations were determined for both WT and Δ*sll1556* cells grown under 20 μmol photons/m^2/s and 200 μmol photons/m^2/s. Under non-stress conditions, WT cells had over 2.5 times the amount of chlorophyll that mutant cells did (Table 4-3). Similarly, the concentration of total carotenoids in WT cells was over 2 times that which was found in Δ*sll1556* cells. This is consistent with the earlier finding that WT cells had ca. 30% more thylakoid membranes than Δ*sll1556* cells (Poliquin et al., 2004) since both chlorophyll and carotenoids are associated with the photosynthetic apparatus in thylakoid membranes (Hall and Rao, 1994).

Growth under high light stress resulted in an overall decrease in the amount of chlorophyll and carotenoids for WT cells as compared to non-stress light conditions

Table 4-3. Chlorophyll and carotenoid concentrations in WT and Δ*sll1556* cells. Results are the average of pigment analyses of 2 or 3 separately grown cultures ± standard deviation.

Light intensity and cell strain	Chlorophyll concentration (fg/cell)	Carotenoid concentration (fg/cell)	Chlorophyll/Carotenoid ratio
20 μmol photons/m^2/s			
WT	9.70 ± 1.32	3.40 ± 0.32	2.85
Δ*sll1556*	3.82 ± 0.19	1.40 ± 0.24	2.73
200 μmol photons/m^2/s			
WT	4.24 ± 0.58	1.71 ± 0.56	2.48
Δ*sll1556*	2.06 ± 0.46	0.98 ± 0.26	2.10

(Table 4-3). The overall reduction in pigments is in agreement with what has been previously found for high light grown *Synechocystis* PCC 6803 (Havaux et al., 2003) and is likely related to having fewer thylakoid membranes, as well as a down regulation of photosynthetic reaction centers and light harvesting antenna pigment complexes (Anderson, 1986). In comparison to WT cells grown at 200 μmol photons/m^2/s, Δ*sll1556* cells had fewer chlorophyll and carotenoids per cell. Again, this is consistent with the noticeable disruption in thylakoid membranes seen in the mutant under high light (Figure 4-5B) and provides further evidence supporting the importance of Sll1556 under light stress. It is interesting to note that the chlorophyll to carotenoid ratio is rather uniform for both WT and Δ*sll1556* cells except in Δ*sll1556* cells grown at 200 μmol photons/m^2/s, where there was less chlorophyll.

Although carotenoids have two basic functions within photosynthetic organisms (light harvesting and photoprotection), their role may vary depending on where they are located (Hirschberg and Chamovitz, 1994). For that reason, it was of interest to examine the carotenoid composition of Δ*sll1556* cells to get a better understanding of how the mutation affected the synthesis of particular carotenoids. *Synechocystis* contains the carotenoids β-carotene, echinenone, zeaxanthin, and myxoxanthophyll (Takaichi et al., 2001). HPLC analysis of pigments extracted from WT cells grown under 20 μmol photons/m^2/s results in an elution profile typical of Figure 4-7, whereby chlorophyll, myxoxanthophyll, zeaxanthin, echinenone, and β-carotene are all evident. Analysis of extracted pigments from Δ*sll1556* cells resulted in a similar HPLC elution profile (not shown), indicating all carotenoids were being synthesized in the mutant. However, the change of individual carotenoids relative to

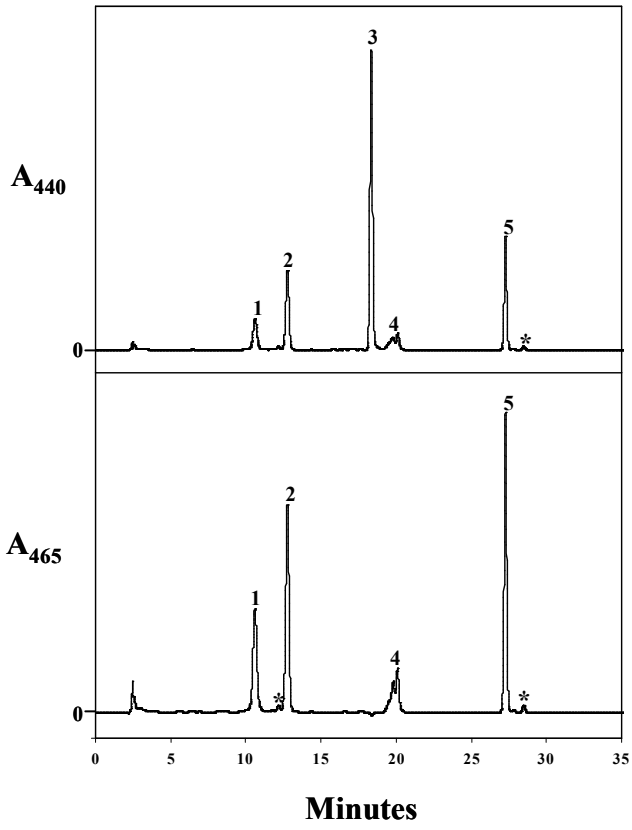


Figure 4-7. HPLC elution profile of pigments extracted from *Synechocystis* WT cells grown at 20 μmol photons/m^2/s. The absorption at 440 nm (chlorophyll and carotenoids) and 465 nm (carotenoids only) are shown. Pigments are as labeled: 1, myxoxanthophyll; 2, zeaxanthin; 3, chlorophyll; 4, echinenone; 5, β-carotene. The asterisks denote *cis*-isomers of zeaxanthin and β-carotene.

chlorophyll in WT and Δ*sll1556* cells was surprisingly much less than anticipated (Figure 4-8). Variability did exist in both strains as evidenced by the large standard deviations in some samples, even though HPLC was done on 3 separately grown cultures of each strain (for both light intensities), all similarly harvested in the log phase of growth. This probably correlates with the pleiomorphic nature as noted earlier, which tends to be more prevalent in the mutant.

