Proteomic Analysis of Plasma Membranes of Cyanobacterium Synechocystis sp. Strain PCC 6803 in Response to High pH Stress

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Cyanobacteria are unique prokaryotes possessing plasma-, outer- and thylakoid membranes. The plasma membrane of a cyanobacterial cell serves as a crucial barrier against its environment and is essential for biogenesis of cyanobacterial photosystems. Previously, we have identified 79 different proteins in the plasma membrane of Synechocystis sp. Strain PCC 6803 based on 2D- and 1D- gels and MALDI-TOF MS. In this work, we have performed a proteomic study screening for high-pH-stress proteins in Synechocystis. 2-D gel profiles of plasma membranes isolated from both control and high pH-treated cells were constructed and compared quantitatively based on different protein staining methods including DIGE analysis. A total of 55 differentially expressed protein spots were identified using MALDI-TOF MS and MALDI-TOF/TOF MS, corresponding to 39 gene products. Twenty-five proteins were enhanced/induced and 14 reduced by high pH. One-third of the enhanced/induced proteins were transport and binding proteins of ABC transporters including 3 phosphate transport proteins. Other proteins include MinD involved in cell division, Cya2 in signaling and proteins involved in photosynthesis and respiration. Furthermore, among these proteins regulated by high pH, eight were found to be hypothetical proteins. Functional significance of the high-pH-stress proteins is discussed integrating current knowledge on cyanobacterial cell physiology.

Keywords: Synechocystis sp. strain PCC 6803 • high-pH-stress • plasma membrane • subcellular proteomics • two-dimensional electrophoresis • MALDI-TOF • MALDI-TOF-TOF

Introduction

Cyanobacteria are ancient and the only prokaryotic organisms carrying out oxygenic photosynthesis. During their long evolution, cyanobacteria have adapted successfully to all photic habitats with a remarkable capacity to acclimate to a variety of environmental conditions. Today cyanobacteria are abundant constituting the largest group of prokaryotes widely distributed and frequently dominant in adverse environments such as high pH and high salinity.1–3 It is intriguing how cyanobacteria have survived in the harsh environments, and what adaptation mechanisms have evolved in the cyanobacteria coping with the adverse growth conditions. With regards to the latter, unicellular cyanobacterium Synechocystis sp. strain PCC 6803 (henceforth referred to as Synechocystis) has been an attractive model organism due to its distinct characteristics4–6 as well as the following aspects: (i) it represents cyanobacteria with moderate halophilic and alkali-tolerance, (ii) the stress responses can be examined at the subcellular compartments based on the well-established separation methods,7–10 and (iii) new insights can be obtained using global approaches such as proteomics and DNA-microarray supported by the sequenced genome.11 Indeed, the findings based on transcriptomic and proteomic profiling in response to salt stress have improved our understanding of cell acclimation in Synechocystis at both genetic and proteomic level substantially.9,12–16 In contrast, our current knowledge of this model organism in response to high pH stress condition remains modest. Most recently, it was reported that in Synechocystis hundreds of genes were up-regulated above 1.5-fold upon transfer of cells from pH 7.5 to 10.2 At the protein level, however, experimental data are still scarce. Compared to the high number of high pH-regulated genes, only dozens of lower and higher pH stress protein spots were detected in Synechocystis.17 The question arises how close the transcription and translation are correlated in terms of the specific cellular networks.
The plasma membrane of a cyanobacterial cell is crucial as a barrier against the ambient medium. It is also an energy-transducing membrane as well as essential for biogenesis of cyanobacterial photosystems\(^\text{18}\) and the thylakoid endomembrane system.\(^\text{19}\) The presence of the photosynthetic thylakoid membrane distinguishes cyanobacteria from other Gram-negative bacteria such as \textit{Escherichia coli}, and complexes the mechanisms to maintain pH homeostasis under high external pH. In \textit{Synechocystis}, several pH-sensitive mutants bearing mutations in PSII have been identified.\(^\text{20,21}\) These demonstrate mechanisms to maintain pH homeostasis under high external pH. For the growing cells (OD\(_{730}\)), the time of cultivation, and the measurements were performed at 1000°C. The membrane distinguishes cyanobacteria from other Gram-negative bacteria such as \textit{Escherichia coli}, and complexes the mechanisms to maintain pH homeostasis under high external pH. In \textit{Synechocystis}, several pH-sensitive mutants bearing mutations in PSII have been identified.\(^\text{20,21}\) These demonstrate mechanisms to maintain pH homeostasis under high external pH.

