Dynamic changes in gene expression of the cyanobacterium Synechocystis sp. PCC 6803 in response to nitrogen starvation

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Chapter 2

Specific versus general responses in gene expression of *Synechocystis* sp. PCC 6803 exposed to nutrient starvation and salt stress

Specific versus general responses in gene expression of *Synechocystis* sp. PCC 6803 exposed to nutrient starvation and salt stress

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Summary

A gene array composed of clones from the *Synechocystis* sp. PCC 6803 genome sequencing project covering the entire genome was used to monitor changes in gene expression upon high salinity stress, nitrogen starvation, and phosphorus starvation. Gene expression profiles obtained for all three conditions were compared to evaluate whether these changes in gene expression consisted merely of general responses to all three stress conditions or of specific responses to each individual stress condition. Up-regulated genes mostly were of the 'general response' type, although stress-specific responses were observed as well. In contrast, down-regulated genes tended to exhibit stress-specific responses, with only few general responses. General responses were found particularly among genes for shock-induced proteins such as the heat-shock proteins and sigma factors. Genes involved in photosynthesis and respiration were particularly responsive to both salt stress and nitrogen starvation. Genes that were uniquely induced under phosphorus starvation included genes encoding phosphate acquisition proteins and high-affinity phosphate transporters. Our results show that changes in gene expression of cyanobacteria exposed to environmental stress are often not stress specific, but rather can be interpreted as a mixture of general and specific responses, in accordance with the functions of the gene products involved.

Introduction

All organisms are able to adapt to a certain range of changes in the external environment by tuning their physiological processes via qualitative and quantitative changes in their protein composition. These changes generally are achieved by subtle alterations in the global gene expression patterns of the organism. Overlay of expression profiles from different stress conditions provides a methodology to determine the specificity of the responses. Cyanobacteria are known to withstand stress conditions in various extreme environments, including deserts, polar regions, and hypersaline environments. The cyanobacterium *Synechocystis* sp. PCC 6803 (henceforth referred to as *Synechocystis*) is well suited for monitoring comprehensive gene expression under various stresses, as its entire genomic sequence has been determined (Kaneko *et al*., 1996; Cyanobase www.kazusa.or.jp/cyano/cyano.html). Despite of its relatively small genome size (3.6 Mbp with 3264 ORFs), nearly half of the ORFs present in *Synechocystis* still lack an assigned function. Several recent studies have monitored changes in gene expression in *Synechocystis* upon exposure to cold (Mikami *et al*., 2002; Suzuki *et al*., 2001), high light intensity (Hihara *et al*., 2001; Tu *et al*., 2004), salt or osmotic stress (Kanesaki *et al.,* 2002; Marin *et al*., 2003; Marin *et al*., 2004; Shoumskaya *et al*., 2005), light-to-dark transition (Gill *et al*., 2002), UV and white light (Huang *et al*., 2002), iron and phosphate deficiencies (Singh *et al*., 2003; Suzuki *et al*., 2004). Moreover, analysis of redox-responsive genes (Hihara *et al*., 2003) and of histidine kinases (Marin *et al*., 2003) has been reported. Genomic research and its approaches in cyanobacteria have recently been reviewed (Burja *et al*., 2003).

In the present study, we describe the utilization of a DNA array composed of overlapping M13 clones directly acquired from the *Synechocystis* genome-sequencing project. A method was optimised to reduce experimental background in hybridisation by purification of the cDNA probe based on removal of cDNA generated from rRNA. Data analysis was performed with use of a statistical method that allows confident prediction of differentially expressed genes from sets of data with a small number of replicates. We focus the transcriptome analysis on genes with altered expression during adaptation to increased salt concentration as well as upon phosphate and nitrogen starvation. Salt stress has been selected because of our interest in the bioenergetic properties of *Synechocystis*, and nitrogen and phosphorus starvation from the perspective that these nutrients play a key role in the natural environment of cyanobacteria. Known aspects of adaptation to increased salt concentration include an increase in the capacity for CO_2 fixation (Jeanjean *et al*., 1998, Rolland *et al*., 1997, Shibata *et al*., 2001) and induction of proteins for the synthesis of osmoprotective compounds (Ferjani *et al*., 2003, Hagemann *et al*., 1997, Marin *et al*., 2002), as well as activation of sodium export systems and enhanced bioenergetics capacity (Alahari *et al*., 2001, Joset *et al*., 1996). In terms of bioenergetics the adaptation to increased salt concentration primarily affects Na^+/H^+ antiporters as these membrane proteins play a major role in pH and Na^+ homeostasis of cells (Padan *et al*., 2001), and this adaptation enhances respiration (Fry *et al*., 1986) and Photosystem I driven cyclic electron transfer (Jeanjean *et al*., 1993, Matthijs *et al*., 2002, van

Thor *et al*., 2000).

To survive under nitrogen-limited conditions, cells induce systems for high affinity uptake of nitrogen, and adapt physiological processes to enable more economic nitrogen usage. Non-nitrogen fixing cyanobacteria are able to use NO_3^-, NH_4^+, urea and some amino acids as sources of nitrogen (Herrero *et al*., 2001). Cyanobacteria can even adapt to and recover from long-term nitrogen starvation (Sauer *et al*., 2001). A number of genes are known to be involved in survival under conditions of low nitrogen availability, including: (1) genes that encode proteins essential for nitrogen assimilation and metabolism such as NirA and NarB (Richardson *et al*., 2001), (2) genes that encode regulatory proteins such as NtcA and NtcB (Aichi *et al*., 2001, Paz-Yepes *et al*., 2003), PphA (Irmler and Forchhammer 2001), and P_{II} (Forchhammer and Tandeau de Marsac 1995, Hisbergues *et al*., 1999), and (3) genes coding for proteins that contribute to the mobilisation of nitrogen reserves such as through phycobilisome degradation (Baier *et al*., 2001).

Phosphorus-containing compounds play a key role in cellular metabolism and in bioenergetics. Biochemical responses of cyanobacteria to phosphorus starvation include increased production and secretion of phosphate acquisition proteins such as nucleases, acidic and alkaline phosphatases, and high affinity phosphate transporters. Two operons encoding high-affinity phosphate uptake systems are present in the *Synechocystis* genome. A two-component signal transduction system is presented by the gene products of *sll*0337 and *slr*0081, which are homologous to the *E. coli* phosphate-sensing histidine kinase PhoR and the response regulator PhoB, respectively (Hirani *et al*., 2001). Scavenging for phosphorous-containing compounds is often catalysed by alkaline phosphatases, for which three putative genes are present in the genome of *Synechocystis*.

In the present work we describe gene expression patterns in response to salt addition, nitrogen starvation and phosphorus starvation. Responsive genes for each of these three stress conditions are sorted according to the functional category assigned to these genes by Cyanobase. The gene expression patterns obtained have been overlaid to reveal specific responses versus general responses in gene expression upon environmental change.

Materials and Methods

Growth conditions. *Synechocystis* sp. strain PCC 6803 was grown at 30ºC in liquid BG11 medium (Rippka *et al*., 1979) supplemented with 10 mM $NaHCO_3$ at a light intensity of 50 µmol photons $m^{-2}s^{-1}$ in ambient air. Besides these control cultures, three different stress conditions were applied. Salt stress was induced by the addition of NaCl to a final concentration of 0.5 M to cultures growing at an optical density at 750 nm (OD_{750}) of 0.5; cells were harvested after 8 h. Optical densities of all cultures used in this work were determined on a NovaspecII spectrophotometer (Amersham-Pharmacia, UK).

To induce nitrogen starvation, cells were harvested by centrifugation, washed with BG11 medium lacking nitrogen, and resuspended in BG11 containing 500 µM $NaNO_3$ to an OD_{750} of 0.03. Cells were returned to normal growth conditions (temperature, light intensity) and harvested by centrifugation after 42 h. Nitrogen starvation was verified by absorbance spectrometry (Aminco DW2000, SLM

Instruments., US), monitoring phycobilisome disappearance by means of absorbance at 628 nm. Cells were harvested near the midpoint of phycobilin degradation (Baier *et al*., 2001) corresponding to an OD_{750} of 0.3.

To induce phosphorus starvation, cells from a normal culture were used to inoculate a 10-fold larger volume of BG11 medium lacking K_2HPO_4 and the culture was allowed to grow for at least 5 days. Phosphorus starvation was verified by an alkaline phosphatase assay (Ray *et al*., 1991), which revealed that alkaline phosphatase activity increased about 100-fold compared to the control. The cell density at the time of harvest corresponded to an OD_{750} of 0.3.

DNA array blots design. Genomic DNA blots consist of sheared *Synechocystis* chromosomal DNA fragments cloned in M13 and comprise all 3264 ORFs in 5540 spots arranged in square grids (6 x 6) spots in each, with empty spots in the upper left and lower right corners of the square, on two 8 x 10 cm positively charged nylon membranes. A number of additional empty spots on the membranes were used for background correction.

