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Directed analysis of cyanobacterial membrane phosphoproteome using stained phosphoproteins and titanium-enriched phosphopeptides[§]

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Gel-free shotgun phosphoproteomics of unicellular cyanobacterium *Synechocystis* sp. PCC 6803 has not been reported up to now. The purpose of this study is to develop directed membrane phosphoproteomic method in *Synechocystis* sp. Total *Synechocystis* membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and phosphoprotein-stained gel bands were selectively subjected to in-gel trypsin digestion. The phosphorylation sites of the resulting peptides were determined by assigning the neutral loss of [M-H_3PO_4] to Ser, Thr, and Tyr residues using *nano*-liquid chromatography 7 Tesla Fourier transform mass spectrometry. As an initial application, 111 proteins and 33 phosphoproteins were identified containing 11 integral membrane proteins. Identified four unknown phosphoproteins with transmembrane helices were suggested to be involved in membrane migration or transporters based on BLASTP search annotations. The overall distribution of hydrophobic amino acids in pTyr was lower in frequency than that of pSer or pThr. Positively charged amino acids were abundantly revealed in the surrounding amino acids centered on pTyr. A directed shotgun membrane phosphoproteomic strategy provided insight into understanding the fundamental regulatory processes underlying Ser, Thr, and Tyr phosphorylation in multi-layered membranous cyanobacteria.

Keywords: cyanobacterial membrane phosphoprotein, phospho-tagging, phospho-trapping, FT-MS

Introduction

Cyanobacteria are prokaryotic microorganisms that perform plant-like photosynthesis to obtain intracellular energy and assimilate carbon and nitrogen. Cyanobacteria have been used as an ideal model organism to study photosynthesis and the abiotic stress/response (Douglas, 1998). The entire genome sequence of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (referred to as *Synechocystis*) facilitates gene manipulation with the genome database (Kaneko *et al.*, 1996). Post-genomics tools have been applied to *Synechocystis*, but the cyanobacterial phosphoproteome has not been globally analyzed.

Protein phosphorylation of serine, threonine, and tyrosine residues is a key post-translational regulatory modification and is involved in many metabolic and signal transduction pathways (Deutscher and Saier, 2005). Thus, a systematic proteomic analysis for *in vivo* protein phosphorylation is needed to understand physiological functions of most organisms. Although 10–30% of total cellular proteins are phosphorylated, it is very difficult to analyze phosphoproteins due to their limited dynamic range, high complexity, very low stoichiometry, and unreliable quantification (Macek *et al.*, 2007). Highly selective enrichment of phosphopeptides and sensitive mass spectrometric strategies have been attempted to identify dynamic phosphoproteins. Phosphoamino acid-specific antibodies have been widely used to identify phosphoproteins, as shown in the murine brain (Ballif *et al.*, 2008). However, this strategy is quite restricted due to availability, such as high cost and specificity. Thereby, a series of site-specific chemical modifications to convert the phosphate moieties of phosphoproteins into biotin derivatives and subsequent phosphopeptide purification have been conducted (van der Veken *et al.*, 2005). However, these strategies have several weaknesses in terms of reproducibility, sensitivity, and unwanted side reactions.

Immobilized metal ion-affinity chromatography (IMAC) has been introduced to enrich phosphorylated peptides (Nuwaysir and Stults, 1993) and allows negatively charged phosphorylated peptides to be purified by binding to cationic metal ions such as Fe^{3+} or Ga^{3+}. However, non-phosphorylated peptides containing multiple acidic residues are enriched simultaneously by IMAC. Another method enriches phosphorylated peptides using titanium dioxide (TiO_2) followed by electrospray ionization liquid chromatography tandem mass spectrometry (ESI-LC-MS/MS) (Pinkse *et al.*, 2004; Misra *et al.*, 2011; Yan *et al.*, 2014). An on-line TiO_2 precolumn has been coupled directly to a reversed-phase capillary column, and various phosphorylated peptides were successfully analyzed (Pinkse *et al.*, 2008; Cho *et al.*, 2012). Additionally, quantitative phosphoproteomic analysis has been improved using a TiO_2-based enrichment method coupled with label-free LC-MS/MS (Montoya *et al.*, 2011).

In the present study, the membrane phosphoproteome of

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Synechocystis sp. PCC 6803 was analyzed using phosphoprotein staining and enrichment of phosphopeptides using titanium magnetic beads followed by *nano*-LC 7 Tesla Fourier transform tandem mass spectrometry (7T-FT-MS/MS). Our results indicate that this membrane phosphoproteomic strategy provides insight into understanding the regulatory mechanism of cellular events in multi-layered membranous cyanobacteria.

Materials and Methods

Cyanobacterial cell culture and total membrane protein preparation

Synechocystis sp. PCC 6803 cells were cultured in BG-11 inorganic liquid medium with 10 mM Tes-NaOH (pH 8.0) at 28°C under light intensity of 30 μmol/m^2/sec. Total membrane proteins were isolated using a procedure described previously (Kwon *et al.*, 2010). Cells were harvested from a 2 L culture at the early-log phase of growth (OD750 =1.2) by centrifugation at 3,500 × *g* for 15 min and washed with 20 mM Tris-HCl (pH 8.0) buffer containing a protease inhibitor cocktail (Roche). Then, the cell pellet was resuspended in 20 mM Tris-HCl (pH 8.0) buffer and disrupted at 20,000 psi by two passages through a French Press (SLM AMINCO). After DNase (150 units, Roche) treatment for 30 min at room temperature, cell-free crude extracts were obtained by two centrifugations at 15,000 × *g* for 20 min at 4°C. The membrane fractions were separated from the crude extracts by ultracentrifugation at 150,000 × *g* for 90 min at 4°C. The total membrane fraction was resuspended in 20 mM Tris-HCl (pH 8.0) buffer. Protein concentrations were determined according to Peterson's method using bovine serum albumin (BSA) as the standard (Peterson, 1977).

