Myxoxanthophyll Is Required for Normal Cell Wall Structure and Thylakoid Organization in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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Myxoxanthophyll is a carotenoid glycoside in cyanobacteria that is of unknown biological significance. The sugar moiety of myxoxanthophyll in *Synechocystis* sp. strain PCC 6803 was identified as dimethyl fucose. The open reading frame *sll1213* encoding a fucose synthetase orthologue was deleted to probe the role of fucose and to determine the biological significance of myxoxanthophyll in *Synechocystis* sp. strain PCC 6803. Upon deletion of *sll1213*, a pleiotropic phenotype was obtained: when propagated at $0.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, photomixotrophic growth of cells lacking *sll1213* was poor. When grown at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, growth was comparable to that of the wild type, but cells showed a severe reduction in or loss of the glycocalyx (S-layer). As a consequence, cells aggregated in liquid as well as on plates. At both light intensities, new carotenoid glycosides accumulated, but myxoxanthophyll was absent. New carotenoid glycosides may be a consequence of less-specific glycosylation reactions that gained prominence upon the disappearance of the native sugar moiety (fucose) of myxoxanthophyll. In the mutant, the N-storage compound cyanophycin accumulated, and the organization of thylakoid membranes was altered. Altered cell wall structure and thylakoid membrane organization and increased cyanophycin accumulation were also observed for $\Delta\text{sllr0940K}$, a strain lacking ζ -carotene desaturase and thereby all carotenoids but retaining fucose. Therefore, lack of myxoxanthophyll and not simply of fucose results in most of the phenotypic effects described here. It is concluded that myxoxanthophyll contributes significantly to the vigor of cyanobacteria, as it stabilizes thylakoid membranes and is critical for S-layer formation.

Cyanobacteria are a large and diverse group of oxygenic, photosynthetic bacteria. Several cyanobacterial strains are amenable to genetic engineering and have a known genome sequence. Among these is the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. Some cyanobacteria, including *Synechocystis* sp. strain PCC 6803, use fucose to synthesize cell wall components (18) as well as the carotenoid glycoside myxoxanthophyll (53), which is specific for cyanobacteria. In *Synechocystis* sp. strain PCC 6803, typical carotenoids accumulated are β -carotene, the dicyclic xanthophylls echinenone and zeaxanthin, and the monocyclic xanthophyll glycoside myxoxanthophyll.

Carotenoids generally are found in membranes (9, 19), with their chemical and physical properties strongly influenced by surrounding molecules, such as proteins and membrane lipids. In turn, the carotenoids influence the properties of membranes, including their fluidity and polarity. Cyanobacteria have three major membrane systems: the thylakoids, the cytoplasmic membrane, and the outer membrane. In *Synechocystis* sp. strain PCC 6714, which is closely related to *Synechocystis* sp. strain PCC 6803, myxol glycosides are located in the cytoplasmic membrane and the outer membrane (25). In cytoplasmic membranes, accumulation of a xanthophyll, zeaxanthin, has been observed in high-irradiation-grown cells of *Synechococcus*

sp. strain PCC 7942 (30). In this and other membranes, carotenoids contribute to the recycling of lipid peroxides and provide an early protection against lipid peroxidation in the membrane (9, 55). The outer membrane is considered to be the first protection barrier against oxidative stresses of UV and excess light intensity, and carotenoids appear to play a vital role in this process. In *Nostoc commune*, the levels of both myxoxanthophyll and echinenone increased in the outer membrane after irradiation with high-intensity light and/or UV (15). In thylakoids, carotenoids play an essential role in photoprotection of photosynthetic membranes (55) in that they provide photoprotection against the potentially damaging combination of light, photosensitizers, and oxygen by reacting with and quenching both the triplet state of chlorophylls and the singlet state of oxygen. In addition, some carotenoids can transfer energy to chlorophyll and thereby have a light-harvesting function (10). Moreover, according to a Fourier transform infrared spectroscopic study of thylakoid membranes from the cyanobacterium *Cylindrospermopsis raciborskii* (58), polar carotenoids in this cyanobacterium appear to be clustered in rigid patches. Local rigidity may protect important components of the membrane, such as the photosynthetic machinery located in such areas. In all membranes, carotenoids may stabilize the membrane, just as with cholesterol and other membrane-spanning lipids (37).

Carotenoids are synthesized in all wild-type photosynthetic organisms (9). They are also synthesized in some heterotrophically growing bacteria, archaea, and fungi. Some carotenoids

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in nonphotosynthetic and photosynthetic bacteria (4, 52) as well as in archaea are glycosylated. The sugar moiety of carotenoid glycosides differs from species to species, and additional modification by acetylation and methylation has been found (48, 50, 51). Among these carotenoid glycosides is myxoxanthophyll, which is a xanthophyll glycoside found in cyanobacteria (22). The structure was determined as a mixed carotenoid glycoside in which rhamnose is the dominant sugar moiety and a hexose is a minor component (17). Until now, several myxol glycosides with fucose derivatives have been found, including myxol 2'-(3-*O*-methyl- α -L-fucoside) in *Oscillatoria bornetii* (16), the nonmethylated myxol 2'- α -L-fucoside from *Oscillatoria limnetica* (1), and myxol 2'-dimethyl fucoside in *Synechocystis* sp. strain PCC 6803 (53). In all cases, the carotenoid moiety was myxol (1',2'-dihydro-3',4'-didehydro-3,1',2'-trihydroxy- γ -carotene) (22).

Fucose is a common sugar used in protein glycosylation, cell wall formation, and synthesis of exopolysaccharides. Fucose has been found in many prokaryotic glycoproteins (34). This deoxy sugar has been found to be a component of the cyanobacterial hydrophilic capsule (S-layer) (18). The glycocalyx, or S-layer, is the outermost layer coating the cyanobacterial cell (44, 56). This crystalline assembly of glycoprotein represents one of the most common cell surface structures in bacteria and archaea (44, 45). The protein lattice of the S-layer covers the cell surface at all stages of cell growth and division and is anchored to the outer membrane underneath. The carbohydrate moiety of the S-layer is a polymer of two to six monosaccharide units that include hexoses, deoxy and amino sugars, uronic acid, and sulfate or phosphate residues as constituents (31, 32, 43). The S-layer is multifunctional in that it appears to function as protective coat, molecule/ion trap, and molecular sieve and is involved in cell adhesion and surface recognition (45).