DNA isolation and PCR. Isolation of *Synechocystis* chromosomal DNA was performed as described in Ermakova-Gerdes and Vermaas (1999). To purify the cDNA probe, 3 pairs of oligonucleotides for amplification of the 23S, 16S and 5S ribosomal genes were designed based on the sequences available in CyanoBase:

B1 (5'-TTGAGAGACAGAAACCAGACCCTTG-3'), N1 (5'-GCTAATAGCCCTTGCCTTTTACCTC-3');
B2 (5'-TCAGAGGGAATGCTGGATGTAAGTC-3'), N2 (5'-CCATATTTTCGCTACTCAAGCCGAC-3');
B3 (5'-CAGGTGTGGACAGTACAGAAGTGAG-3'), N3 (5'-GCCAAAAGCGAAACCCTCCAACTAC-3').

Oligonucleotides B1, B2 and B3 carried biotin on the 5'-end (Sigma, US). Biotinylation enables affinity binding of the oligonucleotides to a streptavidin-covered matrix, which permits specific separation of these primers and their corresponding extension products in later steps of the experimental protocol. Each 50 µl of reaction mix for amplification consisted of 5 ng of chromosomal DNA, 25 pmol of both oligonucleotides, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 5 µl of 10x reaction buffer provided with HotStart polymerase (Qiagen, Germany), and 5 units of HotStart polymerase (Qiagen). PCR amplification was performed on an Uno II Thermoblock (Biometra, Germany) under the following conditions: 1 cycle of denaturation at 94ºC for 7 min; 30 cycles consisting of incubation at 94ºC (1 min), 55ºC (2 min) and 72ºC (3 min) finally followed by 7 min elongation at 72ºC. PCR products were purified using a PCR Purification Mini Kit (QIAquick, Qiagen) according to the instructions of the manufacturer. Quality and quantity of PCR products were estimated according to their absorbance spectra (Aminco DW 2000) and electrophoresis in a 0.8% agarose gel.

Total RNA isolation and cDNA synthesis. RNA was isolated using a protocol modified from that described in Mohamed and Jansson (1989). Briefly, a cell pellet from 50 ml of culture (control or treatment) was resuspended in 0.8 ml 0.3 M sucrose, microcentrifuged, frozen in liquid nitrogen and placed on ice. This was followed by subsequent addition of 60 µl 0.5 M EDTA pH 8.0, 60 µl of 50 mM NaAc pH 4.5, and 60 µl 20% SDS with mixing after each step. Cells were transferred to a 65ºC water bath for 5 min with intense mixing and phenol-chloroform extraction was performed using preheated phenol at 65ºC. After the first addition of hot phenol, cells were incubated at 65°C for 5 min, shaken and quickly cooled down to -80°C for 1 min. RNA was precipitated by 3 volumes of ethanol and 2/9 volume of 10 M LiCl, washed with 70% ethanol and resuspended in TE buffer. Purification of extracted

RNA was performed using RNeasy Midi kit (Qiagen) according to the instructions of the manufacturer. Quality and quantity of extracted RNA was estimated by measuring the absorbance spectrum (240-340 nm, Aminco) and by denaturing formamide-formaldehyde electrophoresis in a 0.8% agarose gel. Isolated total RNA was used to prepare [^{32}P]-labelled cDNA probes using a reverse transcriptase reaction. Each 25 µl of reaction mix consisted of 33 µg of total RNA, 1.8 nmol of random hexamer pd(N)$_6$ (TaKaRa, Kyoto, Japan) (RNA and primer were denatured at 95ºC and chilled on ice), 0.8 mM each of dTTP, dCTP and dGTP and 0.03 mM dATP, 4 mM DTT, 5 µl of 5x reaction buffer provided with reverse transcriptase (TaKaRa), 40 units of RNase inhibitor (TaKaRa), and 40 µCi of [α-^{32}P]-dATP (3000 Ci/mmol, Amersham-Pharmacia, UK). The mix was preheated at 42ºC for 2 min and 200 U of SuperScript II (TaKaRa) was added. Reverse transcription was carried out at 42ºC for 2 h. To stop reverse transcription and to degrade total RNA, 1 µl of 2% SDS and 6 µl of 1 M NaOH were added to the reaction mixture followed by incubation for 15 min at 65ºC. After phenol-chloroform extraction 28 µl of 7 M ammonium acetate and 154 µl of ethanol were subsequently added to the probe and cDNA was precipitated for 2 h at -20ºC, washed with 70% ethanol, and dissolved in 20 µl of TE buffer. A total of 100 µg of RNA was used for cDNA preparation generating three 20 µl aliquots.

Removal of cDNA transcribed from rRNA. To avoid relatively high background upon hybridisation that may result from the predominant presence of rRNA, a protocol for the removal of cDNA produced from rRNA has been optimised. 1.2 ml of streptavidin-coated Dynabeads (Invitrogen, US) was washed three times with washing buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA, 1 M NaCl). Separation of the magnetic beads from the washing buffer was performed by a magnetic stand supplied by the manufacturer. After resuspension in an equal volume of the washing buffer the beads were divided into three 400 µl aliquots. Each Dynabeads aliquot was hybridised at 37ºC for 30 min with 25-40 µg of one of the rDNA PCR products generated with a biotinylated primer. Then the mix was denatured with 0.1 M NaOH and 1 M NaCl for 5 min at room temperature to separate the two strands of the PCR product, washed with the same solution, and subsequently washed three times with washing buffer. Three portions of labelled cDNA generated in the previous section (20 µl each) were mixed with 20 µl of hybridisation buffer (20 mM Tris-HCl pH 8.0, 0.2 mM EDTA, 1.5 M NaCl), denatured at 95ºC for 5 min, quickly cooled on ice, and hybridised with three portions of Dynabeads bearing single-stranded DNA with a sequence corresponding to either 5S, 16S, or 23S rRNA for 2 h at 65ºC. Then unbound cDNA was removed from Dynabeads using the magnetic stand, denatured at 95ºC for 5 min, cooled on ice, and transferred to Dynabeads carrying another rRNA sequence, and hybridised again. This step was repeated once more so each portion of 20 µl of cDNA had been hybridised with 5S, 16S, and 23S rRNA sequences. This led to a virtually complete removal of cDNA sequences corresponding to rRNA. For inspection, cDNA before and after purification procedure was separated in 0.8% agarose gel, transferred onto nylon membranes and probed with ^{32}P labelled 16S rRNA gene under standard hybridisation conditions (Sambrook *et al*., 1989). ^{32}P labelled cDNA before and after purification was separated in 5% PAGE (acrylamide:bisacrylamide 29:1). The autoradiographic images were quantified densitometrically (Quantity-One, Bio-Rad, US).

DNA array hybridisation conditions. Before hybridisation, DNA array blots were prehybridised for at least 3 h at 37°C in 10 ml of prehybridisation mix (30% (v/v) formamide, 1% (w/v) SDS, 10% (w/v) dextran sulfate, 1 mM EDTA, 30 mM Tris/HCl pH 7.5, and 3 x SSC). Dynabeads-treated cDNA

generated from 100 μg of total RNA was added to 1 ml of 7 mg/ml salmon sperm DNA, mixed and incubated at 95°C for 5-10 min to denature, then cooled on ice and added directly to the blots in prehybridisation mix. Hybridisation was performed for 48 h at 37°C while rotating at 60-100 rpm. After hybridisation blots were washed twice in 2 x SSC for 30 min, rinsed with 0.1 x SSC, and transferred to a PhosphorImager screen.

Image acquisition and data analysis. Following exposure of the blots to a PhosphorImager screen and scanning on a STORM 840 PhosphorImager (Molecular Dynamics, Amersham-Pharmacia, UK), the intensity of each spot on the array was quantified using ImageQuant software (Molecular Dynamics) and transferred to an Excel spreadsheet. After background subtraction, data were normalised by calculation of each signal as a percentage of the total signal on the blot. To obtain reliable genomic expression patterns we used two different methods. The ratio method calculates the ratio of expression of each spot under treatment conditions versus control conditions. The non-parametric method "*Patterns from gene expression*" (PaGE 2.1 freeware software) was used to determine differently expressed spots (Manduchi *et al*., 2000). Further details about this program and algorithm can be found at www.cbil.upenn.edu/PaGE. We quantified the magnitude of the gene response by the log ratio of gene expression, which is defined as $^{2}\log(I_{treatment}/I_{control})$, where $I_{treatment}$ and $I_{control}$ are the normalized spot intensities of the treatment and the control, respectively.

Northern hybridisation. 10 μg of total RNA isolated from cultures grown under control and treatment conditions were loaded on a gel and blotted. RNA was visualised after transfer to the nylon membrane by methylene blue staining. Northern blotting analysis was performed according to Sambrook *et al*. (1989). Labelled probe for hybridisation was obtained as PCR product by amplification of chromosomal DNA in the presence of ^{32}P-dATP using the following oligonucleotides:
psbA1(1) (5'-CACGGTTCTGTTGTTGGATTA-3'), psbA1(2) (5'-CCGTTGAGGTTGAAAGCGAAA-3'),
psbD2(1) (5'-CGGTTGGTCTGGTTTGCTA-3'), psbD2(2) (5'-GTCATAAGCCCGTAGGTTCAA-3'),
ccmM(1) (5'-GCTGGTTGTTGGCTGTATCTA-3'), ccmM(2) (5'-GTTGCTATCACTTCTGGGGTTA-3').