WT and Δ*sll1556* cells grown under 20 μmol photons/m^2/s had no significant difference in the amount of myxoxanthophyll, echinenone, zeaxanthin, or β-carotene (Figure 4-8). Growth under 200 μmol photons/m^2/s, however, resulted in an increase of myxoxanthophyll in both WT and Δ*sll1556* cells as compared to those grown under 20 μmol photons/m^2/s, while echinenone remained rather constant. Zeaxanthin, a potential photoprotectant, remained relatively the same in the high light even in Δ*sll1556* cells and a conclusion is difficult due to the wide range in standard deviation. Similarly, β-carotene may have increased slightly in WT cells grown under 200 μmol photons/m^2/s, but a large standard deviation exists. These results are similar to those of Havaux et al. (2003) in that while overall carotenoid concentration decreased in *Synechocystis* PCC 6803 WT cells under high light stress, the amount of myxoxanthophyll increased (ca. 20%). They also reported a slight increase in zeaxanthin under high light, but our results suggest that there is no significant difference. It is important to note that HPLC analysis of pigment extracts from 200 μmol photons/m^2/s grown Δ*sll1556* cells yielded mixed results. While the average of 3 separate experiments suggests that the levels of each carotenoid analyzed did not change much as compared to WT, the variability of the cells within the culture

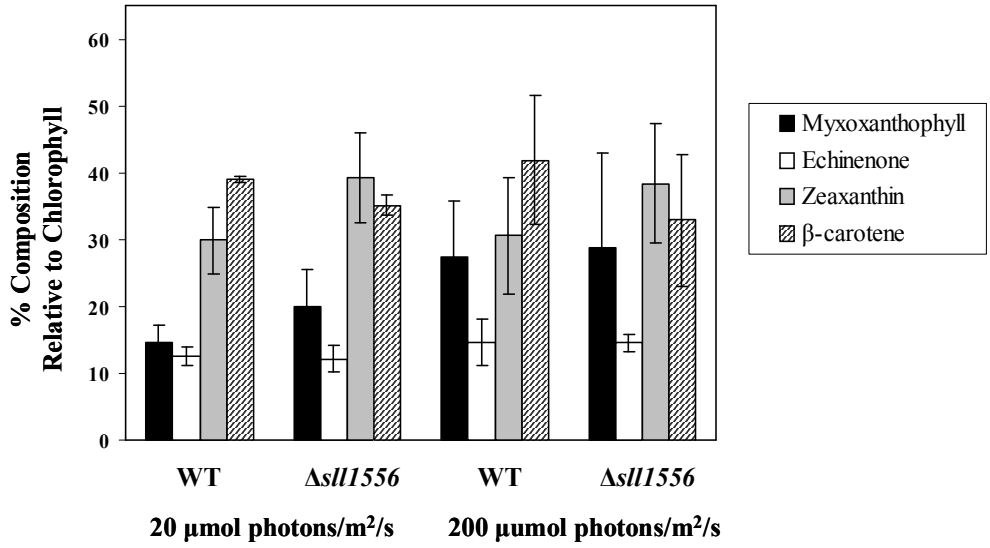


Figure 4-8. Carotenoid composition is variable in WT and Δ*sll1556* cells grown at 20 µmol photons/m^2/s and 200 µmol photons/m^2/s. Extracted pigments were analyzed by HPLC and carotenoid content expressed relative to chlorophyll. Non-stressed WT and Δ*sll1556* cells had comparable overall carotenoid composition. At 200 µmol photons/m^2/s, WT and mutant cells had greater variability in the amount of myxoxanthophyll, zeaxanthin, and β-carotene they contained, but overall did not significantly differ from one another. Both strains had a slight increase in myxoxanthophyll at this light intensity. Results are the average of 3 experiments, each done with separately grown cultures. Standard deviations are indicated.

resulted in large standard deviations in the case of myxoxanthophyll and zeaxanthin. It is difficult to say whether any of these changes are significant. In general, though, the results indicate that mutant cells are making fewer pigments than WT cells, especially in the amount of chlorophyll, but also in carotenoids.

Discussion

Synechocystis is an obligate photoautotroph, meaning it depends entirely on light as its energy source. As such, it has had to adapt to changing environmental conditions since it first evolved over 2.5 billion years ago in order to survive (Olson and Blakenship, 2004). The results from this study indicate Sll1556 has an important role in *Synechocystis* PCC 6803, especially under high light stress. The Sll1556 protein was more highly expressed under high light (Figure 4-3), and WT cells had a significant advantage over Δ*sll1556* cells grown at 200 μmol photons/m^2/s and out-competed them when grown together in a mixed culture (Figures 4-1, 4-2). The impairment in growth and overall fitness under high light stress is not simply due to cell death, however. Removal of mutant cultures from high light stress routinely restored normal cell growth. Furthermore, approximately 20-30% of high light grown cells (both WT and Δ*sll1556*) viewed by electron microscopy were dividing, indicating a viable culture. Instead, this impairment was likely the result of modified cell structure and reduced pigment concentrations, which are likely due to a deficiency in isoprenoid production in the mutant. This deficiency would be particularly detrimental for cells exposed to high light since more isoprenoids are required (such as those involved in membrane synthesis and photosynthesis) in

response to the rapid growth that occurs at this light intensity when the doubling time decreases by more than 50% (Hihara et al., 2001).

The pleiomorphic nature that we noted is not unusual and has also been reported elsewhere (Mohammed et al., 2005). Although the Δ*sll1556* mutation caused changes in morphology under stressed and non-stressed conditions (smaller cells, reduced number of thylakoids, enlarged inner periplasmic layer) as well as a decrease in the amount of chlorophyll and carotenoids produced, the degree of severity of these changes varied (as seen by large standard deviations). This was true for WT cells as well. This is indicative of an organism that is well suited to responding to stresses, whether caused by mutation or environmental change.