Proteomic analysis is a powerful tool to study stress responses. With the use of the proteomic approach, we have earlier studied the effect of salt stress on plasma membrane proteins in \textit{Synechocystis} and identified 25 different proteins that significantly changed in abundance under salt stress conditions.\(^\text{14}\) In the present work, we have investigated the cell growth profiles of \textit{Synechocystis} under high pH stress followed by a comparative proteomic analysis of cells grown at optimal pH and high pH that represses cell growth. With the use of completely purified plasma membranes from \textit{Synechocystis}, we have examined the changes in protein expression profiles based on 2D-gels stained with different protein staining methods including Dige. With the use of MALDI-TOF and MALDI-TOF/TOF-MS coupled with database search, we have identified 39 proteins that changed in abundance after shifting cells from pH 7.5 to 11.0. We found that transport proteins are dominant among the high pH stress-enhanced proteins identified. Other proteins include MinD involved in cell division, PsaF and CoxB involved in photosynthesis and respiration, as well as several regulatory proteins. Furthermore, we found that 8 hypothetical proteins with unknown function were expressed and their levels were regulated by external pH.

Materials and Methods

**Chemicals and Reagents.** CHAPS, DTT, IPG buffer (pH 3–10), IPG DryTrij, Iodoacetamide, Thiourea and Fluorescent dye Cy3 and Cy5 are from GE Healthcare (GE Healthcare, Piscataway, NJ). Urea and sequencing-grade modified trypsin were obtained from Promega (Madison, WI), ASB-14, PEG 3350, β-DM (n-dodecyl-β-D-maltoside), CBB G250 were from Sigma (St Louis, MO). All other chemicals and reagents used in the study were of analytical grade unless indicated otherwise. Deionized water produced by a Milli-Q system (Millipore, Billerica, MA) was used for all buffers.

**Strain, Culture Condition, and High-pH Stress Treatment.** Cells of \textit{Synechocystis} sp. PCC 6803 were grown phototrophically in AA/8 medium (5 mM KNO\(_3\) contained) buffered with 25 mM HEPES (pH 7.5) at 30°C under 60 μE·m\(^{-2}\)·s\(^{-1}\) of continuous cool white fluorescent light. Liquid cultures were grown with vigorous bubbling with air and this was used for all experiments. The pH of medium was measured using Sartorius PB-10 pH meter (Goettingen, Germany) and adjusted to the desired pH with KOH or HCl, that is, pH 6.0, 7.5, 11.0 (buffered with 25 mM Bis-Tris, 25 mM HEPES and 25 mM CAPS, respectively). Growth was measured as OD\(_{730}\) at different time of cultivation, and the measurements were performed at least 3 times. For proteomic and RT-PCR analysis, exponentially growing cells (OD\(_{730}\) = 0.9–1.0) were collected by centrifuging at 1000g for 5 min, and resuspended in fresh pH 7.5 (control) and pH 11.0 (high pH) media at the same initial cell density. Cells were harvested by centrifuging at 5000g and 4°C for 5 min after 2 and 24 h of cultivation. Cell pellets were washed once with 20 mM potassium phosphate buffer (pH 7.8) and were immediately frozen at −70°C.

**Preparation of Plasma Membrane Fractions.** Frozen cells from 2 L of culture were used for independent membrane preparations. Pure plasma membranes from cells grown under both control (pH 7.5) and high pH (pH 11.0) conditions were isolated by a combination of sucrose density centrifugation followed by two-phase partitioning as described by Norling et al.\(^\text{7}\) with minor modifications. Cells were broken with glass beads (diameter 0.17–0.18 mm, Sigma). Cell debris was removed by centrifugation at 3500g and 4°C for 15 min. Total membranes were collected by centrifugation at 203 000g for 60 min. Separation of membranes by sucrose density centrifugation was performed using the method as described previously.\(^\text{7,22}\) The two fractions from 10 to 30% and from 38 to 42% sucrose were collected separately and diluted at least 3-fold with 20 mM potassium-phosphate buffer (pH 7.8) followed by a centrifugation at 203 000g for 60 min at 4°C. The pellets membranes were resuspended in 5 mM potassium phosphate buffer (pH 7.8) supplemented with 0.25 M sucrose and the plasma membrane was isolated by aqueous two-phase partitioning.\(^\text{7,22}\) The plasma membranes were extracted with 0.1 M sodium carbonate on ice for 40 min.\(^\text{23}\) The pellet that resulted after the centrifugation was washed twice with 40 mM Tris to remove excess sodium carbonate. The final pellet of the membrane was resuspended in the buffer with 0.25 mM sucrose and 5 mM potassium phosphate (pH 7.8). To prevent protein degradation, 1 mM PMSF (Amresco) was added during membrane preparations and subsequent storage of the membranes. Protein concentration was determined according to Peterson\(^\text{24}\) using BSA as a standard. The purity of the plasma membrane was verified by immunoblot analysis described by Norling et al.\(^\text{7}\) using antibodies against CP43 and NrtA. The purified membranes were stored at −70°C until use.