Results

Array hybridisation and statistical analysis of the array data. All array hybridisations with cDNA probes were repeated three times in independent experiments. The treatments consisted of one control and three stress incubations based on exposure to 0.5 M NaCl, starvation for nitrogen, and starvation for phosphorus. Alteration in profiles of 5540 normalised spot densities (relative transcript abundance) of the control relative to other conditions is depicted in Fig. 1. The results demonstrated reproducibility within a factor of about 2 for the control (Fig. 1A). Most spot densities contributed between 0.01 and 0.1 % to the sum of all densities. Comparison of the relatively flat distribution in Fig. 1A with the more scattered appearance of Fig. 1B-D indicates that the various treatments changed the abundance of specific transcripts by an order of magnitude or more. Of the three treatments, adaptation to increased salt concentration caused the most substantial differences in global expression relative to the control (Fig. 1B). The results obtained upon nitrogen starvation (Fig. 1C) also showed substantial regulation, whereas phosphorus starvation (Fig. 1D) gave

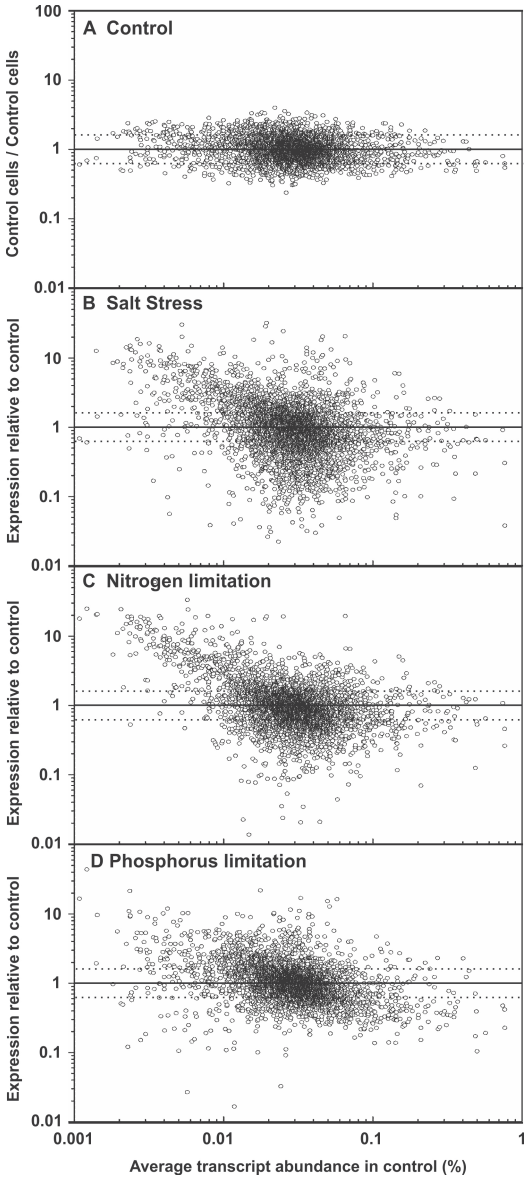

Figure 1. Logarithmic-scale plots of normalized spot intensities. (**A**), The ratio of the transcript intensities of two independent control replicates plotted against the relative transcript abundance in control cells, as a measure of the reproducibility of the experiments. (B-D), The transcript intensities after a particular treatment divided by those in control cells plotted against the relative transcript abundance in control cells. Treatments were: (**B**), 0.5 M NaCl addition to the culture; (**C**), nitrogen starvation; (**D**), phosphorus starvation. The area within the dashed lines (between ratios 1.6 and 1/1.6) represents the approximate experimental error in the data if expression is not significantly altered.

Table 1. Comparison of Ratio and PaGE methods.

The number of up- or down-regulated spots identified by the Ratio method and the PaGE method, respectively, for each of the three applied stress conditions. For the PaGE-analysis, the value of the 'false positive rate' (fpr) is set to 0.005 and confidence of the prediction for each case is shown. For the ratio method, a ratio of more than 1.6 or less than 1/1.6 was considered to be up- or down-regulated. The column named 'Genes' indicates the number of genes within selected spots that were judged to have altered expression by the PaGE method. This is less than the number of spots because one gene generally is distributed over more than one spot on the blot.

Stress	Ratio Method	PaGE Method		
	Spots	Spots	Confidence	Genes
		Up-regulation		
Salt	460	358	0.932	238
Nitrogen$^-$	438	335	0.926	205
Phosphorus$^-$	411	327	0.926	168
		Down-regulation		
Salt	630	450	0.971	203
Nitrogen$^-$	556	380	0.938	124
Phosphorus$^-$	232	159	0.848	81

rise to a relatively mild global response.

Following normalisation of the three complete data sets for control and treatment experiments, the statistical reliability of the data was evaluated. The results of the data analyses, including number of ORFs retrieved from regulated spots are presented in Table 1. The ratio method predicted about 30% more spots to be up-regulated and 48% more to be down-regulated than the PaGE method. All spots with significantly altered relative intensity identified by the PaGE method were also identified by the ratio method.

Conversion from spot densities to the level of expression of individual genes. The genomic array blot used was composed of overlapping M13 clones directly acquired from the *Synechocystis* genome-sequencing project; many of the clones contained multiple complete or partial genes, and most genes were present in multiple clones. The statistically relevant differences in spot density could therefore be translated to resolve the differential expression of given genes present in a particular clone spot. This assignment was made according to the length of genes, their relative length within given clones, and their presence in neighbouring clones. About 10 to 12 % (depending on the stress applied) of all spot data were excluded from the analysis, because of interference of genes and occurrence of simultaneous up- and down-regulation coinciding in a single spot. Also, a number of spots with strong hybridization signal affecting adjacent spots were excluded from the analysis. An example of the analysis is shown in Fig. 2. Fig. 2A and B illustrate overall blots probed with cDNA from control and salt treatment experiments; frames C and D show the hybridisation signals in a selected grid. Fig. 2E depicts the geometry of spot sites in the selected grid. Only spots on the filters judged by PaGE as altered were taken into consideration. The signal in spot d6 containing *sll*1308

that encodes a probable oxidoreductase was strongest in samples isolated from the treatment experiment with salt stress (cf. Fig. 2C, D). From analysis of all three replicates expression of *sll*1308 was judged as induced with a factor of about 3 in cells that were exposed to salt stress. Similarly, the signal from *slr*2135 in spot f3 encoding a hydrogenase accessory protein was also significantly stronger in samples isolated after salt treatment. Finally, in this quadrant, the signal in spots containing *slr*0876 that encodes a hypothetical protein was significantly lower in the treated cells. This observation was confirmed by statistical analysis of all data.

A selection of the regulated genes detected by our study is presented in Table 2, and compared with regulated genes observed in previous *Synechocystis* microarray studies. Changes in gene expression in response to the treatments were quantified using the log ratio of gene expression. The complete data set is listed in the Supplementary Materials, both for up-regulated genes (Apendix 2A) and down-regulated genes (Apendix 2B).

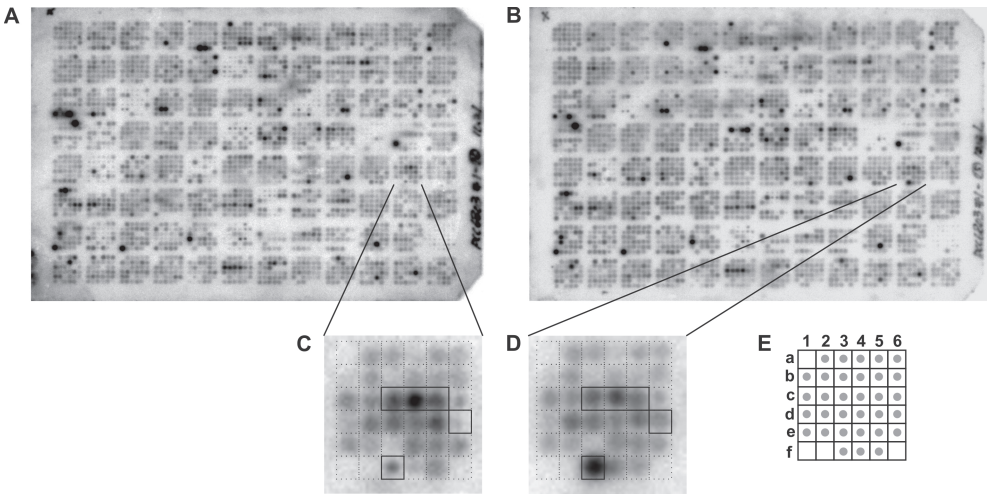

Figure 2. Autoradiograph of the DNA arrays. Arrays probed with cDNA derived from control (**A**) or salt treatment (**B**) cells. Magnification of selected grids of the DNA array from control (**C**) and treatment (**D**) growth conditions; (**E**), a square grid example of spot organisation (grey circles represent the actual position of spots within the grid). Rectangles highlighted in **C** and **D** represent spots with significantly up- or down-regulated signal: c3, c4 and c5 contain ORF *slr*0876, d6 contains *sll*1308, and f3 contains *slr*2135.

