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Yeremenko, N.

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Chapter 2

A DNA array technique directly acquired from gene sequences to monitor changes in gene expression profiles of *Synechocystis* sp. PCC 6803

**A DNA array technique directly acquired from gene sequences to
monitor changes in gene expression profiles of
Synechocystis sp. PCC 6803**

N. Yeremenko^{1*}, V. Krasikov^{1*}, T. Kaneko², S. Tabata², W. F. J. Vermaas³, and
H. C. P. Matthijs^{1*}

¹*Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of
Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, the Netherlands*

²*Kasuzo DNA Research Institute, Kisarazu, Japan*

³*School of Life Sciences and Center for the Study of Early Events in Photosynthesis, Arizona
State University, Box 874501, Tempe, AZ 85287-4501 USA*

*These two authors contributed equally to this work

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transcriptome analysis

Summary

DNA array display provides a unique way to explore the metabolic and genetic regulation of gene expression on a global genome scale. Here we describe the implementation of a DNA array composed of overlapping M13 clones directly acquired from the *Synechocystis* genome-sequencing project. The approaches include experimental design, image acquisition and analysis, normalisation, and data evaluation. 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. The data obtained suggest that this readily applicable approach for DNA array analysis permits monitoring of differential gene expression without a demand for the time consuming and costly design associated with PCR or oligonucleotide-based arrays.

Introduction

The ability to determine conditional changes in transcript levels simultaneously for all the genes in a cell is an extremely powerful tool in cell physiology. Regardless of the state of cell growth, it is possible to measure the relative expression levels for each gene under various growth conditions, different genetic states or over a time course during environmental change. These types of studies help to disclose identities of new sets of genes involved in specific physiological responses. Although regulation of protein abundance in a cell is accomplished solely by regulation of mRNA, virtually all differences in cell type or state are correlated with changes in the transcript levels of many genes. However, traditional methods in molecular biology generally work on a "one gene one experiment" basis, which means that the throughput is very limited and the "whole picture" of gene function is hard to obtain. During the last decade, application of DNA microarray technology has become a powerful tool for studying gene expression and regulation on a very large scale (Schna *et al.*, 1995; DeRisi *et al.*, 1997; Spellman *et al.*, 1998; Iyer *et al.*, 1999; White *et al.*, 1999), also for a variety of prokaryotic organisms (Richmond *et al.*, 1999), including cyanobacteria (Suzuki *et al.*, 2001; Mikami *et al.*, 2002; Hihara *et al.*, 2001, 2003; Kanesaki *et al.*, 2002; Gill *et al.*, 2002; Singh *et al.*, 2003; Marin *et al.*, 2003). This approach for large-scale expression monitoring has been used in a number of different formats including spotted cDNA microarrays and oligonucleotide arrays.

The progression from a completed sequence analysis to applicable microarrays is still a time-consuming and costly process, whereas a spotted DNA library is directly available

during the sequencing project. This strategy of gene expression analysis has originated from application of λ clone spot blots to monitor gene expression in *Escherichia coli* (Chuang and Blattner, 1993), revealing 26 new heat shock genes.

In this report, we describe the procedures for the application of DNA arrays composed of clones from the cyanobacterium *Synechocystis* sp. PCC 6803 genome-sequencing project (Kaneko *et al.*, 1996) covering the entire set of genes, and, we discuss the technical challenges that were met and resolved during the project.

Experimental Procedures

DNA array blots. 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 presented on the membranes were used for background correction.

Growth conditions. *Synechocystis* sp. strain PCC 6803 wild type (hereafter referred to as the control) was grown at 30°C in liquid BG11 medium (Rippka *et al.*, 1979) at a light intensity of 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ in ambient air. Salt-adaptation was used as the stress condition (hereafter referred to as the treatment culture). A salt-adapted culture was obtained by the addition of NaCl to a final concentration of 0.5 M to growing cultures (van Thor *et al.*, 1998) at an OD_{750} of 0.5, and cells were harvested after 8 h.