Phosphoprotein staining

Membrane proteins (100 mg) were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by phosphoprotein staining using Phos-tag™ (PerkinElmer). The gels were fixed in 50% (v/v) methanol/10% (v/v) acetic acid and washed three times with deionized water. The phosphoproteins were revealed as bands during gel scanning using the proXPRESS Proteomic Imaging System (PerkinElmer) at excitation of 540 nm and emission of 570 nm.

Phosphopeptide enrichment with titanium magnetic beads

Total gel proteins were stained with a solution containing 30% (v/v) methanol, 7.5% (v/v) acetic acid, and 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250, followed by destaining with 20% (v/v) methanol and 10% (v/v) acetic acid in deionized water. Bands corresponding to phosphoproteins were sliced and subjected to in-gel digestion, as described previously (Kamal *et al.*, 2012). The phosphopeptides were enriched with the Phos-trap™ Phosphopeptide Enrichment system (PerkinElmer) using titanium dioxide-coated magnetic beads, according to the manufacturer's instruction. The elution buffer was dried in an Eppendorf tube for *nano*-LC FT-MS analysis.

nano-LC FT-MS analysis

A 7T LTQ-FT mass spectrometer (Thermo Scientific) equipped with a *nano*-ESI source was used to separate and identify the peptides, as described previously (Kwon *et al.*, 2010). The peptide mixture produced by trypsin was loaded on a home-made trapping C_{18} silica-packed column (length, 5 mm; particle size, 5 μm; LC Packings, Nieuwerkerk a/d IJssel) to remove salts and concentrate the peptides. The trapped peptides were applied directly on an analytical C_{18} column (75 μm × 150 mm; particle size, 5 μm; LC Packings) at a flow rate of 20 ml/min. An LC gradient program and operation described previously was followed (Pisareva *et al.*, 2011). The mass spectrometer was operated in a data-dependent mode to automatically switch between MS and MS/MS acquisition. Xcalibur™ software (Thermo Scientific) was enabled for the MS/MS spectra. Target ions selected for MS/MS were dynamically excluded for 60 sec. The general MS conditions were: spray voltage, 2.2 kV; no sheath or auxiliary gas flow; ion transfer tube temperature, 220°C; collision gas pressure, 1.3 mTorr; normalized collision energy using wide band activation mode, 35% for MS/MS. Ion selection thresholds were 500 counts for MS/MS. An activation q of 0.25 and an activation time of 30 ms were applied for MS/MS acquisition.

Database search

The proteins were identified using the internal MASCOT Server (ver. 2.2, Matrix Science). The database search used Cyanobase (http://genome.kazusa.or.jp/cyanobase/Synechocystis), included 3,661 entries, and was performed with .dta file from LTQ-FT-MS. Peptide tolerance of the parent ion was set to 1.2 Da, and MS/MS tolerance was 0.8 Da. Variable and fixed modification were chosen for methionine oxidation and cysteine carbamidomethylation, respectively, during the Mascot search. Redundant peptides were excluded by matching the candidate proteins using the PROVALT validation tool (Graham *et al.*, 2006). One missed cleavage was allowed to identify the peptides. Phosphorylation sites were determined when the addition of a phosphoryl group (+98 Da) was detected at a serine, threonine, or tyrosine peptide site. The membrane spanning regions were predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). *In silico* serine or threonine phosphorylation sites were predicted in bacterial proteins with the NetPhosBac 1.0 (http://www.cbs.dtu.dk/services/NetPhosBac-1.0) web-based software.

Results and Discussion

Isolation of total membranes and membrane phosphoprotein staining

We adopted titanium-based phosphopeptide enrichment to efficiently identify the phosphorylated membrane proteins (Larsen *et al.*, 2005). As described above, total cyanobacterial membrane proteins were concentrated by ultracentrifugation, separated on SDS-PAGE, and stained with CBB. The phosphoproteins were stained with Phospho-tag™. A standard phosphopeptide mixture containing the bovine casein α/b subunit, albumin, and chicken ovalbumin were sepa-

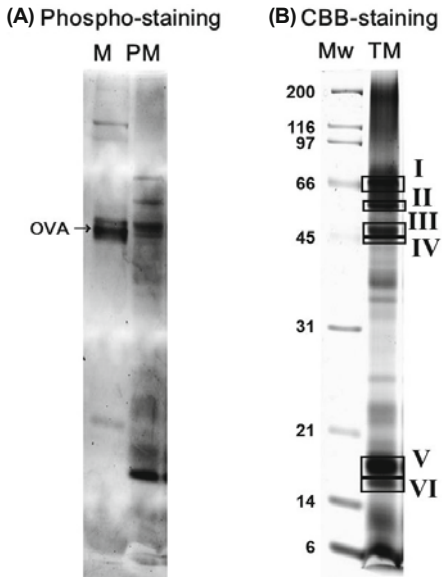

Fig. 1. Gel-based phosphoprotein staining and in-gel digestion followed by Phos-trap™ for tandem mass spectrometry analysis. *Synechocystis* cells were cultured photoautotrophically, and total membrane proteins were isolated followed by the phosphoproteome analysis. (A) Total proteins were fractionated into soluble and membrane proteins followed by staining for phosphoproteins using Phos-tag™ (PM). The phosphoprotein marker (M) ovalbumin (Ova) was positively stained, as indicated. (B) Total membrane proteins (TM) stained with Coomassie Brilliant Blue. Mw, molecular weight.

