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Acetylome Analysis Reveals the Involvement of Lysine Acetylation in Photosynthesis and Carbon Metabolism in the Model Cyanobacterium *Synechocystis* sp. PCC 6803

Ran Mo,^{†,‡,§} Mingkun Yang,^{†,§} Zhuo Chen,^{†,§} Zhongyi Cheng,[§] Xingling Yi,^{||} Chongyang Li,[†] Chenliu He,[†] Qian Xiong,[†] Hui Chen,[†] Qiang Wang,^{*,†} and Feng Ge^{*,†}

[†]Key Laboratory of Algal Biology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

[‡]University of Chinese Academy of Sciences, Beijing 100039, China

[§]Advanced Institute of Translational Medicine, Tongji University, Shanghai 200092, China

^{||}Jingjie PTM Biolabs (Hangzhou) Co. Ltd, Hangzhou 310018, China

Supporting Information

ABSTRACT: Cyanobacteria are the oldest known life form inhabiting Earth and the only prokaryotes capable of performing oxygenic photosynthesis. *Synechocystis* sp. PCC 6803 (*Synechocystis*) is a model cyanobacterium used extensively in research on photosynthesis and environmental adaptation. Posttranslational protein modification by lysine acetylation plays a critical regulatory role in both eukaryotes and prokaryotes; however, its extent and function in cyanobacteria remain unexplored. Herein, we performed a global acetylome analysis on *Synechocystis* through peptide prefractionation, antibody enrichment, and high accuracy LC–MS/MS analysis; identified 776 acetylation sites on 513 acetylated proteins; and functionally categorized them into an interaction map showing their involvement in various biological processes. Consistent with previous reports, a large fraction of the acetylation sites are present on proteins involved in cellular metabolism. Interestingly, for the first time, many proteins involved in photosynthesis, including the subunits of phycocyanin (CpcA, CpcB, CpcC, and CpcG) and allophycocyanin (ApcA, ApcB, ApcD, ApcE, and ApcF), were found to be lysine acetylated, suggesting that lysine acetylation may play regulatory roles in the photosynthesis process. Six identified acetylated proteins associated with photosynthesis and carbon metabolism were further validated by immunoprecipitation and Western blotting. Our data provide the first global survey of lysine acetylation in cyanobacteria and reveal previously unappreciated roles of lysine acetylation in the regulation of photosynthesis. The provided data set may serve as an important resource for the functional analysis of lysine acetylation in cyanobacteria and facilitate the elucidation of the entire metabolic networks and photosynthesis process in this model cyanobacterium.

KEYWORDS: post-translational modification, acetylome, cyanobacterium, *Synechocystis*, photosynthesis, carbon metabolism

INTRODUCTION

Post-translational modifications (PTMs) are a vital cellular control mechanism modulating diverse protein properties, including folding, conformation, and activity, and consequently, regulation of protein function.¹ Almost all proteins undergo appreciable PTMs to attain biological activity, and this dynamic process occurs in various cell compartments to dictate the fate of the modified proteins.² Among the hundreds of different PTMs, lysine acetylation is a highly dynamic and tightly regulated PTM.³ Acetyl phosphate as a critical regulator acts nonenzymatically to regulate acetylation levels.^{5,8} It was first identified in eukaryotic histones about half a century ago and has since been found to regulate histone functions and affect chromatin structure and gene expression.^{4,5} In addition to histones, lysine acetylation also occurs in nuclear, cytoplasmic, and mitochondrial proteins.⁶ There is increasing evidence

suggesting that lysine acetylation is one of the most prevalent PTMs in both eukaryotes and prokaryotes and that it regulates diverse cellular processes.^{3,7} Comprehensive so-called “acetylome” studies have involved humans,⁸ mouse,⁹ *Drosophila*,¹⁰ plants,¹¹ protozoa,¹² eukaryote,^{19,20} and bacteria.^{13–18}

The first system-wide studies involving lysine-acetylated peptide immunoprecipitation and liquid chromatography–mass spectrometry (LC–MS/MS) measurements of bacterial acetylomes of *Escherichia coli* were by Yu¹⁷ and Zhang et al.¹⁸ The same approach has since been employed to analyze the model organisms *Bacillus subtilis*,¹⁴ *Salmonella enterica*,²¹ *Thermus thermophilus*,²² *Erwinia amylovora*,¹³ *Geobacillus kaustophilus*,¹⁶ and *E. coli*,²³ and this approach has become a

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standard procedure in bacterial acetylome analysis.^{7,24} These acetylome studies have generated large data sets of lysine-acetylated proteins in bacteria and demonstrated the diverse cellular functions of this PTM. Notably, lysine acetylation was found to play crucial roles in metabolic control and coordination of different metabolic pathways in both bacterial and human cells.^{21,25,26} It is now widely accepted that lysine acetylation is an evolutionarily conserved and widespread PTM and that lysine acetylation in bacteria can be functionally important and ubiquitous across genera and families.^{3,7,27,28} To the best of our knowledge, however, no study of protein lysine acetylation in cyanobacteria has yet been reported.

Cyanobacteria are a widespread and morphologically diverse group of Gram-negative bacteria and the only prokaryotes capable of oxygenic photosynthesis.^{29,30} They exist in almost every habitat and are believed to make a substantial contribution to global CO₂ assimilation, O₂ production, and N₂ fixation and be the progenitors of chloroplasts in higher plants.^{29,31} It is estimated that more than half of the primary production on Earth is by the photosynthetic activity of cyanobacteria.³² Recently, cyanobacteria have attracted great interest due to their crucial roles in global carbon and nitrogen cycles and their ability to produce clean and renewable biofuel such as hydrogen.^{33–35} *Synechocystis* is a model cyanobacterium that has been extensively studied with respect to photosynthesis, adaptation to environmental changes, and other metabolic processes.³⁶ *Synechocystis* can grow under conditions ranging from photoautotrophic to fully heterotrophic, making it an ideal model for studying fundamental processes such as photosynthesis and/or carbon metabolism.^{37–40} Photosynthesis and carbon metabolism are two physically and functionally interconnected processes, and intricate mechanisms have been developed to control and coordinate different metabolic pathways in *Synechocystis*.^{41–43} In both bacteria and human cells, lysine acetylation has been shown to dynamically regulate enzymes involved in carbon metabolism.^{21,25,26,44} We thus hypothesize that lysine acetylation plays an important regulatory role in metabolic processes such as photosynthesis and/or carbon metabolism in *Synechocystis*.

To test this hypothesis, we analyzed the acetylome of *Synechocystis* by using high accuracy nano-LC–MS/MS in combination with the enrichment of acetylated peptides from digested cell lysates and subsequent peptide identification. We identified 776 unique lysine acetylation sites on 513 proteins in this important model cyanobacterium. The identified acetylated proteins are involved in various biological processes and render particular enrichment to metabolic process and photosynthesis, two processes that are intimately linked to cellular energy status. Lysine acetylation is notably observed at the active sites of many key proteins involved in photosynthesis, including photosystem I subunits (PsaA, PsaB, PsaD, and PsaF), photosystem II subunits (PsbA, PsbB, PsbC, PsbO, PsbP, PsbV, and PsbU), phycocyanin subunits (CpcA, CpcB, CpcC, and CpcG), and allophycocyanin subunits (ApcA, ApcB, ApcD, ApcF, and ApcE). To the best of our knowledge, this work provides the first extensive data set on lysine acetylation for any cyanobacterium and provides novel insights into the range of functions regulated by lysine acetylation in *Synechocystis*.