In general, though, Δ*sll1556* cells displayed a characteristic structural phenotype under both stress and non-stress growth conditions that was not apparent in WT cells (Figure 4-4; 4-5), which is not unusual for an organism impaired in isoprenoid synthesis. As recently reported, *Synechocystis* mutants impaired in the production of the carotenoid myxoxanthophyll had altered cell structure (Mohamed et al., 2005), a finding that has also been observed in the chloroplasts of isoprenoid-deficient mutants in plants (Hsieh et al., 2005; Nagata et al., 2002; Page et al., 2004). The obvious reduction in the number of thylakoid membranes in Δ*sll1556* cells, especially at high light, was consonant with a deficiency in isoprenoid production. Isoprenoids such as hopanoids (similar to eukaryotic sterols), chlorophyll, and carotenoids are present within thylakoid membranes, and the latter two are important for the assembly of the photosynthetic apparatus and thylakoids (Jürgens et al., 1992; Simonin et al., 1996; Masamoto et al., 2004; Anderson, 1986). Carotenoids may also

have an additional function in stabilizing membranes (Havaux, 1998). Therefore, it was in agreement that Δ*sll1556* cells had less chlorophyll and carotenoids per cell at both 20 μmol photons/m^2/s and 200 μmol photons/m^2/s as compared to WT cells (Table 4-3).

One interesting feature characteristic of Δ*sll1556* cells grown under high light stress was an enlargement of the inner periplasmic layer (Figure 4-5, 4-6). In *Synechocystis*, the periplasmic layer contains proteases, transport proteins, and numerous proteins of unknown function (Fulda et al., 2000). Recently, Klinkert et al. (2004) found that a *Synechocystis* PCC 6803 mutant lacking a gene encoding a periplasmic protein of unknown function exhibited a reduction of photosystem II, one of the pigment/protein complexes of the photosynthetic machinery. Their results led them to conclude that this protein may be involved in the initial processing steps of photosystem II, which has been reported to occur within the plasma membrane, not the thylakoid membrane as once thought (Zak et al., 2001). The proteins that make up this early photosystem complex within the plasma membrane were already found to be associated with chlorophyll (Zak et al., 2001). Similarly within the plasma membrane, the existence of a cyanobacterial carotenoid-binding protein has been reported, with a possible role in protection against high light (Masamoto et al., 1987; Reddy et al., 1989). Perhaps the reduction in chlorophyll and carotenoids (as well as other isoprenoids) in the Δ*sll1556* mutant results in the accumulation of proteins or compounds that are normally involved in plasma membrane-associated processes, causing an extension of the inner periplasmic layer. Although no such alteration was seen in the outer periplasmic layer or outer wall layers (Figure 4-6), carotenoid-

binding proteins have also been localized to the outer membrane (Engel et al., 1991). It is possible that a lack of transport to the outer wall layers could cause an enlarged inner periplasmic extension.

The Δ*sll1556* mutant had fewer carotenoids than WT cells under both light stress and non-stress growth conditions (Table 4-3). In cyanobacteria, carotenoids have been identified in each of the membrane systems of the cell. In the thylakoid membranes, β-carotene has been found in the reaction centers of both photosystems (Kamiya and Shen, 2003; Jordan et al., 2001), zeaxanthin in photosystem II of *Synechocystis* PCC 6803 (Tracewell et al., 2001), and echinenone in the cytochrome b_6f complex (Boronowsky et al., 2001; Wenke et al., 2005). In addition, myxoxanthophyll is thought to be important for its stabilization (Mohamed et al., 2005). The plasma membrane of *Synechocystis* PCC 6714 has been reported to contain β-carotene, echinenone, and zeaxanthin (Omata and Murata, 1984). These carotenoids, along with myxoxanthophyll, have also been detected in the outer cell membrane of this organism (Jürgens and Weckesser, 1985). The composition of carotenoids found within Δ*sll1556* cells was not significantly influenced by the impairment in isoprenoid production, taking into consideration the high degree of variability that was found (Figure 4-8). Under 20 μmol photons/m^2/s and 200 μmol photons/m^2/s, Δ*sll1556* cells were comparable to WT cells in that they had similar overall proportions of myxoxanthophyll, echinenone, zeaxanthin, and β-carotene when compared to chlorophyll. This indicates that the impairment in carotenoid synthesis was general and not specific to any particular carotenoid.

The overall carotenoid concentration in WT cells decreased slightly in high light (ca. 1.5 fold; Table 4-3), which was also seen by Havaux et al. (2003), and is readily attributable to an overall down regulation of the photosynthetic apparatus since more light energy is available. High light grown WT cells had more myxoxanthophyll, which has recently been reported to play a role in thylakoid and outer membrane structure in *Synechocystis* PCC 6803 (Mohamed et al., 2005). Interestingly, zeaxanthin did not accumulate in WT cells grown under 200 μmol photons/m^2/s (Figure 4-8). This is not in agreement with its suggested role in photoprotection within the photosynthetic apparatus of *Synechocystis* PCC 6803 cells (Schäfer et al., 2005).

It is worth noting that much remains to be determined regarding the role of particular carotenoids within this organism. In addition, it is sometimes difficult to make comparisons with the findings of other laboratories because of the variability in growth conditions used. Because this strain of *Synechocystis* is glucose-tolerant, cells are sometimes grown mixotrophically (using glucose as an alternate carbon source; as in Mohamed et al., 2005). Also, in studies measuring stress responses to high light (such as pigment composition or photoinhibition) it is not uncommon for cells to be grown at irradiances of 450 μmol photons/m^2/s to 1200 μmol photons/m^2/s and harvested after only a few hours (as in Havaux et al., 2003 and Schäfer et al., 2005). Since these conditions are not always physiological, it can be difficult to make conclusions about what might actually be occurring under favorable conditions within this photoautotrophic cyanobacterium.