Table 2. Selected list of regulated genes.

Numbers represent the log ratio of gene expression, which is defined as $^2\log(I_{treatment}/I_{control})$. Up-regulations are highlighted in dark gray, and down-regulations in light gray. The column 'other studies' indicates regulation of the corresponding ORF in other *Synechocystis* microarray studies.

ORF	Gene	Product	Stress			Other studies*	
			Salt	N-	P-	Up	Down
*slr*1564	*sigF*	group III RNA polymerase sigma factor SigF	1.09	-0.37	-0.50		
*slr*0653	*sigA*	principal RNA polymerase sigma factor SigA	1.06	-0.31	-0.24	15	2ab
*slr*0542	*clpP*	ATP-dependent protease ClpP	1.56	0.17	-1.28		
*sll*0057	*grpE*	heat shock protein GrpE	-0.23	0.88	-0.12		
*slr*0164	*clpP*	ATP-dependent Clp protease proteolytic subunit	0.12	-0.32	1.70	13, 15	
*sll*0170	*dnaK*	DnaK protein	0.12	0.13	0.89	1, 2ab, 4, 5a, 6, 8b, 11b, 13	
*sll*0416	*groEL2*	60kD chaperonin 2	1.61	2.53	0.46	2ab, 4, 5a, 6, 8b, 10, 11ab	13, 15
*sll*1933	*dnaJ*	DnaJ protein	2.61	2.80	2.04		
*sll*0897	*dnaJ*	DnaJ protein	2.65	2.58	0.96	11b	
*slr*0093	*dnaJ*	DnaJ protein	1.62	1.67	1.88	1, 2ab, 4, 5a, 6, 8b, 11b, 13	
*ssr*1789	*hliD*	CAB/ELIP/HLIP-related protein HliD	4.06	2.54	1.74		
*sll*0184	*sigC*	group 2 RNA polymerase sigma factor SigC	1.23	1.10	0.86	11ab, 15	
*slr*0156	*clpB*	ClpB protein	1.64	1.16	2.19	8b	15
*sll*0430	*htpG*	heat shock protein HtpG	-2.76	-2.37	-2.24	2b, 3b,5a, 6, 8b, 10, 11b, 13, 15	
*sll*0199	*petE*	Plastocyanin	1.30	0.26	0.15	11b	15
*sll*1317	*petA*	apocytochrome f, comp. of cyt. b6/f complex	3.10	2.45	0.06		7
*slr*0342	*petB*	cytochrome b6	1.56	2.12	0.75		
*slr*0343	*petD*	cytochrome b6-f complex subunit 4	1.23	1.51	0.54	15	
*slr*0927	*psbD2*	photosystem II reaction center D2 protein	1.22	1.61	0.05	10	
*slr*1181	*psbA1*	photosystem II D1 protein	2.07	2.26	1.44		
*sll*0427	*psbO*	photosystem II manganese-stabilizing polypeptide	-0.96	-0.06	0.07		2ab, 11ab, 15
*sll*1297		probable dioxygenase, Rieske iron-sulfur component	2.64	2.19	2.14		11b
*slr*1185		Rieske Fe-S protein with unknown function	3.14	2.48	1.44		
*sll*0634	*btpA*	photosystem I biogenesis protein BtpA	2.38	1.89	1.62		
*ssl*2559		Ferredoxin	2.48	2.61	2.67		
*slr*1380	*cydB*	cytochrome oxidase d subunit II	0.10	-1.69	-0.76		
*slr*1138	*ctaE*	cytochrome c oxidase subunit III	-1.11	0.80	-0.06		
*sll*1471	*cpcG2*	phycobilisome rod-core linker polypeptide	-1.06	0.12	-0.11		13, 15
*slr*0335	*apcE*	phycobilisome core-membrane linker polypeptide	-1.43	-0.09	-0.44		2ab, 7,10, 11ab, 15
*slr*0171	*ycf37*	photosystem I assembly related protein Ycf37	-1.91	-0.10	0.12		
*slr*1655	*psaL*	photosystem I subunit XI	0.82	-1.17	-0.21		
*slr*1204		Protease	1.78	0.05	-0.35	1, 2ab, 4, 5a, 6, 8b, 15	
*slr*0261	*ndhH*	NADH dehydrogenase subunit 7	-1.18	-0.43	-0.72		
*slr*1280	*ndhK*	NADH dehydrogenase subunit NdhK	-2.28	0.03	-0.22		
*slr*2007	*ndhD5*	NADH dehydrogenase subunit 4	0.83	0.02	0.02	11a	
*sll*1733	*ndhD3*	NADH dehydrogenase subunit 4	1.56	1.15	0.14	5a, 10, 11a	11b
*sll*0521	*ndhG*	NADH dehydrogenase subunit 6	2.68	1.59	0.01		
*sll*0520	*ndhI*	NADH dehydrogenase subunit NdhI	2.96	1.86	0.21		
*sll*0519	*ndhA*	NADH dehydrogenase subunit 1	2.61	1.65	0.36		
*sll*1732	*ndhF3*	NADH dehydrogenase subunit 5	1.26	1.19	-0.04	10, 11a	
*slr*0844	*ndhF1*	NADH dehydrogenase subunit 5	1.91	2.21	1.99		
*ssr*1386	*ndhL*	NADH dehydrogenase subunit NdhL	0.88	1.30	1.62		

Table 2 (continued). Selected list of regulated genes.

ORF	Gene	Product	Stress			Other studies*	
			Salt	N-	P-	Up	Down
slr1302	*cupB*	involved in constitutive low affinity CO2 uptake	1.86	0.44	-0.60		
sll1685	*ycf10*	light-induced Na+-dependent proton extrusion	2.09	-0.06	-0.41		
sll0273	*nhaS2*	Na+/H+ antiporter	0.80	-0.14	0.06		
slr0415	*nhaS5*	Na(+)/H(+) antiporter	1.16	1.34	0.89		
sll0556		Na+/H+ antiporter	3.26	2.25	2.04	1, 3b, 5a	
slr1595		Na+/H+ antiporter	-0.32	-0.85	-1.42		
slr1908		probable porin; major outer membrane protein	1.70	0.13	-1.16		13
slr1272		probable porin; major outer membrane protein	-3.09	-2.53	-0.80		2b
sll1271		probable porin; major outer membrane protein	-1.69	-0.39	-0.85		
slr0898	*nirA*	ferredoxin-nitrite reductase	0.46	1.43	0.50	3b, 16	
sll1499	*glsF*	ferredoxin-dependent glutamate synthase	-0.10	1.25	-0.23		
sll0450		probable nitric oxide reductase	1.04	1.05	-0.53		11a
sll1453	*nrtD*	nitrate/nitrite transport system ATP-binding protein	1.12	1.05	1.23	13, 16	2a, 11b
sll1452	*nrtC*	nitrate/nitrite transport system ATP-binding protein	1.72	1.14	0.93	13, 16	
sll1387	*pppA*	serine/threonine protein phosphatase PppA	-2.32	0.15	1.29		
slr1622	*ppa*	soluble inorganic pyrophosphatase	-0.74	-1.56	1.08		
sll0789		two-component response regulator OmpR subfamily	-0.62	0.25	1.17	2ab, 5a, 13	11a
sll1334		two-component sensor histidine kinase	0.40	-0.02	2.25		
slr0915		putative endonuclease	-0.76	-1.07	1.81		
slr1248		phosphate transport system permease PstC hom.	0.24	0.27	1.18	14	
slr1249		phosphate transport system permease PstA hom.	0.15	-0.01	0.94	14	
slr1250		phosphate transport ATP-binding protein PstB hom.	0.04	0.03	1.05	14	
sll0681		phosphate transport system permease PstC hom.	1.92	2.14	1.72	5a, 10, 14	
sll0682		phosphate transport system permease PstA hom.	1.07	2.17	1.43	14	
sll0683		phosphate transport ATP-binding protein PstB hom.	1.73	2.17	1.94	10, 14	

* 1, high salinity (Marin *et al*., 2003); 2a and 2b, high salinity and cold (Kanesaki *et al.*, 2002); 3a and 3b, short-term and long-term exposure to high salinity (Marin *et al.*, 2004); 4, high salinity (Shoumskaya *et al*., 2005); 5a and 5b, osmotic and cold (Mikami *et al*., 2002); 6, osmotic (Paithoonrangsarid *et al.*, 2004); 7, cold (Suzuki *et al*., 2001); 8a and 8b, cold and heat (Inaba *et al*., 2003); 9, high light (Hihara *et al*., 2001); 10, high light (Tu *et al*., 2004); 11a and 11b, high light and UV (Huang *et al.*, 2002); 12, oxidative stress (Kobayashi *et al*., 2004); 13, redox (Hihara *et al.*, 2003); 14, phosphate starvation (Suzuki *et al*., 2004); 15, iron starvation – recovery (Singh *et al*., 2003); 16, *Anabaena* sp. PCC 7120 nitrogen limitation (Ehira *et al*., 2003).