■ EXPERIMENTAL PROCEDURES

Cell Culture and Protein Extraction

The wild-type strain of *Synechocystis* was obtained from the Pasteur Culture Collection and cultured in BG11 medium⁴⁵ bubbling with filtered air at 30 °C under continuous illumination (40 μmol m⁻² s⁻¹). For nitrogen-deficient conditions,⁴⁶ cells were grown to exponential phase (OD₇₃₀ = 0.8–0.9) and immediately resuspended in lacking nitrate medium. For heterotrophic conditions, cells in the exponential phase were resuspended in medium with additional 5 mM glucose. For high light conditions (HL), cells were grown to exponential phase and immediately prior to harvest were illuminated at 250 μmol photons m⁻² s⁻¹ for 2 h. To inhibit the activities of endogenous protein deacetylases, nicotinamide (10 mM) was added into the cultures. Cells at different treatment were incubated for an additional 30 min before harvesting by centrifugation (6000g at 4 °C for 5 min). Subsequently, the cells were resuspended in lysis buffer containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1× protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The mixture was applied to sonication (2 s on, 2 s off) for about 30 min on ice with an output of 135 W (JY92-IIIN, Ningbo Scientz Biotechnology, Ningbo, China). The whole cell lysate was centrifuged (12000g at 4 °C for 10 min) to remove the cell debris. Proteins were precipitated using 5 vol of ice-cold acetone, dried at room temperature, and then dissolved in 50 mM ammonium bicarbonate. The protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA).

In-Solution Trypsin Digestion, HPLC Fractionation, and Affinity Enrichment of Acetylated Peptides

The precipitated proteins (1 mg) were redissolved in 50 mM ammonium bicarbonate, and then in-solution digested by trypsin according to previously described.^{23,47} The sample was then prefractionated into fractions by high pH reverse-phase HPLC using Agilent 300 Extend C₁₈ column (5 μm particles, 4.6 mm I.D., 250 mm length). Briefly, peptides were separated with a gradient of 2% to 90% basic RP-solvent B (acetonitrile in 10 mM ammonium formate, pH 8.0) over 80 min. The resulting peptides were combined into six fractions and dried by vacuum centrifuging.

Acetylated peptides were then enriched using agarose-conjugated antiacetyl lysine antibody (PTM Biolabs, Chicago, IL, USA). Briefly, tryptic peptides were redissolved in NETN buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and then incubated with antiacetyllysine antibody conjugated protein A agarose beads at 4 °C for 6 h with gentle rotation. The supernatant was removed and the beads were washed three times with NETN buffer, twice with ETN buffer (50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA), followed by three times wash with water. The bound peptides were eluted by washing three times with 1% trifluoroacetic acid. Elutes were combined and dried in a SpeedVac. The resulting acetylated peptides were loaded onto self-packed C₁₈ STAGE tips according to the manufacturer's instructions to desalt the sample, prior to nano-HPLC–MS/MS analysis.

LC–MS/MS Analysis

The enriched peptides were dissolved in the HPLC buffer A (0.1% (v/v) formic acid in 2% ACN), and analyzed by online

nanoflow LC–MS/MS using an easy nLC-1000 system (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Q-Exactive (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer. Briefly, samples were injected onto the analytical C₁₈-nanocapillary LC column (5 μm particle size, 100 Å pore diameter) and eluted at a flow rate of 300 nL/min with a 40 min gradient from 6% solvent B (98% ACN/0.1% formic acid, vol/vol) to 90% solvent B. The peptides were then directly ionized and sprayed into a Q-Exactive mass spectrometer by a nanospray ion source. Mass spectrometer was operated in a data-dependent mode with an automatic switch between MS and MS/MS acquisition. Acquisition of MS and MS/MS data was carried out using Xcalibur 2.2 (Thermo Fisher Scientific, Waltham, MA, USA). Full MS spectra from *m/z* 350 to 1800 were acquired with a resolution of 70 000 at *m/z* = 200 in profile mode. Following every survey scan, up to 20 most intense precursor ions were picked for MS/MS fragmentation by higher energy C-trap dissociation (HCD) with normalized collision energy of 28%. Lock mass at *m/z* 445.12003 was enabled for full MS scan. The dynamic exclusion duration was set to be 10 s with a repeat count of 1 and ±10 ppm exclusion window. The electrospray voltage applied was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 5 × 10⁴ ions were accumulated for generation of MS/MS spectra.

Data Analysis

The resulting MS/MS data were processed using MaxQuant with integrated Andromeda search engine (v. 1.3.0.5).⁴⁸ Tandem mass spectra were searched against the *Synechocystis* protein database downloaded from Cyanobase database (<http://genome.microbedb.jp/cyanobase/Synechocystis/>, 3672 sequences) concatenated with a reverse decoy database and protein sequences of common contaminants. Enzyme specificity was set as full cleavage by trypsin, with two maximum missed cleavage sites permitted. The precursor and fragment ion mass tolerances were 6 ppm and 0.02 Da, respectively. Carbamidomethylation (Cys) was set as a fixed modification, whereas oxidation (Met), deamidation (Asn/Gln), acetylation (Lys), and acetylation (protein N-terminal) were set as dynamic modifications. Minimum peptide length was set at 6. The estimated false discovery rate (FDR) thresholds for modification site, peptide, and protein were specified at maximum 1%. All the peptides identified on the basis of an acetylated Lys residue were manually inspected using a modified method for the neutral loss ion MH⁺-59, which can be used as a unique marker for trimethylation, to exclude isobaric trimethylation⁴⁹ as described by Mann et al.^{50,51} Peptides containing this marker were removed from the analysis. Furthermore, to improve the reliability of result, all acetylation sites assigned to the peptide C-terminal were removed, prior to bioinformatics analysis. All the raw data were deposited in a publicly accessible database PeptideAtlas⁵² and can be accessed using the identifier PASS00622.

Bioinformatics Analyses

The identified acetylated proteins were grouped into biological process and molecular function class based on the gene ontology (GO) terms by Blast2GO software.⁵³ The protein subcellular localization was analyzed with PSORTb 3.0 program.⁵⁴ GO term, protein domain, and KEGG pathway enrichment were performed using the DAVID bioinformatics resources 6.7.⁵⁵ Secondary structures were predicted using NetSurfP.⁵⁶ The mean secondary structure probabilities of

modified lysine residues were compared with those of control residues for all acetylated proteins identified in this study, and *p* values were calculated using a previously described method.⁵⁷ To analyze lysine acetylation sites, the ratios of six amino acids upstream and downstream flanking the identified acetylation sites were calculated, and a position-specific heat map was generated by plotting the log₁₀ of the ratio. Further, to evaluate their conservation across species, orthologs of acetylated proteins we identified were searched using BLASTP as previously described.⁸ The functional interaction network analysis was performed using interaction data from the *Synechocystis* PPI database (<http://bioportal.kobic.kr/SynechoNET>), and the network was visualized by Cytoscape v2.8.3.⁶¹ This interaction network was further analyzed for densely connected regions using a graph theoretic clustering algorithm Molecular Complex Detection (MCODE).⁶²

Production of Polyclonal Antibodies against PsaC, PsaD, ApcA, CpcB, RbcL, or NrtA

