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REVIEW

Serine-Threonine Protein Kinases of Cyanobacteria

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Abstract—Protein phosphorylation is a pivotal mechanism for signal transduction, regulation of biochemical processes essential for reproduction, growth, and adaptation of organisms to changing conditions. Bacteria, which emerged more than 3.5 billion years ago, faced the need to adapt to a variety of ecological niches from the very beginning of their existence. It is not surprising that they developed a wide range of different types of kinases and target amino acid residues for phosphorylation. To date, many examples of phosphorylation of serine, threonine, tyrosine, histidine, arginine, lysine, aspartate, and cysteine have been discovered. Bacterial histidine kinases as part of two-component systems have been studied in most detail. More recently eukaryotic type serine-threonine and tyrosine kinases based on the conserved catalytic domain have been described in the genomes of many bacteria. The term "eukaryotic" is misleading, since evolutionary origin of these enzymes goes back to the last common universal ancestor – LUCA. Bioinformatics, molecular genetics, omics, and biochemical strategies combined provide new tools for researchers to establish relationship between the kinase abundance/activity and proteome changes, including studying of the kinase signaling network (kinome) within the cell. This manuscript presents several approaches to investigation of the serine-threonine protein kinases of cyanobacteria, as well as their combination, which allow to suggest new hypotheses and strategies for researchers.

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INTRODUCTION

Phosphorylation in bacteria is an important regulatory mechanism of adaptation of cells to the changing conditions. Phosphorylation is involved in signal recognition, signal amplification, and cellular response to various types of stress; it regulates DNA replication, formation of biofilms, including substrate adhesion; suppression of the host defense, and many other processes. In cyanobacteria phosphorylation participates in regulation of such vital biochemical processes as photosynthesis [1], CO_2 and nitrogen fixation [2-4], regulation of lipid biosynthesis [5], cell mobility [6], cold-stress adaptation [7].

Phosphorylation in bacteria is much more versatile than in eukaryotes. The following amino acid residues could undergo modification: serine (Ser), threonine (Thr), tyrosine (Tyr), histidine (His), arginine (Arg), lysine (Lys), aspartate (Asp), and cysteine (Cys) [8-10]. Several types of protein kinases are recognized in bacteria – histidine kinases as a part of two-component systems, bacterial tyrosine kinases (Bacterial Y-kinase, BY), atypical serine kinases, arginine kinases, serine-threonine protein kinases of eukaryotic

Abbreviations: 2DE, two-dimensional gel electrophoresis; HPLC, high-performance liquid chromatography; IMAC, Immobilized Metal Affinity Chromatography; iTRAQ, isobaric Tags for Relative and Absolute Quantitation; LFQ, label free quantification; MALDI, Matrix-Assisted Laser Desorption/Ionization; MOAC, Metal Oxide Affinity Chromatography; MS^1 or MS, scanning mass spectrometry; MS^2 or MS/MS, tandem mass spectrometry, fragmentation of isolated peaks; PMF, Peptide Mass Fingerprinting; STPK, serine-threonine protein kinases; TCA, tricarboxylic acid cycle; TMT, Tandem Mass Tag.

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type (STPK) [9]. More than 2400 and 4400 phosphorylation sites were discovered in the *Escherichia coli* and *Staphylococcus aureus* model organisms, which, correspondingly, indicates very complex and intricate network of signal transduction [11, 12].

Processes of phosphorylation of different amino acid residues have its own features: stable phosphoester bonds are formed in phosphorylation of Ser/Thr/Tyr, which allow application of standard proteomics techniques for their purification and detection. On the contrary, phosphorylation of His/Lys/Arg results in formation of thermodynamically unstable phosphoramidates, which are hydrolyzed in the course of sample preparation [10]. This fact leads to underestimation of phosphorylation of these amino acids and requires further optimization and development of new protocols for sample preparation and detection.

The ratio of phosphorylation of different amino acids Ser/Thr/Tyr in different bacteria is approximately the following: 70-80/10-15/5-10 in *Clostridioides difficile*, *Bacillus subtilis*, *Escherichia coli*, and 40-50/35-40/5-10 in *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Listeria monocytogenes* [13]. The ratio of Ser/Thr/Tyr phosphorylation in the cells of cyanobacteria *Synechocystis* sp. PCC 6803 is 42/52/6, moreover, the ratio of mono-, di-, and triphosphorylated peptides is 90/8/2 [14]. Approximately the same ratio of Ser/Thr/Tyr phosphorylation has been reported for *Nostoc flagelliforme* – 56/38/6 and the ratio of mono-, di-, and triphosphorylated peptides – 96/3/1 [15].

APPROACHES FOR INVESTIGATION OF SERINE-THREONINE PROTEIN KINASES IN CYANOBACTERIA

At present several strategies are realized for investigation of protein kinases based on bioinformatics, biochemistry, molecular genetics, physiology, and proteomics [8].

The first strategy involves employment of bioinformatics tools for classification and prediction of functions of proteins and their domains. The biochemistry-based strategy involves isolation of homogenous protein samples followed by investigation of parameters of their activity using “standard” (myelin basic protein, MBP, histone H1, H1, and casein, Cas) and potential endogenous substrates *in vitro*. Next strategies are based on generation of organisms with changed levels of expression of the kinase gene (overexpression or knockout) or deletion of the potentially significant domain(s) in the protein followed by investigation of the changes in transcriptome and cell physiology. The last, most recent proteomics-based strategy aims at establishing association between the amount/activity of kinase and quantitative changes in the proteome including phosphorylation patterns. Use of this strategy allows investigating kinase signaling network (kinome) at the whole-cell level.

All abovementioned approaches individually or in combination were successfully used for studying STPK of cyanobacteria.

Bioinformatics-based approach for investigation of domain organization in protein kinases. It should be mentioned that the term eukaryotic protein kinases or protein kinases of eukaryotic type reflects more history of discovery and investigation of the described enzymes [16, 17].

At present, common evolutionary origin of STPK of eukaryotes, bacteria, and archaea has been demonstrated, which traces back to the Last Universal Common Ancestor (LUCA) [18-20]. Tyrosine kinases represent a later evolutionary branch evolved after separation of pro- and eukaryotic organisms; furthermore, their origin and evolution in these two domains of life occurred independently [21, 22].

Identification of potential STPK in different kingdoms has become possible due to conservatism of the catalytic domain [23, 24]. Three structural parts have been recognized in its composition that perform different functions: N-terminal lobe (β-sheet predominantly) and large-size C-terminal lobe (alpha-helical predominantly) connected via a linker. The catalytic active center *per se* is located in a deep groove between the two lobes [25]. The smaller N-terminal part participates in binding and orientation of nucleoside triphosphates, while the C-terminal part binds protein substrate and initiated transfer of the phosphate group [19, 26].

The kinase domain is typically organized into 12 subdomains, with several consensus motifs recognized in its composition that are required for catalytic functions. Catalysis becomes possible, when the nucleotide binding sites, magnesium binding sites, protein-substrate binding sites, and catalytic center are in ‘correct’ orientation [27]. Furthermore, STPKs have additional subdomains responsible for activity regulation or changes in subcellular enzyme localization [28].

Twelve potential STPK genes were annotated in the genome of *Synechocystis* sp. PCC 6803 based on homology, which are subdivided into two groups (Fig. 1). The first group includes serine-threonine protein kinases of the N2-like type (PKN2): SpkA, SpkB, SpkC, SpkD, SpkF, SpkE, SpkG [29, 30]. The second group including SpkH, SpkI, SpkJ, SpkK, SpkL [30] has several names: ABC1K (activity of BC1 complex kinases), ADCK (ABC1 domain-containing protein kinase), or UbiB-kinases (ubiquinone biosynthesis protein B) [18]. ABC1K is the most commonly used name.

Alignment of human and *Synechocystis* protein kinase sequences demonstrated that SpkG, SpkH, SpkI, SpkJ, SpkK, and SpkL are grouped together and are

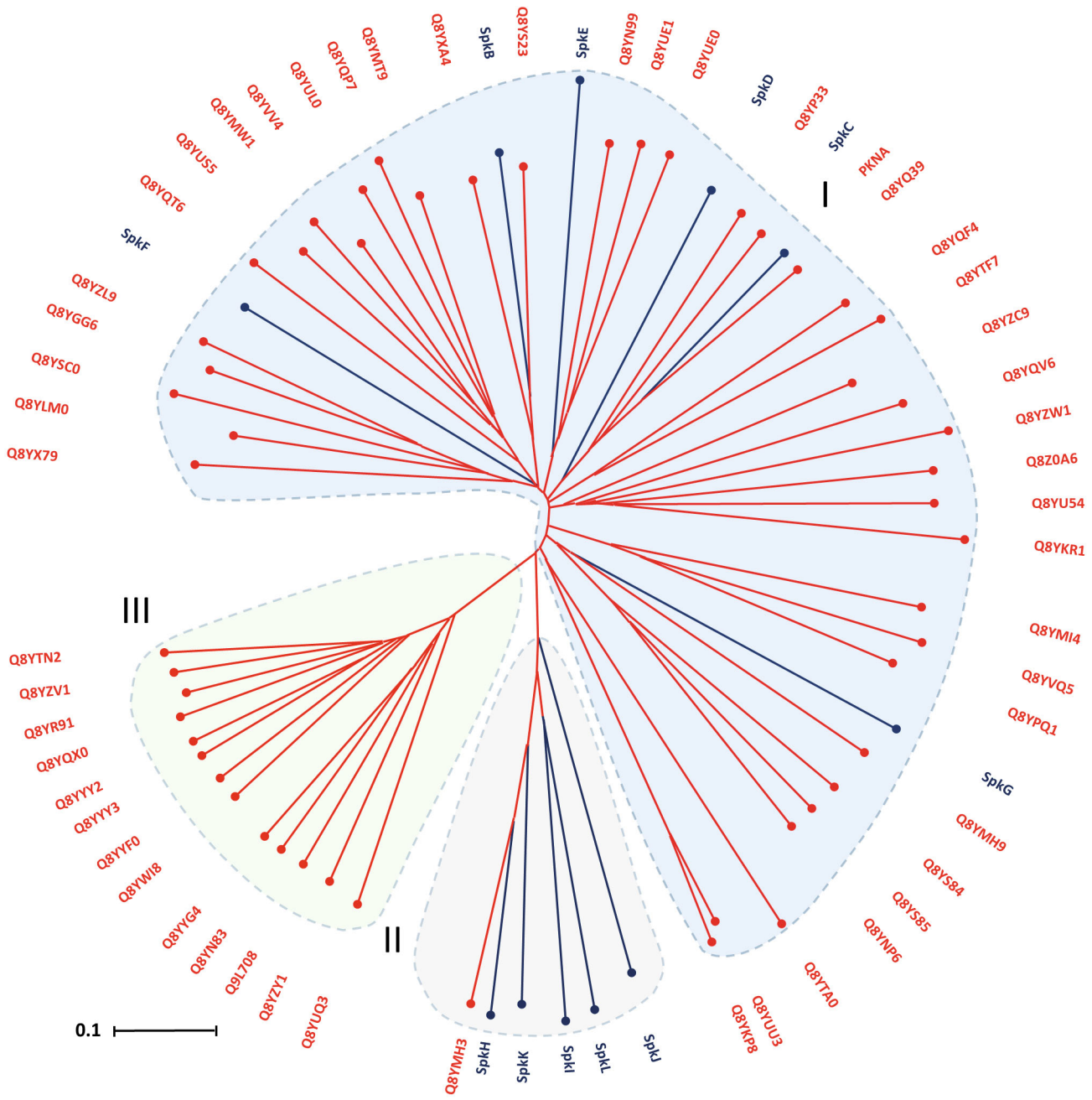

Fig. 1. Phylogenetic tree of serine-threonine protein kinases of *Synechocystis* sp. PCC 6803 (marked with blue) and *Nostoc (Anabaena)* sp. PCC 7120 (marked with red). I and II – PKN2- and ABC1K-like kinases, III – two-domain protein kinases carrying serine-threonine and histidine domains.

close to tyrosine-like and serine-like protein kinases. SpkB, SpkC, SpkD, and SpkF form another group close to the CaMK family. SpkA and SpkE stand apart from other protein kinases of cyanobacteria and could be assigned to tyrosine-like and serine-threonine kinases, respectively [31].