DNA isolation and PCR. Isolation of *Synechocystis* chromosomal DNA was performed as described by Ermakova-Gerdes *et al.* (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'-GCTAATAGCCCTTGCCCTTTACCTC-3');

B2 (5'-TCAGAGGGAATGCTGGATGTAAGTC-3'), N2 (5'-CCATAATTTTCGCTACTCAAGCCGAC-3');

B3 (5'-CAGGTGTGGACAGTACAGAAGTGAG-3'), N3 (5'-GCCAAAAGCGAAACCCTCCAACCTAC-3').

Oligonucleotides B1, B2 and B3 carried biotin on the 5'-end (Sigma). 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, Westburg) under the following conditions: 1 cycle of denaturation at 94°C for 7 min; 30 cycles consisting of incubation at 94°C (1 min), 55°C (2 min) and 72°C (3 min) finally followed by 7 min elongation at 72°C. 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 by Mohamed *et al.* (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 next placed on ice. This was followed by subsequent addition of 60 μ l 0.5 M EDTA pH 8.0, 60 μ l of 50 mM Na acetate pH 4.5, and 60 μ l 20% SDS with mixing after each step. Cells were transferred to a 65°C water bath for 5 min with intense mixing and phenol-chloroform extraction was performed using preheated phenol at 65°C. 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 [³²P]-labelled cDNA probes using a reverse transcriptase reaction. Each 25 μ l of reaction mix consisted of 33 μ g of total RNA, 1.8 nmol of random hexamer pd(N)₆ (TaKaRa, Kyoto, Japan) (RNA and primer were denatured at 95°C and chilled on ice), 0.8 mM each of dTTP, dCTP and dGTP and 0.03 mM dATP, 4 mM DTT, 5 μ l of 5x reaction buffer provided with reverse transcriptase (TaKaRa), 40 units of RNase inhibitor (TaKaRa), and 40 μ Ci of [α -³²P]-dATP (3000 Ci/mmol, Amersham-Pharmacia). The mix was preheated at 42°C for 2 min and 200 U of SuperScript II (TaKaRa) was added. Reverse transcription was carried out at 42°C for 2 h. To stop reverse transcription and to degrade total RNA, 1 μ l of 2% SDS and 6 μ l of 1 M NaOH were added to the reaction mixture followed by incubation for 15 min at 65°C. After phenol-chloroform extraction 28 μ l of 7 M ammonium acetate and 154 μ l of ethanol were subsequently added to the probe and cDNA was precipitated for 2 h at -20°C, washed with 70% ethanol, and dissolved in 20 μ l of TE buffer. A total of 100 μ g of RNA was used for cDNA preparation generating three 20 μ l aliquots.

Removal of cDNA transcribed from rRNA. 1.2 ml of streptavidin-coated Dynabeads (DynaL Co.) 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 μ l aliquots. Each Dynabeads aliquot was hybridised at 37°C for 30 min with 25-40 μ g 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 μ l each) were mixed with 20 μ l of hybridisation buffer (20 mM Tris-HCl pH 8.0, 0.2 mM EDTA, 1.5 M NaCl), denatured at

95°C 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 65°C. Then unbound cDNA was removed from Dynabeads using the magnetic stand, denatured at 95°C 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 ³²P labelled 16S rRNA gene under standard hybridisation conditions (Sambrook *et al.*, 1989). ³²P 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).

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), 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 normal 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.

Results and Discussion

Extraction of cDNA corresponding to mRNA

An issue in studies of gene expression in prokaryotic organisms is the lack of an easy method to purify messenger RNA out of a total RNA pool extracted from cells that is dominated by ribosomal RNA. It is impossible to apply the strategy of purification of mRNA by binding to an oligo(dT) column as routinely used for eukaryotic organisms due to the absence of the polyA-tail on prokaryotic mRNA. To avoid 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. Removal of cDNA from rRNA origin has been based on hybridisation to the complementary strands of rRNA genes and separation of hybridised and non-hybridised cDNA. The complementary strands of rRNA genes prepared from genomic DNA were bound in advance to magnetic Dynabeads (see Experimental procedures). Mixing of total cDNA rendered partition of cDNA from rRNA origin through binding to the beads in the pellet whereas the non-bound cDNA that corresponded to mRNA and tRNA was retained in the supernatant. The data presented in Fig. 1 demonstrate the efficiency of the purification; frame A depicts that virtually all labelled cDNA that hybridised to 16S rRNA was removed. Densitometrical quantification and comparison of lanes 2 and 3 showed that only 12% of incorporated radioactive label remained after purification. Note that the new hybridisation signal in lane 3 corresponding to 16S rRNA in terms of its mobility is an artefact due to

