Functional Proteomic Discovery of Slr0110 as a Central Regulator of Carbohydrate Metabolism in Synechocystis Species PCC6803*[S]

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The unicellular photosynthetic model-organism cyanobacterium Synechocystis sp. PCC6803 can grow photoautotrophically using CO₂ or heterotrophically using glucose as the sole carbon source. Several pathways are involved in carbon metabolism in Synechocystis, and the concerted regulation of these pathways by numerous known and unknown genes is critical for the survival and growth of the organism. Here, we report that a hypothetical protein encoded by the open reading frame slr0110 is necessary for the growth and development of the organism. The slr0110-deletion mutant is defective in glucose uptake, heterotrophic growth, and dark viability without detectable defects in autotrophic growth, whereas the level of photosystem II and the rate of oxygen evolution are increased in the mutant. Quantitative proteomic analysis revealed that several proteins in glycolysis and the oxidative pentose phosphate pathway are down-regulated, whereas proteins in photosystem II and phycobilisome are significantly up-regulated, in the mutant. Among the down-regulated proteins are glucose transporter, glucokinase, glucose-6-phosphate isomerase, and glucose-6-phosphate dehydrogenase and its assembly protein OpcA, suggesting that glycolysis, oxidative pentose phosphate, and glycogen synthesis pathways are significantly inhibited in the mutant, which was further confirmed by enzymatic assays and quantification of glycogen content. These findings establish Slr0110 as a novel central regulator of carbohydrate metabolism in Synechocystis, and shed light on an intricate mechanism whereby photosynthesis and carbon metabolism are well concerted to survive the crisis when one or more pathways of the system are impaired. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.033803, 204–219, 2014.

Cyanobacteria are a diverse group of prokaryotes that are capable of oxygenic photosynthesis and are believed to have played a critical role in changing Earth’s atmosphere from ancient anaerobic conditions to the present aerobic conditions (1–3). It is widely accepted that the chloroplasts of higher plants are derived from the endosymbiotic events between cyanobacteria and eukaryotic cells (4). The photosynthetic activity of cyanobacteria is estimated to account for the production of more than half of the biomass on Earth (2). More recently, cyanobacteria have been shown to have great potential as cell factories for the production of clean and renewable biofuels, such as hydrogen (5, 6). Therefore, understanding the physiology and metabolism of cyanobacteria is of great importance not only in basic sciences, but also in biotechnologies dealing with the worldwide crises of energy shortage and environmental pollution.

The unicellular cyanobacterium Synechocystis sp. PCC 6803 (hereinafter referred to as Synechocystis) has been widely used as a model system for the study of photosynthesis and other metabolic processes. It is highly transformable, and its genome has been completely sequenced (7), making it an excellent system for studying the functions of unknown proteins that may participate in pathways in central metabolism by means of targeted mutagenesis. The organism can grow under a number of different conditions ranging from photoautotrophic to fully heterotrophic modes, making it a great tool for the study of fundamental processes such as photosynthesis and carbon metabolism (8, 9). Synechocystis contains an outer membrane, a plasma membrane, and large amounts of thylakoid membrane (10), providing an ideal model for functional proteomics aiming at the discovery of novel proteins involved in many fundamental processes, including molecule transport, photosynthesis and respiration, and signal transduction.

Photosynthesis and carbon metabolism are two physically and functionally interconnected processes in Synechocystis.
The light reaction of photosynthesis provides reductants and energy for the assimilation of inorganic carbon via the Calvin cycle, the net product of which, glyceraldehyde 3-phosphate, can either be further catabolized through the lower energy-conserving phase of glycolysis and the tricarboxylic acid (TCA) cycle, to produce energy, reductants, and precursors for the biosynthesis of other important biomolecules such as amino acids and lipids, or be used as the primary source for the synthesis of glucose and glycogen through gluconeogenesis and glycogenogenesis. Endogenously synthesized or exogenously supplied glucose can also be catabolized through the oxidative pentose phosphate pathway (OPPP) and/or glycolysis to supply carbon and energy for the growth of Synechocystis (11, 12).

As the Calvin cycle, OPPP, and glycolysis take place within a single cellular compartment, many reversible reactions, enzymes, regulators, and intermediate metabolites are shared by the three processes. Moreover, the processes are physically and functionally connected with the TCA cycle, nitrogen assimilation, amino acid and protein synthesis, lipid biosynthesis, and many other metabolic processes. A single perturbation of one process may lead to significant effects on the activities and outcomes of the others. For example, the addition of glucose to Synechocystis culture can enhance the activity of OPPP while partly repressing the activity of photosynthesis (13). Similarly, perturbation of any NADPH-utilizing pathways that generate NADP⁺, an allosteric stimulator of the first and the rate-limiting enzyme of the OPPP (i.e. glucose-6-phosphate dehydrogenase (G6PDH)), can significantly affect the activity of the OPPP (14). In this regard, it is of great importance to understand the regulatory mechanism of metabolism at a system level, rather than at the level of a single gene or pathway.

The Synechocystis genome contains 3672 putative protein-coding open reading frames (ORFs). Many of these are known to be involved in photosynthesis and carbon metabolism (7). However, nearly 50% of the ORFs encode hypothetical or unknown proteins whose expression and function have not been experimentally determined yet. The lack of functional information on these proteins has seriously hindered the progress toward comprehensive understanding of the mechanism whereby balanced anabolism and catabolism take place in a nonseparated compartment at a system level. Fortunately, well-established and highly time- and cost-effective techniques for generating gene-deletion mutants through insertional mutation allow for the study of functions of hypothetical proteins in a relatively high-throughput way. Moreover, recent progress in proteomics allows the quantitative identification of Synechocystis proteins affected by gene deletion at a system scale (15). In a large-scale screening of the gene-deletion mutants of Synechocystis, we obtained several mutants showing the phenotype defective in heterotrophic or autotrophic growth. Of those, the mutant of the ORF encoding the hypothetical protein Str0110 (Δslr0110) exhibited completely inhibited heterotrophic growth but did not show any observable phenotype under autotrophic conditions. Here, we describe the details of the phenotype of Δslr0110 and the results obtained from physiological and proteomics studies addressing the functional significance of Str0110 in the heterotrophic growth of Synechocystis.

MATERIALS AND METHODS

Cell Culture—For autotrophic growth, Synechocystis cells were grown in liquid BG-11 medium on a shaker (200 rpm) at 30 °C with a photosynthetic photon flux density of 50 μmol m⁻² s⁻¹. For mixotrophic growth, 5 mM glucose was supplemented to the medium. For heterotrophic growth, cells were cultured in the same condition as that used for mixotrophic growth, except that 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea was added to the culture medium.