rated on SDS-PAGE. In particular, BSA, as a phosphoprotein marker, was stained clearly by Phospho-tag™ at 45 kDa (Fig. 1A). The cyanobacterial membrane protein fractions were separated and subjected to phospho-staining, resulting in six clear phosphorylated protein bands (Fig. 1A). The CBB-stained bands corresponding to phosphoproteins were marked as protein bands I (66 kDa), II (55 kDa), III (45 kDa), IV (43 kDa), V (17 kDa), and VI (15 kDa) (Fig. 1B).

Identification of membrane phosphoproteins

The tryptic digests of the phosphopeptides were subjected to *nano*-LC 7T-FT-MS analysis to examine the TiO_2-enriched phosphopeptides mapped with standard proteins. Nine, twelve, and five known phosphorylation sites were detected on bovine α-S1 casein, α-S2 casein, and b-casein serine residues, respectively (Wu *et al.*, 2005; Imanishi *et al.*, 2007). Twenty-one experimentally identified phosphorylation sites were at a serine including one extra phosphotyrosine peptide from BSA (Table 1), and eight phosphopeptides completely coincided with those of a previous study (Thingholm *et al.*, 2008). For example, MS/MS fragmentation and internal sequencing of the casein α-S1 peptide from *Bos taurus* was identified as the fragment ^{58}DIGpSEpSTEDQAMEDIK^{73}, in which the phosphorylation site was positioned at serine residues 61 and 63. Moreover, we identified the monophosphorylated fragment of ^{58}DIGpSESTEDQAMEDIK^{73} of which the MS/MS spectrum is given in Fig. 2A. Besides the fragment derived from the bovine casein α-S2 peptide ^{141}EQLpSTpSEENSKK^{152}, other monophosphorylated peptides specifically located at serine residues 144 and 146 were identified in our analysis. In contrast to the abundance of monophosphopeptides enriched by titanium-based microbeads, the TiO_2-enriched peptides and high resolution *nano*-LC FT-MS analysis enabled us to identify two to five phosphorylation sites.

Based on identifying the standard phosphopeptides, the cyanobacterial membrane proteome was applied to identify the phosphorylation sites. As summarized in Table 2, each phospho-stained protein band contained an average of 2,177 MS/MS spectra, producing a total of 13,060 MS/MS spectra. Finally, 362 peptides were identified, and 80 were phosphorylated, corresponding to 22% phosphopeptide enrichment using Phospho-trapping based on titanium microbeads. A total of 80 phosphopeptides were assigned to 33 phosphoproteins, representing an average of 2.4 phosphorylation sites per protein (Table 2 and Supplementary data Table S1). Figure 2B and C show the MS/MS spectrum of toxin secretion ABC transporter ATP-binding protein (Sll1180) and

Table 1. List of phosphorylated peptides identified from the standard peptide mixture (α/β casein and bovine albumin) using *nano*-liquid chromatography Fourier transform mass spectrometry

Peptide sequence (location of peptide)	Identified protein[a]	No. of phosphorylation site	$[M+H]^+$ (Da)
DIGSEpSTEDQAMEDIK (58-73)	α-S1	1	1846.73
DIGpSEpSTEDQAMEDIK[b] (58-73)	α-S1	2	1927.66
QMEAEpSIpSpSpSEEIVPNpSVEQK[b] (74-94)	α-S1	5	2719.92
VPQLEIVPNpSAEER[b] (121-134)	α-S1	1	1659.79
YKVPQLEIVPNpSAEER[b] (119-134)	α-S1	1	1950.95
EQLSTpSEENSK (141-151)	α-S2	1	1330.53
EQLpSTSEENSKK (141-152)	α-S2	1	1458.62
EQLpSTpSEENSKK (141-152)	α-S2	2	1538.59
TVDMEpSTEVFTK[b] (153-164)	α-S2	1	1465.61
TVDMEpSTEVFTKK[b] (153-165)	α-S2	1	1593.71
ELEELNVPGEIVEpSLpSpSpSEESITR (17-40)	β-C	4	2965.16
FQpSEEQQQTEDELQDK[b] (48-63)	β-C	1	2060.83
GLVLIAFSQpYLQQCPFDEHVK (55-75)	ALB	1	2514.18

[a] α-S1, bovine alpha-S1 casein (UniProt P02662); α-S2, bovine alpha-S2 casein (UniProt P02663); β-C, bovine β-casein (UniProt P02666); ALB, bovine albumin (UniProt P02769).
[b] These phosphopeptides were also identified in a previous study (Thingholm *et al.*, 2008).