Fucose is a deoxygalactose ($C_6H_{12}O_5$), synthesized as GDP-fucose from GDP-mannose in a three-step reaction catalyzed by two enzymes, GDP-mannose-4,6-dehydratase and a dual-function 3,5-epimerase-4-reductase known as GDP-fucose synthetase (7, 46). The genome sequence of *Synechocystis* sp. strain PCC 6803 contains two adjacent open reading frames (ORFs) expected to encode GDP-mannose-4,6-dehydratase (sll1212) and GDP-fucose synthetase (sll1213). Clustering of these two genes is common to all fucose-producing organisms examined so far. A wide range of phenotypes results from defects in GDP-fucose biosynthesis, including adhesion defects in pathogenic bacteria (33), wall deformation in plants (38, 57), and leukocyte adhesion deficiency in humans (6).

The function of the carotenoid glycosides in both cyanobacteria and photosynthetic bacteria remains unresolved. Therefore, in this study *Synechocystis* sp. strain PCC 6803, a spontaneously transformable cyanobacterium with a sequenced genome, is used to probe the role of fucose, and thereby myxoxanthophyll, in this cyanobacterium. With the intent to remove the ability to synthesize fucose, sll1213 was cloned from *Synechocystis* sp. strain PCC 6803 and most of the sll1213 coding region was replaced by a zeocin resistance cassette (24). In the resulting mutant, myxoxanthophyll disappeared, novel carotenoid glycosides were synthesized, and thylakoid organization as well as the ultrastructure of the cell wall was altered.

MATERIALS AND METHODS

Strains and growth conditions. *Synechocystis* sp. strain PCC 6803 was cultivated on a rotary shaker at 30°C in BG-11 medium (40), buffered with 5 mM *N*-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid-NaOH (pH 8.2), and for growth under photomixotrophic or light-activated heterotrophic growth conditions, supplemented with 5 mM glucose. For growth on plates, 1.5% (wt/vol) Difco agar and 0.3% (wt/vol) sodium thiosulfate were added. Flux densities of 0.5, 40, and 100 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ from cool-white fluorescent tubes were used for growth in continuous light in liquid medium. For growth in liquid under light-activated heterotrophic growth conditions (3), cells were kept in complete darkness with the exception of one 15-min light period (40 μmol photons $\text{m}^{-2} \text{s}^{-1}$) every 24 h. The chlorophyll content was analyzed at 665 nm after methanol extraction. Growth was monitored by measuring the optical density of the cells at 730 nm with a Shimadzu UV-160 spectrophotometer.

Gene cloning from *Synechocystis* sp. strain PCC 6803. The putative fucose synthetase gene of *Synechocystis* sp. strain PCC 6803, sll1213, was amplified by PCR based on the available *Synechocystis* genomic sequencing (CyanoBase [www.kazusa.or.jp/cyano/cyano.html]) (27). The forward primer was 5' TCAACCTC AACCTGCAGAACTATGT 3' with an engineered PstI restriction site and a sequence corresponding to base numbers 3988 to 4010 in CyanoBase; the reverse primer was 5' GAACGCTCCAGGAATTCCACGAA 3' with an engineered EcoRI site and a sequence corresponding to CyanoBase bases 5778 to 5802 (base changes to introduce restriction sites have been italicized). The PCR-amplified sequence corresponds to sll1213 with 400- to 450-bp flanking sequences on both sides of the ORF. A PCR product of the expected size was purified and treated with restriction enzymes according to the restriction sites created on each primer. The sll1213 gene and its flanking regions were cloned into pUC19 by using its EcoRI and PstI sites, creating psl1213. The sll1213 gene was deleted by restriction with the BsaAI (nucleotide 4419 in CyanoBase) and AvrII (nucleotide 5430) sites near the beginning and end of the sll1213 open reading frame and replacement of the sll1213 fragment by a 1.4-kb zeocin resistance cassette digested with PvuII and XbaI. This created p Δ sll1213Z, which was used for transformation of *Synechocystis*.

Transformation and segregation analysis of *Synechocystis* sp. strain PCC 6803. Transformation of *Synechocystis* sp. strain PCC 6803 was carried out according to Vermaas et al. (59). Transformants were propagated on BG-11 agar plates supplemented with 5 mM glucose and increasing concentrations of zeocin. The segregation state of the transformants was monitored by PCR of transformant DNA using primers recognizing sequences upstream and downstream of the sll1213 coding region. *Synechocystis* sp. strain PCC 6803 genomic DNA used for PCR analysis of mutants was prepared as described by He et al. (21).

Fucose analysis. To extract total cellular sugar of low molecular weight from *Synechocystis* sp. strain PCC 6803, cell pellets were resuspended in 1 M NaOH, heated for 15 min at 70°C, and then centrifuged at 35,000 $\times g$ for 30 min at 5°C. High-molecular-weight cellular materials were precipitated by mixing the supernatant with 1 volume ice-cold absolute ethanol, incubating overnight at -20°C, and centrifuging as described above. The alkaline, ethanolic supernatant containing sugars was concentrated by freeze-drying. The sugar extracts from the wild-type and Δ sll1213Z strains were analyzed by thin-layer chromatography using a mixture of *n*-butanol, pyridine, and water (10:3:3, vol/vol/vol) as the mobile phase and run for 20 h at room temperature. Sugars were visualized by diphenylamine-aniline-*o*-phosphoric acid (1.8:1.8:8.5% in 90% acetone) (11). A fucose-specific lectin (UEA-I, from *Ulex europaeus*; EY Laboratories, Inc., San Mateo, CA) was also used to specifically isolate fucose from the sugar extract. Affinity chromatography for fucose was carried out according to recommendations of the supplier.