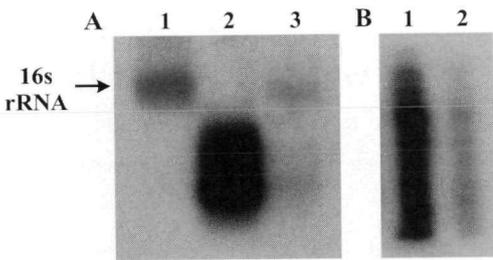


Figure 1. cDNA purification. After reverse transcription of total RNA, cDNA corresponding to rRNA was removed. **A.** Blotted agarose gel, hybridized with ^{32}P labelled probe for the 16S rRNA gene. Lane 1, 10 μg of total RNA; lane 2, cDNA, corresponding to 10 μg of RNA before purification; lane 3, the same cDNA, after purification. **B.** Polyacrylamide gel electrophoresis of ^{32}P labeled cDNA obtained from 10 μg of total RNA. Lane 1, before purification; Lane 2, after purification.

the partial release during washing steps of the 16S DNA that was attached to the Dynabeads, and should be excluded from comparison. This method routinely removed about 90% of cDNA transcribed from 23S, 16S and 5S rRNA. Fig. 1B illustrates the quantity of label that was retained in cDNA probe after removal of cDNA from rRNA origin. In densitometric analysis it corresponded to about 25% (lane 2 versus lane 1). The signal in lane 2 represents labelled mRNA, tRNA and a remainder of unremoved 23S, 16S and 5S rRNAs. The combined results of Frame A and B reflect the effective enrichment of mRNA (and tRNA) in the cDNA probe rendered by the purification protocol.

Array hybridisation and statistical analysis of the data

Each experiment was carried out three times in fully independent experiments under well-defined conditions for cell culture, RNA isolation, ^{32}P label incorporation into cDNA probes, cDNA purification, and array hybridisation. Background subtraction and normalisation of PhosphorImager signals from the blots rendered three sets of tabular data

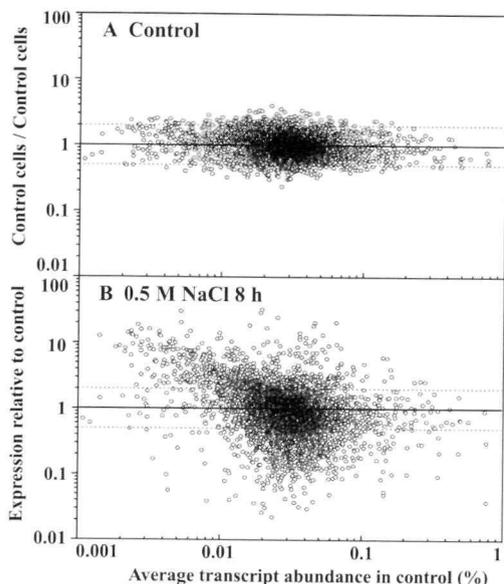


Figure 2. Logarithmic-scale plots of normalized spot intensities. In Frame (A) the ratio of two independent control experiments was plotted against the relative transcript abundance in control cells; the result represents a measure of reproducibility of the experiments. In Frame (B), the transcript intensities on the blots in treatment cells divided by those in control cells were plotted against the relative transcript abundance in control cells. The area between the dashed lines (indicating the ratio range of 0.5 to 2.0) represents the approximate experimental error of expression that was taken as not regulated.

for the control and treatment conditions. Alteration in profiles of 5540 normalised spot densities for control and treatment conditions is depicted in Fig. 2. The results demonstrated reproducibility within a factor of about 2 for the control (Fig. 2A). Most spot densities contributed between 0.01 and 0.1 % to the sum of all densities. It is of interest to observe that also low-abundance transcripts showed high reproducibility, which provided confidence for the quantitative validity of the method. Comparison of the relatively flat distribution in Fig. 2A with the more scattered appearance of Fig. 2B indicated that the treatment could change the abundance of specific transcripts by an order of magnitude or more. In the treatment condition spots corresponding to low abundant cDNA showed a tendency to be more up-regulated than the highly abundant ones. Down-regulation in contrast was more abundant in highly expressed spots.