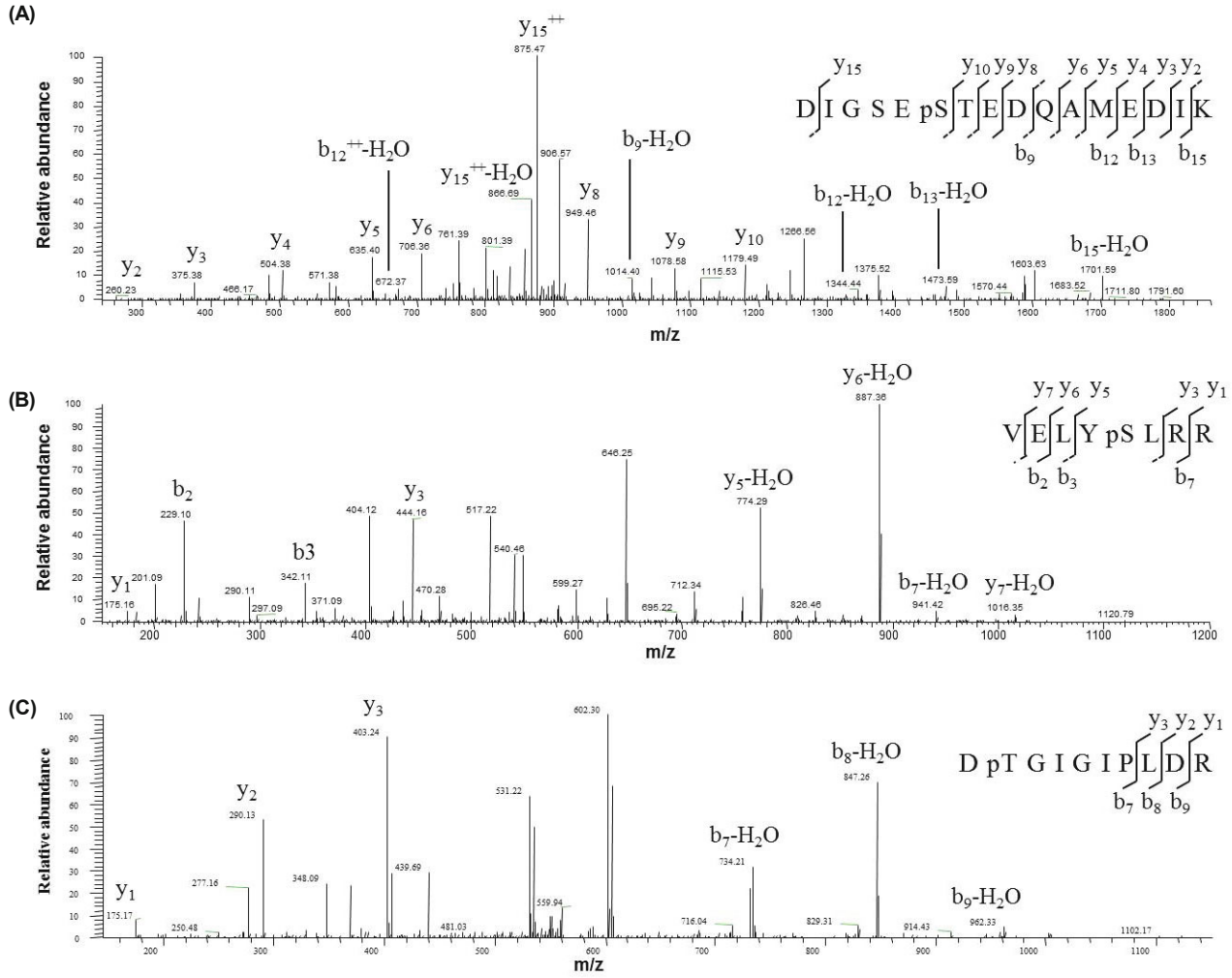

Fig. 2. Tandem mass spectrometry (MS/MS) spectra of (A) the standard casein α S1 *Bos taurus* fragment, (B) double-charged phosphorylated peptide from toxin secretion ABC transporter ATP-binding protein (Sll1180) and (C) triply charged phosphorylated peptide from the two-component hybrid sensor and regulator (Slr1759) of *Synechocystis*. MS/MS spectra resulted from isolation and fragmentation of the precursor ions.

the two-component hybrid sensor and regulator (Slr1759), respectively, as a representative example of a phosphorylated peptide. The number of cyanobacterial phosphopeptides was comparable to previous eubacterial phosphoproteomes, in which 105, 103, and 102 phosphopeptides were identified from *Bacillus subtilis*, *Escherichia coli*, and *Lactococcus lactis*, respectively (Macek *et al.*, 2007, 2008; Soufi *et al.*, 2008). The distribution of phosphorylated amino acid residues was 39 pSer (38.6%), 39 pThr (38.6%), and 23 pTyr (22.8%). This distribution pattern was similar to previous

Table 2. Tandem mass spectrometry (MS/MS) spectra, identified phosphoproteins, and phosphorylation sites from the cyanobacterial membrane

Band no.	MS/MS spectra no.	Identified peptide no.	Phospho-peptide no.[a]	Identified protein no.	Phospho-protein no.	Identified phosphorylation sites no.		
						pSer (%)	pThr (%)	pTyr (%)
1	2,382	31	20	17	6			
2	1,841	22	19	13	10			
3	1,851	16	9	12	6			
4	2,606	122	15	38	11	39 (38.6%)	39 (38.6%)	23 (22.8%)
5	2,534	87	10	35	5			
6	1,846	84	7	47	5			
Sum	13,060	362	80	111	33			

[a] Mascot search rule was applied to MOWSE score corresponding to $P < 0.05$ to select specifically under-represented phosphopeptides from non-phosphopeptides. The phosphoproteins identified were manually validated with MS/MS spectra to assign phosphorylation sites. The numbers of identified phosphoproteins represent the non-overlapped ones.