It should be noted that protein kinases of the PKN2- and ABC1K-types differ significantly both in size and in domain organization [31, 32]. Proteins of the SpkA-G group (PKN2) are more conserved in the N-terminal lobe in comparison with the C-terminal lobe, while the representatives of the SpkH-L group (ABC1K) have the central region as the most conserved. These differences are associated with location of the kinase domain [31, 32].

Independent of the size of catalytic domain, several consensus motifs are recognized in its composition, which are typical for all protein kinases and required for binding ATP and protein substrates, as well as for performing catalysis. The most significant are

the GxGxFGxV, VAVK, HRDx3N, DFG, W/YxAPE, and DxWSxC sequences [28, 33]. In addition to those main sequences, the proteins of ABC1K family have seven other motifs (I, II, V, VI, VIIa, IX, X), which do not have homologous counterparts in the protein kinases of the PKN2 group. Significance of these motifs in the protein composition is unknown so far, and, likely, would require further structural studies and analysis of functions with the help of point mutations [18].

Existence of putative transmembrane domains could be considered as an important structural feature. Their presence in the protein sequence could provide information on localization of the protein in the cell, as well as on potential functions [34]. Major part of the available information is based on the data of structure predictions. In one of the first studies on protein kinases of cyanobacteria [32] only SpkF was assigned to transmembrane proteins from the group of PKN2 STPKs of *Synechocystis*. In the study by Zorina et al. [35] domain architecture of three protein kinases was predicted using SMART modeling [36]: SpkF (four transmembrane domains at C-end), SpkC (one transmembrane domain), and SpkK (no transmembrane domains). Based on this information a cascade mechanism of GroES phosphorylation was suggested (SpkF → SpkC → SpkK). Based on the currently available modeling data the following STPKs of *Synechocystis* could be assigned to the membrane proteins: SpkA-D, SpkF-I, SpkL [7, 31, 37].

One of the first classifications of cyanobacterial STPKs, based on the presence and number of transmembrane domains, the position of the kinase domain and additional structural-functional domains was proposed in the study by Zhang et al. [32]. In particular, from the seven described protein kinases of *Synechocystis* five are assigned to the cbSTK I-other enzymes, which are characterized with size more than 400 amino acid residues and non-identifiable C-end regions that do not contain domains with exception of protein kinase domain.

Presence of additional domains is typical for the proteins of the cbSTK II group. SpkB has Pentapeptide Repeat Protein (PRP) [38], SpkD – SH3-like domain of bacterial type (src Homology-3) [32], and SpkG – sequences with WD40/YVTN repeats [31]. Proteins containing PRP are widely represented among cyanobacteria [39]. However, their potential role in the cell is still poorly understood, hence, it is difficult to suggest functional significance of PRP in the SpkB structure. It is known that the SH3-like domain is capable of binding ligands enriched with proline [40], hence, it could be suggested that SpkD is capable of participation in protein-protein interactions [41]. The WD40 repeats act as adapters during formation of protein complexes or protein-DNA complexes, thus participating in very diverse cellular processes [42].

SpkE has a special place among all protein kinases of *Synechocystis*. Initially SpkE was considered inactive due to the absence or replacement of amino acid residues in the conserved DFG sequence that participates in catalysis [43]. However, later in the *in vitro* experiments SpkE was shown to have enzymatic activity with respect to the histone H1 [7]. More detailed consideration of amino acid sequence of SpkE revealed differences not only in the DFG site, but also in some others: the glycine-enriched repeat GxGxFG has the sequence GxNxLG, motif HRDx$_3$N is changed to HGQx$_3$N. Such kinases with changes in the sequence of key amino acids are called pseudokinases – proteins that lost the canonic phosphate transferase function. Nevertheless, they may also play other important roles in the cell regulation [44], for example by allosterically regulating other active kinases or by acting as scaffolding proteins [45]. It is important to note that the loss of canonical residues does not always prevent the binding of nucleotides or the transfer of phosphoric acid residue [46, 47]. Considering that terminology has not yet been established, SpkE could be assigned both to atypical protein kinases [19], and to pseudokinases [45].

Genome of another cyanobacterium *Nostoc* including around 6000 genes contains 52 genes encoding STPKs [29]. It is possible that the number of kinase genes correlates with the size of cyanobacterial genome, as well as with the number of elements of signaling systems. Protein kinases in *Nostoc* have a more complex structure: according to the classification by Zhang et al. [32] they have been identified in all three groups. This includes proteins with only a kinase domain (cbSTK I), proteins with various additional domains (PAS, PAC, GAF, ANF, WD40, FHA, GUN4, TPR, DUF323, PbH1, CHASE2, PRP, RDD, and SH3b) (cbSTK II) [48, 49], as well as proteins of the cbSTK III group, which are absent in *Synechocystis* [32, 50] (Fig. 1). The so-called dual specificity protein kinases belong to the latter group as they contain an N-terminal STPK domain and a C-terminal histidine kinase domain [32, 50, 51]. The site located between these domains is also sufficiently conserved and often contains GAF- or PAS-motif corresponding to the putative sensory domain of the two-component systems. It is likely that the similar proteins ensure interaction of two-component systems and STPKs during signal transduction. All these proteins are large in size ranging from 1777 (Alr2258) to 2021 (All3691) amino acid residues [50] and are found not only in *Nostoc*, but also in a large number of filament-forming strains.

Despite the rather simple organization of the cyanobacterial cells, proteins could be divided into several subproteomes [52]: cytoplasmic proteins, proteins of thylakoid (TM), plasma (PM), and outer membranes, and proteins of periplasm and peptidoglycan

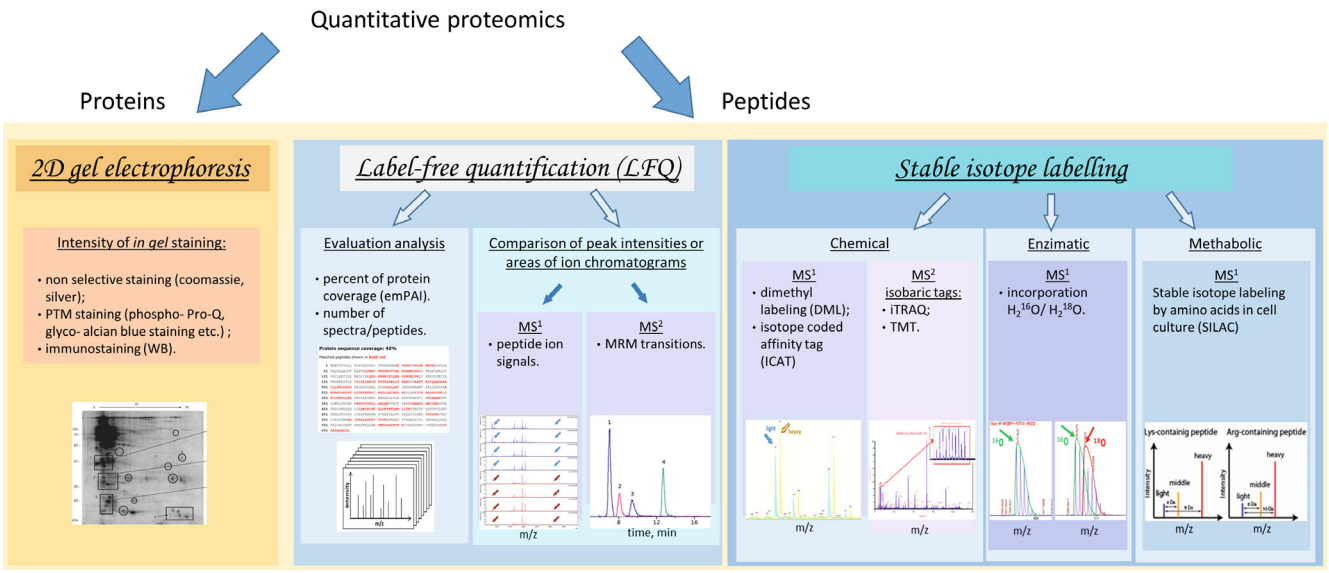

Fig. 2. Main strategies of quantitative proteomics (see text). These strategies can be applied in the bottom-up approach with identification based on peptides obtained via the protein cleavage (chemical or enzymatic). Quantitative information could be based on peak intensities of the peptides in MS^1 (scanning MS) or on the intensities of their fragments in MS^2 (tandem MS).

layer [53]. Several microcompartments are identified in cytoplasm such as carboxysomes, where carbon dioxide fixation occurs [54], various inclusion bodies containing glycogen, cyanophycin, polyhydroxybutyrate, lipids, and polyphosphate [55, 56]. The presence of a large number of compartments implies the existence of a complex system of transport regulation and protein sorting.

Although modern methods of bioinformatics allow rather accurate prediction of the protein structure, its possible localization and functions, experimental verification of such predictions is required.

Proteome-based approach for investigation of kinome. The wide spectrum of proteomics approaches currently available to the researcher very often only complicates the selection of the most appropriate method for a particular problem. In our review we decided to highlight some of the most popular approaches of quantitative phosphoproteomics, their advantages and limitations.