Northern blots substantiating the gene array analysis. The expression level of several genes was evaluated by Northern hybridizations (Fig. 3). Fig. 3A shows that the hybridization signal for the *psbD* gene encoding the photosystem II D2 subunit is higher under salt stress (lane 2) and substantially higher under nitrogen starvation (lane 3); as there are two very similar *psbD* genes in the *Synechocystis* genome, the work presented here cannot unambiguously establish which of the *psbD* genes is induced. Fig. 3B illustrates the specifically increased expression of the *ccmM* gene after salt addition (lane 2) relative to the control (lane 1). The signal on the Northern blot from nitrogen-starved cells in lane 3 was lower than expected from the array data. Fig. 3C presents the case of hybridization with *slr*1181 (*psbA1*), which showed the strongest *psbA* hybridization signal under conditions of nitrogen starvation (lane

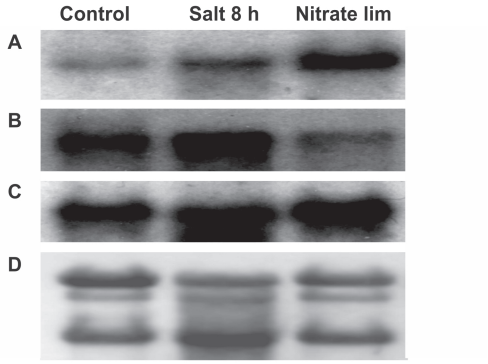

Figure 3. Northern blot analysis of selected genes. Northern blots probed with (**A**), *psbD2* (*slr*0972); (**B**), *ccmM* (*sll*1031); or (**C**), *psbA1* (*slr*1181). (**D**), Methylene-blue staining of total RNA separated on a formaldehyde agarose gel and blotted to a nylon filter. 10 µg of total RNA was loaded per lane. Lane 1, RNA from control cells; lane 2, RNA isolated from cells 8 h after the addition of 0.5 M NaCl; lane 3, RNA isolated from nitrogen starved cells.

3). These data are all qualitatively in good agreement with the results from the gene array analysis. Note that there are three *psbA* genes in the *Synechocystis* genome, of which *psbA1* is silent (Salih and Jansson 1997); the experiments presented in Fig. 3C reflect transcripts from all three *psbA* genes. Methylene-blue staining of the total RNA transferred to the nylon membrane was performed to verify the quality and quantity of the total RNA used for the Northern blotting. Samples from control, high salinity, and nitrogen stress conditions showed similar concentrations of total RNA (Fig. 3D). However, we obtained a 10-50 fold lower amount of total RNA from phosphate-starved cells (data not shown).

Regulation of metabolic categories. Study of the regulation of gene transcription may support expansion of knowledge about cell physiology and metabolic pathways. The results obtained for altered gene expression were analysed with respect to the metabolic category to which regulated genes are assigned according to CyanoBase. The number of regulated ORFs as well as their distribution over the various metabolic categories differed substantially for each of the applied stress conditions. Table 3 presents the absolute number of regulated genes for each metabolic category, and Figure 4 depicts a graphical representation of the relative changes per category.

Most prominent in salt stress were regulated genes belonging to categories 8 (photosynthesis and respiration), 11 (DNA replication etc.), 12 (transcription) and 13 (translation). However, important information is included in changes in other categories as well (Fig. 4). For example, the arrest in cell growth upon exposure to salt stress is accompanied by up-regulation of the gene for the 'cell death suppressor' protein FtsH (*slr*0228) (usually known as a chaperonin) and a down regulation for the cell division protein FtsW (*slr*1267) (both are in category 4 – cellular processes). It is known that under salt stress *Synechocystis*

Table 3. Regulation of expression sorted according to the functional categories to which ORF products have been assigned in CyanoBase.

Number of up- and down-regulated genes per treatment condition per category is listed, together with the total number of genes in each category.

Category	Gene category description	Number of genes	Salt stress		N-starvation		P-starvation	
			up	down	up	down	up	down
1	amino acid biosynthesis	97	10	6	12	1	8	3
2	biosynthesis of cofactors, prosthetic groups and carriers	124	8	6	7	5	8	2
3	cell envelope	67	7	5	5	2	3	3
4	cellular processes	78	10	6	11	9	6	2
5	central intermediary metabolism	31	2	2	2	2	1	0
6	energy metabolism	93	7	8	7	2	3	3
7	fatty acid, phospholipid and sterol metabolism	39	3	2	1	1	2	2
8	photosynthesis and respiration	141	22	16	19	26	7	12
9	purines, pyrimidines, nucleosides and nucleotides	41	1	5	2	0	6	0
10	regulatory functions	146	20	10	16	1	9	4
11	DNA replication, restriction, modification, recombination and repair	60	7	8	5	7	3	3
12	Transcription	30	4	4	2	4	1	2
13	Translation	168	24	20	17	7	16	6
14	transport and binding proteins	196	21	22	21	9	25	6
15	other categories	312	17	17	16	12	17	5
16	similar to hypothetical proteins	1133	53	48	40	23	36	21
17	function not known	508	22	18	22	13	17	7
	total	3264	238	203	205	124	168	81
	% of total	100	7.3	6.2	6.3	3.8	5.1	2.5

accumulates the compatible solute glucosylglycerol (GG) and sucrose (Mikkat *et al*., 1996). Recently, the connection between osmolyte synthesis and cell division has been described. Electron microscopy revealed that mutants deficient in GG synthesis displayed inhibited cell division and had significantly increased cell size (Ferjani *et al*., 2003).

The gene categories that were prominently up-regulated under nitrogen starvation are found in categories 1 (amino acid biosynthesis), 4 (cellular processes), 8 (photosynthesis and respiration), 10 (regulatory functions), and 14 (transport and binding proteins). Relatively many genes with decreased expression levels under nitrogen starvation were observed in category 8 (photosynthesis and respiration), which is in a good agreement with the expected physiological response of cells to nitrogen starvation. Genes that were relatively frequently up-regulated under phosphorus starvation were found in category 10 (regulatory functions), 13 (translation) and category 14 (transport and binding proteins). Note that the number of regulated genes encoding hypothetical and unknown proteins (category 17) was high in all treatments (Table 3), but as the number of genes in this category is very high the percentage of regulated genes in this category is relatively small (Fig. 4).

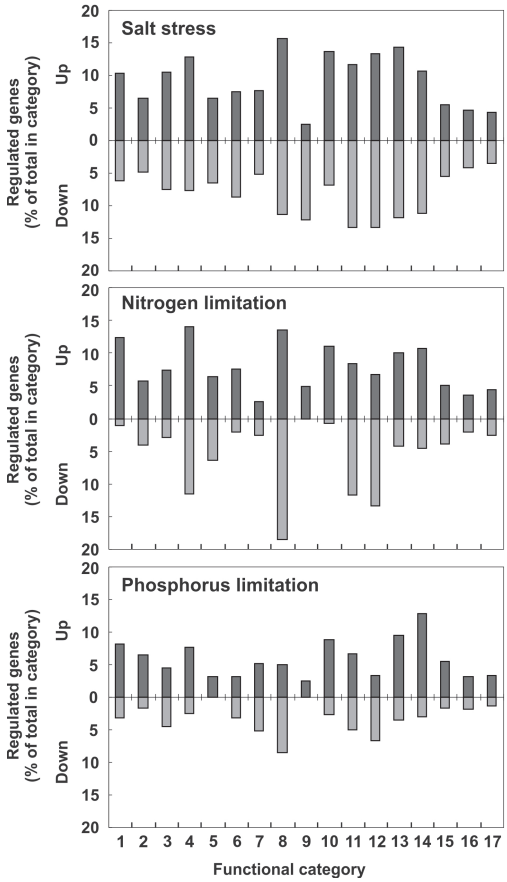

Figure 4. Distribution of regulated genes over different gene categories. Relative number of genes that show a change in gene expression for the 17 different metabolic categories distinguished by Cyanobase. Definition of metabolic categories as in Table 3.

Discussion

A DNA array of M13 clones comprising the entire set of *Synechocystis* genes permitted monitoring of thousands of genes simultaneously in a transcriptome analysis. Knowledge of changes in gene expression will help to improve understanding of metabolic traits of cyanobacteria, and their response to changes in the natural environment. Some changes in gene expression are rather specific, as they occur only under specific environmental conditions. Other changes in gene expression are more general, and may occur in response to several different environmental stresses. This implies that adaptation of gene expression in response to environmental change can be interpreted as a mixture of both specific and general responses (Fig. 5). To judge the specificity of gene regulation, we grouped all genes for which the transcriptome analysis revealed significant changes in gene expression according to their specificity towards the selected stress conditions. Many up-regulated genes showed a general response to all three stress conditions, whereas a few up-regulated genes showed specific responses to particular stress conditions (Fig. 5A). The highest number of up-regulated genes that were specifically induced by a single stress was found after salt addition. In contrast, most down-regulated genes were specifically affected by a single stress only. Remarkably few down-regulated genes were generally affected by all three stress conditions (Fig. 5B).