Table 3. List of cyanobacterial membrane phosphoproteins. The membrane proteins were predicted by the TMHMM 2.0 transmembrane helix prediction program (http://www.cbs.dtu.dk/services/TMHMM). Bold and underline indicates confirmed phosphorylation sites predicted by NetPhosBac 1.0 (http://www.cbs.dtu.dk/services/NetPhosBac-1.0).

ORF/ Gene symbol	Protein name	TM no.[a]	Peptide sequence	*m/z* (Da)	NetPhos-Bac	Reference[b]
Slr0737/*psaD*	Photosystem I subunit II	0	^{11}FGGS**pT**GGLLSK^{21}	1102.51	^{15}pThr	Pisareva *et al.* (2011), Piven *et al.* (2005), Ryu *et al.* (2004)
Sll1039/-	Hypothetical protein	0	^{203}pSApSApYEMYR^{211}	1332.35		
Slr1759/*hik14*	Two-component hybrid sensor and regulator	2	^{948}DpTGIGIPLDR^{957}	1135.53		
Sll1635/*dgt*	Thy1 protein homolog	0	^{92}pYpSCApTEYECpYEARR^{105}	2062.55		
Slr2048/*pratA*	Periplasmic protein	0	^{9}SFTpTSFpSMNLR^{19}	1449.54		Huang *et al.* (2004)
Slr1837/*rre16*	Two-component system response regulator OmpR subfamily	0	^{69}ILGHS**pT**PVLFLTAK^{82}	1575.88	^{74}pThr	
Slr1406/ *fhuA*	Ferrichrome-iron receptor	1	^{92}AFTLITLGPITGQDSpTGAIAQKMpTPAPL PVG**pS**VPLAPNPIpTGL**pS**FSTGGSAIK^{144}	5560.65	^{123}pSer, ^{135}pSer	Ryu *et al.* (2004)
Sll0844/-	tRNA (5-mthylaminomethyl-2-thio uridylate)-methyltransferase	0	^{69}DLFQNHIIDpYLVQGYGEGVTPLPCpSQC NKAVK^{100}	3765.73		
Slr1728/*kdpA*	Potassium-transporting P-type ATPase A chain	10	^{25}pYMARVFLGQSTWLDK^{39}	1909.89		Liu *et al.* (2013)
Sll0252/-	Unknown protein	1	^{123}**pS**APApTPpTGPAGNISNR^{138}	1749.66	^{123}pSer	
Ssr3571/-	Hypothetical protein	0	^{71}pSIDQApTSWpTK^{80}	1375.45		
Slr2131/*acrF*	RND multidrug efflux transporter	12	^{999}QALGTAVFGGMLVATFLSLFVVPVLpYI VVKpTISGR^{1033}	3826.06		Pisareva *et al.* (2011), Matsuda and Uozumi (2006)
Slr1322/*tldD*	Putative modulator of DNA gyrase	0	^{53}VNpYV**pS**CLAEDDAITSLTPRLSSGCGVR^{79}	3099.33	^{57}pSer	
Sll1883/*argJ*	Arginine biosynthesis bifunctional protein	0	^{413}INADpYpTpT^{419}	1036.27		
Sll1180/*hlyB*	Toxin secretion ABC transporter ATP-binding protein	4	^{836}VELY**pS**LRR^{843}	1114.56	^{840}pSer	Kwon *et al.* (2010), Pisareva *et al.* (2011), Matsuda and Uozumi (2006)
Sll0162/-	Hypothetical protein	0	^{149}RAADpTMGWNQGSpTGETpYR^{166}	2255.80		
Sll0771/*glcP*	Glucose transporter protein	12	^{2}NPSpSSPpSQpSpTANVK^{15}	1722.54		Pisareva *et al.* (2007)
Sll0022/-	Unknown protein	0	^{232}p**S**pSNKGFGSpTFK^{242}	1398.46	^{233}pSer	Piven *et al.* (2005)
Slr0009/*rbcL*	Ribulose bisphosphate carboxylase large subunit	0	^{462}FEFEAMDpTL^{470}	1197.43		
Slr0066/*ribD*	Riboflavin biosynthesis protein	0	^{11}CLpTLAKTAIGK^{21}	1197.62		
Slr0640/*hik27*	Two-component sensor histidine kinase	2	^{338}EQpTEPpSVALpTLEHCTADHGALRPR^{361}	2927.19		
Slr0645/-	Hypothetical protein	0	^{19}pS**pS**pSMApTADDPNGPpTR^{33}	1921.48	^{20}pSer	
Sll0369/-	Unknown protein	4	^{49}QDKLL**pS**pYFLFLGSIFTLATGITLVLASR^{76}	3246.69	^{54}pSer	
Sll0473/-	Unknown protein	1	^{309}LIIRDDIVIN**pS**R^{320}	1505.79	^{319}pSer	
Sll0283/-	Hypothetical protein	4	^{369}IRPQD**pS**pYCPHCGpYpYQpYVECV**pS**CH ALpTpYQHLPHCKECGApTQEPNE^{412}	5989.97	^{374}pSer, ^{389}pSer	
Slr0311/*hik27*	Two-component sensor histidine kinase	0	^{467}LLCTIKGTS**pS**pYGEECpTGK^{484}	2242.82	^{476}pSer	
Sll1342/*gap2*	NAD(P)-dependent glyceraldehyde-3- phosphate dehydrogenase	0	^{175}GpTMpTTTHSpYpTGDQR^{188}	1890.54		Huang *et al.* (2002), Piven *et al.* (2005)
Slr0665/*acnB*	Aconitate hydratase	0	^{65}AGFLpTAIAKGEVTCPLISGQGAVDLLGp TMIGGYNVQSLIELLK^{107}	4520.32		
Slr0607/-	Hypothetical protein	0	^{1}MpTSIMMNTVQpTpYGK^{14}	1875.63		
Ssl5027/-	Hypothetical protein	0	^{51}MAVHHCDYDPSNNpYPpSNLMALCpSPC HLpYpYHRR^{82}	4279.51		
Slr6014/-	Unknown protein	0	^{2}**pS**LPpTELIESpSCNQ**pS**pSpTpYFIpSR^{22}	3057.89	^{2}pSer, ^{15}pSer	
Slr6073/-	Unknown protein					
Slr6038/-	Hypothetical protein	0	^{52}LRMAVHHCDYDPGNNpSPpSNLMAVCS PCHLYYHR^{84}	4073.64		