From the very beginning of the development of proteomic approaches with the use of mass spectrometry detectors, two main objectives arose: protein identification and their (semi-) quantitative analysis. Currently, two strategies for the quantitative analysis are used as solutions: either at the protein level with subsequent identification (top-down) or at the peptide level, which are obtained by cleaving the proteome with specific proteases or chemical agents (bottom-up). Two methodical approaches are used for the second strategy: label-free quantification (LFQ) or Stable Isotope Labelling [57-59]. Labeling with stable isotopes could be enzymatic, chemical, or metabolic. In addition, all methods of quantitative analysis could be divided into two large groups. In one case amount is determined from the intensity of parent ions without fragmentation in MS^1, in another – from intensities of the fragment or reporter ions (tags) in MS^2 (Fig. 2).

Quantitative proteomics at the protein level. Two-dimensional electrophoresis. Historically two-dimensional electrophoresis (2DE) with protein identification via Edman sequencing was the first revolutionary technique of proteome analysis [60]. In one direction protein extracts were separated based on their isoelectric points, and in the second direction – according to their molecular masses [61]. This approach is still valid especially for organisms for which there is no data on whole-genome sequencing, because in such cases it is possible to determine protein sequences *de novo*.

Emergence of novel ionization techniques for introducing high mass ions to mass spectrometer, ElectroSpray Ionization (ESI) and Matrix-Assisted Laser Desorption/Ionization, MALDI, allowed use of mass spectrometry for routine protein identification [62].

From the beginning the identification strategy was as follows: after 2DE and staining, protein spots were cut of the gel and subjected to specific cleavage (e.g. trypsin) to produce a set of peptides. After mass spectrometry scanning (MS or MS^1) proteins were identified by comparing masses of the obtained peptides with the theoretical ones [62]. Despite the fact that this methodology has been in existence for more than 30 years, its main steps up to now remain the same and form a basis for the bottom-up proteome analysis strategy and peptide mass fingerprinting (PMF) (Fig. 3).

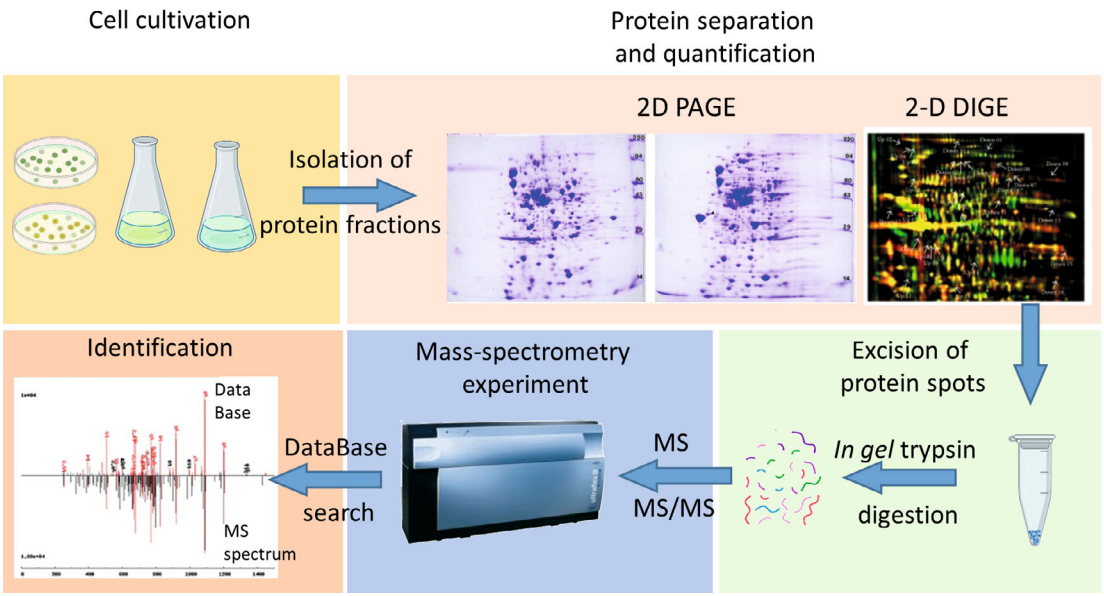

Fig. 3. Strategy of quantitative analysis and identification of proteomes at the protein level using 2D electrophoresis in polyacrylamide gel (2D PAGE) or 2D difference gel electrophoresis (2D DIGE). MS, scanning; MS/MS, fragmentation (tandem MS) of peptides.

The strategy of protein separation in 2DE with MS-detection is still widely used technique. For example, it is employed in the investigation of cytoplasmic proteins, as it allows identification of around 1000-3000 proteoforms in one experiment. In the process, separation occurs at the protein level, therefore the data on molecular masses and their isoelectric points provide additional valuable information on post-translational modifications. The latter is especially important for investigation of protein phosphorylation. This method allows rather simple visualization of the protein phosphoforms from the changes in isoelectric points (p*I* shift) and appearance of a series of characteristic spots (beads) with the same molecular weight [63]. Changes in phosphorylation stoichiometry results in proportional increase of the number of such spots [64]. Staining of the gel with phosphoprotein-specific dyes (such as Pro-Q Diamond) or immunodetection with antibodies specific to phosphorylated amino acids in combination with enzymatic or chemical treatment of the samples with the agents facilitating dephosphorylation allows making an unambiguous conclusion on the presence of modifications [65, 66].

However, this method has significant limitations. Solubilization of a protein sample in a buffer containing chaotropic agents (urea and thiourea) and detergent (CHAPS) results in underestimation of many membrane and membrane-associated proteins, proteins of extracellular matrix and cell wall in the investigated proteome. Moreover, the proteins with extreme p*I* values could be lost during isoelectric focusing being outside of pH range of the ampholytes. Other drawbacks of the method are its relatively high labor intensity, which results in poor scalability for routine analysis of tens of samples, as well as mediocre reproducibility [67]. To overcome these limitations a method of difference gel electrophoresis (DIGE) was introduced. The main idea of this method involves covalent labeling of the protein samples with fluorescent dyes with different spectral characteristics. The combined samples are subjected to separation according to the standard technique. Scanning of the gel is conducted in separate channels corresponding to spectral parameters of the used fluorophores [68].

The aforementioned approaches were utilized for investigation of the *Synechocystis* proteome. In particular, two-dimensional separation followed by MS identification allowed to determine 55 differentially changed proteins in the plasma membrane in response to pH stress [69]. Comparison of the wild type and the Hik34 histidine kinase mutant at the proteome level confirmed accumulation of the proteins induced by heat shock including a number of chaperones [70]. Comparative analysis of proteomes of the plasma membranes of the wild type and of the Hik33 mutant grown under normal conditions and under conditions of salt stress revealed serious rearrangements in the membrane composition [71]. In particular, the abundance of a number of the transport protein, such as GgtB and FutA1, and of the regulatory proteins, such as MorR and Rre13, was decreased in the mutant.

Analysis based on the results of 2DE of three *Anabaena* species (*Anabaena doliolum*, *Anabaena* sp. PCC 7120, and *Anabaena* L31) in the control cultures and after 1, 3, 5, and 7 days of salt stress revealed 256 significantly different protein spots [22].

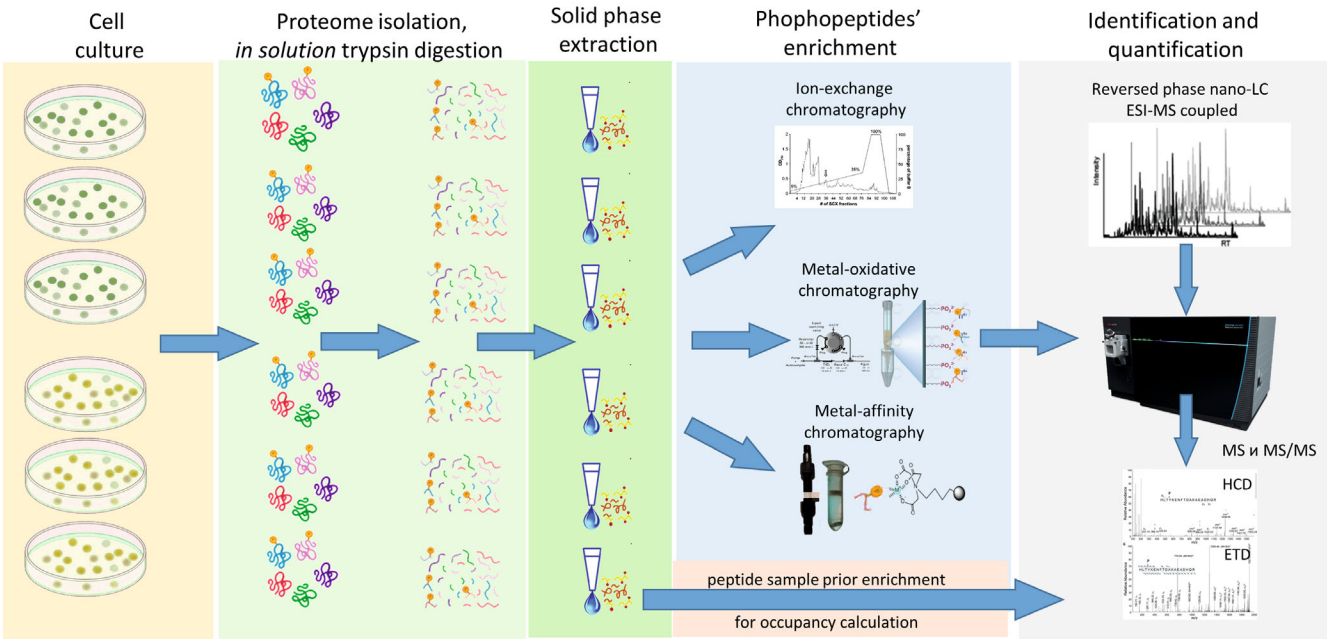

Fig. 4. Main steps of identification protocol for phosphopeptides using label-free quantitate analysis (LFQ). To estimate the degree of protein phosphorylation (occupancy) additional quantification of the initial sample not enriched with phosphopeptides is conducted. To localize phosphorylated amino acid residue the following fragmentation techniques are used: Higher Energy Collision Dissociation (HCD) or Electron Transfer Dissociation (ETD).

Around half of them (134) corresponded to 29 proteins and found to be the same across all species. This group of proteins predominately consisted of the proteins of basic metabolism, stress proteins, and factors regulating translation. Moreover, distinct proteins were identified for each genotype. The authors hypothesized that these differences were associated with adaptation processes in the cyanobacteria species that exhibited different levels of stress resistance.

As demonstrated in the study [72], the combination of 2DE with gel staining using phosphospecific dye ProQ Diamond followed by MS-analysis, resulted in the first phosphoproteome of *Synechocystis* characterization in addition to the identification of alteration in response to stress. Thirty-two phosphoproteins were identified, and for 8 of them phosphorylation sites were determined.