Generation of Knockout and Knockin Mutants—A 1031-base pair (bp) DNA fragment covering a large fraction of the ORF Str0110 and part of its upstream sequence was amplified from genomic Synechocystis DNA via polymerase chain reaction (PCR). The amplified DNA fragment was purified from agarose gel prior to being inserted into pGEM-T vector (Promega, Madison, WI) by ligation. The resulting construct was digested with HpaI and inserted into a ~2.0 kilobase pair (kb) spectinomycin-resistant gene cassette to disrupt the coding region of str0110. All knockout constructs for the other ORFs were generated using a similar procedure. To create a 6× His-tagged slr0110 knockin construct, a 552-bp DNA fragment at the 3′ terminal and before the stop codon of slr0110 and a 505-bp region of its downstream DNA sequence were amplified. The 6× His was then fused to the C terminus of the 552-bp DNA fragment. A final construct was then generated including the 6× His-tagged sequence and the 505-bp downstream DNA sequence with a kanamycin-resistant gene cassette inserted between the two DNA fragments. All knockout and knockin constructs were introduced into the genome of wild-type cells via homologous recombination as described previously (16). After several generations of segregation on BG-11 medium plates with 20 μg/ml spectinomycin or 10 μg/ml kanamycin under low light intensity (2 to 3 μmol photons m⁻² s⁻¹), the stable mutant strains were generated and confirmed by PCR analysis. Sequences of all primers used in this study are shown in supplemental Table S1.

RT-PCR and Quantitative Real-time PCR—Total RNA was prepared from exponentially growing cells according to a published method (17). About 5 μg of total RNA was dissolved in a solution containing 40 mM Tris, 6 mM MgCl₂, 10 mM NaCl, 10 mM CaCl₂, and 0.1% diethylpyrocarbonate prior to being treated with RNase-free DNase at 37 °C for 1 hour. The reverse transcription reaction was carried out using the ThermoScript RT-PCR System (Promega, Madison, WI). Quantitative real-time PCR (qPCR) was carried out with TransStart Top Green qPCR SuperMix (Transgen, Beijing, China). The sequences of the forward and reverse PCR primer sets used in the RT-PCR and qPCR are listed in supplemental Table S1. mBP, which
encodes a subunit of ribonuclease P and is not subject to variable expression under different trophic conditions (18), was used as the internal control for qPCR. All PCR experiments were performed in triplicate.

**Measurement of Chlorophyll Concentration, 77K Fluorescence, and Rate of Oxygen Evolution**—The chlorophyll of 1 OD (730 nm) *Synechocystis* cells was extracted using 95% methanol and the absorbance at 665 nm was measured. 77K fluorescence emission spectra were measured using an F-2500 Fluorescence Spectrophotometer (Hitachi, Ibaraki, Japan). Cells were adjusted to a concentration of 15 μg chlorophyll ml⁻¹, and the fluorescence emission spectra (excitation at 435 nm, bandwidth of 5 nm) were recorded in the range of 650–800 nm and were normalized at 726 nm. Oxygen evolution rates were measured with a Chlorolab-2 oxygen electrode (Hansatech, Norfolk, UK) following the manufacturer’s instructions. For the determination of net photosynthetic oxygen evolution, cells were suspended in BG-11 medium (10 mM NaHCO₃ and measured at 30 °C. For PSII-mediated activity measurements, 1 mM potassium ferricyanide (KFe(CN)₆) and 1 mM 2,6-dichloro-2-benzoquinone were added as electron acceptors.

**Protein Preparation**—*Synechocystis* cells at a late exponential phase were harvested and resuspended in a buffer containing 0.4 M sucrose, 50 mM 3-(N-morpholino) propanesulfonic acid, pH 7.0, 10 mM NaCl, 5 mM EDTA, and 0.5 mM PMSF. Cells were broken using a bead beater, and the whole cell lysate was obtained via centrifugation for 30 min at 5000 × g at 4 °C to remove glass beads and insoluble cell debris. The total proteins from the whole cell lysate were then precipitated with 10% trichloroacetic acid in ice-cold acetone, washed with acetone, and resolubilized with 4% SDS in 0.1 M Tris-HCl, pH 7.6. The membrane proteins and soluble proteins were prepared as previously described (19, 20). Briefly, the whole cell lysate was centrifuged at 76,000 × g for 30 min. The pellet was collected as the membrane fraction, and the supernatant was collected as the soluble fraction. The membranes were then resuspended with the same buffer used for the whole cell lysate. Proteins in both fractions were precipitated and resolubilized in the same way as the total proteins.

**Quantitative Protein Identification**—Proteins were digested using the filter-aided preparation method as previously described (21). Each sample was reduced and alkylated on Microcon YM-30 centrifugal filter units (EMD Millipore Corporation, Billerica, MA); the denaturing buffer was replaced with iTRAQ dissolution buffer, and then digested with sequencing grade trypsin (Promega, Madison, WI) at 37 °C overnight. The resulting tryptic peptides were collected and labeled with iTRAQ reagents (AB Sciex, Inc., Framingham, MA) according to the manufacturer’s instructions, with slight modifications. Briefly, the labeling reaction was performed by incubating peptides with ethanol-dissolved iTRAQ reagents for 2 h at room temperature, and it was terminated by adding H₂O to a final concentration of 30% to inactivate the labeling reaction. The labeled samples were mixed together with equal amounts, and 20 μg of the mixture was then desalted and subsequently fractionated with C18 strong cation exchange stop-and-go extraction tips (StageTips). Six strong cation exchange fractions were obtained from the stepwise gradient elution using 10 mM, 20 mM, 50 mM, 100 mM, 300 mM, and 500 mM NH₄AcO in 0.5% AcOH and 20% acetonitrile as previously described (22). Each fraction was further desalted with C18 StageTips and dried with a SpeedVac, and the peptides were then resuspended in 0.1% formic acid by analyzing a TripleTOF 5600 mass spectrometer (AB Sciex) coupled online to an Eksigent nanoLC Ultra in information-dependent mode. Briefly, 2 μl of peptide sample (1.0 μg/μl peptides) was injected into a 15-cm-long, 75-μm inner diameter capillary analytic column packed with C18 particles of 5-μm diameter. The mobile phases consisted of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in acetonitrile). The peptides were separated using gradients of 5%–8% B for 2 min, 8%–20% B for 63 min, 20%–30% B for 15 min, 30%–90% B for 1 min, 90% B for 7 min, 90%–5% B for 0.1 min, and 5% B for 8 min at a flow rate of 300 nl/min. The ion spray voltage floating and declustering potential were set at 2.4 KV and 100 V, respectively. The interface heater temperature was set at 150 °C, and the curtain gas and ion source gas were set at 25 and 15, respectively. MS spectra were acquired across the mass range of 350–1500 m/z in high-resolution mode (>30,000) using a 250-ms accumulation time per spectrum. A maximum of 25 precursors per cycle were chosen for fragmentation from each MS spectrum with a 100-ns minimum accumulation time for each precursor and dynamic exclusion for 18 s. Tandem mass spectra were recorded in high-sensitivity mode (resolution > 15,000) with rolling collision energy on and iTRAQ reagent collision energy adjustment on (23).