Anti-PsaC (photosystem I subunit III), PsaD (photosystem I subunit II), ApcA (allophycocyanin alpha subunit), CpcB (phycocyanin beta subunit), RbcL (ribulose bisphosphate carboxylase large subunit), or NrtA (nitrite transport system substrate-binding protein) polyclonal antibodies were produced and purified via affinity chromatography by (ABclonal, Wuhan, Hubei, China). Briefly, polyclonal antibody of ApcA and CpcB were generated against the synthetic peptides DRIKAFVTG-GAARLR and PNGITRGDCSAIVAEIAGYF, respectively. To produce antibodies against PsaC, PsaD, NrtA and RbcL, the full-length cDNA of *psaC*, *psaD*, *nrtA*, and *rbcL* gene were amplified using specific primers (*psaC*-Forward: 5'-GTGG-TAACGCTCTCTAATACCTCG-3'; *psaC*-Reverse: 5'-ACGTTGTTCATGCCTGACCTCGAC-3'; *psaD*-Forward: 5'-ATGACAGAACTCTCTGG ACAACC-3'; *psaD*-Reverse: 5'-GACCTCGTAGGGGGCTTACCGG-3'; *nrtA*-Forward: 5'-ATGAGTAATTTTCCCGAAGCAC-3'; *nrtA*-Reverse: 5'-AGCTTTAATAGACTTAATT TTCA-3'; *rbcL*-Forward: 5'-ATGGTACAAGCCAAAGC-3'; *rbcL*-Reverse: 5'-TTAGAGG GTATCCATG-3'), PCR products were cloned into the pGEX-4T expression vector (Pharmacia) at the *Bam*H-I-XhoI restriction sites, and the resulting plasmid was transformed into *E. coli* strain BL21 (DE3) for overexpression of PsaC, PsaD, NrtA, or RbcL. Cells growing logarithmically were treated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 30 °C. The fusion proteins were then purified by performing His-tag affinity chromatography. Following purification of these antigens, immunization and sampling of the antisera from rabbit were performed by ABclonal, according to standard operating procedures. The specificity of the generated antibodies was determined by the manufacturer using ELISA and Western blotting.

Immunoprecipitation and Western Blotting

Immunoprecipitations (IPs) were performed to verify the acetylation of PsaC, PsaD, ApcA, CpcB, RbcL or NrtA. For IP, aliquots (20 μg) were incubated with 1 μL of each antibody (PsaC, PsaD, ApcA, CpcB, RbcL, or NrtA) and 20 μL of Dynabeads (Life Technologies, Oslo, Norway) in incubation buffer (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8 mM K₂HPO₄, pH 7.4) overnight at 4 °C. The conjugated beads were washed three times with PBS and incubated with the whole cell lysates overnight at 4 °C. The beads were then washed three times with PBS to remove the unbound proteins. Bound proteins were boiled in SDS loading buffer for 5 min

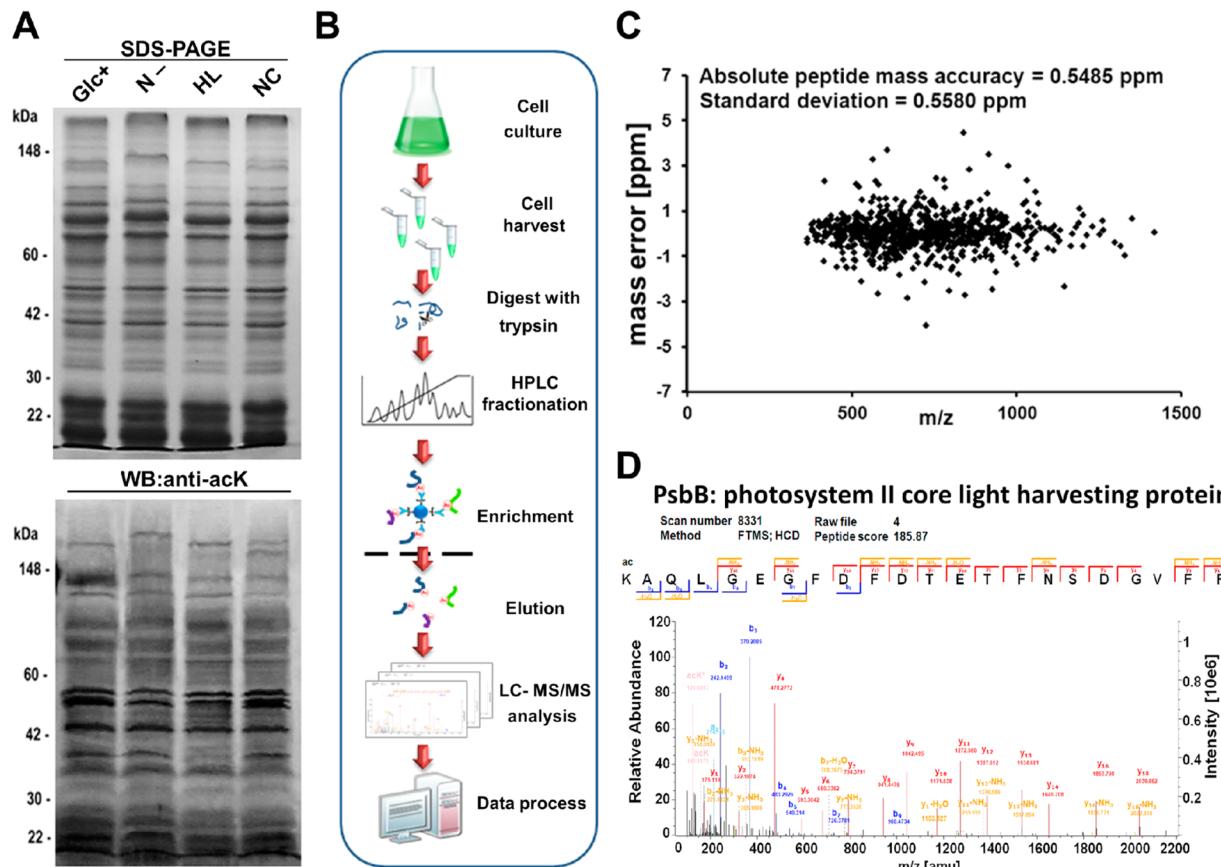


Figure 1. Overview of the experimental design and workflow for the acetylome analysis of *Synechocystis*. (A) Verification of acetylated proteins presented in *Synechocystis*. The SDS-PAGE gel was stained with Coomassie Brilliant blue or transferred to a polyvinylidene fluoride membrane and incubated with antiacetyllysine antibody. Total proteins ($10\ \mu\text{g}$) were extracted from the cells cultured under the glucose condition (Glc+), nitrogen-starvation (N-), high light condition (HL), or normal condition (NC). (B) Overview of experimental procedures used in this study. The extracted proteins were digested with trypsin, and lysine-acetylated peptides were enriched by immunoprecipitation and analyzed by nano-LC–MS/MS. (C) The distribution of peptides mass error (ppm) based on m/z of 774 identified acetylation peptides. (D) A typical example of MS/MS spectra for an acetylated peptide. The peptide K (ac) AQLGEGFDFTETFNSDGVFR is from PsbB. The acetylation site was indicated by (ac).