Quantitative proteomics at the peptide level. Quantitative proteome analysis at the protein level has a number of significant limitations. The main problem is a relatively low resolution of the methods of protein separation followed by the detection with mass spectrometry. Preliminary specific cleavage of proteins to peptides eliminates this limitation.

Accumulation of the data of MS-analysis and the data of genome sequencing revealed that the PMF method based on MALDI-MS has its own limitations – in complex samples peptides with the same measured mass could have different primary structure. To resolve this issue the sequence tags method was suggested; strategy of this method involves combination of information on accurate mass of the whole peptide (parent ion) obtained with the scanning MS (MS^1) with information on the masses of its fragments (tags) obtained with the tandem MS (MS/MS or MS^2) [73]. At present this identification strategy known as Data Dependent Acquisition (DDA) is widely used in proteomic studies.

It must be emphasized that quantification at the peptide level requires quantitative cleavage of all proteins to peptides, which is especially important for insoluble proteins including membrane ones. To improve efficiency of enzymatic cleavage, a combination of enzymes LysC/trypsin is used [74], cleavage in the detergents compatible with mass spectrometry protocols (PPS, ProteasMAX, RapiGest) [75], or cleavage on the surface of filters (Filter-Aided Sample Preparation, FASP) [76].

Separation and enrichment of phosphopeptides from mixtures. High-performance liquid chromatography (HPLC) for the separation of peptides on reversed-phase columns was used in the earliest proteome studies [77, 78]. This separation allows to decrease analytical complexity of the samples, decrease their dynamic range, and to concentrate individual peptides. The utilization of nano-liquid chromatography (nano-LC) coupled with the MS instrument via an ESI interface (ESI-MS) facilitates the real-time separation and identification of peptides. Using this method for analysis of prokaryotic proteome allows identifying approximately 10,000 peptides (1500 proteins)

in a single experiment, which comprises approximately 50% of the proteome [79].

Phosphorylation is a dynamic process determined by the ratio of kinase and phosphatase activities. The level of phosphorylated proteins in the cell remains usually low, which presents a particular problem for the proteome analysis. In order to achieve reliable identification of the phosphopeptides at proteome level the amount of protein in a sample should be 100-1000-fold higher (milligrams) compared to the routine shotgun proteome analysis (micrograms). Furthermore, such low amount of phosphopeptides requires their preliminary enrichment and use of highly sensitive detection techniques (Fig. 4).

Two highly specific enrichment techniques are used in phosphoproteomic analysis: Immobilized Metal Affinity Chromatography (IMAC) on immobilized Fe^{3+} and Ti^{4+}, and Metal Oxide Affinity Chromatography (MOAC) on TiO_2 and ZrO_2 oxides [3, 80-83]. Other techniques are used for either enrichment of phosphosites (phosphotyrosine and phosphosite specific immunoaffinity chromatography), or as a step of peptide fractionation prior to IMAC or MOAC (ion-exchange chromatography, hydrophilic interaction chromatography). It should be emphasized that despite the widespread use of IMAC and MOAC techniques for phosphopeptide enrichment, these methods are complementary – only a part of the phosphopeptides could be enriched with either of them [84-86].

Label-free quantification (LFQ). Firstly, it is important to note that mass spectrometry is not an absolute quantitative method. This is due to the fact that the measured intensity of the peptide peak cannot be directly converted into its concentration (i.e., quantity). Hence, for the correct semi-quantitative analysis of proteins from different samples comparison of the same peptides (with equal mass to charge ratio and, for the case HPLC separation, the same elution time) should be performed. In the process, either the number of spectra obtained or the intensity/area under the MS^1 peak of the individual peptide-ions should be compared. Such comparison of intensities is the basis of label-free quantification or LFQ [87]. The employment of this method in conjunction with high-resolution mass spectrometers and of ultra-short gradients allows scaling up the whole-proteome analysis up to hundreds of samples without additional costs associated with time and reagents required for sample preparation, as well as instrumental time for acquiring MS^2 spectra [88].

One variant of LFQ with tandem peptide fragmentation is the method of counting number of spectra (or number of identified peptides) or percent of protein coverage by the identified peptides based on the exponentially modified Protein Abundance Index (emPAI) [89, 90]. However, these methods are not very accurate and provide approximate estimate of protein abundances in the samples.

A separate group of methods is constituted by those based on measurement of intensity/area of the fragments in MS^2 spectra – Data Independent Acquisition (DIA) and targeted methods. One of the examples of DIA methods is the SWATH-MS (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra), and of target methods – Single/Multiple Reaction Monitoring (S/MRM) [91-94]. These methods have high levels of reproducibility and a wide dynamic range of peptide concentrations for quantitative analysis. The SWATH-MS method utilizes the mass scanning in MS^1, in contrast to DDA approach, peptide fragmentation is carried out not by peaks, but in the wide mass overlapping windows covering the entire scanning range. In the process, both the data on intensities of parent ions and on intensities of fragments could be used for quantitative retrospective analysis. In the MRM method, which is a mass spectrometric analogue of Western blotting, quantitative analysis of unique peptides characterizing a specified protein is carried out based on estimates of the areas under chromatographic peaks of ion-fragments (MRM transitions). This method exhibits the widest dynamic range of concentrations and could be used as a complementary technique to confirm amounts of individual proteins obtained in shotgun proteomics [91].

The LFQ method in combination with multistep fractionation of proteome was used for creation of the *Synechocystis* sp. PCC 6803 proteome atlas [92]. The authors were able to identify 2167 proteins, which comprised 71.3% of the whole proteome, and evaluate distribution of 2027 proteins in the cells of cyanobacteria. Using the FASP method for sample preparation facilitated identification of 504 proteins that have transmembrane domains according to bioinformatics data. According to the results of this study, STPKs of *Synechocystis* belong to different membrane fractions, which could reflect possible different functions of the proteins in the cell and could form a basis for the development of a new hypothesis related to their functions.

Combination of complementary LFQ approaches (large-scale proteomic study and targeted quantification) was used for investigation of the *Anabaena* sp. PCC 7120 proteome under nitrogen-deficient cultivation conditions [39]. This approach revealed changes in the proteome associated with metabolic pathways of nitrogen and carbon assimilation in the case of cultivation under nitrogen deficit for 3, 12, and 24 h; in the process quantities of 363, 603, and 669 proteins changed differentially at each time point, respectively.

Similar complementary approach was used for comparison of the proteomes of fresh-water

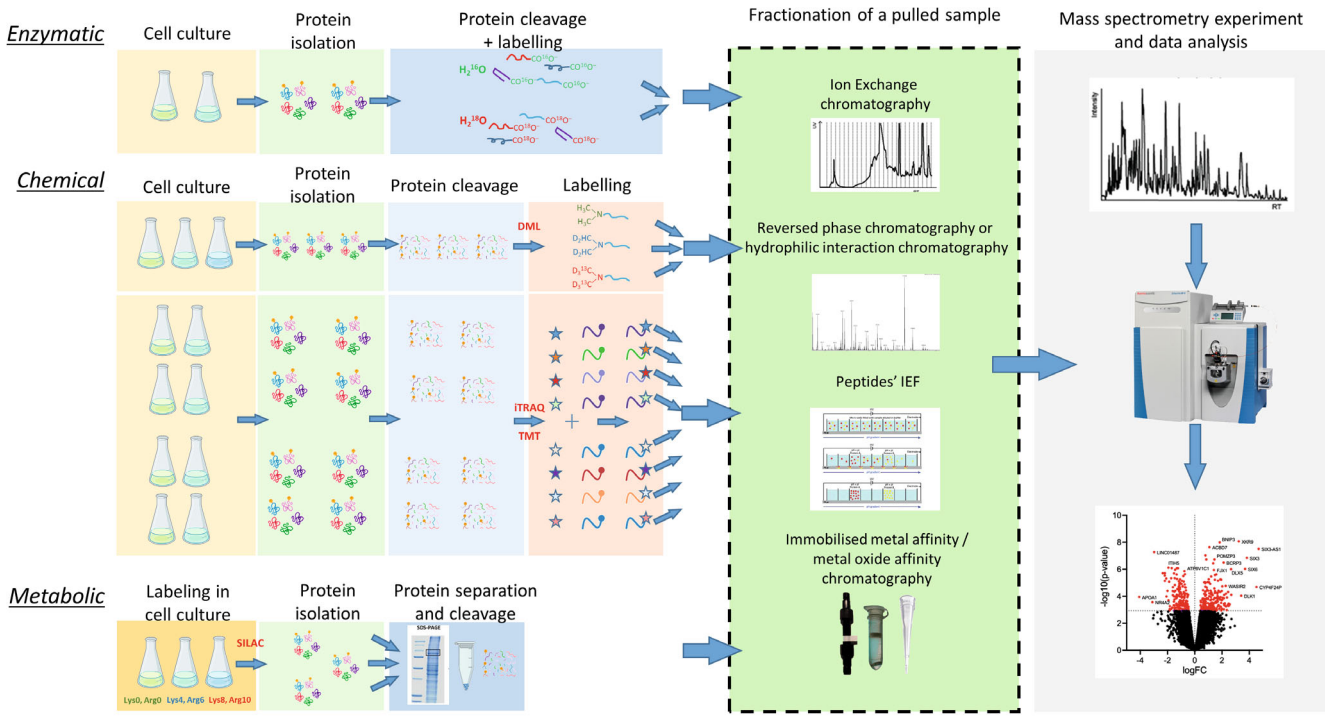

Fig. 5. Stable isotope labeling as a strategy of quantitative proteomics. It should be emphasized that the use of enzymatic or chemical approach for label introduction imply labeling at the level of peptides: at the stage of enzymatic cleavage or immediately after it. Metabolic labeling differs from other methods by introduction of the label at the protein level, which allows protein separation of the pooled sample. Block of the optional stages of the procedure is marked with dashed line.

Synechocystis sp. PCC 6803 and marine *Synechocystis* sp. PCC 7338 [95]. At the first step, the use of DDA approach allowed identification of the proteins of these two strains and creation of the spectral library for quantitative comparison of the proteomes. As the second step the Data Independent Acquisition (DIA) method was used. This approach allows detection of a large number of low abundance proteins in the proteome. In particular, while in the DDA mode 10 parent ions with highest intensity were selected from the entire spectrum for fragmentation, in DIA – the ions from one of the 20 mass windows in the range from 500 to 900 m/z were examined. In total 2049 proteins from both strains were evaluated, from those contents of 396 were different in both strains. The highest difference in content was observed for KdpB, protein of ATP-binding subunit of potassium transporting ATPase. It is assumed that exactly presence of high-affinity ion transporters forms the basis of cell adaptation to living in marine environment.

It should be emphasized that all LFQ could be made absolutely quantitative by using either isotope-labels, or unlabeled standard peptides with known concentration as a spike [96, 97].