Protein identification and quantification were performed using the ProteinPilot 4.5 software (AB Sciex) with the Paragon database search algorithm (4.5.0.1654) (24). The MS/MS spectra were searched against a Synechocystis proteome database (3672 entries, CyanoBase) that is concatenated to a reversed-sequence decoy database derived from the target database and is supplemented with 162 frequently observed contaminants, including human keratin and bovine serum proteins. The mass tolerances for both precursor and fragment ions were set at 0.05 Da, and the maximum number of missed cleavages was set at 2. The modification parameter was set to “biological modifications,” which allows the majority of known modifications to be searched in a single search step with a probability-based approach (24), and a “thorough ID” search effort was used. The detected protein threshold (Unused ProtScore) was set at 0.05. Protein identifications were evaluated via false discovery rate (FDR) analysis through a target-decoy database search. The FDR analysis was performed using the Proteomics System Performance Evaluation Pipeline integrated with ProteinPilot (25). For protein quantitation, the iTRAQ report ion peak areas were used for peptide ratio calculation, and bias correction and background correction in ProteinPilot software were implemented to fix any sampling errors so that the median value of iTRAQ ratios of protein distribution was equal to 1.

**Measurement of Glucose Concentration, Glucokinase, and G6PDH Activities**—The glucose concentration of culture medium was determined using a glucose assay kit (Sigma-Aldrich). Glucokinase (GLK) activity was measured as previously described (26). Briefly, the cells were harvested via centrifugation, suspended in 50 mM HEPES/NaOH, pH 7.5, and broken with glass beads. Protein extracts were cleared via centrifugation for 10 min at 20,000 × g at 4 °C. The enzymatic reactions of GLK were initiated by the addition of 10 μl of protein extracts to a solution containing 50 mM HEPES/NaOH, pH 7.5, in the presence of 5 mM MgCl₂, 2 mM ATP, 0.5 mM NADP⁺, 2 μM 1,2-G6PD, and 20 mM glucose and then allowed to proceed at 30 °C for 0 min, 15 min, and 30 min. The reactions were stopped by heating at 100 °C for 90 s followed by centrifugation to remove denatured proteins. Enzyme activity was determined by measuring the change of the absorbance of the reaction mixture at 340 nm, which represents the change in the NADP level. The activity of G6PDH was measured using a Glucose-Phosphate Dehydrogenase Assay Kit (Sigma-Aldrich) following the protocol provided by the manufacturer. All measurements were performed in triplicate.

**Determination of Glycogen Content**—*Synechocystis* cells were harvested, resuspended in 400 μl 30% (w/v) KOH, and incubated at 95 °C for 2 h. Glycogen was then precipitated with 75% (v/v) ice-cold ethanol, pelleted via centrifugation at 10,000 × g for 10 min, sequentially washed with 70% and 98% (v/v) ethanol, and finally dried at 60 °C for 10 min. The isolated glycogen was resuspended in 100 mM sodium acetate (pH 4.5) and enzymatically hydrolyzed to glucose with
2 mg/ml amylglucosidase (Sigma-Aldrich) at 60 °C for 2 h. The glycogen content was determined with a glucose assay kit (Sigma-Aldrich) used according to the manufacturer's instructions.

Bioinformatics and Statistics—The transmembrane domain (TM) prediction was performed using the software TMHMM, which can correctly predict 97% to 98% of the transmembrane helices (27). The sequence homology and phylogenetic analyses were performed using the software package MEGA 5.10 (28). Specifically, the sequence homology analysis was performed using the tool ClustalW, and phylogenetic tree analysis was performed using the maximum-likelihood algorithm. The one-sample t test was performed using the bioinformatics software Perseus, which is freely available from the Mathias Mann group (29).

RESULTS

Slr0110 Is a Highly Conserved Protein of Unknown Function—The hypothetical protein Slr0110 was identified as a peripheral membrane protein in our previous study (20). Functional domain and motif analysis revealed that Slr0110 contains an ATP/GTP binding site motif A (P-loop) at its N terminus that is highly conserved in coenzyme a-c-diamide synthetase (CbiA) and in chromosome partitioning ATPase (ParA) (supplemental Fig. S1A). Homology analysis revealed that Slr0110 has close homologs in cyanobacteria and thermophilic bacteria (supplemental Fig. S1B). These homologs are annotated as CbiA, ParA, or simply hypothetical protein. To clarify the inconsistency of the annotations, we compared the amino acid sequences of Slr0110 and one of the CbiAs in Salmonella typhimurium LT2 with confirmed CbiA activity (30). Unexpectedly, little sequence similarity was found between Slr0110 and the known CbiA aside from the ATP/GTP binding site. The confirmed CbiA in Salmonella typhimurium LT2 is also highly conserved in prokaryotes and has a homolog (Sll1597) in Synechocystis. Again, little sequence similarity was found between Slr0110 and Sll1597. We then asked whether Slr0110 is the homolog of ParA. The analysis of sequence similarity revealed that Slr0110 has little homology with known ParAs in Synechocystis, including Slr1597, Sll6093, and Sll6036, except for the ATP/GTP binding site. Therefore, we concluded that Slr0110 is a protein of unknown function, and assignment of CbiA or ParA to its homologs in other prokaryotic organisms may be misled by the ATP/GTP binding site, which is highly conserved from higher plants to cyanobacteria (supplemental Fig. S1B).

Disruption of the ORF slr0110 in Synechocystis—An slr0110-deletion mutant (Δslr0110) was generated using the construct shown in Fig. 1A through insertional mutation, a fast and effective approach to generate gene-deletion mutants through homologous recombination between the host chromosomal DNA and the plasmid construct that carries an antibiotic resistance gene cassette flanked with two DNA fragments from each side of the target site of insertion on the chromosome (31–33). The complete segregation of the mutant was confirmed by PCR using the same pair of primers amplifying a 1.0-kb wild-type (WT) slr0110 DNA fragment. The size increase of the PCR products from 1.0 kb for the WT to 3.3 kb for the mutant is ascribed to the insertion of a 2.0-kb spectinomycin resistance gene cassette within the same DNA fragment amplified for WT (Fig. 1B).

To investigate whether the insertional mutation of slr0110 affects the expression of the other ORFs localized in the same genomic place with slr0110, a polar effect that usually occurs in prokaryotes whereby multiple closely localized genes co-express within the same operon, we examined the transcription of the ORFs slr0111, slr0112, slr0114, and slr0115 in the downstream and sll0094 in the upstream of slr0110 with opposite direction (Fig. 1C). The transcription of the selected ORFs was detected using RT-PCR (Fig. 1D) and qPCR (Fig. 1E). The results showed that the mRNA for slr0110 was completely depleted in Δslr0110 relative to the WT (Fig. 1D), whereas no significant difference in transcription was detected for the other ORFs (Figs. 1D and 1E). Thus, an slr0110-deletion mutant was successfully generated without significantly altering the expression of its neighboring ORFs.