Pigment analysis. *Synechocystis* sp. strain PCC 6803 cells were harvested by centrifugation using cultures in exponential-growth phase (optical density at 730 nm of ~0.5). Cell pellets were frozen in liquid nitrogen and freeze-dried. Pigments were extracted from freeze-dried cells with 100% methanol, and extracts were kept under nitrogen. Carotenoids were separated by high-pressure liquid chromatography (HPLC) on a Spherisorb ODS2 4.0- by 250-mm C_{18} column, using a linear 18-min gradient of ethyl acetate (0 to 95%) in acetonitrile-water-triethylamine (9:1:0.01, vol/vol/vol) at a flow rate of 1 ml/min (35). Absorption spectra of individual peaks were obtained with an online photodiode array detector.

Mass spectroscopy. Carotenoid fractions collected after HPLC analysis were evaporated under nitrogen. Mass spectra were obtained by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Voyager DE STR Biospectrometry Workstation; Applied Biosystems, Foster City, CA). Carotenoid species were identified by their absorption spectra, typical retention times, and molecular masses, as well as their fragmentation patterns.

Light microscopy. Cultures of cells of *Synechocystis* sp. strain PCC 6803 were placed in 50-ml centrifuge tubes and spun down at 1,200 rpm in a medical centrifuge for 10 min. A drop of cells from the resulting pellet was transferred by glass pipette to the surface of a glass slide coated with a thin layer of 12% gelatin in BG-11 culture medium. The cells were covered with a 22- by 22-mm coverglass (no. 1.5) and viewed with an Axioskop microscope (Carl Zeiss, Thornwood, NY) using differential interference contrast optics (Plan-Neofluar 100 $\times$; 1.3-numerical-aperture oil immersion objective lens and 1.4-numerical-aperture oil immersion condenser lens). For imaging, a Hamamatsu C3200-07 video camera coupled to an analog camera control unit (Hamamatsu Photonic Systems Corporation, Bridgewater, NJ) was used and an Argus 10 image processor digitally enhanced the contrast in real time. The images were then digitally acquired with a Sony UP-5600MD video/digital printer (Sony Electronics, Montvale, NJ) and uploaded to a Macintosh 7100/80AV Power PC (Apple Computer, Cupertino, CA). Images were prepared for publication by using Photoshop 6.0 (Adobe Systems, San Jose, CA).

Transmission electron microscopy. Wild-type and mutant cells of *Synechocystis* sp. strain PCC 6803 in the logarithmic growth phase were concentrated into a thick paste. The cells were then cryofixed by high-pressure freezing. Approximately 20 μ l of the concentrated cells was placed in interlocking planchets (13) and frozen immediately with liquid nitrogen under high pressure (210,000 kPa) by using a Bal-Tec HPM 010 high-pressure freezing machine system (Bal-Tec Corporation, Middlebury, CT). Following cryofixation, the samples were transferred into a -85°C freeze substitution solution containing 1% glutaraldehyde (Electron Microscopy Sciences, Washington, PA) and 1% tannic acid in HPLC-grade anhydrous acetone for 72 h. The samples were rinsed in three changes of acetone at -85°C for a total of 45 min and then transferred to 1% OsO_4 in acetone for 1 h at -85°C . The samples were warmed slowly to room temperature over a period of 6 h. After being rinsed in acetone, the samples were infiltrated with epoxy resin (47), embedded, and heat polymerized. Serial thin sections of 70 nm were cut with a Leica Ultracut R microtome (Leica, Vienna, Austria) and collected onto Formvar-coated copper slot grids. The sections were poststained with 2% uranyl acetate in 50% ethanol for 5 min and lead citrate (39) for 5 min. The sections were viewed at 80 kV by using a Philips CM12S transmission electron microscope (Philips Electronic Instruments, Co., Mahwah, NJ). Images were captured with a Gatan 689 CCD 1,024- by 1,024-pixel-area digital camera (Gatan, Inc., Pleasanton, CA) using Gatan Digital Micrograph 3.7.4 software and prepared for publication using Photoshop 6.0 (Adobe Systems).

RESULTS

GDP-fucose synthetase gene orthologues. Orthologues of GDP-fucose synthetase genes are found in almost all organisms, including cyanobacteria. In Fig. 1, a sequence alignment between known GDP-fucose synthetases of *Arabidopsis thaliana* and *Escherichia coli* K-12 and cyanobacterial orthologues of *Synechocystis* sp. strain PCC 6803, *Thermosynechococcus elongatus* BP-1, *Anabaena* sp. strain PCC 7120, and *Nostoc punctiforme* ATCC 29133 is shown, indicating 24% identity between the six proteins. The conserved Ser-Tyr-Lys catalytic triad (46, 54) that is characteristic for the majority of fucose synthetases in *Synechocystis* and other cyanobacteria is instead Thr-Tyr-Lys, indicating a conservative mutation in the first residue of the triad. GDP-mannose-4,6-dehydratase shares similarity with GDP-fucose synthetase, and genes for these proteins are found together in all species that produce GDP-fucose examined so far.

GDP-fucose synthetase gene paralogues. *Synechocystis* contains five other genes that are similar in sequence to *sll1213* (Table 1). The products of these genes also contain the catalytic triad (Thr-Tyr-Lys). The gene most closely resembling *sll1213* is *slr0583*, which is most similar to the *Xanthomonas campestris* *rmd* gene encoding GDP-4-dehydro-D-rhamnose reductase, which converts GDP-mannose to GDP-rhamnose. Therefore, the *sll1213* and *slr0583* gene products are expected to use the same substrate and to produce two closely related deoxyhexose sugars, fucose and rhamnose, respectively. The

slr0836 and *slr0809* gene products show more than 60% identity with the known dTDP-glucose 4,6-dehydratase from *Xanthomonas campestris* pv. *campestris* involved in rhamnose metabolism. The last two paralogues, *sll0244* and *slr1067*, code for proteins with the same conserved epimerase domain as in the *sll1213* gene product. These proteins have more than 50% identity with known UDP-glucose 4'-epimerase. As the five *Synechocystis* genes that are similar to *sll1213* code for proteins that are far more similar to orthologues with known function than to fucose synthetase, *sll1213* is the most likely candidate to code for fucose synthetase.