Quantification using stable isotope labeling. Labeling of the samples with stable isotopes allows conducting multistep (multidimensional) fractionation without losing quantitative information. Moreover, this strategy allows to increase throughput of the analysis, several proteomes, as a pooled sample, could be analyzed simultaneously in one experimental run. However, this approach requires costly consumables and lengthy sample preparation, which limit application of this method.

Several protocols for stable isotope labeling are used in practice nowadays (Fig. 5): enzymatic, chemical, and metabolic [57].

Enzymatic labeling. Enzymatic method for labeling involved specific cleavage of the samples with the help of trypsin conducted in $H_2{}^{16}O$ and $H_2{}^{18}O$; in the process, the label is introduced to the C-end of the generated peptides. The peptides from different samples will differ in MS^1 by 2 Da, and the ratio of their intensities will reflect their content in the initial samples [98]. This method has a significant drawback – the obtained difference in the masses of peptide with light and heavy label is insufficient to separate their isotope clusters. Starting from the second isotope of the light peptide, the clusters overlap, which could cause significant errors in quantification.

Chemical labeling using isobaric labels. Chemical introduction of stable isotope labels could be realized using several methods. The so-called isobaric labels are commonly used with mass of reporter groups (tags) balanced by the linker mass. Peptides from different samples labeled at $–NH_2$ groups with different tags

have the same mass (isobaric tags) and are indistinguishable both in multidimensional chromatographic separation (LC/LC), and in MS^1. During the peptide fragmentation in MS^2 reporter tags with unique mass are released and amount of peptide in the initial sample is determined from their intensities [99]. Currently isobaric tags are represented with two kits including 8-plex isobaric Tags for Relative and Absolute Quantitation, (iTRAQ) and 16-plex Tandem Mass Tag (TMT).

The iTRAQ technique was used to investigate the response of *Synechocystis* to prolonged phosphorous starvation by using medium with different concentrations of inorganic phosphorous for cultivation. Proteome rearrangement under these conditions was similar to the response to nitrogen deficiency in the nutrient medium, but the effect was less pronounced [100].

Analysis of the *Synechocystis* proteome changes in response to nitrogen starvation using iTRAQ demonstrated specific response depending on the stress duration (24 and 48 h) [101]. Only a small number of proteins were found that did not exhibit changes in their quantities in both stages. On the first day, the level of proteins associated with the biosynthesis of aspartic acid and histidine was found to decrease, while the amount of proteins involved in glutamic acid synthesis decreased only on the second day. Decrease of the amount of enzymes of tricarboxylic acid cycle (TCA) was observed during both stages, while the amount of the glycolytic enzymes decreased only in the second stage. The response of cyanobacterial cells to the short-term nitrogen starvation was manifested as a significant transient rearrangement of carbohydrate and nitrogen metabolism.

In the study investigating the response of *Spirulina platensis* to cold stress [102] a total of 3786 proteins were identified, of which the content of 1062 changed. For the majority of these proteins (749) the decrease in abundance during the stress was shown, while the number of proteins with increased levels was twice as low. These data are in good agreement with already known data on the changes in the transcriptome. The most significant changes were demonstrated for the proteins associated with carbohydrate metabolism, protein translation, and amino acid synthesis.

The iTRAQ labeling was used by Flores et al. [103] for investigation of contribution of the alternative sigma factor SigF to synthesis of extracellular polysaccharides, processes of protein secretion, and the formation of cell wall in *Synechocystis*. In comparison with the wild type, the levels of 313 proteins (from 1654 identified) were differentially changed in the ΔSigF mutant. Comparison of the search results for the sequences in the genome, which potentially could be SigF binding sites and identified proteins, revealed 12 potential protein targets of σ-factor predominantly involved in photosynthesis and carbohydrate metabolism.

In one of the studies dedicated to the *Synechocystis* sp. PCC 6803 proteome mapping [52], the authors used localization of organelle proteins by isotope tagging (LOPIT) approach. This approach combined multistep fractionation of protein in sucrose density gradient followed by labeling with 10-plex TMT and two-dimensional (LC/LC) reversed-phase HPLC under alkaline and acidic conditions. The authors identified 2445 protein (67% of the proteome). Use of quantitative data and protein markers of cellular fractions allowed assigning 1712 proteins to the particular cellular compartments.

Use of 6-plex isobaric TMT label helped to reveal regulatory role of the Hik8 histidine kinase in phosphorylation of the PII protein [69]. In the experiment the authors compared quantitative proteomes of the wild type *Synechocystis* with five mutants carrying mutant proteins participating in regulation of carbon metabolism (histidine kinases Hik8 and Hik31, their paralog Slr6041, response regulator Rre37, as well as σ-factor of RNA polymerase SigE) with the goal to establish specificity and interrelation of the regulatory pathways. In the series of mass spectrometric experiments, the authors identified more than 2500 proteins, from which 2189 were used for the following quantification. As a result of proteome comparison, it was found that in all 5 mutants the content of only 4 proteins changes: the levels of Sll7085, Slr6074, and Slr1161 were decreased, and the level of glucosylglycerol-phosphate synthase (GgpS) was increased. In addition, the use of TMT label helped the authors measure quantitatively the degree of phosphorylation at S49 of the PII protein in the mutant strain Hik8, which was shown to be increased under auxotrophic, and decreased under mixotrophic conditions of cultivation.

Despite all the advantages offered by the method of isobaric labeling, its limitations should be mentioned; these include the high cost of commercial kits and reduction of the dynamic range of measured intensities. Use of modern high-resolution instruments and peptide separation in gas phase helped to resolve the latter issue [99, 104].

Chemical labeling using "anisobaric" labels. Another approach for quantification involves introduction of "anisobaric" labels into peptides, which provide equal difference in mass. In MS^1 peptides in the pooled sample generate doublet or triplet peaks with different intensities, based on which quantification is performed. Chemical agents modifying –SH or $–NH_2$ are used for labeling.

Labeling of –SH group is commonly carried out with the only available 2-plex commercial kit ICAT

(Isotope-Coded Affinity Tag). Despite the possibility of enrichment of the labeled peptides at the stage of affinity chromatography and compatibility with the whole-proteome analysis [105], ICAT has only one advantage – high selectivity with respect to thiol groups, which is especially important for quantification of redox status of proteins in the cell [106, 107].

DiMethyl Labeling (DML) 3-plex protocol has been developed for labeling $–NH_2$ groups [108]. This method allows peptide labeling both in solution and on the reversed-phase column during the process of sample preparation. The deuterated cyanoborohydride and ^{13}C-formaldehyde are the least costly among the all-abovementioned reagents, hence, this method attracts attention of more and more researchers. It must be mentioned that all isotope methods with quantification based on MS^1 have an effect of increasing analytical complexity of the pooled sample, which results in the lower depth of proteome coverage. This drawback is counterbalanced by the possibility of employing complex separation and enrichment schemes, such as phosphopeptide enrichment, without losing quantitative information.

DML was used in proteome and phosphoproteome analysis to investigate of the response of a collection of STPK mutants of *Synechocystis* to abiotic stress. Depth of proteome coverage was around 2500 proteins (67% of proteome), and 2200 proteins were selected for quantification. The phosphoproteome analysis was conducted using a multistage fractionation protocol, which involved a series of chromatographic steps. These steps included reversed-phase HPLC under alkaline conditions, TiO_2-based chromatography, HPLC under acidic conditions. As a result, 115 phosphorylated proteins were identified. It was also established by the authors that some peptides are present only as doubly phosphorylated (such as sites pT148 and pS155 in RpoD), while in other peptides phosphorylation of only one residue was predominantly observed (such as either pT384, or pT387, pT387 site in Sll0103).

Metabolic labeling. Metabolic stable isotope labelling is carried out at present using the method of Stable Isotope Labelling by/with Amino acids in Cell culture (SILAC) [109]. In this method light, medium, and heavy variants of Lys and Arg amino acids are added to the culture medium. These amino acids labeled with heavy isotopes are eventually incorporated into the synthesized proteins. At the stage of enzymatic protein cleavage with the help of trypsin (or LysC/trypsin), the generated peptides contain one terminal amino acid Lys or Arg, intensity of which are detected in MS^1 as doublets or triplets (2- and 3-plex SILAC). The main advantage of this method is the fact that isotope labelling occurs at the level of proteins, which could be separated (using, for example, single-dimension SDS electrophoresis) without losing quantitative information even in the process of in-gel protein cleavage.

The significant drawbacks of this method are high cost, increase of analytical complexity of the pooled sample, as well as impossibility of its application for autotrophic organisms synthesizing Lys and Arg [110]. As an alternative to amino acids, introduction of the nitrogen isotope ^{15}N often in the form of $Na^{15}NO_3$ or $K^{15}NO_3$ has been suggested. This approach was used for investigation of *Microcystis aeruginosa* toxins metabolism in the course of revival after prolonged nitrogen starvation [111]. ^{15}N-ammonia, ^{15}N-urea, ^{15}N-nitrate, and ^{15}N-alanine were used as nitrogen sources. Analysis was carried out based on incorporation of ^{15}N into microcystins produced by cyanobacterium. It was shown in the course of the study that the microcystin-LY was accumulated only under stress conditions, at the stage of revival in the presence of various nitrogen sources, predominantly in the medium with ^{15}N-alanine. The most abundant microcystin-LR was synthesized in the presence of all nitrogen sources.

INVESTIGATION OF THE STPK FUNCTIONS IN CYANOBACTERIA EXEMPLIFIED BY INDIVIDUAL PROTEINS

Despite the fact that recently proteomes and types of posttranslational modifications, such as phosphorylation, have been actively investigated in different species of cyanobacteria, *Synechocystis* and *Nostoc* still remain the most studied among all.

PKN2 family in *Synechocystis* sp. PCC 6803. SpkA (Sll1574-Sll1575). SpkA is the first protein kinase of *Synechocystis*, for which kinase activity was demonstrated *in vitro* towards all “standard” exogenous substrates: MBP, H1, and Cas [6]. SpkA also demonstrated autophosphorylation ability. Deletion of *spkA* was manifested as the loss of cell motility, as well as increase of expression of the *pilA1–pilA2* and *pilA5–pilA6* operons encoding protein of pilus apparatuses and decrease of expression of the *pilA9–pilA10–pilA11–slr2016* operon [6, 112]. The researchers associated these exactly changes with the disruption of thick pili, structures participating in cell movements. Search for intracellular substrates of this protein kinase revealed SpkA-dependent phosphorylation of the proteins of membrane fraction with molecular weight 30 and 90 kDa. Presence of intensive phosphorylation signal in the low molecular weight region around 17 kDa in the mutant cells without addition of exogenous protein provided the basis for suggesting that activity of other STPKs depends on the presence of SpkA [6]. To date, no direct substrates of this protein kinase have been identified.