Depletion of Str0110 Inhibits Heterotrophic Growth of Synechocystis—To investigate whether slr0110 deletion causes growth defects in Synechocystis, we measured the growth rate of the WT and the mutant in BG11 culture medium supplemented with (photomixotrophic condition) or without
Fig. 2. *Δslr0110* is defective in heterotrophic growth with an increased level of PSII. A, growth curves of *Δslr0110* and the WT were measured in the presence (+ G) or absence of 5 mM glucose under medium light intensity (50 μmol m⁻² s⁻¹) (left-hand panel). The images of cell cultures were taken at the 72-h time point (right-hand panel). B, the growth curves of *Δslr0110* and the WT cultured in the medium with 5 mM glucose and in the presence or absence of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). The images of cell cultures were taken at the 72-h time point. C, determination of glucose concentration in the culture medium for the mutant and the WT *Synechocystis*. The glucose concentration was measured at the time points as indicated after the cells were seeded in BG11 medium supplemented with 5 mM glucose. The bar graph is representative of the results from more than five repeated experiments performed at different times. D, low-temperature (77K) fluorescence emission spectra of *Δslr0110* and the WT cells cultured under photoautotrophic conditions. E, the chlorophyll contents of *Δslr0110* and the WT. The chlorophyll of 1 OD (730 nm) *Synechocystis* cells was extracted at the indicated time points after the cells were seeded in liquid culture. F, the growth curves of the WT and *Δslr0110* in the presence of both glucose and B12.

(photomixotrophic condition) 5 mM glucose under medium light intensity (50 μmol m⁻² s⁻¹). The growth of *Δslr0110* was significantly slower than that of WT under photomixotrophic conditions, whereas no significant difference was observed under photoautotrophic conditions (Fig. 2A). This result suggests that the mutant might not be able to utilize exogenous glucose for photomixotrophic growth. If we inhibit photosynthesis of the mutant in the presence of glucose (photomixotrophic condition), the cell might die as a result of its inability to utilize glucose as the sole carbon source for growth. Indeed, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, a photoautotrophic growth inhibitor that blocks photosynthetic electron transfer from PSII to the plastoquinone pool, completely inhibited the growth of the mutant in the presence of glucose, but not of the WT, though the growth rate of the latter was reduced to the same level as that cultured without glucose (Fig. 2B). To further confirm the inability of *Δslr0110* to utilize glucose for heterotrophic/mixotrophic growth, we measured the concentration of the glucose in the culture medium 48 or 96 h after seeding the cells. The results show that the glucose concentration in the culture medium remained unchanged at 48 or 96 h for the mutant but was reduced by about 50% at 48 h and was undetectable at 96 h for WT (Fig. 2C). These data suggest that exogenously supplied glucose cannot be consumed by the mutant cells, whereas it can be efficiently transported and utilized by the WT cells.
To test whether the photosystems of the mutant were significantly altered, we measured the 77K fluorescence of the WT and the mutant cells cultured under photoautotrophic conditions. The emission spectra, as indicated by the intensities of the peaks at 685/695 nm and 725 nm, corresponding to PSII and photosystem I (PSI), respectively, show that Δslr0110 had a greater amount of PSII than WT, whereas PSI was not altered (Fig. 2D). In line with this observation, Δslr0110 had higher PSI activity, as indicated by the higher rate of oxygen evolution relative to that of WT (Table I). The chlorophyll a content, an indicator of the PSI amount, was nearly identical between WT and Δslr0110 (Fig. 2E), further confirming that the PSI level was not significantly altered in Δslr0110. These data, together with the observation that Δslr0110 and the WT had identical photoautotrophic growth rates, suggest that depletion of Slr0110 impairs heterotrophic growth of *Synechocystis* without inhibition, if not with an increase, of photosynthesis.

To further exclude the possibility that Slr0110 might have CbiA activity and be involved in coenzyme B₁₂ (B₁₂) biosynthesis, we measured the growth rate of the Δslr0110 in the presence of both glucose and B₁₂. As expected, B₁₂ could not rescue the reduced growth rate of the mutant under the photomixotrophic growth condition (Fig. 2F), confirming that the inhibited photomixotrophic growth of Δslr0110 is not due to defective B₁₂ biosynthesis.

**Depletion of Slr0110 Alters the Protein Expression Pattern of Synechocystis**—The inability to utilize exogenously supplied glucose as the sole carbon source for heterotrophic growth indicates that glucose transport or catabolism in Δslr0110 could be impaired. Therefore, we sought protein changes that were potentially attributable to the defective heterotrophic growth of Δslr0110. The WT and the mutant cells cultured in the presence or absence of glucose were lysed and separated into membrane and soluble fractions to allow more low-abundant proteins to be visualized by means of SDS-PAGE and Coomassie Blue staining. As expected, some protein bands exhibited different intensities of staining for the mutant and the WT, although the majority of the bands did not show obvious differences (supplemental Fig. S2). This result suggests that the protein profile was altered to some extent due to the depletion of Slr0110, and it prompted us to quantify the differences in protein expression using iTRAQ-based quantitative mass spectrometry. The experimental design of the quantitative proteomics and the iTRAQ labeling scheme are diagrammed in Fig. 3. In total, we did three quantitative analyses. In experiment 1, two biological replicates of both WT and Δslr0110 that were cultured in autotrophic conditions were included. In experiment 2, one biological replicate of WT grown in the absence or presence of glucose and two technical replicates of Δslr0110 grown in the absence of glucose were included. The design and the labeling scheme of experiment 3 were the same as in experiment 2, except that the whole cell lysates were further separated into membrane and soluble fractions.

**Quantitative Identification of Differentially Expressed Proteins in Δslr0110**—Though we included some cells grown in the presence of glucose for quantitative proteomic analysis (Fig. 3, experiments 2 and 3), the analysis described hereinafter was performed only on the comparison of the proteome between the mutant and the WT cells grown in the absence of glucose, in order to focus on the differences caused only by the depletion of Slr0110 and not by the differential uptake of glucose. In total, 855,787 high-resolution spectra were collected, and 2040 unique proteins were identified with FDRs of 1%, including 228 proteins with single-peptide identification (annotated spectra are shown in supplemental Fig. S3). The identified proteins represent 55.6% of the predicted proteome (supplemental Table S2). Among these, 1185 proteins were identified by all three experiments (Fig. 4A). Proteomic identification of the expressed proteins for *Synechocystis* has been performed in more than 25 independent studies in the past 15 years, and 2218 distinct proteins were identified in total, as summarized by two recent articles (20, 34), including 1827 proteins that were also identified in the current study. In addition, 213 proteins from the current study had never been identified by any previous studies (supplemental Table S3). This dataset is the largest protein catalogue generated in a single study to date from *Synechocystis* and will provide a wealth of information regarding gene expression for this organism.