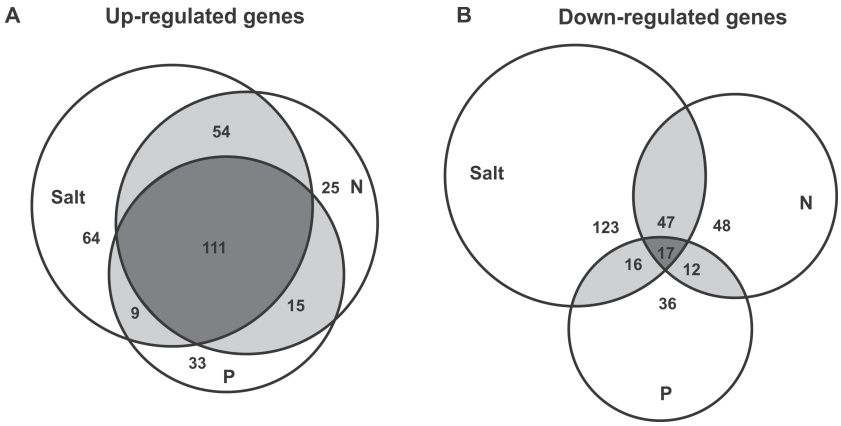

Figure 5. Specific responses versus general responses. Venn diagrams illustrating that some changes in gene expression are highly specific; they occur in response to one specific stress condition only. Conversely, other changes in gene expression are more general; they may be induced by several different stress conditions, as indicated by the overlapping circles. (**A**), up-regulated genes; (**B**), down-regulated genes. The size of the circle indicates the number of regulated genes; the numbers inside the circles provide the number of genes for each response type.

Our set of data is consistent with *Synechocystis* microarray data from various previous studies (Table 2). For instance, our results confirm earlier findings of Suzuki *et al.* (2004) that several genes involved in phosphate transport are upregulated in response to phosphorus starvation. Our salt stress data overlap only partially with previous studies (Kanesaki *et al.*, 2002; Marin *et al.*, 2003, 2004; Shoumskaya *et al.*, 2005), which might be explained by differences in experimental conditions. In particular, most earlier microarray studies of salt stress investigated short-term changes in gene expression of *Synechocystis* within the first hour of salt stress, whereas we studied long-term changes in gene expression after 8 hours of salt stress.

Interestingly, some of the observed general stress responses in this study were also found in other studies using completely distinct stress conditions. For instance, we found that the genes *pstS* (*sll*0680), encoding part of a phosphate uptake system, and *nrtC* (*sll*1452), encoding the ATP binding protein of the nitrate/nitrite transporter, were induced upon salt stress and both nutrient stresses. These two genes were also shown to be regulated by high light intensity in the study of Bhaya *et al*. (2000). Moreover, expression of *nrtC* was found to be under control of the redox state of the plastoquinone pool (Hihara *et al*., 2003). Conversely, *ycf10* (*sll*1685) encoding a protein involved in light-independent Na^+-dependent proton extrusion that is implicated in CO_2 uptake, and *nhaS2* (*sll*0273) encoding a Na^+/H^+ exchanger illustrate a specific response. Both of these genes were uniquely induced 8 h after the addition of NaCl. However, another set of two Na^+/H^+ antiporters, *nhaS5* (*slr*0415) and *sll*0556 were increased upon all treatments in our array examination. These results might indicate that some of the Na^+/H^+ antiporters can be specifically functional in salt stress whereas others could replace each other under general 'stress' conditions in *Synechocystis* cells, which correlates with previous observations (Allakhverdiev *et al*., 1999; Elanskaya *et al*., 2002).

Global stress responses of shock-induced genes. The so-called shock-induced genes (referred to as global stress response genes) that encode general stress proteins, such as heat shock proteins and sigma factors, constituted a relatively large group of genes regulated for one or more of the treatments used in this work. Examples are homologues of *dnaJ* (*slr*0093, *sll*1933 and sll0897), *sigC* (*sll*0184), *clpB* (*slr*0156) and *hliD* (*ssr*1789) genes that were found to be upregulated, and the heat shock protein gene *htpG* (*sll*0430) that was judged downregulated in all stresses applied. The *groEL-2* gene (*sll*0416) was induced after salt addition and also upon nitrate starvation. However, our data suggest that some other genes representative of the global stress group display strict specificity. As a specific response, *sigF* (*slr*1564), *clpP* (*slr*0542) and *sigA* (*slr*0653) were induced in the salt treatment only; *dnaK* (*sll*0170) and *clpP* (*slr*0164) featured specific enhancement upon phosphate starvation; and *grpE* (*sll*0057) was specific for nitrate starvation.

Effects of salt stress. In cyanobacteria, photosynthetic and respiratory electron transfer in thylakoids intersects at the level of the cytochrome b_6f complex and plastocyanin (Berry *et al*., 2002). Adaptation to increased salt concentration was shown to enhance the bioenergetic functions of respiration (Fry *et al*., 1986) and Photosystem I driven cyclic electron transfer

(Jeanjean *et al*., 1993) in *Synechocystis*. Indeed, our array analysis revealed a substantial number of regulated genes related to photosynthesis and respiration, especially under salt stress and nitrogen starvation. The *petB* gene (*slr*0342) encoding cytochrome b_6, *petD* (*slr*0343) encoding cytochrome b_6f subunit IV, *petE* (*sll*0199) encoding plastocyanin, *petA* (*sll*1317) encoding apocytochrome *f*, *sll*1297 encoding the Rieske Fe-S protein as well as a second Rieske-type gene *slr*1185 were all induced 8 h after NaCl addition. This response may be related to enhancement of photosynthesis and/or respiration during adaptation to increased salt concentration. However, generally no upregulation was observed for the photosynthetic reaction center genes. The *psaL* gene (*slr*1655), encoding a Photosystem I subunit involved in the formation of trimers (Chitnis and Chitnis, 1993), showed upregulation after salt addition but a downregulation upon N-starvation. This observation suggests a possible role of PsaL in the regulation of Photosystem I activity and electron transfer capacity. The *btpA* gene (*sll*0634) encoding the Photosystem I-complex-specific regulatory protein, which appears to stabilise core Photosystem I subunits in stress conditions (Zak and Pakrasi, 2000) is upregulated commonly upon salt treatment and nutrient starvation. The *psaA* (*slr*1834) and *psaB* (*slr*1835) genes as well as genes encoding various subunits of ATP synthase show down-regulation upon N or P-starvation, which correlates with the decrease in photosynthesis activity per cell under these conditions (Sauer *et al*., 2001), and fits also with the decrease in the transcript levels of many genes that contribute to phycobilisome function. Much to our surprise, several genes encoding terminal oxidase subunits, including *ctaE* (*slr*1138) and *ctaDII* (*slr*2082), showed a decreased expression level after salt treatment, suggesting depression of respiration around that transient phase in adaptation, although respiratory activity is known to be enhanced in salt-adapted cells (Fry *et al*., 1986). However, *Synechocystis* contains 2-3 parallel terminal oxidases (Howitt and Vermaas 1998), and therefore a decrease in the transcript level of a couple of components may be easily compensated by other pathways. Another example of a decrease in transcript level of a respiratory-related gene after 8 h of salt incubation is *sll*1625, one of the two genes encoding the succinate dehydrogenase iron-sulphur protein subunit SdhB. The SDH complex is viewed to play a dominant role in respiratory electron transfer into the PQ pool (Cooley and Vermaas 2001). However, the transcript level of *slr*0201, now recognized to be the C subunit of SDH (F. Xiong and W. Vermaas, unpublished observations) rather than subunit B of the heterodisulfide reductase, increased by a factor of two in all stress conditions. Therefore, in situations where multiple (relatively divergent) genes encode functionally similar components of a pathway functionally relevant interpretation of transcript levels may be equivocal. Moreover, at least in yeast, increases in transcript levels do not necessarily imply increases in protein levels (Ghaemmaghami *et al*., 2003). Therefore, situations in which some transcripts for subunits of a protein complex go up and others go down are difficult to interpret functionally, but at least provide information for further experimentation.

Another interesting example of quantitative and qualitative differences in expression upon exposure to stress conditions is provided by the family of *ndh* genes, encoding subunits

of a type I NADPH dehydrogenase (NDH-1). Two- to eight-fold increases in expression were found for about half of the *ndh* genes in response to salt stress. To this group belong *sll*0519 for NADH dehydrogenase subunit 1 (NdhA), *ndhD3* (*sll*1733), *ndhD5* (*slr*2007), *ndhE* (*sll*0522) encoding NDH-1 subunit 4, *ndhF3* (*sll*1732) and *ndhF1* (*slr*0844) encoding NDH-1 subunit 5, *ndhG* (*sll*0521) encoding NDH-1 subunit 6, *sll*0520 encoding NDH-1 subunit NdhI, and *ssr*1386 encoding NDH-1 subunit NdhL. Expression of some of them was enhanced upon nutrient starvation as well. However, transcript levels of three other subunits of NDH-1 (*ndhH* (*slr*0261), *ndhJ* (*slr*1281) and *ndhK* (*slr*1280)) were down-regulated two- to five-fold after 0.5 M NaCl incubation. Reverse genetics approaches have revealed that NdhF3, NdhF4, NdhD3, and NdhD4 subunits contribute mainly to the uptake of CO_2, whereas NdhD1, NdhD2 and NdhF1 are involved in respiration and cyclic electron transfer around Photosystem I (Ohkawa *et al*., 2000).