SpkB (Slr1697). The first data for SpkB were obtained for the recombinant protein [113]. Kinase activity of SpkB towards "standard" substrates without noticeable substrate specificity was demonstrated in the *in vitro* experiments. Similar to SpkA, SpkB also is capable of autophosphorylation, intensity of which depends on the presence of divalent ions in the reaction mixture. Presence of manganese, but not magnesium, enhanced autophosphorylation, no phosphorylation signal was observed on addition of calcium [113]. The study investigating insertion mutant of the *slr1697* gene revealed absence of motility during cultivation of the cells on a solid substrate. However, the data of transmission electron microscopy (TEM) did not show any changes in the pili structure. It was concluded based on these facts that SpkB is an important component in regulation of cell motility, but details of its functioning are still poorly understood.

The presence of four conserved Cys residues in the N-terminal domain of the SpkB molecules allowed to hypothesize possible redox regulation of its activity. Addition of reducers *in vitro* did not affect ability of this protein kinase to autophosphorylation [113]. At the same time, ability of the Cas substrate phosphorylation was suppressed after treatment with oxidizer ($CuCl_2$) and was restored in the presence of dithiothreitol [114]. The truncated form of the protein that did not contain Cys residues at the N-terminus was inactive, which indirectly supported the notion on dependence of the SpkB activity on the changes of redox status.

Comparison of phosphorylation profiles of wild type and SpkB mutant cell lysates showed the disappearance of the signal in the range corresponding to the molecular mass 90 kDa, as well reduced intensity of the bands in the regions of 70 and 150 kDa. Combination of 2DE, radioautography, and PMF-based identification allowed to determine the protein kinase substrate – glycyl-tRNA-synthetase subunit (GlyS). The physiological significance of this modification has not been elucidated yet [114].

Whole-proteome analysis of the SpkB mutant during cultivation under conditions of different levels of available carbon [82] allowed indirectly confirm association of SpkB with the cell motility. From 50 proteins that differ under conditions of high concentration of CO_2 (5%) in gas-air mixture, 4 proteins were from the group of pilin-like proteins (Slr2016, Slr1928, Slr1929, Slr2015), and one protein of cell surface – S-layer protein Sll1951. Contents of pilin proteins in the mutant cells changed in different directions: Slr2015, Slr2016 were present in the mutant at higher amounts, while two others – at lower amounts. At the same time, determination of the functions of the major part of quantitatively different proteins is still requires investigation. Absence of the functional SpkB protein kinase affected only slightly composition of the cellular proteins, and did not explain less intensive growth of the culture at low (0.04%) concentration of CO_2. Analysis of phosphoproteome allowed demonstrating absence or significant decreases of the level of protein phosphorylation in the SpkB mutant. No phosphorylation was detected in the glutathione S-transferase (Sll1545, Gst1) and Slr0483 (protein with unknown function), as well as it was significantly reduced in the carboxysome protein CcmM (Sll1031) and in the important regulatory protein PII (Ssl0707, GlnB). Phosphosites of the glutathione S-transferase (T266) and Slr0483 (T34 or T35) were detected during cultivation under different conditions exclusively in the wild type cells. This enabled the authors to hypothesize that they are substrates of SpkB. The protein PII (GlnB) is a master-regulator of many aspects of nitrogen and carbon metabolism (C/N balance, for example). Kinases that phosphorylate this protein still remain a mystery [115]. Hence, participation of SpkB in phosphorylation of the S49 residue in the PII protein with changing CO_2 concentration is especially important. It should be noted, nevertheless, that the authors observed residual phosphorylation of S49 even in the mutant cells, which implies existence of other kinase(s) targeting it as a substrate.

SpkC (Slr0599). Initially the SpkC protein was expressed and characterized in the *in vitro* experiments [43]. Similar to SpkB, SpkC was capable of autophosphorylation and also did not exhibit any specificity towards "standard" substrates. The first study dedicated to establishing role of this protein (which was then named PknE) in the cell was carried out with the mutant strain [116]. The mutant with inactivated *pknE* gene exhibited resistance to methylamine at concentration approximately one order of magnitude higher than the wild type, especially under conditions of low light. The protonated methylamine penetrates into cells through the same transport systems as ammonium ions [117]. In the cells methylamine is converted into γ-glutamylmethylamide in the reaction catalyzed by glutamine synthetase. It is not metabolized further, and is accumulated in the cells till toxic levels. In the process, no changes in ammonium transport and, respectively, methylamine transport, were observed in the mutant strain, as well as no changes in glutamine synthetase activity. Based on this, it was suggested that the observed resistance is, most likely, associated with functioning of a system transforming methylamine into a non-toxic product. Resistance to another toxic agent – L-methionine-D,L-sulfoximine – was also observed for the mutant strain. This glutamine analog irreversibly inhibits glutamine synthetase. Similarly to the first case, the system of glutamine transport was not affected. Particular role of SpkC in the mechanism of resistance of the *Synechocystis* cells has not been

established, but its participation in regulation of nitrogen metabolism was proposed [116].

This hypothesis was confirmed in the study by Spat et al. using wild type cells [3, 83]. In particular, it was shown that phosphorylation of the SpkC protein kinase itself depends on the changes in C/N balance. The level of phosphorylation decreased under conditions of nitrogen starvation. Four threonine phosphorylation sites have been identified in SpkC using methods of phosphoproteomics, including T291, T297, T312, and T319 [3].

In another study investigating the role of CO_2 level in the cell cultivation, significant increase of SpkC phosphorylation at the T304 and T312 residues was observed, which confirms involvement of SpkC in adaptation to the changes in carbon levels and possible regulation of the SpkC activity via phosphorylation [83]. The proteins were also identified with phosphorylation partially or entirely depended on the presence of SpkC in the cells. No phosphopeptides of the protein response-regulator in the composition of the two-component system (CopR, Slr6040), DnaJ-like protein (Sll1384), Slr1619 protein, as well as of the CmpB protein (Slr0041) as a part of the bicarbonate transporter BCT1 were detected in the mutant. The authors explained retardation of the mutant growth at low CO_2 concentration exactly by the changes in CmpB phosphorylation [83].

An important regulator of C/N balance – PII protein (GlnB) – is also phosphorylated by SpkC [83]. Hence, this protein is target of at least two protein kinases of *Synechcocystis*, SpkB and SpkC, which indicates direct participation of the PKN2-type kinases in regulation of response to the changes in C/N balance.

The protein kinase SpkC also participates in phosphorylation of the co-chaperone GroES [35]. The results of proteome studies identified phosphorylation at the T29 residue [82].

SpkD (Sll0776). Biochemical characterization of the recombinant SpkD protein [43] under *in vitro* conditions allowed establishing autocatalytic kinase activity of this protein. Preferable phosphorylation was observed in the series of the “standard” substrates: H1 ≥ MBP > Cas.

Functional role of the SpkD protein in the cell was investigated with the mutant strain. Differences in the growth rates at low level of inorganic carbon in the medium were demonstrated for the mutant, although activities of the high-affinity bicarbonate transport system were similar in both strains. Addition of organic carbon sources such as glucose, phosphoglyceraldehyde, and pyruvate did not stimulate mutant growth, while addition of metabolites of TCA cycle to the cultivation medium resulted in the growth restoration [118]. The hypothesis that SpkD is involved in coordinating the function of enzymes of the TCA cycle was confirmed by the 20%-decrease of 2-oxoglutarate (2OG) content in the mutant. 2OG is a key metabolite participating in coordination of C/N balance. It interacts with the PII protein, which is subjected to phosphorylation after this interaction [115]. In the process, decrease of the 2OG level in the mutant did not cause any changes in the level of PII phosphorylation, which, probably, indicates existence of alternative regulatory pathways [118].

SpkE (Slr1443). As has been mentioned above, initially SpkE expressed in a heterologous system was considered inactive due to replacement of amino acid residues in the active center [43]. However, changing of the expression system facilitated the production of an active enzyme *in vitro* [7]. It was shown that SpkE was not capable of autophosphorylation, but exhibited substrate specificity phosphorylating H1. Experiments investigating activity of this protein kinase at the level of cells demonstrates differences in phosphorylation profiles between the wild type cells and SpkE mutant cells.

Despite the fact that the target protein subjected to phosphorylation were not identified, it still provides indirect confirmation of the enzyme activity in the *Synechocystis* cells. In particular, the regulatory role of SpkE could be associated with the response of cells to cold stress. Similarity of transcription profiles between the SpkE and histidine kinase Hik33 mutant, which is a known sensor of cold stress [5], allows suggesting participation of SpkE in the regulatory response of the *Synechocystis* to cold stress as an additional element [7].

Possible involvement of SpkE in regulation of cyanobacteria motility was also demonstrated [119]. Deletion of the *spkE* resulted in the loss of thick pili mediating cell motility, but it did not change either the level of expression of the *pilA1* gene (main structural protein of thick pili), or its amount in the cell. The researchers associated the observed change of the PilA1 protein mass with the possibility of post-translational modification. The latter has not been confirmed experimentally. Nevertheless, it was established that in the absence of SpkE assembly of thick pili was disrupted, and the PilA1 protein was accumulated in the extracellular space [119].

SpkF (Slr1225). For the first time SpkF was obtained in the heterologous system as a protein of membrane fraction [43]. The researchers did not perform protein purification, but conducted phosphorylation reaction in the *E. coli* lysate after the induction. It was shown that in the presence of Mg-ATP the enzyme exhibited kinase activity, became autophosphorylated and modified “standard” substrates, while preferring MBP and H1 in comparison with Cas.

Screening of the collection of STPK mutants of *Synechocystis* using co-chaperonin GroES as a potential

protein target revealed the ability of SpkF to phosphorylate GroES. A cascade scheme was suggested in the pathway of phosphorylation signal transduction with participation of SpkF and two other protein kinases – SpkC and SpkK [35].

The data on the changes of the level of autophosphorylation of SpkF in response to fluctuations of the levels and C/N ratio in the cultivation medium were presented in the study dedicated to investigation of adaptation of *Synechocystis* to these changes [3, 82].

SpkG (Slr0152). It must be mentioned that up to now all attempts to express the SpkG kinase in *E. coli* failed, hence, its activity at the protein level in the *in vitro* system has not yet been confirmed. The main data for this enzyme have been obtained at the level of a whole organism.

Investigation of the SpkG functions and its potential role in signal transduction at the cellular level was first conducted using transcriptomics [120]. Initially the levels of expressions of four protein kinase genes (*spkC*, *spkD*, *spkF*, *spkG*) under a number of stress conditions were evaluated. The level of expression of the *spkG* gene increased significantly under the salt stress (0.68 M NaCl). Under these conditions the mutant exhibited slower growth in comparison with the wild type. Transcriptome analysis revealed differential expression of sixty genes in the SpkG mutant strain. Moreover, among the affected genes there were genes expression of which depended entirely of the presence of SpkG, as well as genes expression of which changed only partially. It was concluded based on these data that this protein kinase is an additional regulatory element in the response of cyanobacteria cells to salt stress [120].