Fucose analysis. Deletion of *sll1213* from the *Synechocystis* sp. strain PCC 6803 genome led to full segregation of the Δ *sll1213Z* mutant strain as confirmed by PCR (data not shown). In this mutant, the presence of fucose could no longer be demonstrated, as shown in Fig. 2. However, an as-yet-unidentified sugar with a different mobility is visible in the Δ *sll1213Z* extract. In addition, affinity chromatography analysis of sugar extracts from the Δ *sll1213Z* mutant and the wild type showed that no fucose was bound to the fucose-specific UEA-I lectin in the mutant but bound fucose was detected in the wild-type extract (data not shown). Therefore, the segregated Δ *sll1213Z* strain is no longer able to synthesize fucose, confirming that *sll1213* encodes the sole GDP-fucose synthetase in *Synechocystis* sp. strain PCC 6803.

Mutant phenotypes. Interestingly, transformant colonies showed an obvious phenotype even before full segregation: colonies were very sticky, remained small and dense, and were difficult to fragment. Colonies of the fully segregated mutant were less sticky and sometimes were very loose compared to the wild type, suggesting that the strain has adapted and that other compounds that may functionally complement are produced. A liquid culture of the segregated Δ *sll1213Z* strain had two major phenotypes. Under photomixotrophic conditions at low light intensity (0.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), cells grew slowly and excreted a yellow compound while the chlorophyll content decreased (Table 2), resulting in a distinctly yellow culture. However, when grown photomixotrophically at 40 or 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, growth was vigorous (Table 2). In contrast, when grown photoautotrophically at 40 or 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the doubling time of the Δ *sll1213Z* strain was double that of the wild type. In either case, the chlorophyll content of the mutant was reduced by 30 to 40% and aggregation of Δ *sll1213Z* cells to quickly sedimenting beads was common. Therefore, deletion of *sll1213* appears to have conveyed more hydrophobic properties to the cell surface and impaired photoautotrophic growth.

Identification of carotenoid pigments. Carotenoids were extracted from *Synechocystis* sp. strain PCC 6803 with methanol and were identified based on their absorption spectra and HPLC retention times as previously described (28, 36). Figure 3 shows an HPLC elution profile of pigments extracted from the Δ *sll1213Z* strain grown photomixotrophically at light intensities of 0.5 and 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. For comparison, the wild-type pigment profile of cells grown at 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ is shown. The profile of wild-type cells grown at 0.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was similar to that obtained at 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (not shown). The carotenoid composition of the mutant is similar to that of the wild type except that myxoxanthophyll in the wild type was replaced by apparently

FIG. 1. Multiple-amino-acid-sequence alignment of GDP-fucose synthetases. Four GDP-fucose synthetases from cyanobacterial sources (*Nostoc punctiforme* ATCC 29133 [AB2409], *Anabaena* sp. strain PCC 7120 [ZP_00159041], *Synechocystis* sp. strain PCC 6803 [NP_439904], and *Thermosynechococcus elongatus* BP-1 [NP_681422]) are aligned against the known plant and bacterial GDP-fucose synthetases from *Arabidopsis thaliana* (AAC02703) and *Escherichia coli* K-12 (AAC32346), respectively. (Numbers following all strain names are GenBank accession numbers for GDP-fucose synthetases.) The catalytic-triad (Ser [Thr]-Tyr-Lys) residues are boxed. The six sequences are 24% identical (*). Similarity in alignments is indicated as well (: and ., more and less similarity, respectively). The alignment was obtained with ClustalW (<http://www.ebi.ac.uk/clustalw/>).

more-hydrophilic carotenoids (Fig. 3A, peaks 1 and 2) in the mutant. The compound with the highest HPLC mobility appears to be a myxol isomer, as its absorption spectrum (Fig. 4, spectrum 1) is different from that of myxol whereas the molecular mass (584 *m/z*) corresponds to that of myxol (Table 3). Peak 1 has a spectrum resembling that of myxol (Fig. 4, spectrum 2), but its HPLC mobility indicates that it is glycosylated. Peak 2 (Fig. 3) contains a mixture of different chromophores, including myxol- and lycopene-type compounds (Fig. 3B). Again, the HPLC mobility indicates that these compounds are glycosylated. Different absorption spectra were obtained for different regions of the HPLC peak (Fig. 4, spectra 2 to 10). The carotenoids represented in spectra 4 and 5 in Fig. 4 have a fine structure (the ratio of the right and middle peaks [III/II]

TABLE 1. *Synechocystis* sp. strain PCC 6803 genes that are sll1213 paralogues^a

<i>Synechocystis</i> ORF	Amino acid identity (%) with fucose synthetase from:		Putative function
	<i>A. thaliana</i>	<i>E. coli</i>	
sll1213	37.3	33.2	GDP-fucose synthetase
slr0583	26.1	26.5	GDP-4-dehydro-D-rhamnose reductase
slr0836	19.3	18.8	dTDP-glucose 4,6-dehydratase
slr0809	21.1	18.3	dTDP-glucose 4,6-dehydratase
sll0244	19.1	20.5	UDP-glucose 4'-epimerase
slr1067	22.8	20.2	UDP-glucose 4'-epimerase

^a The similarities of the corresponding proteins to experimentally proven fucose synthetases from *A. thaliana* and *E. coli*, and the putative functions of these *Synechocystis* proteins, are also shown.

FIG. 2. Fucose-containing portion of a thin-layer chromatogram of sugars in extracts from the $\Delta sll1213Z$ and wild-type strains of *Synechocystis* sp. strain PCC 6803. Top, fucose was applied as a standard; middle and bottom, extracts from the $\Delta sll1213Z$ and wild-type strains, respectively. Sugar staining was performed as described in Materials and Methods.

of the spectrum is 91%) similar to that of neurosporene but with an absorption maximum at 500 nm, whereas neurosporene has an absorption maximum at 471 nm. This suggests that these two carotenoids have additional conjugated double bonds relative to neurosporene, similar to myxoxanthophyll.