Table 1. Comparison of *Synechocystis* sp. PCC 6803 Acetylome with Other Published Bacteria Acetylomes^a

organism	strains	proteins	no. (%) of ac-proteins	no. of ac-sites	ref
<i>Synechocystis</i> sp.	PCC 6803	3672	513 (13.96)	776	this work
<i>Escherichia coli</i>	W3110	4146	91 (2.19)	113	18
<i>Escherichia coli</i>	DH5	4146	85 (2.05)	125	17
<i>Escherichia coli</i>	K-12	4146	349 (8.42)	1070	23
<i>Salmonella enterica</i>	LT2	4525	191 (4.22)	235	21
<i>Erwinia amylovora</i>	Ea1189/Ea273	3565	96 (2.69)	141	13
<i>Bacillus subtilis</i>	168	4176	185 (4.43)	332	14
<i>Thermus thermophilus</i>	HB8	2238	128 (5.71)	201	22
<i>Vibrio parahemolyticus</i>	RIMD 2210633	3079	656 (21.31)	1413	15
<i>Geobacillus kaustophilus</i>	HTA426	3653	114 (3.12)	253	16
<i>Mycobacterium tuberculosis</i>	H37Ra	4034	137 (3.40)	226	78
<i>Mycobacterium tuberculosis</i>	H37Rv	4034	658 (16.31)	1128	60
<i>Streptomyces roseosporus</i>	NRRL 15998	6315	667 (10.56)	1143	59

^a“ac” = acetylation.

and then subjected to 15% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. For Western blotting, the membranes were blocked with 5% skim milk powder in phosphate-buffered saline containing 0.1% Tween-20 at room temperature for 1 h and incubated with either the specific antibody (PsaC, PsaD, ApcA, CpcB, RbcL or NrtA; 1:2000 dilution) or the anti-succinyl lysine antibody (PTM

Biolabs, Chicago, IL) (1:2000, in TBS/5% BSA) overnight at 4 °C. After being washed three times with TBST buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween20), the membrane was incubated with horseradish peroxidase-conjugated goat antirabbit antibody (1:5000 dilutions) for 1 h at 37 °C. The membrane was then washed with TBST buffer and visualized with enhanced chemiluminescence (ECL) immuno-

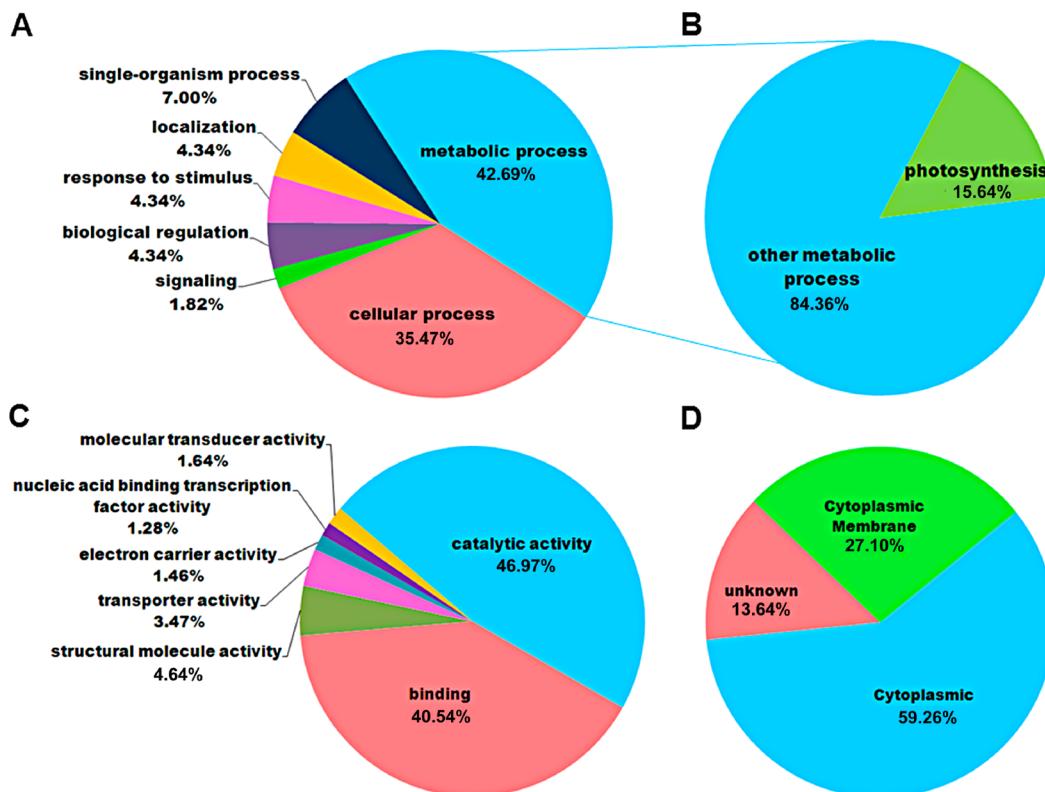


Figure 2. Pie charts show the distribution of all the identified acetylated proteins categorized according to (A) biological process, (B) distribution of functional subcategories in “metabolic process”, (C) molecular function, and (D) cellular component.

blotting detection reagents (Advansta, CA, USA). The density of each band was determined with a fluorescence scanner (ImageQuant TL, GE Healthcare).

RESULTS AND DISCUSSION

Establishment of the *Synechocystis* Acetylome

In order to establish the diversity and relative abundance of lysine acetylation on *Synechocystis* proteins, Western blotting analysis was performed using antiacetyl lysine antibody. Figure 1A showed the global levels of lysine acetylation at different culture conditions, suggesting that lysine acetylation is a dynamic and regulated process in *Synechocystis*. On the basis of this, to gain further insights into the large-scale data set of lysine acetylation sites in this model cyanobacterium, we used immunoaffinity enrichment and a MS-based high-throughput proteomic approach combined with nano-LC to identify acetylated proteins in *Synechocystis* and their modification sites (Figure 1B). In total, we identified 774 acetylated peptides in 513 acetylated proteins with an estimated FDR of less than 1%, corresponding to 776 acetylation sites with localization probability higher than 0.75 (Table S1, Supporting Information). We compared the *Synechocystis* acetylome to the bacterial acetylomes reported by other groups, and the results are presented in Table 1. It is noteworthy that *Synechocystis* has a large quantity of acetylated proteins detected among the bacteria studied so far. This finding probably reflects the crucial role of reversible acetylation in the photoautotrophic cyanobacteria. The overall absolute peptide mass accuracy was 0.5485 ppm (ppm) (standard deviation, 0.5580 ppm) (Figure 1C), confirming the high accuracy of modified peptide data obtained from MS. Taken together, the results of Western blot analysis and proteomics screening using MS/MS spectra

suggest the extensive existence of lysine acetylation in cyanobacteria. To the best of our knowledge, none of these cyanobacterial proteins have previously been associated with lysine acetylation, and this data set provides the first global survey of lysine acetylation in this model cyanobacteria. An example of the analysis of an acetylpeptide sequence from photosystem II core light harvesting protein (PsbB) and assignment of the acetylation site is provided in Figure 1D. Detailed information on all identified acetylated peptides is provided in the Supporting Information as Table S1. All the MS/MS spectra of the identified acetylated peptides are presented in Figure S1.

Functional Annotation and Cellular Localization of Acetylated proteins in *Synechocystis*

To better understand the lysine acetylome in *Synechocystis*, we investigated the GO functional classification of all acetylated proteins based on their biological process, molecular function using Blast2GO (Figure 2 and Tables S2 and S3). Among all the identified acetylated proteins, 343 (66.86%) were annotated on biological process (Figure 2A and Table S2), and a large proportion of identified proteins were involved in metabolic processes 219 (42.69%). Because *Synechocystis* is a model organism for studies of photosynthetic carbon fixation, further classification of metabolic process showed that 28 (15.64%) of the metabolism-related acetylated proteins were involved in photosynthetic pathway (Figure 2B and Table S3). In the molecular function classification, most acetylated proteins were related to the binding of various targets 208 (40.54%) and enzyme catalytic activity 241 (46.97%) (Figure 2C). Within the classification of subcellular localization, the majority of the identified acetylated proteins 304 (59.26%) were proposed to be located in the cytoplasm. Importantly, 139 (27.10%) were