Use of targeted proteomics (SRM) allowed to establish quantitative relationship between the SpkG and the ferredoxin 5 protein (Fd5). It is interesting to note that the Fd5 protein gene (*slr0148*) is located in the same operon (*slr0144-0152*) as the *spkG* (*slr0152*) gene [121]. Phosphorylation at the threonine residues T18 and T72 was demonstrated for Fd5, however, the involved kinase has not been identified [3, 14]. According to the mass spectrometry data the level of phosphorylation of T18 was significantly decreased in the SpkG mutant in comparison with the wild type, and phosphorylation at the T72 residue was absent [121]. These data confirm that Fd5 is one of the protein targets for SpkG, but existence of other protein kinases also modifying the T18 residue also cannot be ruled out.

Group of ABC1K in *Synechocystis* sp. PCC 6803. Despite the long time since identification of potential STPK genes of cyanobacteria and publication of numerous studies devoted to kinases of the PKN2-type, the group of ABC1K kinases for the most part remains *terra incognita*. Only few studies have been dedicated to these kinases, and they have been assigned most often to the proteins with unknown function in the Uniprot data base, or even as potential proteins.

SpkK (Slr1919). The first protein kinase of the ABC1K group in *Synechocystis* that attracted attention of the researchers was SpkK. It was established in 2011 that this enzyme together with two other proteins of the PKN2 group (SpkC and SpkF) is involved in the response to heat shock [35]. Several proteins have been identified with the help of protein separation in 2DE followed by MALDI-TOF analysis, phosphorylation of which changed in the wild type cells in response to increased temperature. Among those was the co-chaperonin GroES. Hence, this protein was used as an exogenous substrate in the *in vitro* reaction with the proteins from the cells of the STPK gene mutants (SpkB-SpkL). Comparison of phosphorylation profiles and the data of complementation assay allowed establishing that the protein kinases of the ABC1K-type (SpkK) and PKN2-type (SpkC and SpkF) are involved in modification of GroES in the cyanobacteria cells. This made it possible for the first time to establish the functional interplay between different STPKs and the cascade nature of their functioning [35].

At present, the fact of co-chaperonin GroES phosphorylation has been confirmed, and the modified residue, T29, was identified [3, 82, 83].

SpkI (Sll1770). The SpkI protein kinase was characterized at the level of organism in the experiments conducted with knockout insertion mutant and the complementation strain [1]. The main physiological differences were observed in the growth parameters and efficiency of photosynthesis under conditions of high sodium chloride concentrations. Under normal conditions, the mutant exhibited almost the same growth rate as the wild type and the complementation strain. However, its growth rate at high salt concentration (0.6 M in the case of solid medium and 0.9 M in the liquid medium) was lower. Furthermore, under stress conditions significant decrease of the maximum quantum yield of photosystem II (PSII) was observed in the mutant in comparison with the wild type and the complementation strain, as well as decrease of the netto-photosynthesis, activity of PSII, and increase of respiration. Mutation in the *sll1770* gene also resulted in the change of the content of the main proteins of photosystems I and II under stress conditions [1].

The obtained data allowed concluding that SpkI is required for coordination of functioning of both photosystems and high efficiency of photosynthesis, which is most important during adaptation under salt stress conditions.

SpkH (Sll0005). The last characterized protein kinase of the ABC1K-group is protein kinase SpkH. Its activity was established under *in vitro* conditions [37]. The only one “standard” substrate phosphorylated

by SpkH was Cas. Intensity of the signal on the autoradiograph increased with the increase of the content of manganese ions in the reaction mixture. The recombinant protein was shown to exist in solution in the form of tetramer.

At present, the SpkH protein kinase is the only one STPK of *Synechocystis*, for which phosphorylation site has been established – XXpSXE. Based on biochemical properties of the SpkH (amino acid sequence of the phosphorylation motif (pSXE), oligomerization of the protein in solution (tetramer)), the authors assigned the SpkH to the representatives of the group of casein kinases (CK). The main effectors were tested, which were capable of affecting activity of various CKs: heparin, spermine, staurosporine, DNA, and RNA. Protein kinase activity of SpkH was inhibited by spermine and heparin, but did not change in the presence of inhibitor of the majority of protein kinases – staurosporine. These data indicate that the protein kinase SpkH of *Synechocystis* is functionally close to one of CK, the "genuine" CK Fam20C, in particular.

Protein kinases of *Nostoc (Anabaena)* sp. PCC 7120. The single-cell cyanobacterium *Synechocystis* is quite convenient object for a wide range of investigation. The filament-forming, nitrogen-fixing cyanobacterium *Nostoc* is a more complex system. It is likely, that the mechanisms of adaptation to the environmental changes correlates with the genome size of cyanobacteria and number of genes encoding elements of signaling systems. In particular, 12 STPK were annotated in the *Synechocystis* genome, while 52 STPK genes were identified in *Nostoc* [29].

Nostoc is especially interesting from the point of view of signal transduction features both at the level of a single cell, as well as cell–cell communication including regulation of heterocyst formation for atmospheric nitrogen fixation. Anaerobic conditions are needed for this process to occur, which require deep morphological, physiological, and biochemical rearrangement of cells [122].

Formation of heterocysts is observed 24 h after removal of inorganic forms of nitrogen from the nutrient medium, which are distributed along the filament at the distance of 10-20 vegetative cells. Such type of organization is retained during the following growth of cells by division using nitrogen from atmosphere. At the later stages of heterocyst formation, synthesis of nitrogenase is initiated, which is responsible to nitrogen fixation. Specific structure of heterocyst envelope that is composed of certain glycolipids and polysaccharide layer create anaerobic conditions required for nitrogenase functioning [123].

It must be mentioned that the studies investigating protein kinases of *Nostoc* started already in the pre-genomics era. The search and selection of the study objects was carried out exclusively based on the search for homologs of protein kinases from even non-related organisms [4, 124, 125].

PknA (Alr4366). The first STPKs in cyanobacteria were found exactly in *Nostoc* in 1993 [126]. With the help of Southern-blotting, presence of a whole group of genes was detected in the cyanobacterium genome that have nucleotide sequence similar to eukaryotic protein kinases.

PknA (Alr4366) was the first described protein kinase among them. It was shown that the *pknA* gene is expressed during cultivation under normal conditions. Transfer of the culture to the conditions of nitrogen starvation results in enhanced expression of this gene in the initial steps of starvation (2.5-5 h), which is followed by its gradual decrease. Mutation of the protein kinase gene was not lethal both in the normal medium and in the medium without nitrogen. However, phenotype of the mutant cells was significantly different in comparison with the wild type. Under the standard conditions the cells were smaller, the filaments formed aggregates, which sedimented even with moderate shaking. Heterocysts were formed after transfer to the nitrogen-free medium, but the process was delayed in time, and heterocysts were formed with less frequency along the entire length of the filament [126].

Hence, it has been shown that PknA (Alr4366) participates in regulation of growth processes and is required for formation of heterocysts. However, up to now there are no data available confirming its kinase activity and no putative intracellular substrates have been suggested.

PknC (All4813). PknC is the first STPK of *Nostoc* that was expressed, purified, and characterized *in vitro* [127]. All enzymes described previously, PknA, PknD, PknE, were investigated at the level of organism using mutant strains. This protein kinase exhibited ability to autophosphorylation at threonine residue, which depended on the presence of Lys41 and Lys44 residues in the subdomain II. The truncated protein forms containing, for example, only kinase domain, did not have enzymatic activity. Considering that PknC has extended unstructured C-terminus, it is most likely that this part of the molecule is subjected to autophosphorylation and regulates its activity.

Several substrates have been tested in the *in vitro* experiments. It has been reported that among the standard exogenous substrates PknC is capable of phosphorylating Cas. In addition, the recombinant protein PII was also tested as a substrate, however, presence of histidine tag resulted in the false-positive signal, PknC phosphorylated serine in the linker domain [127].

No other data have been obtained for this protein so far. There have not been any studies investigating its functions in the cell.

PknD (Alr4368). Protein kinase PknD was discovered via the same approach as in the case of PknA [4]. The *pknD* gene was characterized with stable level of expression independent on the presence of nitrogen in the growth medium or forms of inorganic nitrogen (ammonium or nitrate). The obtained insertion mutant phenotypically did not differ from the wild type, did form normal heterocyst with the same distribution along the filament length. Cultivation of the mutant in the presence of inorganic nitrogen in the medium revealed growth rate similar to that of the wild type, however, nitrogen deficit resulted in the 5-fold decrease of the growth rate. At the same time, inactivation of the *pknD* gene did not cause any changes in activity of nitrogenase under diazotrophic conditions, i.e., the cells did not lose the ability for fixation of atmospheric nitrogen. Evaluation of phosphorylation level of the regulatory protein PII showed accumulation of the highly modified protein forms. Hence, the cells were affected by the nitrogen deficit even with maintaining the ability for heterocyst formation and preservation of nitrogenase activity. It is likely that the protein kinase PknD, which is required for normal growth of cyanobacteria under condition of nitrogen fixation, also participates in the transport of the products of nitrogen metabolism between heterocysts and vegetative cells [4]. This hypothesis requires further validation.

PknE (Alr3732). According to the transcriptome data, expression of the *pknE* (*alr3732*) gene is induced 8 h after introduction of mineral nitrogen deficit [128]. Amount of the protein, on the contrary, decreases after 3 h, and is restored to the initial level after this [124].

The PknE insertion mutant was viable under normal conditions and its growth parameters did not differ from the wild type. Under conditions of nitrogen starvation, the mutant growth slowed down after 4 days. The mutant strain has normal distribution pattern of heterocysts, but their structure was changed [124]. Prolonged cultivation resulted in filament defragmentation followed by the cell death. Different results were reported in the study from other researchers: the obtained mutant has slightly increased frequency of heterocyst formation, although they have normal structure [129]. In this case, overexpression of the PknE protein kinase resulted in disappearance of heterocysts even under conditions of nitrogen starvation.

In addition to the changes in filament structure, activity of nitrogenase also decreased in the mutant cells [124]. This effect was preserved independently on the cultivation conditions, but was more pronounced under anaerobic conditions. This phenomenon could be due to the changes in the composition of heterocyst envelopes, however, composition of glycolipids in the mutant cells did not differ from the wild type strain.

Expression of the *pknE* gene requires presence of the 118-bp-long cis-regulatory element located in the promoter region, which contains a potential binding site for the product of the *hetR* gene (one of the main transcription regulators in cyanobacteria cells required for transition of the cells to heterocysts formation) [129]. In the early stages of heterocyst formation binding of HetR induces expression of the *pknE* (*alr3732*) gene, and in the later stages of differentiation the increased amount of the gene product and/or its activity results in inhibition of the transcription regulator [129].