[a] Transmembrane helix number.
[b] Corresponding proteins were cited previously.

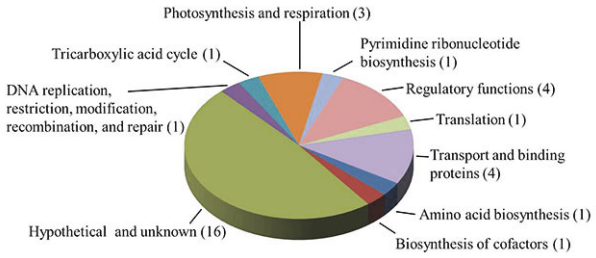

Fig. 3. Functional classification of the cyanobacterial membrane phosphoproteins identified. Thirty-three membrane phosphoproteins were grouped into 10 major categories by their functions assigned in Cyanobase. The total number of proteins in each group is indicated in parenthesis.

observations in the Gram-negative bacterial phosphoproteome (Ge *et al.*, 2011).

Thirty-three phosphoproteins were identified from the cyanobacterial membrane fraction (Table 3). The octaphosphopeptide ^{2}pSLPpTELIESpSCNQpSpSpTpYFIpSR^{22} was commonly identified as Slr6014 and Slr6073, suggesting that this peptide is located in the conserved region of both open reading frames. One-third of identified membrane phosphoproteins had at least one transmembrane helix according to the TMHMM transmembrane prediction program, which helped predict that those proteins were localized in the cyanobacterial membrane. A total of 11 integral membrane proteins possessing transmembrane helix domain were identified. The phosphotyrosine of potassium-transporting P-type ATPase A chain (Slr1728) and the phosphoserine of unknown protein (Sll0369) were located in transmembrane helix domain. Phosphorylation sites of glucose transporter (Sll0771) and hypothetical protein (Sll0283) were positioned in cytoplasmic domain, whereas six phosphopeptides (Slr1759, Slr1406, Sll0252, Sll1180, Slr0640, and Sll0473) were located in the stretched extracellular domain. The phosphorylation site at tyrosine 1024 and threonine 1029 residues of RND multidrug efflux transporter (Slr2131) was positioned in 12th transmembrane domain and extracellular domain, respectively. To better understand the functional diversity and importance of such membrane proteins, we inferred the physiological function of the identified proteins by searching the gene annotations in Cyanobase (Nakamura *et al.*, 1998). According to putative physiological functions, the identified proteins were categorized into 10 different groups (Fig. 3). The largest functional category included proteins with hypothetical and unknown functions (48%). The proteins belonged to the following functional categories: transport and binding (12%), regulatory function (12%), and photosynthesis and respiration (9%). Thus, these proteins may play pivotal roles in signal transduction, regulation, and energy metabolism in the membrane.

Interestingly, the periplasmic PratA factor (Slr2048) was found in the cyanobacterial membrane; Slr2048 interacts with the PsbA (D1) subunit of photosystem II (Schottkowski *et al.*, 2009). Thus, the biogenesis of photosystem II is suggested to occur in the plasma membrane by PratA processing of the D1 protein. The periplasmic protein FhuA (Slr1406), which is a ferrichrome-iron receptor, was also found in the present study. Slr1406 has been detected in the outer membrane component with a single transmembrane helix (Huang *et al.*, 2004), suggesting that these periplasmic proteins are present both in the periplasmic space and the outer membrane to carry out an iron homeostasis role.

A previous study prepared a snapshot of the *Synechocystis* phosphoproteome based on two-dimensional (2D) gel electrophoresis (Mikkat *et al.*, 2014). Two proteins, such as Slr0009 (RbcL) and Sll1342 (Gap2), were found in our shotgun membrane phosphoproteomic approach and the previous 2D gel-based phosphoproteomic data. This result suggests that these two proteins are highly abundant in cyanobacteria and are detectable in both the soluble and membrane fractions. Well-known cyanobacterial phosphoproteins, such as phycobilisome linker proteins, ferredoxin-NADP reductase, P-II protein (GlnB), and KaiC, were not detected in the present study, as they are probably soluble (Forchhammer and Tandeau de Marsac, 1995; Nishiwaki *et al.*, 2000; Piven *et al.*, 2005). However, the phostosystem I subunit PsaD was identified in the membrane fraction. PsaD is a possible substrate of SynPTP protein tyrosine phosphatase (Mukhopadhyay and Kennelly, 2011). A global phosphoproteome analysis of *Synechococcus* sp. PCC 7002 using the shotgun method has been reported (Yang *et al.*, 2013), and the pSer, pThr, and pTyr distribution pattern in *Synechococcus* PCC 7002 was quite similar to that of *Synechocystis* sp. PCC 6803.