**Protein Separation by 2-DE.** 2-DE of plasma membrane proteins was performed as described previously.\(^\text{22}\) For analytic and preparative gels, 100 μg and 1.3 mg of plasma membrane proteins were used, respectively. Plasma membrane proteins were precipitated with methanol/chloroform according to the method described by Wessel et al.\(^\text{25}\) The protein precipitate was solubilized in 350 μL of an electrofocusing solution containing 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 1% (w/v) β-DM, 4% (w/v) CHAPS, 50 mM DTT, and 1% (v/v) IPG buffer (pH 3–10). The mixture was incubated at room temperature for 1 h and then sonicated in the presence of PMSF. After centrifugation at 10 000g for 10 min, the supernatant was applied onto a linear 18 cm IPG strip (pH 4–7). Rehydration loading and IEF were performed as described by Huang et al.\(^\text{22}\) The IEF was performed at 20°C and the running program was 300 V for 40 min, 500 V for 40 min, 1000 V for 1 h, and 7000 V until a total of 120 000 Vh was reached. Following the two-step equilibration, the second-dimension separation was carried out on 12.5% SDS-PAGE gel with Protean II xi cell (Bio-Rad) running at 20 mA/gel. Proteins were detected by silver nitrate as described\(^\text{22}\) or CBB G-250.

**Image Analysis of 2-D Gels.** 2-D gels were scanned using a UMAX PowerLook 2100XL scanner (Willich, Germany) at a resolution of 600dpi. 2-D gel image analysis software ImageMaster 2D Platinum (version 5.0, GE Healthcare) was used for gel to gel matching, identification of differences in spot
intensities between the control and high pH-treated samples, and spot quantification. Protein spots were detected automatically and further edited manually to eliminate errors. Only images for protein patterns from control and high pH-treated cells were compared, which were obtained in the same experimental set. Gel images were normalized using total density method for a maximum correction of the overall image differences caused by minor experimental variations in protein loading and staining. Protein quantification was repeated at least two times for each type of sample.

For DIGE analysis, pairs of Cy3- and Cy5-labeled protein samples were mixed together. The DIGE sample buffer (7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 1% (w/v) β-DM, 4% (w/v) CHAPS, 50 µM DTT, 30 mM Tris, and 1% (v/v) IPG buffer (pH 3–10) was added to bring the volume to 350 µL and the samples were then applied to 18 cm IPG strips (pH 4–7). IEF and SDS-PAGE were carried out as described above. The gels were scanned using a Typhoon 9400 image scanner (GE Healthcare) according to manufacturer’s recommendation. The DIGE images were analyzed using DeCyder v6.5 software (GE Healthcare) as described in the user manual. Protein spots with an expression level greater than 1.5-fold were defined as being differentially expressed.

Protein Identification by Mass Spectrometry. MALDI-TOF and MALDI-TOF/TOF analysis was performed on a mass spectrometry instrument Ultraflex III from BrukerDaltonics (Bremen, Germany). Excision of protein spots and sample preparation for MALDI-TOF analysis was done as described by Huang et al.8 For acquisition of mass spectra, 0.5 µL samples were spotted onto a MALDI plate, followed by 0.5 µL of matrix solution (4 mg/mL α-cyano-4-hydroxycinnamic acid in 35% acetonitrile (ACN) and 1% TFA). Mass data acquisitions were piloted by flexcontrol Software v3.0 using batched-processing and automatic switching between MS and MS/MS modes. All MS survey scan were acquired over the mass range 700–5500 m/z in the reflectron positive-ion mode and accumulated from 2000 laser shots with acceleration of 23 kV. The MS spectra were externally calibrated using PeptideCalibStandard II (BrukerDaltonics) ([M + H]+ = 1046.542, [M + H]2+ = 1296.685, [M + H]3+ = 1347.735, [M + H]+ = 1619.822, [M + H]+ = 2093.086, [M + H]+ = 2465.198, [M + H]+ = 3147.471) and resulted in mass errors of less than 50 ppm. The MS peaks were detected on minimum S/N ratio ≥ 20 and cluster area S/N threshold ≥ 25 without smoothing and raw spectrum filtering. Peptide precursor ions corresponding to contaminants including keratin and the trypsin autolytic products were excluded in a mass tolerance of ±0.2 Da. The filtered precursor ions with a user-defined threshold (S/N ratio ≥ 50) were selected for the MS/MS scan.