The light intensity at which the mutant was grown greatly altered the amplitude ratio of peaks 1 and 2 (Fig. 3A). Peak 1 (myxol glycosides) was found predominantly when cells were grown at low light intensity, while growth at higher light intensity resulted in an accumulation of more peak 2 containing a mix of carotenoid glycosides. The wavelengths of maximum absorption of the carotenoids in peaks 1 and 2 are identical to those of myxoxanthophyll, but the carotenoid glycosides in peak 2 had a different absorption fine structure (III/II of 71.6% for the major constituent of peak 2 [Fig. 4, spectrum 3]) than that expected for myxoxanthophyll (III/II of 57.6% for myxoxanthophyll [Fig. 4, spectrum 2]). Peak 2 in the $\Delta sll1213Z$ mutant appears to be even more heterogeneous, as different absorption spectra (Fig. 4) are found between peaks 2C and 2D

FIG. 3. HPLC chromatogram of methanol extracts of the wild type and the $\Delta sll1213Z$ mutant. (A) The wild type was grown at a light intensity of 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and the $\Delta sll1213Z$ mutant strain was grown at a light intensity of 0.5 (bottom) or 40 (middle) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Cultures were grown photomixotrophically, and methanol extracts were analyzed by HPLC using a C_{18} column; detection was at 510 nm (A) and 440 nm (B). The peak at 2.5 min is of an unknown (U) carotenoid in both the wild type and the $\Delta sll1213Z$ mutant. Novel carotenoids of the $\Delta sll1213Z$ mutant strain are peaks 1 and 2. (B) Separation of mixed glycosides in peak 2 (Fig. 3A) with a linear 18-min gradient of ethyl acetate (30 to 70%) in acetonitrile-water-triethylamine (9:1:0.01, vol/vol/vol) at a flow rate of 1 ml/min. Known carotenoids are myxoxanthophyll (M), zeaxanthin (Z), echinenone (E), and β -carotene (β). Ch, chlorophyll.

(Fig. 3B). The absorption wavelengths of peak III of these carotenoids (Fig. 4, spectra 6, 7, 8, 9, and 10) vary from 501 nm to 507 nm, with variation in their absorption fine structures as well. These carotenoids presumably represent different geometric isomers of myxol/lycopene glycosides. The β -carotene peak of the $\Delta sll1213Z$ strain was mixed with phytofluene and ζ -carotene (Fig. 4, spectra 11 and 12). The accumulation of early carotenoid precursors in cells of the $\Delta sll1213Z$ strain indicates that the carotenoid biosynthesis pathway was impaired.

Mass spectroscopic analysis. The carotenoids in peaks 1 and 2 (Fig. 3A) were subjected to MALDI-TOF MS. The molecular masses are listed in Table 3. Peak 1 has a main molecular mass of 744 m/z , which corresponds to myxol dimethyl pentoside or myxol methyl deoxyhexoside (Fig. 5). However, a molecular mass of 584 m/z was observed for this sample as well. This mass corresponds to free myxol (Fig. 5A), which may be a degradation product generated upon mass spectroscopic analysis. The carotenoids accumulated in the mutant upon growth at 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ showed masses of 744, 748, 762, and 776 m/z (Fig. 3B, peaks 2A to 2D). In view of spectral characteristics of these compounds (Fig. 4), the masses of the last three compounds are most consistent with a linear, hydroxylated lycopene-type (presumably 1',2',4-trihydroxy-1',2'-dihydro-lycopene) carotenoid glycosylated with a

TABLE 2. Doubling time and chlorophyll content of the $\Delta sll1213Z$ mutant compared to those of the wild type under different growth conditions^a

Growth condition and light intensity ^b	Doubling time (h)		Chlorophyll content ^c	
	Wild type	$\Delta sll1213Z$	Wild type	$\Delta sll1213Z$
LAHG	34 $\pm$ 2	40 $\pm$ 1	16 $\pm$ 2	10 $\pm$ 2
Photoautotrophic				
40	15 $\pm$ 2	32 $\pm$ 3	35 $\pm$ 4	24 $\pm$ 3
100	15 $\pm$ 2	30 $\pm$ 4	33 $\pm$ 3	20 $\pm$ 3
Photomixotrophic				
0.5	15 $\pm$ 4	39 $\pm$ 5	24 $\pm$ 4	14 $\pm$ 2
40	12 $\pm$ 1	16 $\pm$ 3	45 $\pm$ 3	35 $\pm$ 5
100	12 $\pm$ 2	11 $\pm$ 1	38 $\pm$ 4	24 $\pm$ 5

^a The mean values ($\pm$ standard deviations) were calculated from replicates of three different experiments.

^b Light intensity is expressed in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Photoautotrophic growth at 0.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ could not be attempted as the light intensity was too low. LAHG, light-activated heterotrophic growth.

^c Chlorophyll content is expressed in pmol/million cells.

FIG. 4. Absorption spectra of carotenoids recorded for the Δ sll1213Z mutant. Spectrum 1 represents the unknown carotenoid accumulated in peak U (Fig. 3A) in both the wild type and the Δ sll1213Z mutant of *Synechocystis* sp. strain PCC 6803 (maximum absorption wavelengths [$\lambda_{\max}$], 476 and 504 nm; III/II, 18.6%). The spectra characteristic of the major myxoxanthophyll-like components in the Δ sll1213Z mutant were of a myxol-type chromophore (spectrum 2: $\lambda_{\max}$, 453, 475, and 509 nm; III/II, 57.6%) found in peaks 1 and 2A (Fig. 3) and of a lycopene-type chromophore (spectrum 3: $\lambda_{\max}$, 453, 477, and 509 nm; III/II, 71.6%) in peaks 2B to 2D (Fig. 3). Absorption spectra of the carotenoid glycosides synthesized at 40 μ mol photons $m^{-2} s^{-1}$ in the Δ sll1213Z mutant of *Synechocystis* sp. strain PCC 6803 between peak 2C and peak 2D (Fig. 3) are depicted in spectra 4 ($\lambda_{\max}$, 442, 468, and 500 nm; III/II, 91%) and 5 ($\lambda_{\max}$, 444, 470, and 500 nm; III/II, 92%). Absorption spectra of myxol/lycopene *cis*-isomers present in the margin of peaks 2A to 2D (Fig. 3) synthesized at a light intensity of 40 μ mol photons $m^{-2} s^{-1}$ in the Δ sll1213Z mutant of *Synechocystis* sp. strain PCC 6803 are shown in spectra 6 ($\lambda_{\max}$, 298, 365, 446, 469, and 499 nm; III/II, 31%), 7 ($\lambda_{\max}$, 298, 365, 447, 471, and 502 nm; III/II, 34%), 8 ($\lambda_{\max}$, 297, 365, 447, 473, and 503 nm; III/II, 40%), 9 ($\lambda_{\max}$, 297, 365, 450, 474, and 505 nm; III/II, 35%), and 10 ($\lambda_{\max}$, 296, 366, 452, 476, and 507 nm; III/II, 41%). Carotenoids that coeluted with the β -carotene peak in the Δ sll1213Z mutant grown at low light intensity are ζ -carotene ($\lambda_{\max}$, 334, 350, and 368 nm) mixed with β -carotene ($\lambda_{\max}$, 433, 457, and 482 nm) (spectrum 11) and phytofluene ($\lambda_{\max}$, 384, 406, and 428 nm) mixed with β -carotene ($\lambda_{\max}$, 433, 457, and 482 nm) (spectrum 12).