The results of this study leave little doubt that *Synechocystis* PCC 6803 Sll1556 plays an important role in isoprenoid biosynthesis, particularly under high light when isoprenoids may be needed for rapid growth. The question remains, however, regarding its function. Sll1556 was first annotated as a type 2 IPP isomerase based on 32% sequence identity with the *Streptomyces* enzyme (Kaneda et al., 2001). Initially, our lab could not demonstrate IPP isomerase activity or that of other enzymes with low homology such as glycolate oxidase and lactate dehydrogenase, but instead found that Sll1556 was required for *in vitro* pentose phosphate cycle (PPC) substrate stimulated DMAPP biosynthesis in *Synechocystis* (Poliquin et al., 2004; Chapter 3). Recently, however, Barkley et al. (2004b) demonstrated type 2 IPP isomerase activity for purified recombinant Sll1556. The enzyme was found to have optimal activity in the presence of 10 mM NADPH, a concentration that is higher than what has been previously used in activity assays of other type 2 IPP isomerases (Kaneda et al., 2001; Takagi et al., 2004; Laupitz et al., 2004; Yamashita et al., 2004). This would explain why IPP isomerase activity for Sll1556 could not be demonstrated in our earlier attempts, when only 1 mM NADPH was used in the assay based on reported requirements (Poliquin et al., 2004). The need for high concentrations of NADPH for IPP isomerase activity, as well as our finding that PPC stimulated isoprenoid synthesis in this organism requires Sll1556 (Poliquin et al., 2004), leads to the consideration that the addition of PPC compounds to our *in vitro* system stimulates Sll1556 IPP isomerase activity (Figure 4-9), since one of the products of the pentose phosphate pathway is NADPH (Yang et al., 2002). It should be noted that the 10 mM NADPH needed for Sll1556 IPP isomerase activity

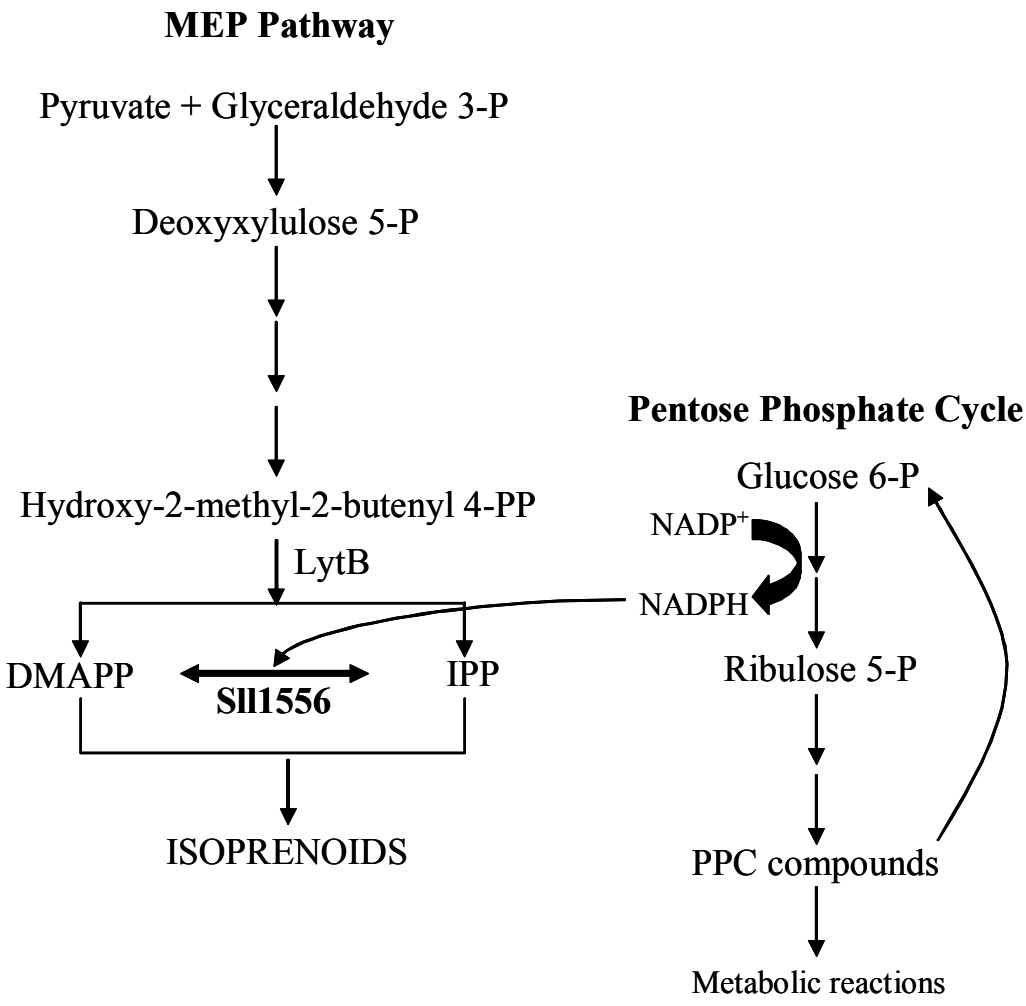


Figure 4-9. Possible role of *Synechocystis* Sll1556 as a type 2 IPP isomerase in isoprenoid biosynthesis. NADPH produced by the conversion of glucose 6-phosphate to ribulose 5-phosphate in the pentose phosphate cycle (PPC) may be used by Sll1556 to interconvert IPP and DMAPP made from the MEP pathway to produce longer chained isoprenoids.

is over 10 times the concentration that is actually found within cyanobacteria and chloroplasts in the light (0.65-0.7 mM) (Tamoi et al., 2005; Latouche et al., 2000). While it is important to consider that the reported requirements for activity were all determined from *in vitro* assays that may not entirely reflect what is happening *in vivo*, the significant contrast in the NADPH concentration needed versus what is available raises the possibility of dual functions for Sll1556.