Fragmentation of precursor ions was performed using LIFT positive mode. MS/MS spectra were accumulated from 4000 laser shots. The MS/MS peaks were detected on minimum S/N ratio ≥ 3 and cluster area S/N threshold ≥ 15 with smoothing. Mass spectra were evaluated using FlexAnalysis software and were subjected to Mascot Searching engine for protein identification. Database searching was performed using Mascot (http://www.matrixscience.com) or MS-Fit (http://prosector.ucsf.edu) using the NCBI database. Search parameters allowed for mass accuracy of ±50 ppm, one mis cleavage of trypsin, oxidation of methionine and carbamidomethylation of cysteine. Proteins were identified as the highest ranking results deduced by searching in the databases of NCBI nr20071010 against all species.

The presence of putative signal peptides and their cleavage sites were predicted using the SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/) and the Lipop 1.0 server (http://www.cbs.dtu.dk/services/LipoP/). The transmembrane helices were predicted using the TMHMM2.0 server (http://www.cbs.dtu.dk/services/TMHMM).

Total RNA Isolation and RT-PCR. Total RNA was isolated from Synechocystis 6803 cells using Redzol reagent and Si-MaxTM membrane spin columns (SBS Genetech, China) using the protocol suggested by the manufacturer. Residual DNA in RNA preparations was eliminated by digestion with RNase-free DNase I (Takara). Semiquantitative RT-PCR was carried out as previously described.27,28 Reverse transcription reactions using random primers were performed with M-MLV RT (Takara). To detect possible DNA contamination, control reactions were performed without RT but with Taq DNA polymerase. Reverse transcription products were amplified by PCR and analyzed by electrophoresis on 1.2% (w/v) agarose gels. The transcripts were amplified using the forward and reverse primers specific for the genes of interest, sll0684-sphX (accession no. NP442272); sll0684-pslB (accession no. NP442267); sll0683-pslB (accession no. NP442268); sl1699 (accession no. NP441060); str1295-sufl (accession no. NP440162); sl1450-nrta (accession no. NP440812); slr0074-ycf24 (accession no. NP442472). The primer sequences were listed in Supporting Information Table 3. The rnpA was used as a positive control.29 The log phase of RT-PCR was determined by measuring the amounts of PCR products at different PCR cycles.

Results and Discussion

Growth Response to High-pH Stress. It is known that, similar to the shift to an acid environment, a shift to an alkaline environment is stressful for bacterial such as E. coli.30–32 In order to investigate how cyanobacterium Synechocystis respond to high pH stress, growth profiles of Synechocystis were initially monitored under different external pH. Since Synechocystis is cultivated at media pH 7.5 in most experimental studies, this pH value (pH 7.5) was chosen as control in our experiments. To eliminate the growth-induced pH shift,29 buffered media were used to maintain the specified media pH at 6.0, 7.5 and 11.0, respectively. Figure 1 shows the growth profiles of
Synechocystis in response to the external pH. Compared to the cells grown at pH 7.5 (control), growth of Synechocystis was significantly repressed or inhibited at both high (pH 11.0) and low pH (pH 6.0) conditions throughout the measured period. Further rise in pH up to 11.3 did not support any sustained growth and the cells perished after 12 h (data not shown). Therefore, pH 11.0 was defined as the high pH value to be applied for the stress treatment of Synechocystis.

As shown in Figure 1, the growth of Synechocystis was affected significantly upon transfer from pH 7.5 to pH 11.0. Compared to the growth of control cells, only 55% of growth (measured as OD_{730} retained during 24 h of culture at high pH. In contrast to the growth pattern at low pH (Figure 1), the growth of the high-pH-stressed cells at 48 h was slightly increased than that at 24 h followed by a recovery gradually at the end of the measured period (Figure 1). These observations imply that Synechocystis cells sense/respond to the varying pH during the first 24 h. Cellular adaptations occurred in the high pH-stressed cells after coping with the prolonged stress conditions. In order to gain molecular insights and identify proteins involved in the response to high pH stress, we therefore determined to collect the Synechocystis cells treated for 24 h for subsequent proteomic studies.

Plasma Membrane Separation, 2-D Electrophoresis, and Protein Identification. Subcellular proteomic analysis represents an efficient way to study stress responses. We have previously carried out subcellular proteomic investigations toward understanding molecular networks in Synechocystis in response to salt stress. More than 70 salt-stress proteins were identified from purified fractions, that is, plasma membrane, periplasm and cytoplasm. In the present project, we applied this approach to study high pH stress response of Synechocystis. As an initial investigation, we examined the changes in the plasma membrane proteome of Synechocystis since the plasma membrane is crucial as a barrier against the surrounding environment. Plasma membranes were isolated and purified from total membranes of Synechocystis by combination of sucrose density centrifugation and aqueous two-phase partitioning. Plasma membranes from 20 preparations were collected from cells cultured at control and high pH stress conditions. The purity of the plasma membranes was verified by immunoblot analysis (Figure 2) using antibodies against plasma-membrane- and thylakoid membrane-specific marker proteins.