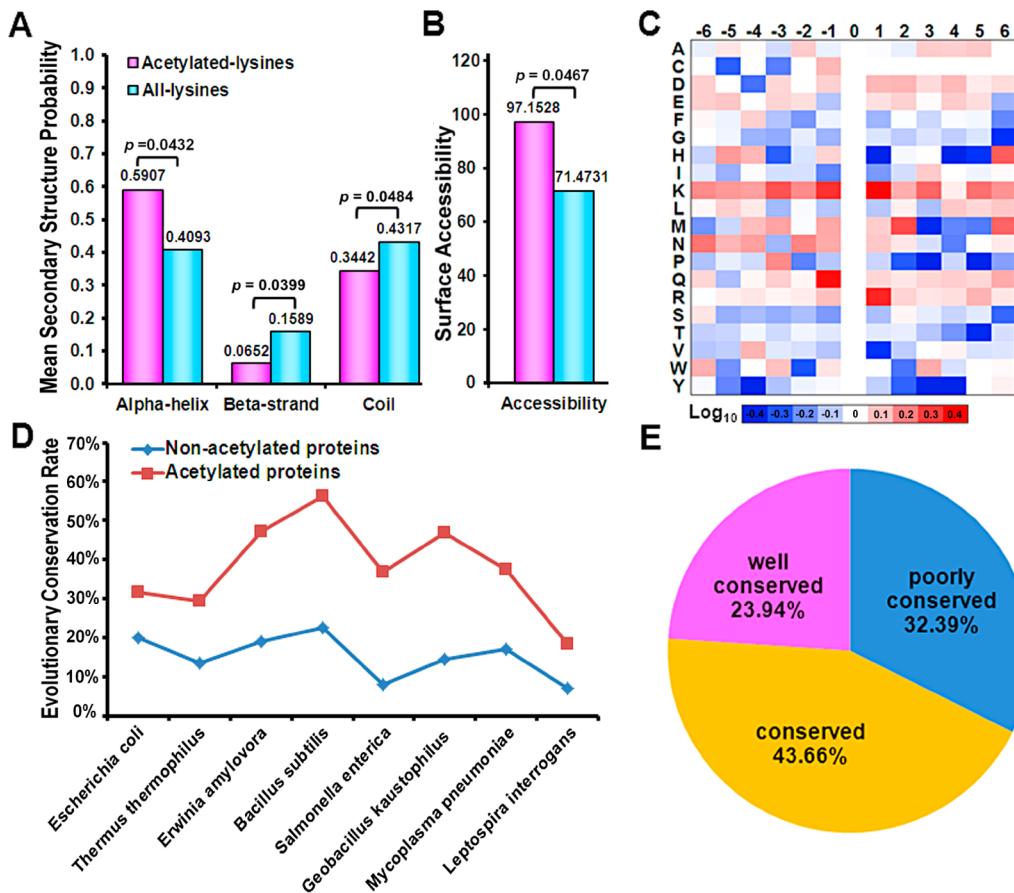


Figure 3. Properties of acetylated proteins and sites. (A) Probabilities of localization to different secondary structures (α helix, beta strand, and coil). Acetylated lysines were compared with all the lysine residues in all the proteins identified in this study. Pink represented acetyl-lysine and blue represented all-lysine. (B) Predicted secondary structures accessibility of acetylation sites. Acetylated lysines were compared with all the lysine residues in all the proteins identified in this study. Pink represented acetyl-lysine and blue represented all-lysine. (C) Intensity map indicating the overrepresentation of amino acids in positions from -6 to $+6$ around the acetylated lysine residue based on all the identified acetylation sites compared to the overall proteome amino acid frequency distribution. Colors were plotted by using intensity map and represent the \log_{10} of the ratio of frequencies within acetyl-13-mers versus nonacetyl-13-mers (red shows enrichment, blue shows depletion). (D) Percentage of *Synechocystis* homologues of acetylated (red) or nonacetylated (blue) proteins in eight bacterial organisms. (E) The pie chart shows the conservation of acetylated proteins across species from bacteria basing on the quantities of ontologies. Each protein in the three groups has orthologs from eight representative species. Grouping was performed as follows: “well conserved”, 5–8 orthologs; “conserved”, 2–4 orthologs; “poorly conserved”, 1 ortholog.

assigned to the cytoplasmic membrane (Figure 2D, Table S2). Because the subcellular compartment of thylakoid was likely classified as a cytoplasmic membrane by PSORTb, it is not surprising that no acetylated proteins were classified as thylakoid proteins in the subcellular localization.

We further conducted GO enrichment analyses (biological process, molecular function, and cellular component categories), KEGG pathway, and protein domain (Figure S2 and Table S4). Our GO enrichment analysis showed that the acetylated proteins were markedly enriched in translation, oxidation reduction, glutamine family amino acid metabolic process, generation of precursor metabolites and energy, cellular amino acid biosynthetic process and photosynthesis. Consistently, the GO enrichment analysis of molecular functions further demonstrated that many functions were enriched in our set, including catalytic activity and binding. Likewise, in the GO cellular component category, we found that a large number of acetylated proteins we identified were significantly enriched in the plasma membrane part ($p = 4.93 \times 10^{-6}$), organelle ($p = 8.66 \times 10^{-6}$), and thylakoid ($p = 3.73 \times 10^{-5}$). This data set implied that acetylation may play an important role in photosynthesis. Furthermore, the identified

acetylated proteins were also mapped to KEGG metabolic pathways and protein domains. We found that acetylation occurs on many proteins involved in valine, leucine, and isoleucine biosynthesis, photosynthesis, RNA degradation, RNA polymerase, and aminoacyl-tRNA biosynthesis, and most acetylated proteins were enriched with the phycobilisome domain.

Analysis of Lysine Acetylation Sites

We further investigated the local secondary structures of acetylated proteins using the algorithm NetSurfP (Figure 3A). As noted, the acetylated lysine residue was more frequently found in structured regions, such as in α helix (0.5907 , $p = 0.0432$) and beta-strand (0.0652 , $p = 0.0399$). We also found that the acetylation sites were enriched on the protein surface (absolute surface accessibility = 97.1528) as compared with 71.4731 of the all lysine residue ($p = 0.0467$) (Figure 3B). Therefore, lysine acetylation may affect the surface properties of modified proteins similar to other PTMs, such as phosphorylation.⁶³

To elucidate the properties of amino acids surrounding the identified acetylation sites in *Synechocystis* proteins, we