The membrane proteins of *Synechocystis* play critical roles in many important processes such as photosynthesis and respiration, and the identification of these proteins has been performed in numerous studies since the year 1997 (19, 20,
Because of the insolubility of the membrane proteins, however, the total number of identified proteins that contain at least one TM from numerous reports is less than 500 (supplemental Table S3), and fewer than 300 membrane proteins have been identified in any single study. In our current data-set, 409 identified proteins are predicted to contain at least 1 TM, and 232 proteins are predicted to contain 2 or more TMs, including 1 with 17 TMs and 1 with 16 TMs (Fig. 4B). The number of total identified membrane proteins is 46% of all predicted membrane proteins encoded by the Synechocystis genome, including 52 membrane proteins that have never been identified before (supplemental Table S3). Therefore, our proteomics dataset is the most comprehensive, and is suitable for quantitative screening of the target proteins that are potentially responsible for the impaired heterotrophic growth of Δslr0110.

The reproducibility of the quantitation was evaluated through correlation of the iTRAQ ratios from two biological replicates (Fig. 3A, experiment 1). The overall reproducibility of the quantitation in two biological replicates was high, with $R^2$ equal to 0.62. The reproducibility can be increased along with the increased stringency of the $p$ value for the iTRAQ ratios. If we include only the proteins with $p$ values of $<0.05$ for iTRAQ ratios from both replicates, a very high reproducibility can be observed, with $R^2$ equal to 0.91. However, higher stringency can also lead to a reduced number of quantitated proteins (supplemental Fig. S4). To accurately quantify the changes of protein levels in Δslr0110 and WT and increase the sensitivity in finding potential targets whose expression could be affected by the deletion of slr0110, we applied multiple criteria to determine confidently quantitated proteins. First, all quantitated proteins that had a total of at least two iTRAQ ratios with $p$ values of $<0.05$ were included, and proteins with only one peptide identified were excluded from analysis. Using this criterion, 349 proteins were included. Second, for the 349 proteins, a one-sample t test was performed, and 156 proteins were shown to be significantly changed between Δslr0110 and the WT ($p < 0.05, n = 3$). The confidences of the 156 proteins were labeled as “high” (supplemental Table S4). Third, from among the rest of the proteins that did not pass the t test, we included only proteins with all log2-transformed iTRAQ ratios uniformly greater than 0.58 or less than 0.58.
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(1.5-fold change). 37 proteins met this criterion, and the confidences of these proteins were labeled as “medium” (supplemental Table S4). We reasoned that it should be safe to include these proteins for further analysis, as the two ribosomal proteins Sll1810 and Sll1802 had log2-transformed iTRAQ ratios of 1.89, 1.69, and 3.36 and 1.28, 1.79, and 3.31, respectively. Though the t test p values of both were greater than 0.05, both proteins are more likely up-regulated as indicated by their iTRAQ ratios because the majority of the up-regulated ribosomal proteins passed the t test and the ribosomal proteins function as a macroprotein complex in cells that requires appropriate stoichiometry (supplemental Table S4). Finally, for the proteins identified from only the membrane fraction that had only one biological replicate with two technique replicates, we performed target-decoy FDR analysis to select the confidently quantified proteins (supplemental Fig. S5). With a FDR of <5%, seven proteins were included and labeled as “fraction only” (supplemental Table S4). The mean iTRAQ ratios and the corresponding variances for all confidently quantitated proteins were plotted (Fig. 4C). To further ensure that all proteins were truly up-regulated or down-regulated, we excluded all the proteins with a fold change of less than 1.5, as shown by the two proteins outside of the dashed boxes in Fig. 4C, even though they were confidently quantitated as indicated by the p value of the t test. In total, 123 proteins were up-regulated and 75 proteins were down-regulated in Δslr0110 relative to WT (supplemental Table S4).

Depletion of Slr0110 Differentially Affects Expression of Proteins with a Wide Spectrum of Functions—All up- or down-regulated proteins were functionally categorized according to the first-class functions annotated by CyanoBase (Fig. 5) (39). In some functional groups, including translation, photosynthesis and respiration, and biosynthesis of cofactors and prosthetic groups, more proteins were elevated in Δslr0110 than in WT (Fig. 5A), whereas in other groups such as regulatory functions, transport and binding proteins, cellular processes, and other categories, more proteins were reduced in Δslr0110 than in WT. In addition, nearly equal numbers of hypothetical and unknown proteins were quantitated as elevated or reduced in Δslr0110 (Fig. 5A), suggesting that protein quantitation in the current study was not biased toward or against certain functional groups of proteins and truly reflects the expression levels of proteins in different functional categories, either elevated or reduced, because the hypothetical and unknown proteins should have the same divergent functions as exhibited by those known proteins. Considering that Δslr0110 is impaired in heterotrophic growth with glucose as the exogenous carbon source and has an elevated PSII level and oxygen-evolving capability, we focused our analysis on the functional categories that are presumably more relevant to the phenotypic or physiological observations (i.e., the categories of photosynthesis and respiration and carbon metabolism). It is worth noting that translation-related proteins are most predominant among the up-regulated proteins in Δslr0110. Subdividing these proteins according to the second-class function annotated by CyanoBase revealed that the vast majority of the proteins in this group are ribosomal proteins, and no ribosomal proteins were found to be reduced in Δslr0110 (Fig. 5B, left-hand panel, and supplemental Fig. S6). Though the biological significance of the elevated ribosomal proteins remains to be addressed, it is probably a strategy developed by Synechocystis to acclimate to the loss of Slr0110 function.

Proteins in Photosynthesis Are Differentially Regulated in Δslr0110—Three, six, and three proteins in photosystems, phycobilisome, and CO2 fixation, respectively, are up-regulated in Δslr0110, whereas three and one subunits of ATP synthase and NADH dehydrogenase, respectively, are down-regulated (Fig. 5B, middle panel, and supplemental Fig. S6). Up-regulation of photosystem subunits is consistent with the observation that Δslr0110 has a higher 77K emission peak of PSII and elevated photosynthetic activity (Table I). This, together with the up-regulation of phycobilisomes, explains the increased efficiency of Δslr0110 in light harvesting and oxygen evolution (Table I). The up-regulation of CO2 fixation proteins may be due to the increased CO2 necessity of the elevated photosynthetic activity. Interestingly, ATP synthase, which synthesizes ATP driven by the proton gradient generated from photosynthetic and respiratory electron transport, is down-regulated in the mutant. Down-regulation of ATP synthase suggests that the mutant may have a reduced rate of electron transport generating the proton gradient for ATP synthesis. Considering that ATP synthase is shared by both electron transport chains on the thylakoid membrane and the photosystem is not down-regulated, it is reasonable to presume that the rate of respiratory electron transport is reduced in the mutant. Indeed, one subunit of NADH dehydrogenase, an integral component of the respiratory electron transport chain, was down-regulated in the mutant. Down-regulation of NADH dehydrogenase is a strong indicator that carbon metabolism, including OPPP, glycolysis, and the TCA cycle, is also down-regulated in the mutant. In fact, the proteins in these processes were differentially regulated in the mutant (Fig. 5B, right panel). Therefore, we focused our analysis on these proteins to address whether their differential regulation is relevant to the phenotype of Δslr0110.