Plasma membrane proteins from both control and high-pH-stress-treated cells were solubilized and separated in duplicates using analytical (100 µg, silver stained) and preparative 2D-gels (1.3 mg, CBB stained) in the pH range of 4–7. Reproducible gel patterns were observed between the replicates. Figure 3 shows representative CBB-stained 2D-gel images of plasma membrane proteins from control (Figure 3A) and from high-pH-stress-treated (Figure 3B) cells. Approximately, 200 intensive spots were visualized on the CBB-stained gels resolving plasma membrane proteins (Figure 3). Comparison of the 2D profiles using ImageMaster version 5.0 revealed that about 80 spots were differentially expressed under high pH stress. Several protein spots also appeared to be newly induced under high pH conditions, since the corresponding position in 2D gels with proteins from control cells was empty. To verify this, we analyzed protein samples prepared in parallel using 2-D DIGE (Supporting Information Figure 1), which allows accurate quantification of relative protein abundance of the spots. DIGE labeling experiments were carried out according to the manufacturer’s instruction (GE Healthcare). Briefly, 50 µg of plasma membrane proteins from control and high-pH-stress-treated cells was labeled with DIGE specific Cy3 or Cy5, respectively. The Cy3- and Cy5-labeled protein samples were mixed pair-wise and then separated in the same gel as described above. The DIGE images were analyzed using DeCyder software version 6.5 as described in the user manual. Spots differentially expressed more than 1.5-fold were determined (Supporting Information Table 2). As expected, the
differentially expressed protein spots revealed by DeCyder 6.5 software were largely consistent with those determined by ImageMaster software. Gel spots of interest were therefore excised from the reproducible 2D-gels stained with CBB and subjected to in-gel digestion according to Huang et al. The proteins were then identified using peptide mass fingerprints (PMF) obtained by MALDI-TOF MS in combination with protein database search. For some of them, identifications were confirmed by combining PMF and sequence tag obtained by MALDI-TOF/TOF MS. Figure 4 showed an example of the identification of the phosphate transport ATP-binding protein, Sll0683, annotated in CYORF.

Differentially Expressed Proteins in the Plasma Membrane. In total, 55 differentially expressed protein spots were identified, corresponding to 39 different gene products as annotated in Figure 3 and summarized in Table 1. Eleven proteins identified were for the first time found to be present in the plasma membrane of *Synechocystis* (Table 1, denoted with "a" as superscript on the spot no.). This slight modification of our previous plasma membrane proteome can be due to several proteins only appearing in the high-pH-stressed cells. Other reasons could be slight differences in cultivation of cells as well as inclusion of an additional detergent solubilizing membrane proteins (for details see Section 2). Prediction of signal peptide using SignalP 3.0 and LipoP showed that two of the differentially expressed proteins newly identified in the present work, that is, SII1482 and SII0679, have a putative Sec-signal and a lipoprotein-signal in the N-terminus, respectively (Table 2). This predicts their location at the periplasmic side of the plasma membrane. Two proteins, that is, the guanylyl
cyclase Cya2 (Sll0646, spot 1) and the ABC transporter permease protein (Sll1482, spot 7), both have 4 predicted transmembrane helices (Table 2). It is known that hydrophobic fragments obtained after trypsin digestion are not easily ionized for mass spectrometric analysis. In the present study, we found that all the matched fragments are located in the loop regions of the integral membrane proteins mentioned above. This yields high confidence in the protein identification as well as

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<th>mass theo/appa.</th>
<th>Mowse score</th>
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Table 1. Differentially Expressed Proteins Identified in Plasma Membrane Fractions of Cells from *Synechocystis* Grown under Control or High-pH Conditions
the topology prediction. Interestingly, 9 of the high pH-responsive proteins produced multiple spots probably due to post-translational modifications (Figure 3). The type of the modification was, however, not clear. In most cases the shift in position is horizontal, suggesting that the modification only affects the pI without changes in molecular mass. Therefore, the modifications are mainly in the side chains of the amino acids rather than the differential processing of the precursor molecules. Interestingly, five of the 9 high-pH-stress responsive proteins with multiple spots were transport proteins (Table 1). Recent proteomic work in *Salmonella enterica* showed that the majority of the transport proteins identified by 2-D-gels was present in more than one isoform.‡ Further studies should be directed to determine the nature of each isoform and to examine if the isoforms exhibit differential affinity to their substrates.