One such function could be in the enhancement of DMAPP production using intermediates of the MEP pathway (Figure 4-10). Evidence indicates PPC compounds are not directly incorporated into isoprenoids in our *in vitro* system (Chapter 3), but perhaps products of the PPC, such as NADPH, stimulate the synthesis of MEP pathway intermediates, which are then acted upon by Sll1556. LytB, the terminal enzyme of the MEP pathway, would not be involved because it is not required for PPC substrate stimulation (Chapter 3). This scheme seems less likely, though, given that pyruvate, deoxyxylulose 5-phosphate, methyl-erythritol 5-phosphate (Ershov et al., 2002; Poliquin et al., 2004), and hydroxy-2-methyl-2-butenyl-4-diphosphate (unpublished) do not stimulate isoprenoid synthesis in our *in vitro* assay.

Of course, the possibility remains that *in vitro* PPC stimulation of isoprenoid synthesis occurs through an alternate shunt to DMAPP involving as yet unknown substrate(s). Whatever the mechanism may be, however, it is likely important for balancing the pool of low molecular weight isoprenoids (i.e. IPP and DMAPP), especially under high light growth conditions when enhanced production of isoprenoids is needed.

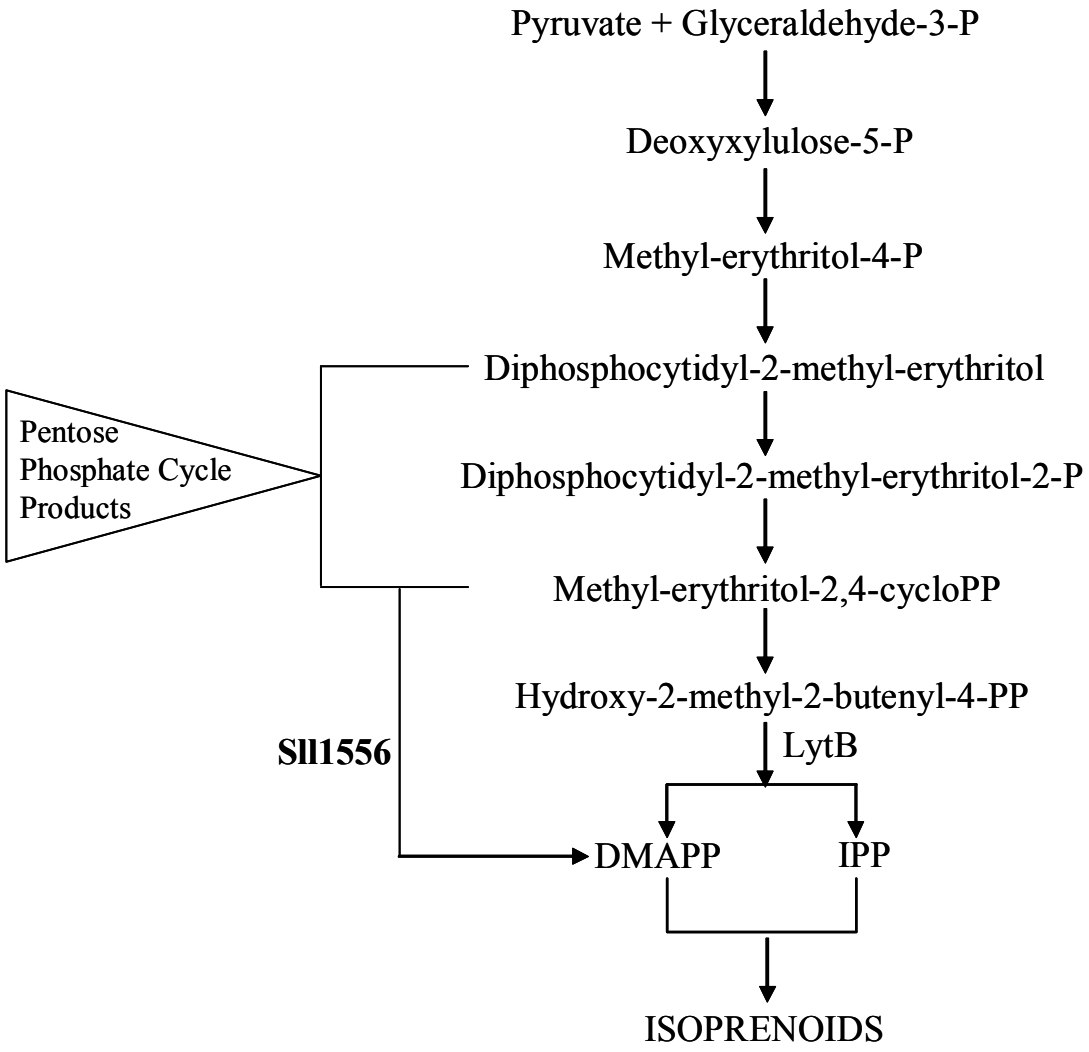


Figure 4-10. Possible role of *Synechocystis* Sll1556 in the utilization of MEP pathway intermediates for DMAPP synthesis. Products of the pentose phosphate cycle may stimulate the production of intermediates of the MEP pathway (excluding pyruvate, deoxyxylulose-5-phosphate, methyl-erythritol-4-phosphate, and hydroxy-2-methyl-2-butenyl-4-diphosphate, which do not stimulate *in vitro* isoprenoid synthesis). These are then utilized to make DMAPP through the action of Sll1556.

CHAPTER 5

Summary and Future Research

Summary

In this dissertation, isoprenoid biosynthesis in the photosynthetic cyanobacterium *Synechocystis* PCC 6803 was assessed in view of the operation of the MEP pathway, which is the primary pathway by which isoprenoids are synthesized. It is has been generally presumed that it is a linear pathway where the final enzyme LytB gives rise to IPP and DMAPP, which upon isomerization produces the C_5 building blocks for isoprenoids (Rodríguez-Concepción and Boronat, 2002; Bouvier et al., 2005). This work, however, provides new insight into isoprenoid biosynthesis in *Synechocystis*. The results obtained support previous research in the laboratory that found isoprenoid production in *Synechocystis* differs from that seen in the bacterial model used to elucidate the steps of the MEP pathway (Ershov et al., 2000; 2002).