hexose sugar. These masses are different by 14 mass U, consistent with a methyl group substitution in the sugar moiety. Therefore, these three carotenoid glycosides appear to have a hexose sugar with different degrees of methylation. The tentative identifications of the new glycosides are provided in Table 3; the presumed molecular structures of these carotenoids are shown in Fig. 5.

Photosynthesis. The amount of chlorophyll per cell decreased in the Δ sll1213Z deletion mutant (Table 2). We examined the consequences of the Δ sll1213Z deletion on the stoichiometry of photosystems by means of 77-K fluorescence emission analysis. The spectrum of photomixotrophically grown Δ sll1213Z cells cultured at a light intensity of 0.5 or 40 μ mol photons $m^{-2} s^{-1}$ was qualitatively normal (not shown), suggesting that the stoichiometry between the two photosystems had remained unchanged under these conditions. The sensitivity of the Δ sll1213Z strain to exposure to high light intensity (100 μ mol photons $m^{-2} s^{-1}$) under photoautotrophic

TABLE 3. Mass spectroscopic analysis of myxol, myxoxanthophyll new peaks characteristic for the Δ sll1213Z mutant^a

Peak	Mass (m/z)	Formula	Tentative identification
U	584	$C_{40}H_{56}O_3$	Trihydroxy lycopene (or myxol)
M	758	$C_{48}H_{70}O_7$	Myxoxanthophyll
1	744	$C_{47}H_{68}O_7$	Myxol dimethylpentoside or myxol methyldeoxyhexoside
2A	744	$C_{47}H_{68}O_7$	Myxol dimethylpentoside or myxol methyldeoxyhexoside
2B	748	$C_{46}H_{68}O_8$	Trihydroxy carotenoid hexoside
2C	762	$C_{47}H_{70}O_8$	Trihydroxy carotenoid monomethylhexoside
2D	776	$C_{48}H_{72}O_8$	Trihydroxy carotenoid dimethylhexoside

^a Peaks 1, 2A, 2B, 2C, and 2D were collected from the mutant grown at 0.5 and 40 μ mol photons $m^{-2} s^{-1}$. Peak U was present in all strains. Peak numbering is according to Fig. 2. Tentative identifications of the novel carotenoid glycosides are provided.

conditions is apparently normal compared to that of the wild type, as the doubling time of the Δ sll1213Z strain remained stable relative to growth at 40 μ mol photons $m^{-2} s^{-1}$ (Table 2). However, this does not necessarily imply that the absence of native myxoxanthophyll has no effect on the light sensitivity of the mutant. The formation of cell aggregates in liquid medium decreases the average amount of light that reaches the cells. Therefore, cell aggregation may provide an alternative mechanism to avoid high light intensity and may help to protect cells against photodamage despite the lack of myxoxanthophyll.

Structure. Light and transmission electron microscopy examinations showed significant phenotypic differences between wild-type and Δ sll1213Z cells grown at light intensities of 0.5 or 40 μ mol photons $m^{-2} s^{-1}$ (Fig. 6). The Δ sll1213Z strain aggregated readily when grown at either light intensity (Fig. 6B and C). However, note that Δ sll1213Z cells grown at low light intensity have a diameter of about one-third less than those

FIG. 5. Molecular structures of myxol and glycosylated carotenoids in *Synechocystis* sp. strain PCC 6803. (A) Myxol (R represents H in the case of myxol and dimethyl fucose in the case of myxoxanthophyll); (B) trihydroxy lycopene (R represents H). Structures labeled 2a to 2d are the proposed structures for the sugar moieties (R) in the as-yet-unknown carotenoid glycosides recorded in peaks 2A to 2D (Fig. 3B), respectively, in the Δ sll1213Z mutant.

FIG. 6. Light (A to D) and electron (E to L) microscopy of *Synechocystis* sp. strain PCC 6803 wild-type cells grown at a light intensity of 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (A, E, and I), $\Delta\text{sll1213Z}$ mutant cells grown at 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (B, F, and J) or 0.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (C, G, and K), and $\Delta\text{slr0940K}$ mutant cells grown at 0.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (D, H, and L). Light microscopy showed that $\Delta\text{sll1213Z}$ mutant cells often aggregated (B and C). Transmission electron microscopy showed thylakoid membrane pairs (arrows in panels E to H) and putative carboxysomes (arrowheads in panels E to H). In wild-type cells, thylakoid membranes were positioned primarily along the cytoplasmic membrane (E), while in $\Delta\text{sll1213Z}$ and $\Delta\text{slr0940K}$ cells, thylakoid membrane organization was disrupted (F, G, and H). The cytoplasm in the $\Delta\text{sll1213Z}$ mutant appeared to be structured differently in some locations (asterisks in panel F) with cyanophycin granules (c) more common in $\Delta\text{sll1213Z}$ and $\Delta\text{slr0940K}$ cells grown at a light intensity of 0.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (G and H). Contact points between adjacent cells (black arrowheads in panel G) were common in the $\Delta\text{sll1213Z}$ mutant, consistent with increased aggregation. Wild-type cells displayed a clear S-layer (asterisk in panel I), which was not apparent in the mutants. In both the wild type and the mutants, the outer membrane (white arrowheads in panels I to L), peptidoglycan layer (arrows in panels I to L), and cytoplasmic membrane (black arrowheads in panels I to L) are apparent. Bars = 3 μm (A to D), 200 nm (E to H), and 50 nm (I to L).

grown at 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Cyanophycin granules, which contain aspartate and arginine polymers (2), were abundant in mutant cells particularly when grown at low light intensity (Fig. 6G). To discriminate between effects due to the absence of just fucose (important for cell wall structure [38, 57]) and of myxoxanthophyll, $\Delta\text{slr0940K}$, a strain lacking ζ -carotene desaturase (CrtQ) that is needed for carotenoid formation (5, 8), was included in this study as well: differences between the wild type and $\Delta\text{sll1213Z}$ that are due to myxoxanthophyll should be visible also in $\Delta\text{slr0940K}$, which lacks myxoxanthophyll, whereas features shared by the wild type and $\Delta\text{slr0940K}$ but not by $\Delta\text{sll1213Z}$ presumably involve just fucose. As shown in Fig. 6A to D, aggregation may be caused primarily by the absence of fucose (12) rather than of myxoxanthophyll.