Protein spots significantly enhanced or induced by high pH were indicated in Figure 3B. Nineteen proteins were enhanced at least 2-fold while 6 were enhanced within 1.5- to 1.9-fold. Fourteen proteins were reduced above 1.5-fold under high pH stress condition (Table 1). To confirm the proteomics data, a semiquantitative RT-PCR analysis was performed using total RNA isolated from *Synechocystis* cultured under control and high pH conditions (Figure 5). The increased level of gene expression was evident for the high-pH enhanced proteins revealed by 2-D gels. Within 2 h of culture in high pH media, all 7 genes of interest were clearly up-regulated. The transcription level at 24 h was somewhat lower than that at 2 h but was still higher than that under control condition (Figure 5). The largest group of the differentially expressed proteins consists of proteins involved in transport processes. It is noteworthy that both the substrate- and ATP-binding proteins of the putative phosphate-specific transport system Pst1 were clearly enhanced by high pH stress. The second largest group was attributed to the hypothetical proteins of unknown function. Other proteins found in response to high pH stress include proteins involved in respiration and photosynthesis, as well as regulatory proteins. The physiological implications of the high pH affected proteins are discussed below. 

**Transport and Substrate Binding Proteins.** Fourteen of the high-pH-responsive proteins identified in the plasma membranes of *Synechocystis* are transport and binding proteins. The majority of them are substrate binding proteins of ABC transporters (Table 1). These include 9 of the high pH-enhanced proteins, that is, Sll1699 (spot 2), oligopeptide-binding protein; Slr0074 (spot 4), ABC transporter subunit ycf24; Sll1450 (spot 6), nitrate/nitrite-binding protein NrtA; Sll1482 (spot 7), ABC transporter permease protein; Slr1295 (spot 9), iron-binding protein FutA1; Sll0679 (spot 13), phosphate transport ATP-binding protein PstB1; Sll0684 (spot 14), phosphate transport ATP-binding protein PstB1; and 5 of the reduced proteins, that is,
Proteomic Analysis of Plasma Membranes of Synechocystis

Slr0040 (spot 29), bicarbonate transporter CmpA; Slr0447 (spot 30), urea-binding protein UrtA; Slr1794 (spot 33), probable anion transporting ATPase; Slr0513 (spot 34), iron-binding protein FutA2; Slr1082 (spot 37), ABC-transport system ATP-binding protein. For 5 of the different substrate binding proteins (Slr1450, Slr1295, Slr0679, Slr0040 and Slr0447), a putative lipoprotein anchor to the plasma membrane was clearly predicted in the N-terminus by the LipoP- predictor \(^{37}\) (Table 2). This has been experimentally proven for NrtA protein. \(^{39}\) All these proteins are believed to be located in the periplasmic side of the plasma membrane. The oligopeptide-binding protein (Slr1699) does not contain a predicted lipoprotein or Sec signal. However, according to Pfam (http://pfam.sanger.ac.uk/), Slr1699 belongs to the bacterial extracellular solute-binding protein family 5 Middle (E-value 3.3 \( \times 10^{-72} \)). Homologous proteins of Slr1699 are found in the majority of the 32 cyanobacterial genomes currently deposited in the CyanoBase. Approximately, about half of them have a predicted lipoprotein signal, whereas 25% of them have a Sec signal, and 25% is similar to Slr1699 with neither a lipoprotein- nor Sec-signal. Those later proteins, which are classified as extracellular solute-binding proteins, must possess an atypical signal peptide that plays a role in protein secretion. Two of the ATP-binding proteins (Slr0683 and Slr0684), as well as the ABC transporter subunit ycf24 (Slr0074), are most likely the peripheral proteins located in the cytoplasmic side since no N-terminal signal sequences were predicted. \(^{46}\) In the present work, we found that one integral membrane protein, that is, the ABC transporter permease protein (Slr1482), was present in the plasma membrane and enhanced more than 2-fold by high-pH stress (Figure 3, Table 1).

High pH appears to cause nutrient deficiency. The detection of increased amounts of the phosphate- and the ATP-binding proteins, as well as decreased amount of UrtA protein (spot 10, 13, 14, and 30 in Figure 3) in high pH-stressed cells indicates severe phosphate deficiency resulting from the high pH. DNA microarray analysis has shown that expression of both \( \text{pstB}^\prime \) and \( \text{pstB}^\prime \)′ genes were enhanced 7-fold, whereas expression of the \( \text{urtA} \) gene was repressed under phosphate limitation. \(^{41}\) In the present work, we have identified, for the first time, the plasma membrane located phosphate-binding protein (SphX) and the ATP-binding proteins (PstB1 and PstB1′) of the ABC-type phosphate transporter. SphX was enhanced 1.7-fold and PstB1 and PstB1′ were enhanced more than 3-fold. Another phosphate-binding protein PstS1 (Slr0680) in Pst1 was previously identified also in the plasma membrane of \( \text{Synechocystis} \). \(^{14}\) This strongly suggests that Pst1 system (Slr0679–Slr0684) is located in the plasma membrane since all the peripheral subunits (Slr0679, SphX; Slr0680, PstS1; Slr0683, PstB1 and Slr0684, PstB1′) in the Pst1 system were found so far in the proteome of the plasma membrane. The other two subunits of the Pst1, that is, the permease protein PstA1 (Slr0681) and PstC1 (Slr0682), have not been found. These are integral membrane proteins possessing 6 transmembrane helices, which could probably be excluded from the 2D-gels. Interestingly, only the proteins of Pst1 were so far identified in the plasma membrane and found to be enhanced significantly by high pH stress (Figure 3), although the Pst1 and Pst2 are encoded by similar clusters of genes. \(^{41}\)