In Chapter 2 it is shown that *in vitro* pentose phosphate cycle (PPC)-stimulated isoprenoid biosynthesis, as measured by [^{14}C]IPP incorporation, was dependent on a protein (Sll1556), an apparent enzyme. Although required for *in vitro* activity, deletion of the *sll1556* gene did not affect cell viability under optimal photoautotrophic growth conditions, perhaps suggesting an alternate source of low molecular weight isoprenoids through the pentose phosphate cycle.

In Chapter 3 it was determined that *in vitro* PPC-stimulated isoprenoid biosynthesis resulted in the production of C_5, C_{10} and C_{20} compounds. Interestingly, unlike Sll1556,the terminal enzyme (LytB) of the MEP pathway was not required for isoprenoid synthesis. PPC compounds appear to only stimulate isoprenoid formation, however, since they were not directly incorporated into isoprenoid compounds.

The physiologically importance of Sll1556 to *Synechocystis* was clearly demonstrated in Chapter 4. From the combined results it is concluded that limited isoprenoid biosynthesis under high light stress makes the Δ*sll1556* mutant less competitive than wild type cells.

The mechanism of PPC-stimulated isoprenoid biosynthesis is still unknown, but it is clear that an alternate method of enhancing isoprenoid production exists in *Synechocystis*. This is not to say that the well-described MEP pathway is not essential in this organism. Instead, it is confirmation of the adaptability of this 'primitive' photoautotrophic cyanobacterium that has been essential for its survival for over 2.5 billion years.

Preliminary Results towards Future Research

Some major issues remain to be addressed in future research: what is the mechanism by which Sll1556 is affected by PPC substrates; does Sll1556 have more than one function; and what interrelationships within the PPC pathway could account for the stimulatory action of multiple phosphorylated PPC compounds (Ershov et al. 2002). Some preliminary results are included here in an attempt to address some of these concerns.

Sll1556 is a soluble enzyme that is dependent on various cofactors, a critical one being NADPH. Barkley et al. (2004b) demonstrated IPP isomerase activity for purified recombinant Sll1556 in the presence of physiologically unrealistic high NADPH concentrations. Under our conditions we sought to verify that the stimulatory effect by PPC compounds *in vitro* was not simply due to an interaction between PPC substrates and Sll1556 that enhanced IPP isomerase activity. Using purified Sll1556, an assay for IPP isomerase activity using [^{14}C]IPP was done (as in Chapter 2 Materials and Methods) in the presence of FR6P and GL6P, but IPP isomerase activity could not be detected.

We next investigated the effect of NADPH, as well as other cofactors and coenzymes, on Sll1556-dependent PPC-stimulated isoprenoid biosynthesis. Using a WT cell-free extract, [^{14}C]IPP incorporation into the isoprenoid fraction in the presence of FR6P was measured (as described in Chapter 2 Materials and Method). Additional reactions were also set up in which components of the standard reaction mixture were omitted: FAD, NADP(H), nucleotide triphosphates (ATP, CTP), thiamine pyrophosphate (TPP), or coenzyme B_{12}. Omitting ATP, CTP, TPP or B_{12} did not have any considerable effect on the amount of incorporation achieved compared to the control (Figure 5-1). In contrast, a significant decrease in the amount of [^{14}C]IPP incorporated was apparent when FAD or NADP(H) was left out (ca. 60% and 40% less than the control, respectively). The requirement of PPC-stimulated isoprenoid biosynthesis for NADPH was most interesting, considering that Sll1556 IPP isomerase activity depended on NADPH, albeit at a high, non-physiological concentration (Barkley et al., 2004; Tamoi et al., 2005; Latouche et al., 2000).

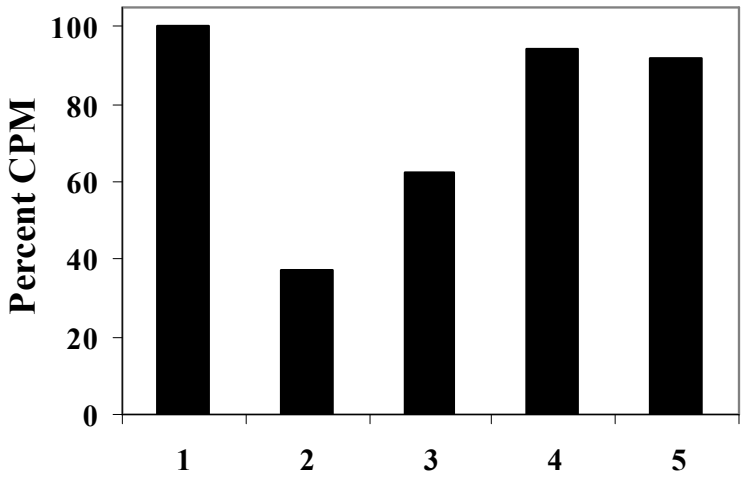


Figure 5-1. PPC stimulated isoprenoid biosynthesis requires FAD and NADP(H). [^{14}C]IPP incorporation into a WT cell-free extract with the addition of 500 μM FR6P containing the usual cofactor mix (1) (as described in Chapter 2 Materials and Methods) or omitting: 2) FAD; 3) NADP(H); 4) ATP, CTP, TPP; 5) coenzyme B_{12}. Incubation time was 40 min. Results are expressed as percent CPM of the control (1).