In wild-type cells, thylakoid membrane pairs were organized mostly in stacks of three to five pairs separated by approximately 30 nm and localized along the cytoplasmic membrane (Fig. 6E). In $\Delta\text{sll1213Z}$ mutant cells grown at a light intensity of 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, thylakoid membrane pairs were maintained in an organization partially similar to that of the wild type (Fig. 6F) but thylakoid membranes that were dis-

persed in the cytoplasm were visible as well. This lack of thylakoid organization was even more prominent when $\Delta\text{sll1213Z}$ cells were grown at a light intensity of 0.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; under such conditions, thylakoid membrane pairs in the mutant often appeared individually dispersed throughout the cytoplasm (Fig. 6G). The $\Delta\text{slr0940K}$ strain that is unable to synthesize carotenoids showed a thylakoid organization most similar to that of the $\Delta\text{sll1213Z}$ mutant grown at 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 6F and H), indicating that myxoxanthophyll is needed for normal thylakoid organization.

Cell wall layers were well defined in wild-type cells and included an S-layer, the outer membrane, and an electron-dense peptidoglycan layer (Fig. 6I). The cytoplasmic membrane was visible below these layers (Fig. 6I). The cell wall layers of the $\Delta\text{sll1213Z}$ and $\Delta\text{slr0940K}$ strains were less apparent: the S-layer was barely detectable and the peptidoglycan layer was poorly defined (Fig. 6J, K, and L). Interestingly, the layer was thicker (14 nm) in the $\Delta\text{sll1213Z}$ strain upon growth at a light intensity of 0.5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 6K) than when grown at higher intensity. A model of the cell wall structures in the wild type and the $\Delta\text{sll1213Z}$ mutant is shown in Fig. 7.

FIG. 7. Schematic representation of the cytoplasmic membrane and cell wall in wild-type *Synechocystis* sp. strain PCC 6803 and the Δ sll1213Z mutant. The figure represents the cell wall of the wild type grown at a light intensity of $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (A) and that of the Δ sll1213Z strain grown at light intensities of 40 (B) and 0.5 (C) $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. See Fig. 6 for the experimental observations. CM, cytoplasmic membrane; P, inner and outer periplasmic space; PG, peptidoglycan layer; OM, outer membrane; C, carotenoid (myxoxanthophyll); SL, S-layer; and LPS, lipopolysaccharide layer.

DISCUSSION

sll1213 encodes the GDP-fucose synthetase in *Synechocystis*.

Fucose analysis by means of affinity and thin-layer chromatography (Fig. 2) provides direct experimental evidence that sll1213 encodes the fucose synthetase. The five genes with some similarity to sll1213 in the genome of *Synechocystis* sp. strain PCC 6803 are expected to code for proteins that catalyze similar reactions using the same substrate and/or produce similar sugars (Table 1). In further support of sll1213 being the only GDP-fucose synthetase gene in the *Synechocystis* sp. strain PCC 6803 genome, this gene is clustered with sll1212, which appears to encode GDP-mannose-4,6-dehydratase. This clustering is observed with all organisms producing fucose examined thus far (46). Moreover, deletion of sll1213 in the Δ sll1213Z strain led to a disappearance of myxoxanthophyll, which is replaced by novel carotenoid glycosides (Fig. 3). This result provides an internal bioassay confirming that sll1213 encodes the GDP-fucose synthetase in *Synechocystis* sp. strain PCC 6803.

New carotenoids in the Δ sll1213Z mutant. Enzymes involved in the myxoxanthophyll biosynthesis pathway remain largely unknown, but this study clearly demonstrates the requirement of fucose synthetase for myxoxanthophyll production. This reinforces the notion that myxoxanthophyll in *Synechocystis* sp. strain PCC 6803 is fucosylated, and the data presented in this paper show that other sugars can be used for glycosylation but lead to significant phenotypic effects, implying that fucose substitution by other sugars impairs cell function. The newly formed carotenoid in peak 1 (Fig. 3A) in the mutant when grown at low light intensity is myxol glycoside (Table 3), which is equivalent to a cyanobacterial glycoside found in *Oscillatoria limosa* and identified as myxol 2'-O-di-

methylpentoside (17). Upon HPLC analysis, this putative dimethyl pentose glycoside (Table 3) eluted more rapidly than did the C₆-sugar carotenoid glycosides synthesized in the Δ sll1213Z mutant at a light intensity of $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. These carotenoid glycosides (with a C₆-sugar) that accumulated in peaks 2B to 2D (Fig. 3B) presumably have a linear trihydroxy carotenoid moiety: the high III/II in the carotenoid absorption fine structure (Fig. 4, spectrum 2) is characteristic for lycopene and is indicative of a noncyclized polyene chain (48); moreover, the reduced retention time of peak 2 relative to myxoxanthophyll is indicative of a linear polyene chain. Therefore, the spectral properties of the new carotenoids in peaks 2A to 2D (Fig. 3B) indicate a mix of myxol and lycopene derivatives (Fig. 4, spectra 2 and 3) with different glycosylation and methylation patterns, neither of which affect the absorption spectra of the carotenoids (48, 49).