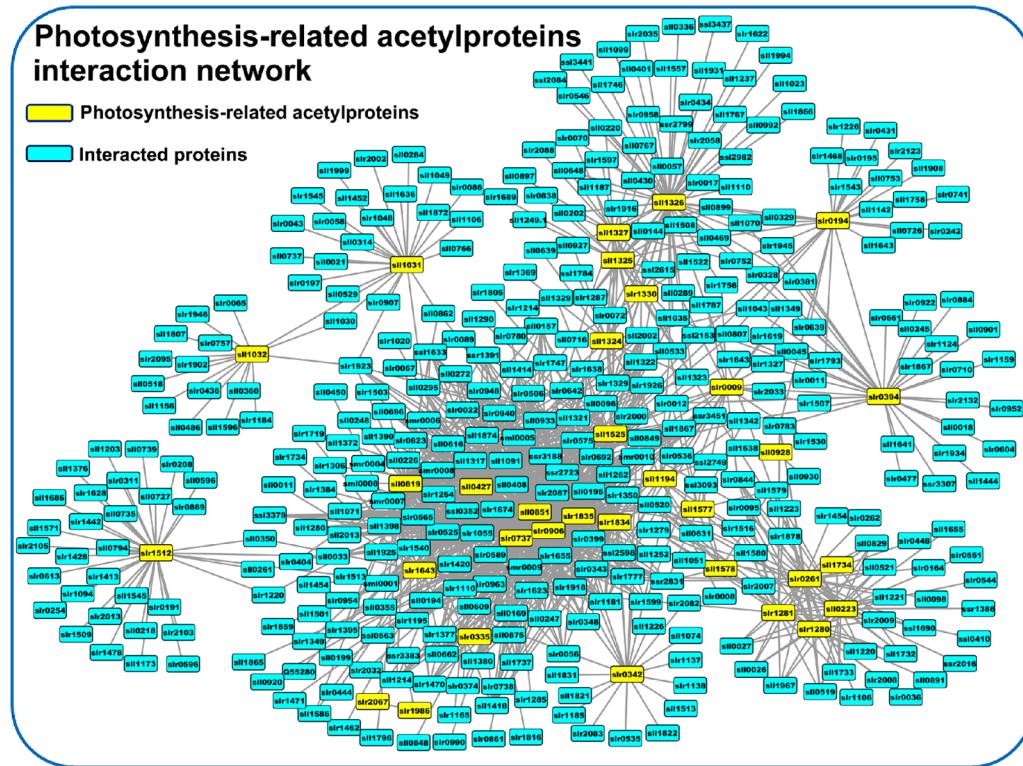


Figure 4. Protein–protein interaction network of acetylated proteins associated with photosynthesis. Identified acetylated proteins involved in photosynthesis are highlighted in yellow and the interacted proteins are highlighted in light blue.

compared the occurrences of neighboring amino acids (six amino acids upstream and downstream of the acetylation site) with the entire set extracted from the *Synechocystis* proteome (Figure 3C). We observed that the residue preferences for acetylated peptides are lysine (K) at the -1 and $+1$ positions, arginine (R) at the $+1$ position, and glutamine (Q) at the -1 position. Colors were plotted by using intensity map and represent the \log_{10} of the ratio of frequencies within acetyl-13-mers versus non-acetyl-13-mers (red shows enrichment, blue shows depletion). The different preference of amino acid residues surrounding lysine sites suggests unique substrate preferences in *Synechocystis*.

Evolutionary Conservation of Lysine Acetylation in *Synechocystis*

Increasing evidence suggests that lysine acetylation is likely to be evolutionarily conserved.¹⁸ In this study, we performed a systematic analysis of the orthologs of cyanobacterial acetylated proteins across species. In the line chart, red line represented the acetylated proteins, and the blue line represented the non-acetylated proteins. Our data showed that the incidence of acetylprotein homologues was significantly higher than that of non-acetylated proteins across species (Figure 3D and Table S5).

To further compare the acetylated proteins among different species, we selected some of the reported bacterial acetylome studies. We analyzed the orthologs of acetylated proteins of *E. coli*,²³ *E. amylovora*,¹³ *B. subtilis*,¹⁴ *S. enterica*,²¹ *T. thermophilus*,²² *Leptospira interrogans*,⁷⁹ *G. kaustophilus*,¹⁶ and *Mycoplasma pneumoniae*⁶⁴ (Table S6). The orthologs were further classified based on the quantity of orthologs, and the results demonstrated that 71 (13.8%) of the identified *Synechocystis* acetylated proteins have orthologous acetylated proteins in

other organisms. Within the cluster of conserved proteins, we found that 17 (23.94%) *Synechocystis* acetylated proteins belong to the “well conserved” category, which has orthologous proteins in more than five organisms. The percentage of “conserved” proteins containing 4 to 2 orthologs and “poorly conserved” proteins containing 1 ortholog was 31 (43.66%) and 23 (32.39%) of total acetylated proteins, respectively (Figure 3E). Moreover, the comparative analysis of acetylated proteins revealed that several proteins involved in energy metabolism, such as glycogen phosphorylase (Sll1356) and nitrite transport system ATP-binding protein (Sll1453), are conserved between cyanobacteria with other bacteria.

Protein–Protein Interaction Network of Identified Acetylated proteins

The protein interaction networks can serve as an alternative strategy to analyze the physical and functional interactions.⁶⁵ To understand the cellular processes regulated by acetylation, we generated a protein interaction network of all acetylated proteins using *Synechocystis* PPI database (<http://bioportal.kobic.kr/SynechoNET>). This bioinformatics analysis will be useful for testing the hypothetical functions of the identified *Synechocystis* acetylated proteins. On the basis of this network, we characterized protein complexes that associate with acetylated proteins and four highly interconnected clusters of acetylated proteins were retrieved using MCODE algorithm in Cytoscape (Figure S3 and Table S7). The top cluster (cluster I) along with cluster IV we identified consists of several acetylated proteins involved in photosynthesis and energy metabolism, whereas the clusters II–III consist of ribosome-associated proteins. We speculate that the physiological interactions among these protein complexes might contribute to the cooperation and/or coordination of their functions in the

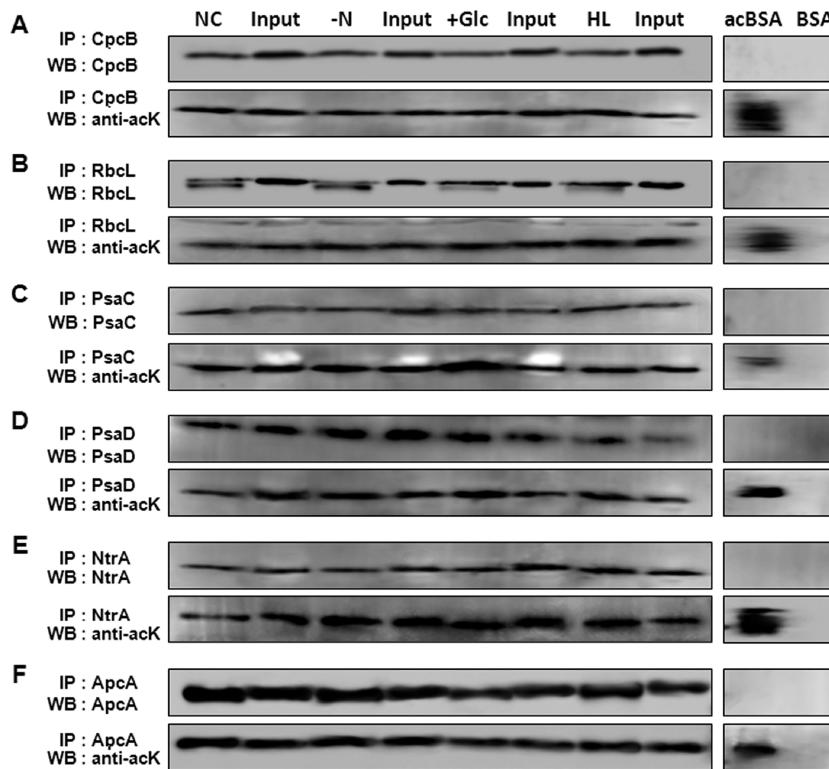


Figure 5. Verification of acetylated proteins in *Synechocystis*. (A) CpcB, (B) RbcL, (C) PsaC, (D) PsaD, (E) NrtA, and (F) ApcA were immunoprecipitated from *Synechocystis* cell lysate and detected by Western blotting. Cell lysate without immunoprecipitated was as input. Immunoblotting was performed using the antiacetyl lysine antibody (Ac-K) or antibodies specific for CpcB, RbcL, PsaC, PsaD, NrtA, or ApcA. Glc +: glucose addition; N-: nitrogen-starvation; HL: high light condition; NC: normal condition.

control of numerous intracellular signaling and regulatory pathways in *Synechocystis*.