Pathways for Glucose Catabolism are Down-regulated by the Disruption of slr0110—In line with the observation that NADH dehydrogenase is down-regulated in the mutant (Fig. 5B), the impaired glucose-dependent heterotrophic growth of Δslr0110 suggests that the glucose catabolism or transport is defective in the mutant, which potentially down-regulates the respiration and hence the NADH dehydrogenase in the mutant. Two pathways are responsible for glucose metabolism, the glycolysis and the OPPP, though the latter is generally considered as the major pathway for breaking down glucose (12). Proteomic analysis revealed that three proteins in glycolysis and two proteins in OPPP were down-regulated in the mutant, whereas three proteins in glycolysis and none in
OPPP were up-regulated in the mutant (Fig. 5B, right-hand panel). The two pathways operate in the same compartment in *Synechocystis* and could actively exchange intermediate or terminal metabolites such as fructose-6-phosphate and glyceraldehyde 3-phosphate (Fig. 6A). A number of reactions and catalytic proteins in the two pathways are also shared by the Calvin cycle for CO₂ fixation. The three down-regulated proteins in glycolysis are GLK (Sll0593), glucose-6-phosphate isomerase (GPI) (Slr1349), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Slr0884). In OPPP, G6PDH (Slr1843) and its assembly protein OpcA (Slr1734) were significantly down-regulated. Whereas the reduced levels of GLK and GPI can significantly inhibit glycolysis, the nearly 10-fold down-regulation of G6PDH and its assembly protein may completely inhibit OPPP. Inhibition of these critical steps in the two pathways of glucose catabolism should be sufficient to prevent the mutant from efficiently utilizing glucose for heterotrophic growth. If this is the case, glucose transport may also be repressed because exogenous glucose cannot be catabolized by the mutant. Prominently, the proteomics and bioinformatics analyses revealed that many transport and binding proteins are down-regulated in the mutant, including the only glucose transporter (GlcP, Sll0771) encoded by the *Synechocystis* genome (Fig. 5A and supplemental Fig. S6)(40, 41). To further confirm the expression of the GlcP, we generated a knockin strain of sll0771 with a 6x His tag fused at the C terminus of its encoded protein in both WT and Δsll0110, and we confirmed via Western blotting that the Sll0771 level was significantly reduced in Δsll0110 relative to WT, irrespective of the presence or absence of glucose in
the culture medium (Fig. 6B). The data indicated that the defect in glucose transport is caused by the down-regulation of Slr0771 in the mutant. Interestingly, RT-PCR results showed that the mRNA levels of the repressed proteins, as well as those of the other proteins in glycolysis or OPPP, were slightly up-regulated in the slr0110-deletion mutant (Fig. 6C). The inconsistency of the protein and mRNA levels is not surprising, as it is well accepted that the levels of mRNA and protein are not well correlated (42). Instead it suggests that the down-regulation of GlcP and catabolism-related proteins in Δslr0110 does not occur at a transcriptional level.

Activities of Glucokinase and G6PDH Are Significantly Reduced in Δslr0110—To examine whether the down-regulation of the proteins in glucose transport and catabolism has a causal relation with the impaired heterotrophic growth of Δslr0110, we generated knockout mutants for sll0771 (Δsll0771) and sll0593 (Δsll0593), the two proteins that are primarily responsible for glucose transport and GLK activity in Synechocystis, respectively, through insertional mutation as described above for slr0110. The growth measurement shows that the completely segregated Δsll0771, as shown by PCR, is defective in heterotrophic growth with glucose as the carbon source (Fig. 7A), a phenotype consistent with previous reports and similar to that of Δslr0110 (26). The glucose concentration in the culture medium for Δsll0771 also remained unchanged after 48 or 96 h (Fig. 7B), further confirming that down-regulation of sll0771 was the direct cause of impaired glucose transport in the Δsll0110 mutant. However, Δsll0110 was not

Fig. 6. Depletion of Sir0110 reduced protein but not mRNA levels of glucose transport and catabolism-related genes in Synechocystis. A, a diagram shows the glucose catabolism pathways and the involved proteins in Synechocystis. The relative abundance of each protein in Δslr0110 compared with the WT (the logarithm transformed mean iTRAQ ratio), if available, is shown in parentheses. A red asterisk indicates the corresponding reaction and proteins are also involved in the Calvin cycle; the dashed double-headed arrows indicate that the corresponding metabolites can be shared by more than one reaction. A red “X” indicates that the corresponding reaction or process is inhibited. B, confirmation of inhibited expression of sll0771 in Δslr0110 by Western blot. The knockin strains of sll0771-His tag were generated from Δslr0110 and the WT, respectively, and subsequently cultured in the presence or absence of glucose. The cells were then lysed and the expression of Sll0771 was probed using anti-His tag antibody. C, the mRNA levels of the indicated ORFs involved in glucose catabolism in Δslr0110 and the WT were detected via RT-PCR. The cells used for RNA preparation were cultured in photoautotrophic conditions.
Depletion of Slr0110 Leads to Reduced Glycogen Content and Impaired Dark Viability—Glycogen is the primary form of storage for excess assimilated carbon (11). The proteomes shared by glycolysis and gluconeogenesis is necessary for glycogen synthesis from the product of the Calvin cycle (Fig. 6A). The proteomics data indicate that GPI is also down-regulated to a lesser extent than GLK or G6PDH in Δslr0110, suggesting that glycogen synthesis may also be partially inhibited in the mutant. Measurement of the glycogen content confirmed that the mutant had a certain but significantly lesser amount of glycogen (Fig. 8). We then asked whether the glycogen storage can be utilized by the mutant to maintain survival and growth when cells are incubated in the dark, a condition under which no inorganic carbon can be fixed and assimilated through photosynthesis. To address this question, we transferred the fast-growing WT and Δslr0110 cells cultured in an autotrophic condition to solid BG11 medium with or without glucose and incubated the cells in the dark for 72 h before exposing them to normal light illumination. We reasoned that long-time dark incubation should allow cells to reduce their glycogen storage and hence their dark viability. Indeed, 72-hour dark incubation seriously reduced the viability of the mutant, and its glycogen storage was almost completely exhausted (Figs. 8A and 8B). In contrast, the viability of the WT was less affected by the dark incubation, and its glycogen content also was reduced but was still maintained at a high level because of the much larger volume of the storage before dark incubation (Fig. 8B). It is worth noting that GPI is necessary not only for gluconeogenesis and glycogen synthesis, but also for glycogen catabolism in Δslr0110 with inhibited OPPP. The breakdown of glycogen produces glucose-6-phosphate that can be further catabolized through glycolysis via the reaction catalyzed by GPI (Fig. 6A). Repression of GPI may inhibit both glycogen synthesis and catabolism. The similarly reduced level of glycogen in both WT and Δslr0110 suggests that glycogen catabolism was operating at a similar rate in both the WT and the mutant under dark incubation (Fig. 8B), which is probably due to down-regulation of the GPI activity and hence glycolysis in the WT to the same levels as those of Δslr0110, as slow growth in the dark does not require high glycolysis activity. Taken together, these data demonstrate that deletion of Slr0110 leads to defective glycogen synthesis and impaired dark viability of Synechocystis.