\( \text{Synechocystis} \) requires 10 times more iron than other Gram-negative bacteria, such as \( \text{E. coli} \), to sustain photosynthetic growth. \(^{32}\) In the present investigation, we found that the iron-binding protein FutA1 (spot 9) was enhanced more than 2-fold under high pH stress condition. Earlier experiments using immunoblot and proteomic analysis have demonstrated that FutA1 was strongly enhanced under high-salt stress. \(^{14}\) These results indicate that FutA1 is subjected to both stress signals. Mutagenesis and \( \text{in vitro} \) studies have demonstrated that FutA1 plays a more significant role in iron uptake than FutA2 (Slr0513), the second iron-binding protein abundant in the periplasm of \( \text{Synechocystis} \). \(^{15,43}\) The increased level of FutA1 observed in the present investigation could be explained by the much lower solubility of Fe(II) at high pH compared to neutral pH. It was also found that the ABC-transporter subunit ycf24 (Slr0074, spot 4) was enhanced by high-pH stress. In cyanobacteria, four \( \text{suf} \) genes (\( \text{sufBCDS} \)) constitute an operon (slr0074/slr0075/slr0076/slr0077) that is proposed to be involved in the biogenesis and assembly of iron–sulfur clusters of PSI. Under iron limitation, the transcription levels of the \( \text{suf} \) genes were found up-regulated in cyanobacteria. \(^{44}\) In the present work, ycf24 (Slr0074, spot 4), the homologue of SuB, was for the first time identified in the plasma membrane and found to be enhanced by high pH (Figure 3, Table 1). We postulate that SUF system encoded by the \( \text{suf} \) operon was activated either directly by high pH or indirectly by iron-stress resulting from the high media pH.

Apart from the nutrient deficiency mentioned above, intracellular nitrogen supply could also be affected by high pH stress treatment. Earlier reports have demonstrated that the nitrate/ nitrite binding protein NrtA increased at both transcriptional and protein level under salt stress condition. \(^{12,14}\) In the present work, we found that the levels of NrtA (Slr1450) and the oligopeptide-binding protein (Slr1699) were elevated by high pH stress treatment (Figure 3). We assume that the enhancement of these solute-binding components of the ABC transporters is one of the adaptive strategies of the \( \text{Synechocystis} \) cell to cope with changed status of nitrogen availability. It is noteworthy that the bicarbonate transporter CmpA was reduced under high pH stress condition (spot 29). CmpA is the closest homologue of NrtA based on the amino acid sequence as well as the recently resolved structures. Comparison of the structures revealed that bicarbonate binds in nearly an identical location compared with nitrate binding to NrtA. \(^{45,46}\) The opposite pattern of NrtA and CmpA under high pH stress condition observed in the present work (Figure 3, Table 1) implies that high pH may interrupt the nitrogen and carbon uptake pathways potentially synchronized by intracellular nitrogen in \( \text{Synechocystis} \). Interestingly, our data showed that the putative SbtB (spot 25) was remarkably increased under high pH stress. Mutagenesis studies have shown that \( \text{sbtA} \) system, the Na+-dependent bicarbonate transporter, plays a central role in bicarbonate uptake and is sensitive to ambient pH level. \(^{47}\) Our previous work demonstrated that SbtA could only be resolved by 1D-gels \(^{48}\) while SbtB are readily detected on both 1D- and 2D-gels resolving plasma membranes of \( \text{Synechocystis} \). \(^{7,22,48}\) This is most likely due to the distinct hydrophobicity of the two proteins. With the use of the TMHMM program, SbtA is predicted to be an integral protein possessing 8 transmembrane helices while SbtB is a peripheral protein. The presence of SbtA and SbtB in the plasma membrane strongly suggests that SbtA system is located in the plasma membrane. Since both \( \text{sbtA} \) and \( \text{sbtB} \) of the SbtA system were up-regulated under mild Cl limitation, \(^{49}\) the clear enhancement of the putative SbtB in high pH-treated cells (Figure 3) may indicate that SbtA system is activated by high pH or by...
CI limitation that could be resulted from the high pH applied in the present experiments.