Because of a potential stimulatory effect by PPC substrates and the effect on Sll1556, it is of interest to look at a selected portion of the PPC pathway. As seen in Figure 5-2, production of NADPH occurs when glucose-6-phosphate is converted to ribulose-5-P. Fructose-6-P can be easily isomerized to glucose-6-phosphate and thus would give a comparable stimulation to that of glucose-6-phosphate. In fact, both phosphorylated glucose and fructose gave equal stimulation of [^{14}C]IPP incorporation (Ershov et al., 2002). If the stimulation were attributable only to NADPH production, however, it may be difficult to rationalize and account for the stimulation that is also obtained with glyceraldehyde-3-phosphate, erythrose-4-phosphate, ribulose-5-phosphate, and 6-phosphogluconate, all of which are PPC compounds.

We further explored the possibility that addition of PPC substrates to our *in vitro* assay system generates NADPH via the PPC, which is then utilized by Sll1556 to enhance isoprenoid production. To determine the effect that NADPH concentration had on isoprenoid biosynthesis in WT cell-free extracts, a [^{14}C]IPP incorporation assay was done (Chapter 2 Material and Methods) using varying amounts of NADPH. This was compared to the incorporation of [^{14}C]IPP with FR6P addition (with the usual coenzyme and cofactor reaction mixture). As shown in Figure 5-3, the amount of [^{14}C]IPP incorporation increased with NADPH concentration, with little incorporation seen in the absence of NADPH. This may suggest that the incorporation seen in the reaction containing FR6P, but lacking NADPH (Figure 5-1, column 3), might be the result of NADPH production by the PPC.

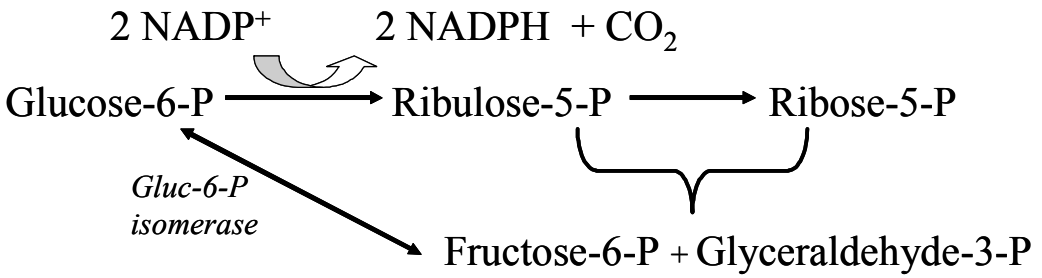


Figure 5-2. Early steps of the pentose phosphate cycle. Glucose-6-P is oxidized, which results in the production of NADPH and carbon intermediates involved in biosynthetic reactions. Fructose-6-P is converted to glucose-6-phosphate by an isomerase.

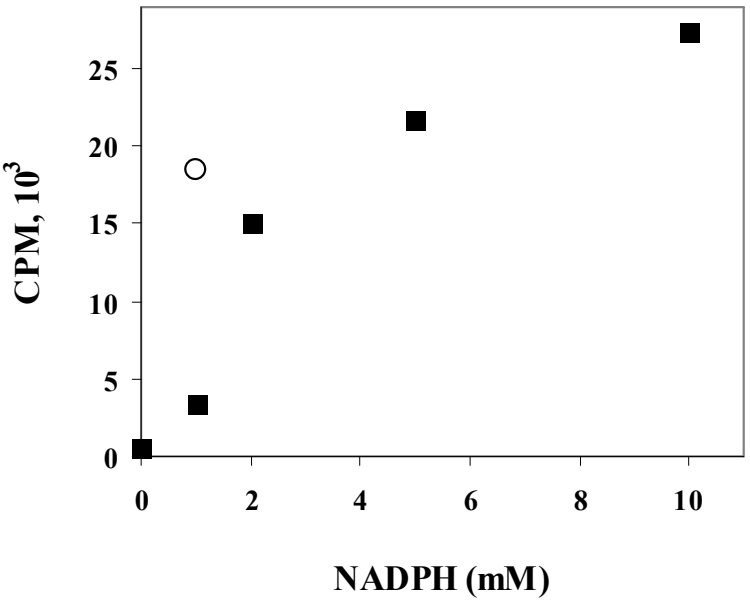


Figure 5-3. *In vitro* isoprenoid biosynthesis in *Synechocystis* increases with addition of NADPH. [^{14}C]IPP incorporation into the isoprenoid fraction of a WT cell-free extract was measured in the presence of varying concentrations of NADPH (■). For comparison, FR6P stimulated [^{14}C]IPP incorporation using the typical incubation mix (which includes 1 mM NADPH; see Chapter 2 Material and Methods for details) is shown (○). Incubation time was 40 min.

The possible production of NADPH through the addition of PPC compounds was further investigated. NADPH is produced through the reduction of NADP. If NADPH is being synthesized by the PPC as a result of FR6P addition, reducing the endogenous pool of NADP should lead to a reduction in the amount of NADPH being produced and thus [^{14}C]IPP incorporation. A [^{14}C]IPP incorporation assay was done using WT cell-free extracts with no added NADP(H). The reaction mixture was preincubated with or without 0.25 units/mL NADase (Sigma-Aldrich), an enzyme that hydrolyzes NAD(P), rendering it unusable for NADP(H) synthesis. [^{14}C]IPP and FR6P were then added as usual (Chapter 2 Material and Methods). Results suggest that the addition of FR6P to our *in vitro* [^{14}C]IPP incorporation assay does indeed lead to the production of NADPH (Figure 5-4). The amount of incorporation in the reaction with FR6P that was pre-incubated with NADase was almost 4 times less than what was obtained with FR6P addition alone. Further experimentation is needed to verify these findings, though. We recently learned of another compound, 6-aminonicotinamide, that eliminates NADPH production from the PPC cycle by inhibiting the enzyme that generates NADPH and plan to test its effect on PPC dependent NADPH synthesis in our *in vitro* assay.