DISCUSSION

The Synechocystis genome is predicted to encode about 50% hypothetical or unknown proteins, and more than 900 have been collectively confirmed as expressed under different conditions through numerous proteomics studies since the first report of proteomics analysis for this organism in 1997.

sensitive to glucose, which is significantly different from Δsll0593, suggesting that down-regulation of Sll0593 is a side effect but not the major cause of defective heterotrophic growth caused by the deletion of Slr0110.

To investigate whether the activity of the key enzyme G6PDH in OPPP is also inhibited by the deletion of Slr0110, we measured its activity in Δslr0110. As expected, the enzyme activity was significantly reduced relative to that of the WT (Fig. 7D), regardless of whether cells were grown in the presence or absence of glucose, which correlates well with the protein level quantified by mass spectrometry. Interestingly, the enzyme activity of G6PDH was not significantly altered in Δsll0771 and Δsll0593 (Fig. 7E), suggesting that down-regulation of G6PDH and its enzyme activity in Δslr0110 is not an adaptive response to the reduced level of Sll0771 and Sll0593, but probably vice versa. Therefore, we presumed that Slr0110 regulates the level of G6PDH through an unknown mechanism—possibly changes in some protease activity targeted at G6PDH—to control the expression of sll0771 and sll0593 at the protein but not the mRNA level, and thus to control the glucose transport and catabolism.

Fig. 7. Confirmation of defective glucose transport and catabolism in Δslr0110. A, growth curves of the WT, Δslr0110, and Δsll0771 strains cultured in the presence of 5 mM glucose. The inset shows the complete segregation of Δsll0771 detected via PCR (right-hand panel). The images of the cell cultures were taken at 72 h post-seeding. B, determination of glucose concentration in the culture medium for the WT, Δslr0110, and Δsll0771 at the indicated time points. The cells were cultured in BG11 medium supplemented with 5 mM glucose. C, determination of GLK activities of the WT and Δslr0110. The cells were cultured in BG11 culture medium supplemented with or without 5 mM glucose under medium light intensity (50 μmol m−2 s−1). D, E, determination of G6PDH activity in WT, Δslr0110, Δsll0771, and Δsll0593. The cells were cultured in either the presence or absence of glucose, as indicated.
Deletion of slr0110 completely inhibited the photoheterotrophic growth of Synechocystis with glucose as the exogenous carbon source without impairing autotrophic growth. The quantitative proteomics analysis revealed that protein levels of key enzymes in glycolysis and OPPP, as well as that of GlnP, were significantly reduced in the slr0110 mutant cultured in autotrophic conditions. Further mutagenesis studies and enzymatic activity measurements confirmed that glycolysis and OPPP were almost completely blocked in the mutant, as indicated by the near-complete inhibition of the enzymatic activities of the respective key enzymes of the two pathways (i.e., GLK and G6PDH). Inhibition of multiple enzymes of the two pathways not only prevented the mutant from using exogenous glucose, but also reduced its volume of glycogen storage and dark viability (Fig. 8). These findings establish Slr0110 as a central regulator of carbohydrate metabolism in Synechocystis. Depletion of Slr0110 also leads to modest up-regulation of PSI activity, and probably of the overall photosynthetic activity as well. This is more likely an adaptation response to the loss of Slr0110 function intended to compensate for the reduced glycogen synthesis and storage in the mutant cells.
significantly different from those of \( \Delta \text{slr0110} \). Deletion of \( \text{hik31} \) completely inhibits GLK activity and induces glucose sensitivity in Synechocystis (26), whereas deletion of \( \text{hik8} \) or \( \text{sigE} \) impairs heterotrophic growth of the mutant and represses the transcription of key enzymes of glycolysis and OPPP such as G6PDH, PFK, and GAPDH to different extents (44). Although \( \Delta \text{slr0110} \) is also unable to utilize glucose for heterotrophic growth and the expression and activities of GLK and G6PDH were significantly repressed, the mutant was not glucose sensitive, and the transcription of the key enzymes of glycolysis and OPPP was not repressed (Fig. 6C). Taken together, our proteomics and transcription data suggest that the known regulators of carbon metabolism, including \( \text{hik31}, \text{hik8}, \) and \( \text{sigE} \), are unlikely to play a major role in mediating the defective heterotrophic growth of \( \Delta \text{slr0110} \), though we cannot rule out the possibility that other proteins of two-component systems or \( \sigma \) factors may be necessary for \( \text{Slr0110} \)-mediated regulation of carbon metabolism.

An inability to take up glucose is the most prominent observation for the \( \text{slr0110} \) mutant; more than 10-fold repression of the GlcP relative to the WT can reasonably account for this defect. It has been documented that \( \text{Slr0771} \) is the main GlcP of Synechocystis (26, 40), and a \( \Delta \text{sll0771} \) mutant exhibited the same phenotypes as \( \Delta \text{slr0110} \) (i.e., inhibited heterotrophic growth and impaired transport of exogenous glucose) (Fig. 7A). However, repression of \( \text{Slr0771} \) might not necessarily be the immediate consequence of \( \text{Slr0110} \) depletion, because repression of other proteins in carbon metabolism can also inhibit glucose uptake via some feedback mechanisms. For instance, glucose uptake can be significantly inhibited in a Synechocystis mutant defective in glycogen synthesis (11). Our quantitative proteomics analysis revealed that many proteins in carbon metabolism pathways, including GLK (\( \text{Slr0593} \) and G6PDH, were repressed in \( \Delta \text{slr0110} \) (supplemental Table S3, Fig. 6A). \( \text{Slr0593} \) has been considered as the major, if not the only, functional GLK in Synechocystis, because the other one encoded by \( \text{slr0329} \) has little GLK activity (26, 45). Depletion of \( \text{Slr0593} \) through insertional mutation can generate a mutant with a stable glucose-sensitive phenotype, which was reproducibly shown by others and us (supplemental Fig. S7) (26). Therefore, it is unlikely that repression of GlcP is caused by feedback inhibition from the repression of \( \text{Slr0593} \) in \( \Delta \text{slr0110} \), because the repression of \( \text{Slr0593} \) alone does not inhibit glucose transport, and cell death is more likely to happen instead of down-regulation of \( \text{Slr0771} \). G6PDH encoded by the gene \( \text{zwf} \) is the first and key enzyme of the OPPP, the major route of glucose catabolism in Synechocystis and in some other photosynthetic cyanobacteria (12, 46). The \( \text{zwf} \)-deletion mutants of the cyanobacteria Nostoc sp. ATCC 29133 and Synechococcus sp. PCC7942 are both impaired in dark viability (14, 47), even when cultured in a medium supplemented with an exogenous organic carbon source such as fructose (14, 48), suggesting that \( \text{zwf} \)-deletion mutants might not be able to use an exogenous carbon source to support heterotrophic growth, a phenotype to some extent similar to that of \( \Delta \text{slr0110} \). However, newer evidence has shown that the deletion of \( \text{zwf} \) does not affect the dark survival of Synechocystis (49), suggesting that OPPP is dispensable for Synechocystis cells cultured in dark, which probably consume endogenous carbon reserves through glycolysis for survival. The paradox was explained by the fact that \( \text{opca} \) (slr1734), a gene that encodes G6PDH assembly protein and is essential for the activity of G6PDH (46, 50, 51), localizes in the same operon with \( \text{zwf} \) in Nostoc sp. ATCC 29133 and Synechococcus sp. PCC7942, and deletion of G6PDH could cause a polar effect that inhibits the expression of \( \text{opca} \) (49). In Synechocystis, the genomic locations of \( \text{zwf} \) and \( \text{opca} \) are distant, and no polar effect of \( \text{zwf} \) deletion would be imposed on \( \text{opca} \) expression. An \( \text{opca} \)-deletion mutant showed more severe effects on dark survival (51), suggesting that \( \text{Opca} \), but not G6PDH, is presumably necessary for the dark survival of cyanobacteria. This view is further supported by the observation that \( \text{Opca} \) has functions in temporarily separated reductant-producing pathways in addition to OPPP (51). Here, more than 10-fold repression in both the protein level and the enzymatic activity of G6PDH, together with the significant down-regulation of \( \text{Opca}, \text{GPI}, \) and GLK, suggests that both OPPP and glycolysis are nearly completely blocked in \( \Delta \text{slr0110} \), which probably generates a feedback inhibition of glucose transport. Considering the observation that the activity of G6PDH in \( \Delta \text{slr0110} \) is significantly inhibited, whereas it is not changed in \( \Delta \text{sll0771} \) and \( \Delta \text{slr0593} \), we reasoned that it is not possible that the repression of GlcP or GLK alone in \( \Delta \text{slr0110} \) can lead to the repression of G6PDH, although the reverse is possible. Therefore, our data better support the hypothesis that the deletion of \( \text{slr0110} \) directly or indirectly inhibits the OPPP and glycolysis, which in turn inhibits GlcP via a feedback loop.