Characteristics of the membrane phosphoproteins identified

Transport and binding

We identified four proteins (Sll0771, Sll1180, Slr1728, and Slr2131) that are involved in transport and binding functions. Toxin secretion ABC transporter ATP-binding protein (Sll1180) and RND multidrug efflux transporter (Slr2131) were identified in our previous shotgun membrane proteomic analysis (Pisareva *et al.*, 2011). The Sll1180 and Slr2131 proteins were located exclusively in the plasma membrane fraction. Glucose uptake and phosphorylation are mediated by a glucose transporter (Sll0771), which induces upregulation of carotenoid genes (Ryu *et al.*, 2004). The Sll1180 protein was identified previously in the integral plasma membrane fraction (Pisareva *et al.*, 2007) and in a shotgun proteomic analysis of cyanobacterial total membranes (Kwon *et al.*, 2010). Slr1728 (KdpA) is a component of the ATP-dependent Kdp system for potassium uptake in *Synechocystis* (Matsuda and Uozumi, 2006). Slr2131 (RND multidrug efflux transporter) constitutes the plasma membrane component of a cation/multidrug efflux pump (Pisareva *et al.*, 2007). The phosphoserine site positioned at 840 of Sll1180 was double-checked by NetPhosBac.

Regulatory functions

Proteins involved in regulatory function contained Slr0311, Slr0640, Slr1759, and Slr1837. These proteins all belong to the two-component sensor histidine kinase and/or response regulator. *Synechocystis* has two-component systems of eukaryotic-type serine and threonine kinases, in which the two-component systems play a key role regulating cyanobacterial physiology under abiotic stress (Liu *et al.*, 2013). Thus, the newly found phosphorylation sites of two-component system proteins may be related to novel cyanobacterial physiology in response to external stress. The phospho-

Table 4. Homologs of hypothetical and unknown *Synechocystis* proteins. BLASTP (http://www.ncbi.nlm.nih.gov/ BLAST) was used to identify homologs of the hypothetical and unknown proteins (Table 3)

ORF	TM no.[a]	Homolog[b]						
		Accession number[c]	Protein name	Species	Ident. (%)[d]	Pos. (%)[e]	Query cover (%)	E-value
Sll0252	1	WP_008274160.1	Sporulation protein SpoIID	*Cyanothece* sp. CCY 0110	66	79	100	4e-111
Sll0369	4	CDH14668.1	Probable Iron transporter SMF3	*Zygosaccharomyces bailii* ISA1307	33	48	52	1.2
Sll0473	1	YP_007107333.1	Nitrate/sulfonate/bicarbonate ABC transporter substrate-binding protein	*Synechococcus* sp. PCC 7502	45	66	96	3e-91
Sll0283	4	WP_002733474.1	Putative membrane protein	*Microcystis aeruginosa*	51	69	99	6e-149

[a] Transmembrane helix number.
[b] Proteins with the highest homology are listed as *Synechocystis* hypothetical or unknown proteins.
[c] NCBI accession number of the homologs.
[d] Percentage of identities with the same amino acids to be aligned.
[e] Percentage of positives with the same group of amino acids to be aligned.

serine positioned at 476 of Slr0311 and the phosphothreonine at position 74 of Slr1837 were double-checked by NetPhosBac. In particular, Slr1759 (1462 amino acids in length) is a hybrid sensor kinase with a single-pass transmembrane domain. Notably, the identified monophosphopeptide ^{948}DpTGIGIPLDR^{957} of Slr1759 was found in the HATase family by a Pfam domain search (http://pfam.sanger.ac.uk). The HATase family is structurally related to the ATPase domain of histidine kinase (Campanella *et al.*, 2005), suggesting that threonine residue 949 is phosphorylated in the ATP-binding domain.

Photosynthesis and respiration

Synechocystis facilitates oxygenated photosynthesis using thylakoid membrane proteins involved in the phosphosystem. *Synechocystis* can also grow heterotrophically, so this cyanobacterium can metabolize glucose to obtain energy by respiration. We identified Sll1342, Slr0009, and Slr0737 with photosynthetic and respiratory functions. PsaD subunit II protein (Slr0737) of photosystem I was also identified from the thylakoid and plasma membrane fractions in shotgun and 2D gel-based proteomics analyses (Huang *et al.*, 2004; Pisareva *et al.*, 2011). Soluble cytoplasmic proteins, such as Sll1342 and Slr0009, were identified in the membrane fraction. These proteins presumably attach to other membrane-bound energy metabolism proteins. In particular, Sll1342, encoded in glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalyzes glycolysis in the cytosol; however, GAPDH and several glycolytic enzymes are closely assembled in complexes on red blood cell membranes (Dutta and Inouye, 2000). Thus, the metabolic enzymes and GAPDH were breakdown glucose in the cyanobacterial membrane. The phosphothreonine positioned at 15 of Slr0737 was also predicted by NePhosBac.