The increased demand for CO_2 under salt stress, presumably for the necessary synthesis of osmolyte, is evident from specific upregulation of the *sll*1734 (*cupA*) gene encoding a carbon uptake protein, which was found to assemble together with NdhD3 and NdhF3 to form a small low-CO_2-induced protein complex (Herranen *et al*., 2004). The observed induction of some genes encoding distinct subunits of the NDH-1 complex might suggest increased cyclic and/or respiratory electron flow via NDH-1 when exposed to salt stress, and could contribute to a better understanding of the complex physiological role of the NDH-1 complex.

Effects of nutrient starvation. To survive in nutrient-limited conditions, cells should induce or enhance systems for high affinity uptake of limiting nutrients, adapt physiological processes to enable more economic usage of the nutrient concerned, and strive to utilise non-traditional resources. Our conditions have been selected to induce a starvation for nitrogen or phosphorus without reaching conditions that approach cell death. A number of genes showed enhanced expression only upon nutrient starvation (for example, *slr*1740 encoding an extracellular solute-binding protein), indicating that the proteins (and possibly pathways) induced in response to nutrient starvation are different from those induced upon salt stress. One of the expected responses to nitrogen starvation is up-regulation of both the *nirA* (*slr*0898) gene encoding ferredoxin-nitrite reductase as well as the high affinity nitrate/nitrite uptake system (gene cluster *nrtABCD-narB*), which is separate from *nirA* in *Synechocystis*. Even though *nirA* is specifically induced under nitrogen starvation conditions, the *nrtC* (*sll*1452) and *nrtD* (*sll*1453) genes were induced under all three treatments studied here.

Biochemical responses to phosphate starvation include increased production and secretion of phosphate acquisition proteins such as nucleases, acidic and alkaline phosphatases, and high-affinity phosphate transporters. During total RNA isolation from phosphate-starved cells we observed a 10-50 fold decreased amount of total RNA that could be extracted compared to what was extracted from cells that had been exposed to the other stress conditions. This observation could be explained by utilisation of nucleic acids as a source of phosphate (Abel *et al*., 2000), as well as by an increased nuclease production by phosphate-starved cells. Furthermore, our data show increased expression of *slr*0915 encoding a putative

endonuclease, which could possibly rapidly degrade/recycle nucleic acids inside cells. An increase in the expression of *slr*1622 encoding an inorganic pyrophosphatase suggests the usage by cells of pyrophosphate as an additional source of P_i. A system for active phosphate uptake is represented by two operons in the *Synechocystis* genome. The genes of one of these operons (*sll*0679-0683) showed an enhanced expression level in all types of stress, whereas the upregulation of the second one (*slr*1247-1250) was unique for phosphate starvation only.

Outer membrane proteins showed substantial differences in regulation in response to the nutrient starvations. Our results demonstrate the versatility of porins that may be involved in nutrient uptake (Sauer *et al*., 2001). We found *slr*1908 (*somB*), *sll*1271 (*somF*) and *slr*1272 to be regulated. The transcript level of other porins remained unchanged under our incubation conditions.

Conclusions. In this work comparative transcriptome analysis of three physiologically distinct stresses has been described. Our observations demonstrate that the largest set of regulated genes was generally induced in response to a variety of stress conditions rather than by a specific stress. Specific gene responses were retrieved as well, however, and the information offers an additional tool for the study of metabolic pathways. Grouping of regulated genes according to their functional category showed that specific responses were generally in line with expectations based on the functions of the genes. Identification of metabolic networks regulated by specific conditions may give an advantage for the design of diagnostic tools to rapidly monitor the response of organisms to changes in their natural environment, for example regarding the nutritional status of cells.

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List of supplemental data

Appendix 2A. List of up-regulated ORFs.

Appendix 2B. List of down-regulated ORFs.

References

Abel S, Nürnberger T, Ahnert V, Krauss GJ, Glund K (2000) Induction of an extracellular cyclic nucleotide phosphodiesterase as an accessory ribonucleolytic activity during phosphate starvation of cultured tomato cells. *Plant Physiol* **122**: 543-552

Aichi M, Takatani N, Omata T (2001) Role of NtcB in activation of nitrate assimilation genes in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **183**: 5840-5847

Allakhverdiev SI, Nishiyama Y, Suzuki I, Murata N (1999) Genetic engineering of the unsaturation of fatty acids in membrane lipids alters the tolerance of *Synechocystis* to salt stress. *Proc Natl Acad Sci USA* **96**: 5862-5867

Baier K, Nicklisch S, Grunder C, Reinecke J, Lockau W (2001) Expression of two *nblA*-homologous genes is required for phycobilisome degradation in nitrogen-starved *Synechocystis* sp. PCC 6803. *FEMS Microbiol Lett* **195**: 35-39

Berry S, Schneider D, Vermaas WFJ, Roegner M (2002) Electron transport routes in whole cells of *Synechocystis* sp. strain PCC 6803: the role of the cytochrome *bd*-type oxidase. *Biochemistry* **41**: 3422-3429

Bhaya D, Vaulot D, Amin P, Takahashi AW, Grossman AR (2000) Isolation of regulated genes of the cyanobacterium *Synechocystis* sp. strain PCC 6803 by differential display. *J Bacteriol* **182**: 5692-5699

Burja AM, Dhamwichukorn S, Wright PC (2003) Cyanobacterial postgenomic research and systems biology. *Trends in Biotechnol* **21**: 504-510

Chitnis VP, Chitnis PR (1993) PsaL subunit is required for the formation of photosystem I trimers in the cyanobacterium *Synechocystis* sp. PCC 6803. *FEBS Lett* **336**: 330-334

Cooley JW, Vermaas WFJ (2001) Succinate dehydrogenase and other respiratory pathways in thylakoid membranes of *Synechocystis* sp. strain PCC 6803: capacity comparisons and physiological function. *J Bacteriol* **183**: 4251-4258

Ehira S, Ohmori M, Sato N (2003) Genome-wide expression analysis of the responses to nitrogen deprivation in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. *DNA Res* **10**: 97-113

Elanskaya IV, Karandashova IV, Bogachev AV, Hagemann M (2002) Functional analysis of the Na^+/H^+ antiporter encoding genes of the cyanobacterium *Synechocystis* PCC 6803. *Biochemistry (Mosc)* **67**: 432-440

Ermakova-Gerdes S, Vermaas WFJ (1999) Inactivation of the open reading frame *slr*0399 in *Synechocystis* sp. PCC 6803 functionally complements mutations near the Q(A) niche of photosystem II. A possible role of *slr*0399 as a chaperone for quinone binding. *J Biol Chem* **274**: 30540-30549

Ferjani A, Mustardy L, Sulpice R, Marin K, Suzuki I, Hagemann M, Murata N (2003) Glucosylglycerol, a compatible solute, sustains cell division under salt stress. *Plant Physiol* **131**: 1628-1637

Forchhammer K, Tandeau de Marsac N (1995) Functional analysis of the phosphoprotein P_{II} (*glnB* gene product) in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol* **177**: 2033-2040

Fry IV, Huflejt M, Erber WW, Peschek GA, Packer L (1986) The role of respiration during adaptation of the freshwater cyanobacterium *Synechococcus* 6311 to salinity. *Arch Biochem Biophys* **244**: 686-691

Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, Dephoure N, O'Shea EK, Weissman JS (2003) Global analysis of protein expression in yeast. *Nature* **425**: 737-741

Gill RT, Katsoulakis E, Schmitt W, Taroncher-Oldenburg G, Misra J, Stephanopoulos G (2002) Genome-wide dynamic transcriptional profiling of the light-to-dark transition in *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **184**: 3671-3681

Hagemann M, Richter S, Mikkat S (1997) The *ggtA* gene encodes a subunit of the transport system for the osmoprotective compound glucosylglycerol in *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **179**: 714-720

Herranen M, Battchikova N, Zhang P, Graf A, Sirpio S, Paakkarinen V, Aro EM (2004) Towards functional proteomics of membrane protein complexes in *Synechocystis* sp. PCC 6803. *Plant Physiol* **134**: 470-481

Herrero A, Muro-Pastor AM, Flores E (2001) Nitrogen control in cyanobacteria. *J Bacteriol* **183**: 411-425

Hihara Y, Kamei A, Kanehisa M, Kaplan A, Ikeuchi M (2001) DNA microarray analysis of cyanobacterial gene expression during acclimation to high light. *Plant Cell* **13**: 793-806

Hihara Y, Sonoike K, Kanehisa M, Ikeuchi M (2003) DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **185**: 1719-1725