Owing to the identification of many photosynthesis-related acetylated proteins, we further constructed an interaction map centered on photosynthesis (Figure 4). This interaction map consisted of a large network covering 412 proteins. Among these connected proteins, 33 acetylated proteins involved in photosynthesis, such as proteins in photosystem I (Slr0171, Slr0226, Slr0819, Slr0737), photosystem II (Slr1194, Slr0906, Slr0851, Slr1867, Slr0427, Slr1418), phycobilisome linker peptide (Slr1459, Slr0335, Slr1580, Slr2051), phycocyanin (Slr1663, Slr1578, Slr1577), phytochrome-like proteins (Slr0041, Slr0821), and the ferredoxin oxidoreductase (Slr0116). Therefore, our results implied direct involvement of lysine acetylation in the regulation of photosynthesis and cellular metabolism in *Synechocystis*. The elucidation of such molecular interactions will provide novel insights into the mechanism of photosynthesis in cyanobacteria.

Validation of Acetylated Proteins by Immunoprecipitation and Western Blotting

To validate our acetylome findings and determine whether environmental stimuli would affect acetylation status *in vivo*, six acetylated proteins, including PsaC, PsaD, ApcA, CpcB, RbcL, and NrtA, were selected for verification. The presence of several acetylated proteins identified through our acetylome approach was validated by Western blotting (Figure 5). These data generated additional confidence in the robustness of the MS data sets. As expected, because the extracts were obtained from different stress treatments, the relative abundances of acetylated proteins also differed according to the immunoreactive signals in *Synechocystis*. The result indicated that the acetylation status

of identified proteins may be affected by different stimuli. We further sought to correlate changes in protein acetylation under specific stress conditions by analyzing the acetylation states of six acetylated proteins *in vivo*. Changes in the acetylation levels of PsaC, PsaD, ApcA, CpcB, RbcL, or NrtA were in the response of cells to heterotrophic conditions, high light and nitrogen starvation (Figure 5). Observed changes in the acetylation status of PsaC, PsaD, ApcA, CpcB, RbcL, and NrtA were not due to changes in protein abundance. Under high light treatment, the acetylation status of CpcB was increased slightly while PsaB was decreased. The acetylation status of NrtA was slightly decreased under nitrogen starvation, and RbcL was increased under heterotrophic condition. The changes in acetylation statuses of six acetylated proteins indicate that lysine acetylation may play regulatory role in response to environmental stimulation and acclimation. Further studies are required to reveal how reversible acetylation contributes to the response of *Synechocystis* to different stimuli.

Acetylated Proteins Involved in Metabolism and Photosynthesis

Emerging evidence shows that lysine acetylation plays a major role in metabolism regulation.^{21,25} We investigated the acetylation of metabolic enzymes in *Synechocystis* by mapping acetylated proteins to KEGG pathways (Table S8). Consistent with previous knowledge on lysine acetylation,^{13,14,20–23} a large proportion of metabolic enzymes involved in central metabolism were found to be acetylated, such as enzymes involved in glycolysis/gluconeogenesis, citric acid cycle, and fatty acid metabolism (Figure 6). Nearly every enzyme in the central metabolism pathway was acetylated, implying that lysine acetylation may regulate cellular metabolic process at multiple

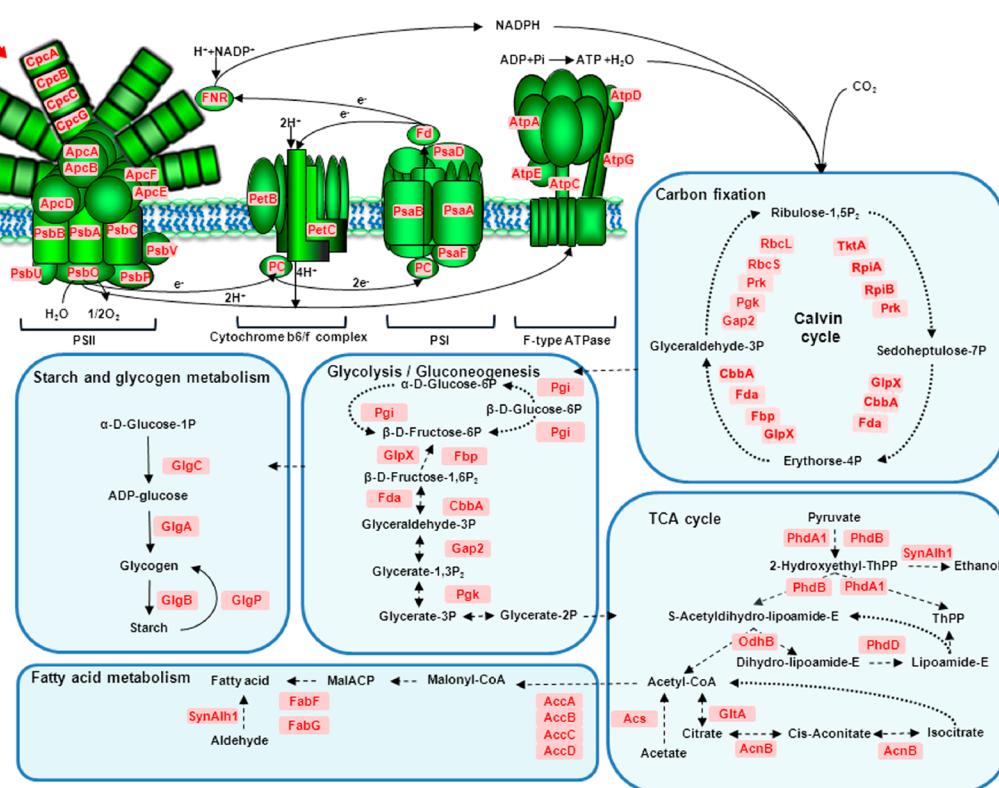


Figure 6. Working scheme of lysine acetylation events involved in photosynthesis and carbon metabolism in *Synechocystis*. Identified acetylated proteins were highlighted in pink. CpcB: phycocyanin beta subunit; CpcA: phycocyanin alpha subunit; CpcG: phycobilisome rod-core linker polypeptide; CpcC: phycocyanin associated linker protein; ApcA: allophycocyanin alpha chain; ApcB: allophycocyanin beta chain; ApcD: allophycocyanin-B; ApcE: phycobilisome core-membrane linker polypeptide; ApcF: phycobilisome core component; PsbB: photosystem II CP47 protein; PsbC: photosystem II CP43 protein; PsbU: photosystem II complex extrinsic protein U; PsbV: cytochrome C-550; PsbO: photosystem II manganese-stabilizing polypeptide; PsbA3: photosystem II D1 protein; PsaA: photosystem I P700 chlorophyll a apoprotein A1; PsaB: photosystem I P700 chlorophyll a apoprotein A2; PsaD: photosystem I subunit II; PsaF: photosystem I subunit III; AtpF: ATP synthase FOF1 subunit beta; AtpD: ATP synthase FOF1 subunit delta; AtpA: ATP synthase FOF1 subunit alpha; AtpC: ATP synthase FOF1 subunit γ ; PetE: plastocyanin; PetB: cytochrome B6; PetC: cytochrome B6-f complex iron-sulfur subunit; FNR: ferredoxin-NADP oxidoreductase; Fd: ferredoxin; RbcL: ribulose bisphosphate carboxylase large subunit; CbbA: fructose-1,6-bisphosphate aldolase; Ppc: phosphoenolpyruvate carboxylase; Tkta: transketolase; Gap2: glyceraldehyde-3-phosphate dehydrogenase; Prk: phosphoribulokinase; RpiA: ribose-5-phosphate isomerase A; Pgk: phosphoglycerate kinase; GlpX: fructose 1,6-bisphosphatase II; AcnB: aconitate hydratase; SynAlh1: oxidize aldehydes; GltA: citrate synthase; PdhB: Pyruvate dehydrogenase E1 component subunit beta; OdhB: branched-chain alpha-keto acid dehydrogenase E2; PhdD: dihydrolipoamide dehydrogenase; PhdA1: pyruvate dehydrogenase E1 component subunit alpha; Acs: acetyl-CoA synthetase; GlgB: glycogen branching protein; GlgA: glycogen synthase; GlgC: glucose-1-phosphate adenylyltransferase; GlgP: glycogen phosphorylase.