Kinase activity of PknE, its potential targets, and its role in the process of heterocyst formation has not been investigated yet.

PknH (Alr1336). Nitrogen starvation for 8 days resulted in the induction of the *alr1336* (*pknH*) gene, expression of which reached maximum 24 h after stress initiation [130]. The PknH protein was detected 3 h after the stress initiation, and after 24 h its amount reached the maximum. Moreover, accumulation of the protein occurred not in all cell, but only in heterocysts. The name assigned to this putative at that time enzyme was associated with this fact – Protein KiNase expressed in Heterocysts (PknH).

The insertion mutant exhibited normal growth in the nitrogen-containing medium, but the growth was significantly hindered under starvation conditions [130]. Microscopic examination and specific staining of polysaccharides conducted after 24 h starvation revealed normal formation of the structures. Expression of the main genes involved in formation of heterocysts was not changed in the mutant. Activity of nitrogenase was also not changed in the mutant strain. However, visually the filaments were shorter than in the wild type strain, and heterocysts were separating from the vegetative cells. Analysis of the complementation strain phenotype confirm association of the observed changes with the presence of this protein. The researched concluded at that time that the protein kinase PknH is involved in maintenance of contacts of heterocysts with vegetative cells, but not in their formation [130].

This study was continued several years later [131]. It was established in the study that formation of shortened filaments in the PknH mutant is most likely associated with the cultivation conditions (intensity of barbotages), rather than with a specific effect of the mutation. Fluorescent microscopy of the cells starving for a long time demonstrated that the *pknH* mutation resulted in disruption of the pattern of heterocyst formation along the filament. Their formation started in the neighboring cells and their frequency increased significantly. Complementation of the gene restored normal realization of the process. Analysis of phenotypes of the cells with point mutation of aspartic

acid in the DFG-motif showed its conservatism in composition of the PknH active center. Hence, this provided indirect confirmation of protein kinase activity of PknH [131].

Similarly to the case of *pknE* (see above), dependence of the transcription induction of the *pknH* gene on the protein regulator HetR was demonstrated [130]. However, there was no HetR-binding sequence in the promoter region of the *pknH* gene. Hence, it was suggested that expression of the *pknH* gene is controlled by some additional transcription factors, which, in turn, is regulated by HetR. It is noteworthy that PknH reciprocally regulates *hetR*. Both genes are actively expressed in the early stages of nitrogen starvation in the differentiating cells. It has been suggested that only the cells with the PknH/HetR balance shifted towards accumulation of HetR normally differentiate into heterocysts, while vegetative cells are formed from the cells with the PknH/HetR balance shifted towards PknH. However, this hypothesis requires further investigation and experimental confirmation.

Pkn22 (Alr2502, PbH1). The role of Pkn22 (Alr2502, PbH1) in *Nostoc* was investigated in the mutant strain under conditions of iron deficiency in the medium and oxidative stress [132]. Expression of the *alr2502* gene increased significantly under conditions of oxidative stress (induced by addition of methyl viologen) after 15 min. This allowed suggesting participation of Pkn22 in recognition and/or response to these stress factors. The mutant demonstrated comparable growth and phenotype to the wild-type strain under standard conditions. However, growth rate of the mutant was much slower under conditions of iron deficiency [132].

Analysis of transcriptome under conditions of iron deficiency revealed that there practically was no induction of the *isiA* gene. This gene encodes the chlorophyll-binding protein CP43, which could function as an additional antenna complex for the photosystem I (PSI), as well as participate in the protection of the photosynthetic apparatus against photoinhibition [133]. Expression of the *isiA* gene was restored in the mutant only after 24 h starvation, which is likely associated with initiation of adaptation process regulated by other signaling systems. In the process, the induction of another gene normally induced under conditions of iron deficiency – *isiB*, did not depend on the presence of the protein kinase gene.

Accumulation of the IsiA protein is an important stage of adaptation of the cells to the conditions of iron starvation. Comparison of the fluorescence spectra of intact cells at 77 K demonstrated absence of the characteristic peak in the cells of Pkn22 mutant, which indicates its participation in the cell response to iron deficiency [132, 134].

Considering that reactive oxygen species are universal triggers of the stress response in cyanobacteria [135], the induction of Pkn22 in the presence of methyl viologen that initiates oxidative stress, suggested its involvement in the cell response to several types of stress. Presence of binding sites of such transcription factors as FurA and NtcA in the promoter region of the *alr2502* gene, which was demonstrated *in vitro*, confirmed the role of Pkn22 as an intersection point in the response to different stress factors [136].

Transcriptome analysis of the *alr2502* mutant revealed significant changes in the expression of 26 genes during oxidative stress caused by treatment with hydrogen peroxide, and changes in expression of more than 100 genes during nitrogen starvation. Remarkably, the spectra of induced by different stress stimuli genes practically did not overlap, which indicated independent nature of recognition and/or signal transduction mediated by this protein kinase [136].

Later the role of Pkn22 in response to nitrogen starvation was investigated in more details [137]. The mutant was not capable of growth under starvation conditions, because the process of heterocyst formation was disrupted, and their formation started later, and their number was significantly lower than in the wild type strain. Importance of the Pkn22 functioning as protein kinase was also demonstrated. Point mutation of the K63 residue in the catalytic center of the protein resulted in the same effects as the deletion of entire gene.

It was shown using yeast two-hybrid system that Pkn22 interacts with the transcription factor HetR. Moreover, based on the phosphoproteomics data, it was established that Pkn22 phosphorylates HetR at the residue S130. Point substitutions of this amino acid with alanine resulted in inability of the strain to form heterocysts, and substitution with aspartic acid resulted in formation twice as much heterocysts. Hence, the role of Pkn22 as an active protein kinase was demonstrated both in *in vitro*, and in *in vivo* experiments [137].

Remarkably, mutation of the protein kinase gene results in the delay of transition of vegetative cells of cyanobacterial into heterocysts, and increase of the level of transcription factor HetR is capable to restore the normal process. Hence, it could be assumed that there are other protein kinases capable of modifying this protein.

HepS (All2760). Screening of the library of *Nostoc* mutants grown in the medium with inorganic nitrogen, but dying during cultivation in the nitrogen-free medium, resulted in the discovery of previously unknown Fox genes (nitrogen Fixation in the presence of OXygen), one of which was the *hepS* (*all2760*) gene [138]. Non-viability was associated with the absence of the wholesome heterocysts and inability to realize nitrogen fixation. Disruption of the process of heterocyst formation was associated with the

absence of the polysaccharide Hep layer with preservation of the glycolipid Hgl layer, which was confirmed at the level of morphology (TEM), synthesis glycolipids (TLC), and polysaccharides (staining of the cell with alcian stain) [138]. At the same time, mineral nitrogen deficiency for 14 h did not lead to induction of glycolipid layer synthesis genes in the all2760 mutant, which could be explained by regulation at the level of activity of the proteins of its biosynthesis [139].

It should be mentioned that the data on the effect of HepS on gene expression indicate its putative regulatory function in the cell during transition to nitrogen fixation. However, these data do not confirm its role as protein kinase, and its protein targets has not been identified yet.

HstK (Alr2258). HstK is the first from the investigated protein kinases in cyanobacteria assigned to the group of "hybrid" protein kinases [50]. Features of their organization have been described above. It should be mentioned that protein kinases of the similar structures have been detected also in the fungus kingdom [140].

The protein kinase HstK was detected accidently in the course of investigation of another protein from the same family (PknB), because both genes are located in close vicinity to each other on the chromosome [51]. HstK and PknB were expressed in the heterologous system and their ability to autophosphorylation was demonstrated. Due to the complex organization of HstK its properties as protein kinase were investigated using not the full-size protein, but only its STPK domain. In this study the method of complementation was used: PknB and kinase domain of HstK expressed in *Saccharomyces cerevisiae* were able to restore functioning of the Hog1p signaling pathway, activation of which is required for the response to osmotic shock in yeasts [141]. In this way their functionality was demonstrated as well as the possibility to participate in signal transduction in eukaryotic cells. Functionality of the second kinase domain has not been shown yet.

Pkn41 (Alr0709) and Pkn42 (Alr0710). Two genes of protein kinases *alr0709* and *alr0710* also belonging to the HstK family are adjacent along the chromosome and co-transcribed. Their expression does not depend on the form of nitrogen present in the medium during cultivation. However, strong induction of both genes is observed in response to iron deficiency in the nutrient medium exclusively in the presence of ammonium. It was shown that their induction is regulated through binding of the transcription factor NtcA with the promoter region of the *pkn41* gene [142].

The strains mutated in both these genes were phenotypically similar to the wild type under all cultivation conditions. The only exclusion were the experiments with combination of two factors – iron deficiency and absence of mineral nitrogen in the medium, which led to the growth slowdown. The mutants were characterized with the reduced level of iron accumulation under normal growth conditions and induction of the genes associated with the response to iron starvation (*nifJ1* and *nifJ2*) [142]. Obviously, further and more detailed studies are needed to confirm protein kinase activity of Pkn41 and Pkn42, as well as biochemical mechanism of their action.

Pkn30 (All3691) and Pkn44 (All1625). Another example of HstK protein kinases in *Nostoc* are proteins Pkn30 and Pkn44 [143]. Deletion of each of these genes did not result in noticeable changes in phenotype. The double mutant was characterized with disruption of growth under diazotrophic conditions. Detailed microscopic examination showed that the mutant belongs to the Hgl^- Hep^+ type, i.e., the mutant is lacking glycolipid layer, but the polysaccharide layer is retained. Thin layer chromatography confirmed presence of only one of the glycolipids specific for heterocysts – minor glycolipid HGL2. Such global morphological changes naturally affect permeability of oxygen across the heterocyst envelope, which inhibits activity of nitrogenase. However, the mechanism of interaction between these protein kinases, which could provide a rationale for the observed effects, has yet to be elucidated.

CONCLUSIONS

The cited studies on investigation of STPKs in cyanobacteria *Synechocystis* and *Nostoc* demonstrate versatility of approaches and methods used for their investigation. However, it must be mentioned that the available data are in general fragmentary and reflect only separate aspects of their structure, physicochemical parameters, their role in the cells, and their interaction with other regulatory systems. The omics-based methods allow deeper understanding of the signal transduction pathways in the live cell and to obtain new information on mechanisms of regulation.

The following aspects remain poorly understood: conserved phosphorylation motifs for each of the kinases, effect of phosphorylation of the kinases themselves on their activity, localization of the kinases in the cell, existence of their regulatory proteins, as well as role of non-catalytic domains. These and a number of other questions are far away from being resolved and require further investigation by researchers.

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