Interestingly, a novel carotenoid in the right shoulder of peak 2D (Fig. 3B) has an absorption fine structure similar to that of neurosporene but has a bathochromic shift of 29 nm (Fig. 4, spectra 4 and 5). These spectral properties suggest that the carotenoid has an asymmetric distribution of 10 conjugated double bonds. This compound appears to be hydrophilic, as its retention time on the hydrophobic C₁₈ column coelutes with the novel carotenoid glycosides and is shorter than that of myxoxanthophyll. The short retention time suggests the presence of OH groups and perhaps of a glycosyl group as in myxoxanthophyll. A C-3,4 desaturase (36) is involved in the formation of such neurosporene derivatives with additional desaturation. Neurosporene is converted by the slr1293 gene product to 3,4-didehydroneurosporene, which is processed further to produce polyhydroxy glycosylated carotenoid derivatives. We interpret the presence of this array of novel compounds as evidence that carotenoid biosynthesis intermediates may have been enzymatically converted in secondary reactions. This interpretation agrees with the concept of a promiscuous nature of carotenogenic enzymes with regard to the structure of their substrates, leading to further metabolism of accumulated precursors to produce a new array of carotenoids (41, 42).

The requirement of a moderate or higher light intensity for normal photomixotrophic growth of the Δ sll1213Z mutant may be related to the formation of a fully methylated glycoside. However, the level of new glycosides in the Δ sll1213Z mutant grown at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was lower than that of myxoxanthophyll in the wild type, suggesting that the enzymes involved in glycosylation are fairly specific toward fucose. Furthermore, the accumulation of phytofluene and ζ -carotene (Fig. 4, spectra 11 and 12) in the Δ sll1213Z mutant grown at a light intensity of $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ indicates that the rate at which these carotenoid species that are early in the carotenoid biosynthetic pathway are consumed is lower in Δ sll1213Z than in the wild type. In addition, these carotenoids are excreted and accumulate in the medium in cultures of the mutant (data not shown). This excretion may contribute to continued carotenogenesis and prevent phytofluene and ζ -carotene from inhibiting phytoene dehydrogenase and ζ -carotene desaturase, respectively (8, 29). Moreover, the excretion may be indicative of a membrane permeability defect. Indeed, a vital role of myxoxanthophyll in the outer membrane has been

postulated (20, 25), and the lack of an S-layer may further contribute to increased permeability.

Cell surface properties of the Δ sll1213Z mutant. The surface properties of the Δ sll1213Z mutant have been altered significantly. Before segregation of the mutant, colonies were dense and sticky, suggesting a defect in a fucose-containing component that plays a role in anchoring or even serving as an antiadhesive agent (e.g., an extracellular polysaccharide). This fucose-containing component may be similar to a fucoid oligosaccharide that was isolated from brown algae and is used as an antiadhesive agent (12). However, the disappearance of the S-layer (glycocalyx) (Fig. 6F and J) in strains lacking myxoxanthophyll may further affect surface properties, as the S-layer has a role in cell adhesion in cyanobacteria (23). The lack of the S-layer in strains lacking myxoxanthophyll was unexpected but may indicate that myxoxanthophyll in the outer membrane provides an anchor for the S-layer. This is similar to the situation for *Mycobacterium tuberculosis* H37Rv, where polyacetyl-trehalose provides an anchor to the hydrophilic capsule protein (14). In *Synechocystis*, the hydrophobic carotenoid moiety of myxoxanthophyll may be embedded in the outer membrane, with the hydroxy and sugar groups being on opposite sides of the membrane and the sugar serving as an anchor for glycocalyx proteins (Fig. 7A). The versatility of sugars to form linkages at multiple positions may make fucose a core molecule connecting myxoxanthophyll with the S-layer protein and may link to other cell wall components like exopolysaccharides as well.

Peptidoglycan layer. A defect in fucose biosynthesis is expected to lead to accumulation of mannose, as this is the precursor for fucose biosynthesis. Mannose and its derivatives are among the polysaccharides that are covalently linked to the peptidoglycan layer in the cyanobacterium *Synechocystis* sp. strain PCC 6714 (25, 26), which is a close relative of *Synechocystis* sp. strain PCC 6803. The changes in the thickness and density of the peptidoglycan layer in the Δ sll1213Z strain grown at a light intensity of $0.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 6K and 7C) may be due to an increase in the amount of the polysaccharides that are covalently linked to the peptidoglycan layer.

Myxoxanthophyll and thylakoid membranes. As shown in Fig. 6, in the Δ sll1213Z mutant the thylakoid membrane organization was disrupted. According to Fourier transform infrared spectroscopy of thylakoid membranes of the cyanobacterium *Cylindrospermopsis raciborskii* (58), polar carotenoids in this cyanobacterium are interpreted to be arranged in local patches, presumably to protect the photosynthetic machinery. The results obtained in the current study show the importance of myxoxanthophyll for thylakoid organization but do not seem to support the photosynthetic machinery protection hypothesis, as the Δ sll1213Z mutant grows about as well photoautotrophically as can be expected from its reduced chlorophyll content (Table 2) in spite of a disorganized thylakoid membrane arrangement (Fig. 6F and G). Myxoxanthophyll, which is a rigid rod-shaped conjugated hydrocarbon with hydroxy groups and a sugar molecule at the ends, may be inserted into the lipid bilayer (with the ends of the molecule on opposite sides of the membrane) and brace and reinforce the two leaflets of the bilayer, thus stabilizing the membranes (Fig. 7A). The carotenoids substituting for myxoxanthophyll do not seem

to be able to fulfill this function efficiently, as the thylakoid organization is similar to that of the Δ slr0940K strain (Fig. 6F to H).

The results presented in this paper indicate that fucose and myxoxanthophyll have a range of functions in *Synechocystis* sp. strain PCC 6803. In the Δ sll1213Z mutant, novel carotenoid glycosides partly substituted for the native myxoxanthophyll. Nonetheless, cell wall properties and thylakoid organization were altered as they were in the Δ slr0940K strain (Fig. 6), indicating that the absence of myxoxanthophyll, and not just of fucose, leads to structural changes in the cell. This suggests that myxol dimethyl fucoside is an essential component for normal membrane organization and stability in *Synechocystis* sp. strain PCC 6803. This work highlights the biological significance of myxoxanthophyll in *Synechocystis* and presents a new approach to engineering secondary glycosides in their native host, enriching the library of biologically active molecules produced in vivo.

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