levels.²¹ Importantly, we also identified four acetylated enzymes involved in the starch and glycogen metabolism responsible for maintenance energy for cell integrity, function, and viability in dark periods. It is widely accepted that the glycogen can serve as a respiratory substrate in periods of darkness and a reserve to survive starvation periods, such as nitrogen depletion in this study of *Synechocystis*.⁴¹ Thus, our results suggested that several acetylation sites on these enzymes may play an important role in the modulation of glycogen accumulation in *Synechocystis*. Because cyanobacteria are the most ancient life form known to inhabit Earth, with a fossil record of over 3.5 billion years, our results support the notion that lysine acetylation may have an anciently conserved role in controlling cellular metabolism.

Notably, a total of 33 acetylated proteins were involved in the photosynthetic pathway (Figure 6). It is well-known that phycobilisome is the major antenna protein complex for photosystem II (PSII), and it transfers light energy to photosystem I (PSI) under conditions of state transition, a temporal energy redistribution mechanism between PSII and PSI.^{66,67} In higher plants, the reversible acetylation of light

harvesting complex II (LHCII) plays important roles in the balance of the absorbed excitation energy distribution between the two photosystems, which contains PSI involved in generation of reductive power and PSII involved in oxidation of water, as well as associated ATPase required for ATP generation.^{11,68} Therefore, it is likely that acetylation of the subunits of phycocyanin (CpcA, CpcB, CpcC, and CpcG) and allophycocyanin (ApcA, ApcB, ApcD, ApcE, and ApcF) in *Synechocystis* may play a key role in the regulation of the association of phycobilisome with PSII similar to that observed in higher plants. Interestingly, the lysine 317 of ApcE, which is cross-linked with PSII components, was found to be acetylated in this study.⁶⁹ The lysine 48 of ApcD was also acetylated and identified as a cross-link between lysine 11 in PsaA located on the edge area of PSI through a domain formed by PsaA.⁶⁹ Thus, the reversible acetylation may play a role in the regulation of assembly/disassembly of phycobilisome and maintains the energy down-transfer in cyanobacteria, similar to phosphorylation process of linking proteins.^{70,71}

PSII is an integral membrane protein complex with more than 20 subunit proteins and numerous cofactors.⁷² Most chromophores of PSII involved in light-harvesting, as well as electron transfer reactions, are bound to four main subunits, known as D1 (PsbA3), D2 (PsbD), CP43 (PsbC), and CP47 (PsbB). In this work, we identified that three subunits were acetylated, including the D1, CP43 and CP47. Notably, lysine 238 of D1, which could serve as a potential binding site for redox active proteins,⁷³ was found to be acetylated in this study. It has been reported that the mutation of lysine 238 results in a strain that grows photautotrophically in *Synechocystis*.⁷³ Therefore, the lysine 238 may be a crucial site and acetylation may play a role in the regulation of function of D1 protein in the electron transfer pathway of PSII. We also identified four acetylated proteins involved in the electron transfer pathway of PSII, such as PsbO, PsbP, PsbU, and PsbV. In addition, PSI is a membrane-embedded multisubunit complex capable of photo-induced electron transfer to soluble electron acceptors like ferredoxin (Fd).⁷⁴ In this study, four photosystem I subunits (PsaA, PsaB, PsaD, and PsaF) were identified as acetylprotein in *Synechocystis*. Among them, PsaD, which is likely to interact with many different PSI subunits,⁷⁵ was found to be acetylated. Previous observation showed that the lysine 106 in PsaD may play a crucial role in cross-linking to Glu 93 in ferredoxin, and the mutation of lysine 106 may lead to a significant decrease of ferredoxin-mediated NADP⁺ reduction rates.^{76,77} We speculate that the acetylation of lysine 106 may affect the interaction between PsaD and Fd in order to regulate the activity of Fd in *Synechocystis*.

Taken together, our findings provided a novel notion that lysine acetylation is an essential mechanism of photosynthetic functional regulation, in a way similar to phosphorylation.^{70,71} Our data set should provide a point of departure for more in-depth functional studies of photosynthetic pathways in cyanobacteria.

In conclusion, our results provide the first extensive data on lysine acetylation in model cyanobacteria, *Synechocystis*. A total of 776 acetylation sites from 513 acetylated proteins were identified in *Synechocystis*. This study is the most comprehensive acetylome analysis of any cyanobacteria and provides a rich resource that can be used to examine the function of reversible lysine acetylation in cyanobacteria, including potential roles in photosynthesis and carbon metabolism. Our results constitute an important advance in understanding the physiological functions of lysine acetylation and facilitate the elucidation of entire metabolic networks in both the specific context of *Synechocystis* and in the case of cyanobacteria in general.

ASSOCIATED CONTENT

Supporting Information

Supporting Information 1: Supplemental Table 1: Detailed information on identified acetylated peptides in *Synechocystis* sp. PCC 6803. Supplemental Table 2: Gene Ontology (GO) classification of acetylated proteins according to biological processes, molecular functions, and cellular localization using Blast2GO. Supplemental Table 3: Detailed classification of acetylated proteins involved in metabolic processes. Supplemental Table 4: Enrichment analysis of GO terms, protein domain and KEGG pathway. Supplemental Table 5: Comparison of the *Synechocystis* sp. PCC 6803 acetylome with other organism proteomes. Supplemental Table 6: Comparison of conservation of acetylated proteins in *Synechocystis* sp. PCC

6803 with those in other organisms. Supplemental Table 7: MCODE analysis of acetylated proteins and their interacting proteins. Supplemental Table 8: Complete list of KEGG pathways represented by the identified acetylated proteins. Supporting Information 2: Supplemental Figure 1: The annotated spectra of all the identified acetylated peptides. Supplemental Figure 2: Enrichment analysis of GO terms, protein domain and KEGG pathway. Supplemental Figure 3: Protein–protein interaction (PPI) network of identified acetylated proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Authors

*(F.G.) E-mail: gefeng@ihb.ac.cn. Phone/Fax: +86-27-68780500.

*(Q.W.) E-mail: wangqiang@ihb.ac.cn. Phone/Fax: +86-27-68780790.

Author Contributions

[#]These authors (R.M., M.Y., and Z.C.) contributed equally to this work.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PTM, post-translational modification; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gelelectrophoresis; PMSF, phenylmethylsulfonyl fluoride; ACN, acetonitrile; PBS, phosphate-buffered saline; OD, optical density; ppm, parts per million; LC–MS/MS, liquid chromatography–mass spectrometry; HCD, higher energy C-trap dissociation; HPLC, high-performance liquid chromatography; AGC, automatic gain control; FDR, false discovery rate; IPTG, isopropyl-β-D-thiogalactopyranoside; IP, immunoprecipitation; ELISA, enzyme linked immunosorbent assay; PVDF, polyvinylidene difluoride; EDTA, ethylenediaminetetraacetic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, gene ontology; PPI, protein–protein interaction network; PSI, photosystem I; PSII, photosystem II; LHCII, light harvesting complex II; Fd, ferredoxin

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