Hirani TA, Suzuki I, Murata N, Hayashi H, Eaton-Rye JJ (2001) Characterization of a two-component signal transduction system involved in the induction of alkaline phosphatase under phosphate-limiting conditions in *Synechocystis* sp. PCC 6803. *Plant Mol Biol* **45**: 133-144

Hisbergues M, Jeanjean R, Joset F, Tandeau de Marsac N, Bedu S (1999) Protein P_{II} regulates both inorganic carbon and nitrate uptake and is modified by a redox signal in *Synechocystis* PCC 6803. *FEBS Lett* **463**: 216-220

Howitt CA, Vermaas WFJ (1998) Quinol and cytochrome oxidases in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochemistry* **37**: 17944-17951

Huang L, McCluskey MP, Ni H, LaRossa RA (2002) Global gene expression profiles of the cyanobacterium *Synechocystis* sp. strain PCC 6803 in response to irradiation with UV-B and white light. *J Bacteriol* **184**: 6845-6858

Inaba M, Suzuki I, Szalontai B, Kanesaki Y, Los DA, Hayashi H, Murata N (2003) Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in *Synechocystis*. *J Biol Chem* **278**: 12191-12198

Irmler A, Forchhammer K (2001) A PP2C-type phosphatase dephosphorylates the P_{II} signalling protein in the cyanobacterium *Synechocystis* PCC 6803. *Proc Natl Acad Sci USA* **98**: 12978-12983

Jeanjean R, Matthijs HCP, Onana B, Havaux M, Joset F (1993) Exposure of the cyanobacterium *Synechocystis* PCC 6803 to salt stress induces concerted changes in respiration and photosynthesis. *Plant Cell Physiol* **34**: 1073-1079

Jeanjean R, Bédu S, Havaux M, Matthijs HCP, Joset F (1998) Salt-induced PSI cyclic electron transfer restores growth on low inorganic carbon in a type 1 NAD(P)H-dehydrogenase deficient mutant of *Synechocystis* PCC 6803. *FEMS Microbiol Lett* **167**: 131-137

Joset F, Jeanjean R, Hagemann M (1996) Dynamics of the response of cyanobacteria to salt stress: deciphering the molecular events. *Physiol Plant* **96**: 738-744

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res* **3**: 185-209

Kanesaki Y, Suzuki I, Allakhverdiev SI, Mikami K, Murata N (2002) Salt stress and hyperosmotic stress regulate the expression of different sets of genes in *Synechocystis* sp. PCC 6803. *Biochem Biophys Res Commun* **290**: 339-348

Kobayashi M, Ishizuka T, Katayama M, Kanehisa M, Bhattacharyya-Pakrasi M, Pakrasi HB, Ikeuchi M (2004) Response to oxidative stress involves a novel peroxiredoxin gene in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol* **45**: 290-299

Manduchi E, Grant GR, McKenzie SE, Overton GC, Surrey S, Stoeckert CJ (2000) Generation of patterns from gene expression data by assigning confidence to differentially expressed genes. *Bioinformatics* **16**: 685-698

Marin K, Huckauf J, Fulda S, Hagemann M (2002) Salt-dependent expression of glucosylglycerol-phosphate synthase, involved in osmolyte synthesis in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J Bacteriol* **184**: 2870-2877

Marin K, Suzuki L, Yamaguchi K, Ribbeck K, Yamamoto H, Kanesaki Y, Hagemann M, Murata N (2003) Identification of histidine kinases that act as sensors in the perception of salt stress in *Synechocystis* sp PCC 6803. *Proc Natl Acad Sci USA* **100**: 9061-9066

Marin K, Kanesaki Y, Los DA, Murata N, Suzuki I, Hagemann M (2004) Gene expression profiling reflects physiological processes in salt acclimation of *Synechocystis* sp. strain PCC 6803. *Plant Physiol* **136**: 3290-3300

Matthijs HCP, Jeanjean R, Yeremenko N, Huisman J, Joset F, Hellingwerf KJ (2002) Hypothesis: versatile function of ferredoxin-$NADP^+$ reductase in cyanobacteria provides regulation for transient photosystem I-driven cyclic electron flow. *Funct Plant Biol* **29**: 201-210

Mikami K, Kanesaki Y, Suzuki I, Murata N (2002) The histidine kinase Hik33 perceives osmotic stress and cold stress in *Synechocystis* sp PCC 6803. *Mol Microbiol* **46**: 905-915

Mikkat S, Hagemann M, Schoor A (1996) Active transport of glucosylglycerol is involved in salt adaptation of the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Microbiology* **142**: 1725-1732

Mohamed A, Jansson C (1989) Influence of light on accumulation of photosynthesis-specific transcripts in the cyanobacterium *Synechocystis* 6803. *Plant Mol Biol* **13**: 693-700

Ohkawa H, Pakrasi HB, Ogawa T (2000) Two types of functionally distinct NAD(P)H dehydrogenases in *Synechocystis* sp. strain PCC 6803. *J Biol Chem* **275**: 31630-316344

Padan E, Venturi M, Gerchman Y, Dover N (2001) Na(+)/H(+) antiporters. *Biochim Biophys Acta* **1505**: 144-157

Paithoonrangsarid K, Shoumskaya MA, Kanesaki Y, Satoh S, Tabata S, Los DA, Zinchenko VV, Hayashi H, Tanticharoen M, Suzuki I, Murata N (2004) Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in *Synechocystis*. *J Biol Chem* **279**: 53078-53086

Paz-Yepes J, Flores E, Herrero A (2003) Transcriptional effects of the signal transduction protein P (II) (glnB gene product) on NtcA-dependent genes in *Synechococcus* sp. PCC 7942. *FEBS Lett* **22**: 42-46

Ray JM, Bhaya D, Block MA, Grossman AR (1991) Isolation, transcription, and inactivation of the gene for an atypical alkaline phosphatase of *Synechococcus* sp. strain PCC 7942. *J Bacteriol*.**173**: 4297-4309

Richardson DJ, Berks BC, Russell DA, Spiro S, Taylor CJ (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cell Mol Life Sci* **58**: 165-178

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* **111**: 1-61

Rolland N, Dorne AJ, Amoroso G, Sultemeyer DF, Joyard J, Rochaix JD (1997) Disruption of the plastid *ycf10* open reading frame affects uptake of inorganic carbon in the chloroplast of *Chlamydomonas*. *EMBO J* **16**: 6713-6726

Salih GF, Jansson C (1997) Activation of the silent psbA1 gene in the cyanobacterium *Synechocystis* sp. strain 6803 produces a novel and functional D1 protein. *Plant Cell* **9**: 869-878

Sambrook J. Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sauer J, Schreiber U, Schmid R, Volker U, Forchhammer K (2001) Nitrogen starvation-induced chlorosis in *Synechococcus* PCC 7942. Low-level photosynthesis as a mechanism of long-term survival. *Plant Physiol* **126**: 233-243

Shibata M, Ohkawa H, Kaneko T, Fukuzawa H, Tabata S, Kaplan A, Ogawa T (2001) Distinct constitutive and low-CO_2-induced CO_2 uptake systems in cyanobacteria: genes involved and their phylogenetic relationship with homologous genes in other organisms. *Proc Natl Acad Sci USA* **98**: 11789-11794

Shoumskaya MA, Paithoonrangsarid K, Kanesaki Y, Los DA, Zinchenko VV, Tanticharoen M, Suzuki I, Murata N (2005) Identical Hik-Rre systems are involved in perception and transduction of salt signals and hyperosmotic signals but regulate the expression of individual genes to different extents in *Synechocystis*. *J Biol Chem* **280**: 21531-21538

Singh AK, McIntyre LM, Sherman LA (2003) Microarray analysis of the genome-wide response to iron deficiency and iron reconstitution in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Physiol* **132**: 1825-1839

Suzuki I, Kanesaki Y, Mikami K, Kanehisa M, Murata N (2001) Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. *Mol Microbiol* **40**: 235-244

Suzuki S, Ferjani A, Suzuki I, Murata N.(2004) The SphS-SphR two component system is the exclusive sensor for the induction of gene expression in response to phosphate limitation in *Synechocystis*. *J Biol Chem* **279**: 13234-13240

Tu CJ, Shrager J, Burnap RL, Postier BL, Grossman AR (2004) Consequences of a deletion in dspA on transcript accumulation in *Synechocystis* sp. strain PCC6803. *J Bacteriol* **186**: 3889-3902

van Thor JJ, Jeanjean R, Havaux M, Sjollema KA, Joset F, Hellingwerf KJ, Matthijs HCP (2000) Salt shock-inducible photosystem I cyclic electron transfer in *Synechocystis* PCC 6803 relies on binding of ferredoxin:NADP(+) reductase to the thylakoid membranes via its CpcD phycobilisome-linker homologous N-terminal domain. *Biochim Biophys Acta* **1457**: 129-144

Zak E, Pakrasi HB (2000) The BtpA protein stabilizes the reaction center proteins of photosystem I in the cyanobacterium *Synechocystis* sp. PCC 6803 at low temperature. *Plant Physiol* **123**: 215-222