Hypothetical and unknown proteins

Approximately 30% of the proteins identified in previous proteomic studies were categorized to hypothetical or unknown functions on the periplasma, plasma, outer, and thylakoid membranes (Huang *et al.*, 2002, 2004, 2006; Srivastava *et al.*, 2005). Similarly, our membrane proteomics analysis identified 16 proteins involved in hypothetical and unknown functions of the 33 total proteins. These hypothetical and unknown proteins were all newly identified and have not been reported. Among them, four proteins containing a transmembrane helix were collected and annotated by homology search. A BLASTP search (http://ncbi.mlm.nih.gov/BLAST) was performed to investigate their homologies with other proteins in the database. The corresponding homologies with the highest scores are shown in Table 4. Sporulation protein SpoIID, which is homologous to Sll0252, is required for membrane migration and the engulfment step during formation of the bacterial endospore (Abanes-De Mello *et al.*, 2002). A probable SM3 iron transporter from *Zygosaccharomyces baillii* ISA1307 was homology-matched with Sll0369 containing four TM domains; however, the

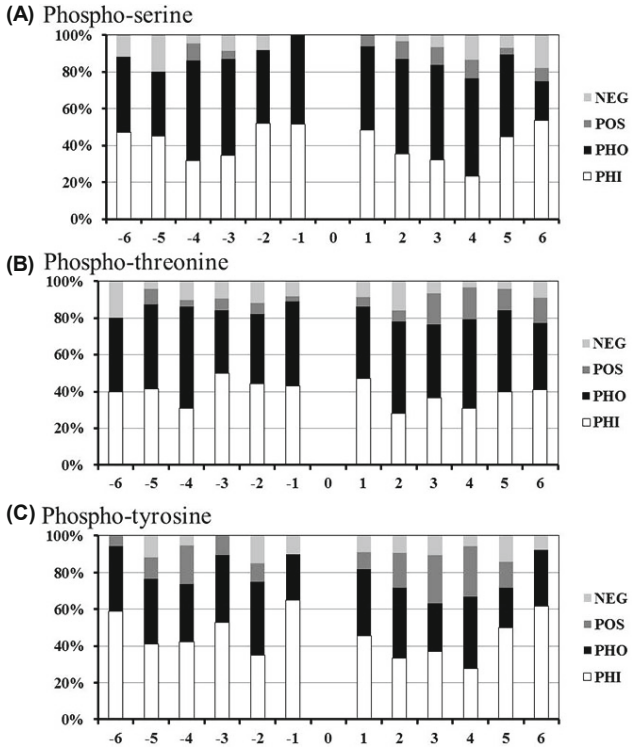

Fig. 4. Distribution of neighboring amino acids around the phosphorylated sites. The number "0" indicates the central phosphorylation site: (A) phosphoserine, (B) phosphothreonine, and (C) phosphotyrosine. Hydrophilic amino acids (PHI): C, G, N, Q, S, T, and Y; hydrophobic amino acids (PHO): A, F, I, L, M, P, V, and W; negatively charged amino acids (NEG): D and E; positively charged amino acids: H, K, and R (POS).

identity score was only 33%. We identified phosphorylation sites at the serine 54 and tyrosine 55 residues of Sll0369. The Sll0639 phosphoserine site was predicted by NetPhosBac; however, the phosphotyrosine site at 55 was not predicted. NetPhosBac is not available to predict tyrosine phosphorylation in prokaryotic systems due to limitations in the database. Unknown protein Sll0473 was highly similar with the nitrate/sulfonate/bicarbonate ABC transporter substrate-binding protein from *Synechococcus* sp. PCC 7502. The Sll0473 monophosphopeptide is in the NMT-1 family and is required for thiamine biosynthesis from pyridoxine (Wightman and Meacock, 2003). Sll0283, with four TM domains, showed high similarity with a putative *Microcystis aeruginosa* membrane protein, in which the Sll0283 phosphopeptide was located in a double zinc-ribbon domain of the homologous protein. The tandem phosphorylated sites at Ser 374 and Ser 389 of Sll0283 coincided with the predicted phosphorylation sites.

Amino acid distribution around the phosphorylated sites

The specific amino acid sequences around the phosphorylation sites, such as serine, threonine, and tyrosine, often determine protein kinase specificity (Zhai *et al.*, 2008). Thus, we examined the frequency of amino acids positioned at -6 to +6 around the phosphorylated sites pSer, pThr, and pTyr according to hydrophilic, hydrophobic, positively charged, and negatively charged amino acids (Fig. 4). The distribution of hydrophilic amino acids was similar among pSer, pThr, and pTyr; however, the distribution of hydrophobic amino acids in pTyr was lower than that of pSer or pThr. Interestingly, the positively charged amino acids, such as Arg, His, and Lys, comprised 56.5%, which was the highest level in pTyr, whereas those of pSer and pThr were 34.8% and 8.7%, respectively. Tyrosine phosphorylation is critical for specific kinase regulatory processes in bacteria (Grangeasse *et al.*, 2007). We found that the negatively charged amino acids, such as Asp and Glu, were not found in pSer. It is notable that the negatively charged amino acids occurred in the neighboring threonine and tyrosine phosphorylation sites.

Conclusion

We analyzed the directed membrane phosphoproteome in *Synechocystis* sp. PCC 6803 for the first time. We identified 80 membrane phosphopeptides, and 33 were membrane phosphoproteins including 11 integral membrane proteins. The proteins were categorized into 10 distinct physiological groups, suggesting that these membrane proteins have diversified functions in the *Synechocystis* membrane system. TiO_2-based phosphopeptide enrichment coupled with high resolution LC-FT-MS analysis was appropriate to identify multiple phosphorylation sites in the *Synechocystis* membrane proteins. In summary, our directed shotgun membrane phosphoproteomic strategy provides insight into understanding cyanobacterial physiology underlying serine, threonine, and tyrosine phosphorylation in multi-layered membranous cyanobacteria.

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