The down-regulation of proteins in glycolysis and OPPP of \( \Delta \text{slr0110} \) more likely occurred at the translational or post-translational level, as many of these genes have a higher level of transcripts, as exhibited by the RT-PCR result (Fig. 6C), which is in contrast to the protein level measured by mass spectrometry. In fact, enzymatic activities in the carbohydrate metabolism of cyanobacteria were considered more likely to be modulated at the post-translational level (12, 26, 52), which is consistent with our observation. Considering the observations of elevated transcription of the majority of the genes in glycolysis and OPPP and the significant increase of translatable proteins in \( \Delta \text{slr0110} \), the dramatic decrease of the proteins governing key reactions of carbon metabolism in the mutant can be reasonably attributed to abnormal activities of proteases. The Synechocystis genome encodes 62 putative proteases (53), including \( \text{HhoB} \) (Slr1427), \text{Prc} (Slr1751), and \( \text{FtsH4} \) (Slr1463), that are significantly repressed in the mutant. Though we did not confidently identify any proteases that were significantly up-regulated in the mutant, it is conceivable that one or more of the key enzymes of glycolysis or the OPPP...
are substrates of the identified and/or unidentified proteases with abnormal activities, and degradation of the substrates might cause the repression of other proteins as a result of potential feedback or feedforward inhibition.

The observation that the expression of ribosomal and other proteins of translational machinery is highly elevated in Δslr0110 is somewhat unexpected. Takabayashi et al. recently generated a protein co-migration database for Synechocystis proteins co-migrating on a blue native PAGE gel that were sliced and identified via mass spectrometry (64). After searching the database, we found that Slr0110 co-migrated with ribosomal proteins on the blue native PAGE gel and formed dominant peaks of migration profile on slices 56, 57, and 58 (supplemental Fig. S9), suggesting that Slr0110 may have strong interactions with ribosomes. Interestingly, Slr0110 also co-migrates with phycobilisome rod linker proteins (supplemental Fig. S9). Both phycobilisomes and a large fraction of ribosomes are membrane-associated macromolecular complexes (10), and thus it is possible that the membrane localization of Slr0110 is mediated by interaction with these complexes. Though the biological significance of the interaction between Slr0110 and the membrane-associated protein complexes is poorly understood, Slr0110 might play an important role, either positive or negative, in the assembly of these complexes, considering that Slr0110 depletion caused significant and coherent elevation of the majority of the ribosomal and phycobilisomal proteins.

In addition to levels of proteins of translational machinery, protein levels in many other functional groups are also remarkably modulated in Δslr0110. One of the interesting observations is that nitrogen regulatory protein PII was up-regulated whereas the glutamate-ammonia ligases (GlnA, Str1756 and GlnN, Str0288) were down-regulated in the Δslr0110 mutant (supplemental Table S4). Modulation of the expression of these genes has been repeatedly observed in either carbon- or nitrogen-limiting growth conditions (55–59). PII protein plays a central role in regulating nitrogen uptake and maintains the carbon/nitrogen (C/N) balance in cells (56). PII serves as a sensor of the intracellular level of 2-oxoglutarate (2-OG) (60), an intermediate metabolite of the TCA cycle and the major acceptor of ammonium. Nitrogen starvation can promote PII expression at both transcription and translational levels (61, 62), whereas expression of the two glutamate-ammonia ligases is differentially regulated in response to nitrogen availability (58), with glnA and glnN highly expressed in nitrogen-supplied and nitrogen-deficient conditions, respectively. In the growth condition explored in the current research, the supply of nitrogen was not limiting, but the intracellular level of 2-OG in Δslr0110 could be higher than in the WT because the majority of assimilated carbon is presumably converted to 2-OG through glycolysis and TCA as estimated from the reduced volume of glycogen in the mutant. The higher level of 2-OG could shift the C/N balance toward a nitrogen limitation and promote the expression of PII protein. Though we currently do not have a satisfying explanation for the repression of both glutamate-ammonia ligases in Δslr0110, it is conceivable that the elevation of PII protein in the mutant was induced by the elevated level of 2-OG and the shifted C/N balance.

In conclusion, we quantitatively compared the proteomes of the WT and the Δslr0110 strain of Synechocystis that is defective in glucose transport and phototrophic growth and found that the deletion of slr0110 leads to significant down-regulation of key enzymes in glycolysis and OPPP in addition to the glucose transporter. We showed that a new player such as slr0110 that is essential in the regulation of carbohydrate metabolism, an extensively studied subject, can still be identified through the combined methods in reverse genetics and proteomics, and opened a gate toward uncovering a novel mechanism that regulates carbohydrate metabolism. We believe that more novel regulators can and need to be identified in order to better mechanistically understand carbohydrate metabolism from a system rather than a single pathway perspective. Our work not only built the basis for future experimental studies elucidating the mechanism underlying Slr0110-regulated carbohydrate metabolism, but also provided a wealth of information regarding the expression and intracellular localization of Synechocystis proteins. The proteomics data presented in the current study represent the largest protein catalogue of Synechocystis generated so far from a single study, and will serve as an important resource for the research community using Synechocystis as a model organism.

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