Two putative porins (Slr1908 and Slr1272) as well as TolC (Slr1270) were also found to be responsive to high pH stress. These proteins have β-barrel structures and are outer membrane components of different transport systems.8,50 On their way to the final localization, they are captured in the plasma membrane.22,48

MinD. In the present investigation, we identified the septum site-determining protein MinD (Slr0289) in the plasma membrane of Synechocystis (Figure 3, spot 15) and found to be increased under high pH stress condition. In Synechocystis, it has been demonstrated that MinCDE system affects cell division at all potential division sites.53 Since MinD is the most conserved and widespread nonspecific inhibitor of cell division at all potential division sites, MinD was suggested.58 Our finding of the enhancement of CoxB under high pH (Figure 3) as well as their enhancement by high pH, provides important clues for further studies toward a better elucidation of these proteins.

Photosynthetic and Respiratory Proteins. The presence of several subunits of photosystems and respiratory chain in the plasma membrane has been demonstrated in different cyanobacterial species.18,22,56 In this work, we found that PSI subunit III PsAF (Slr0819, spot 24) was clearly enhanced under high pH stress condition, indicating that PSI could be highly susceptible to the stress signal (Figure 3). Apart from the proteins involved in photosynthesis, we found that cytochrome c oxidase subunit II CoxB (Slr0813, spot 16) increased under high pH (Figure 3). This implies that the respiratory chain mediated by cytochrome c oxidase is also involved in the high pH stress response process. Earlier experiments have demonstrated that cytochrome c oxidase is bioenergetically active in Synechocystis57 and a function in protecting photosynthesis from salt stress was suggested.58 Our finding of the enhancement of CoxB under both salt- and high pH-stress14 indicates that CoxB is subjected to broad stress signals, although the exact mechanism remains to be further elucidated.

Regulatory Proteins. Among the high pH-enhanced proteins of the plasma membrane fraction, several of them exhibit regulatory functions. Most interestingly, the guanylyl cyclases Cya2 (Slr0646, spot 1) was for the first time identified in the plasma membrane (Figure 3, Table 1). Our data confirmed the prediction of Cya2 being connected through putative transmembrane segments to a regulatory CHASE2 domain.59 Deletion of the cya2 gene caused a significant loss of cGMP,60 an important signaling molecule mediating responses of living organisms to their environment.61,62 Most recently, the crystal structure of Cya2 from Synechocystis has been resolved to 2.3 Å resolution and revealed that Cya2 is highly specific for GTP.63 In this work, we found that Cya2 was enhanced clearly under high pH stress condition (Figure 3). This is, to best our knowledge, the first report regarding physiological significance of Cya2 in cyanobacteria, although the exact mechanism is to be elucidated. Another regulatory protein, LexA (Slr1626, spot 19), appeared also involved in the high pH stress response of Synechocystis. We found that the level of LexA increased significantly under high pH stress (Figure 3). In addition, the NADH dehydrogenase NdbB (Slr1743) was found present in the plasma membrane (spot 31) and reduced in the high pH-treated cells.

Concluding Remarks

The present study revealed, for the first time, the major changes in the proteome of plasma membranes of Synechocystis in response to high pH stress. In total, 39 high pH-responsive proteins were identified using highly reproducible 2-D gels coupled with mass spectrometry. The largest group of proteins enhanced by high pH consists of transport and substrate-binding proteins of ABC transporters as well as proteins with unknown function. Most remarkably, the enhancement of both substrate- and ATP-binding proteins of the phosphate transporter was observed, suggesting that phosphate uptake system is highly susceptible to the elevated media pH. Though the antiporers have been proposed to play important roles in bacterial pH homeostasis,64 none of them were so far identified. Analysis of Synechocystis antiporers using the TMHMM program predicts those are integral membrane proteins possessing multitransmembrane helices, which are well-known to be easily excluded from the 2-D gels. Nevertheless, our present investigation provides new insights into the plasma membranes in terms of coping with high pH stress. Our data also offers valuable information for in-depth studies into the mechanisms of alkali tolerance. Importantly, we identified a substantial number of high-pH-stress proteins currently annotated as hypothetical proteins in the genome. Further studies are initiated toward understanding the specific roles of these proteins using complementary approaches.

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**Supporting Information Available:** Supplemental Table 1, 2-D DIGE analysis of high-ph stress-responsive proteins in plasma membranes of *Synechocystis*; Supplemental Table 2, differentially expressed proteins detected by DIGE analysis; Supplemental Table 3, specific primers used for RTPCR of genes encoding the changed proteins identified by 2-DE analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

**References**


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