The results of these preliminary experiments indicate NADPH is involved in PPC-stimulated isoprenoid biosynthesis in *Synechocystis*. It is possible that NADPH produced from the PPC may be used for Sll1556 IPP isomerase activity, although optimal activity is achieved with 10 mM NADPH and the reported in vivo concentration of NADPH is 0.65-0.7 mM for cyanobacteria and chloroplasts (Tamoi et al., 2005; Latouche et al., 2000). It is interesting to note that the results of our

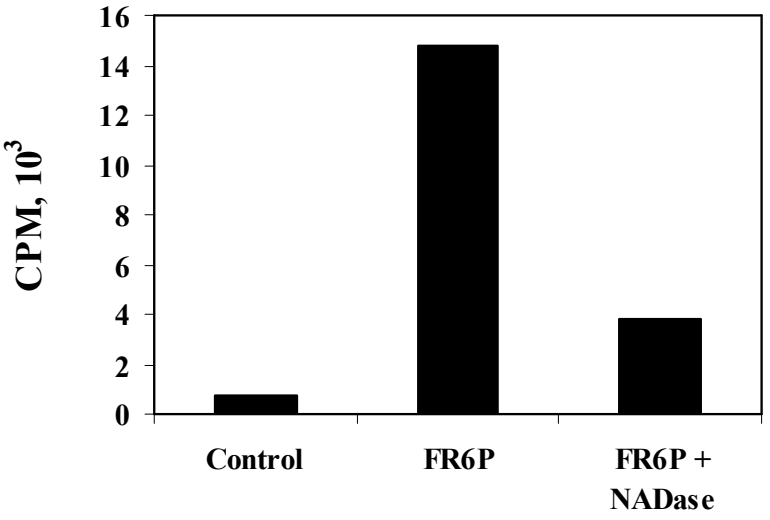


Figure 5-4. FR6P addition to WT cell-free extracts results in the production of NADPH. WT cell-free extracts were preincubated with or without 0.25 units/mL NADase as indicated, followed by addition of FR6P and [^{14}C]IPP. Control, no FR6P addition. Incubation time was 40 min.

work with *Synechocystis* Sll1556 are consonant with what has been reported for studies on plant IPP isomerases. For example, Page et al. (2004) recently described their work using virus-induced gene silencing of the plastid localized type 1 IPP isomerase in *Nicotiana benthamiana* (tobacco). The amount of chlorophylls and carotenoids, which are derived from the MEP pathway in chloroplasts, each decreased by approximately 80% in leaves with a deficiency in IPP isomerase as compared to the control and chloroplasts exhibited altered thylakoid membrane organization. In addition, Nakamura et al. (2001) reported that in *N. tabacum*, an increase in the transcript of plastid-targeted IPP isomerase was evident in plants grown under high light stress. This is similar to what we found for Sll1556 protein expression (Figure 4-3). However, it still remains that for IPP isomerase activity, Sll1556 requires non-physiological NADPH concentrations.

Instead, it is possible that Sll1556 has a dual function, both of which require NADPH. Sll1556 activity may be involved in an enhancement of DMAPP production through the already established pathway to isoprenoids. This mechanism, however, would not involve LytB, the terminal enzyme of the MEP pathway that makes both IPP and DMAPP, as it has been previously shown that LytB activity is not required for PPC substrate stimulated isoprenoid synthesis (Figure 3-4). Another possibility is that Sll1556 has an unknown function, one in which DMAPP is produced through an as yet undiscovered shunt involving alternate substrates. It is not unrealistic to consider that a photoautotrophic organism would use alternate means in the form of energy and metabolites from photosynthesis to enhance cellular processes such as membrane synthesis, pigment production, etc., for growth and

development, something that would not be apparent by studying a non-photosynthetic organism.

Future Research

While much was discovered about isoprenoid biosynthesis in *Synechocystis* during the course of this work, there are still many aspects to consider. One obvious question is how PPC substrate stimulated isoprenoid production occurs, as discussed above. Further investigation is needed to determine the function of Sll1556 and how NADPH is involved. One way in which to learn more about the activity and regulation of Sll1556 is to determine whether it exists in a complex with other proteins. This can be investigated by affinity purifying epitope-tagged Sll1556 and any associated proteins from *Synechocystis* cells (as described in Rigaut et al., 1999), which can then be identified by mass spectrometry. The identification of possible Sll1556-associated proteins may provide information about its function. This technique has been attempted in our lab but results were difficult to obtain because of the low abundance of Sll1556 within the cell. Recent attempts, however, at purifying epitope-tagged LytB have been met with some success using variations on the original technique. Affinity purification of epitope-tagged LytB may in itself provide new insights about Sll1556, since these two enzymes are involved in C_5 synthesis and may be associated.

Another aspect of this project that would be interesting to explore is whether PPC-stimulated isoprenoid biosynthesis occurs in plants. Cyanobacteria are thought to be the progenitors of plant chloroplasts and as such, make a good model for

studying isoprenoid biosynthesis in a photosynthetic organism. No homologs to Sll1556 have been identified in plants thus far, but it would be a novel discovery if chloroplasts were similarly able to utilize PPC compounds to enhance isoprenoid production. The one difficulty with testing this is the presence of an active type 1 IPP isomerase that interconverts IPP and DMAPP within chloroplasts that is not found within *Synechocystis*. To determine if PPC substrates stimulate isoprenoid synthesis in chloroplasts using our *in vitro* [^{14}C]IPP incorporation assay, IPP isomerase activity needs to be eliminated. We first attempted to do this by adding iodoacetamide, a reported inhibitor of IPP isomerases (Ramos-Valdivia et al., 1997), to the assay. Results indicate, however, that iodoacetamide not only inhibits IPP isomerase activity, but also that of prenyltransferases as well. Not knowing what other enzymes might be affected by this non-specific inhibitor, IPP isomerase activity will have to be eliminated in another way, possibly through immunodepletion using antibodies to a green algal IPP isomerase already available in the lab.

In short, there is still much work to be done and many avenues to explore in understanding isoprenoid biosynthesis in *Synechocystis*.

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