Statistical evaluation of observed differences in spot densities

Following normalisation, of the three complete data sets for control and treatment experiments the statistical reliability of the data has been evaluated. Two methods for evaluation were used. In the direct ratio method treatment and control data are directly divided. The ratio method suffered a limited statistical foundation due to the low number of replicate experiments and rendered large standard deviation. The "Patterns of Gene Expression" method (PaGE) (Manduchi *et al.*, 2000) provided a more adequate analysis. This program permitted treatment of data from a relatively low number of replicates with an assignment of statistical significance to the predicted differential expression. The PaGE program allowed simultaneous comparison of several sets of data with reference data. It permits the user to

define a single parameter, the false-positive rate, after which the number of statistically reliable regulated cases (both up and down) is calculated. The false positive rate (fpr) is defined as the probability of predicting a gene to be differently expressed when it actually is not. When trying to find a small set of differentially expressed genes from a large pool a seemingly reasonable false-positive rate, say 0.05, can lead to a set of predictions with a high proportion of false positives among them. For example, if 50 out of 1000 genes are differentially expressed, a false positive rate of 0.05 will give $950 \times 0.05 = 47.5$ genes falsely predicted to be regulated, nearly as many as are truly regulated. So, in this example, the confidence in the prediction that a gene is regulated is not much higher than 50%, even with a fpr of 0.05.

As a first step, we explored several fpr values ranging from 0.00025 to 0.03. This means that in actual predictions of regulated spots (for our data set of 5540 spots) on average 1.4 to 166.2 false predictions could be obtained. The lower the fpr the higher the confidence of the results obtained (Fig. 3A), and ideally a fpr of zero should give a confidence of 100%. However, a lower fpr also reduces the number of data points of truly regulated genes that pass the test with the verdict regulated (Fig. 3B). After setting of a desired fpr the program determines the upper and lower limits (ratio limits, Fig. 3C) between which spots were judged neither up- nor down-

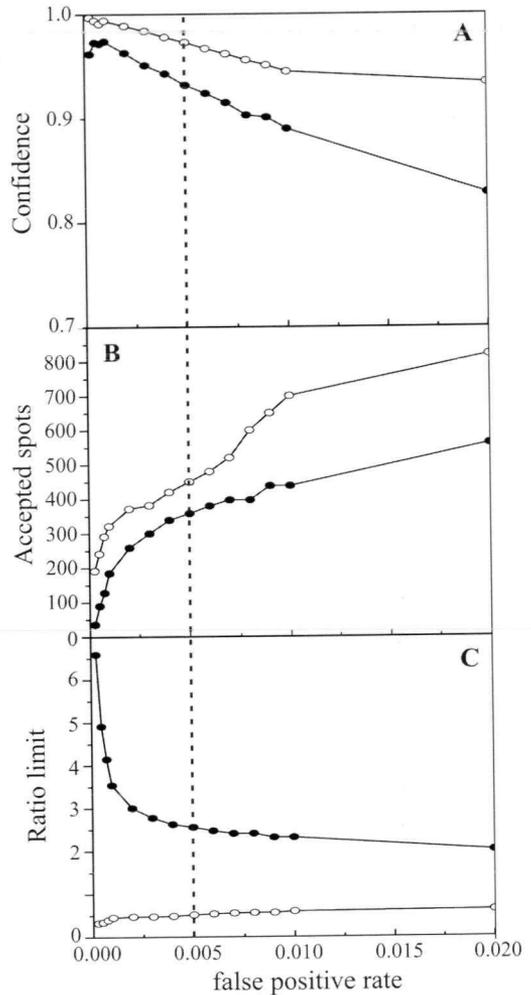


Figure 3. Concept of the PaGE method. (A), Predicted confidence that a gene selected by the PaGE method is indeed differentially expressed; (B), number of spots that are accepted to have an altered expression in the treatment experiment. (C), correlation between 'false positive rate' and upper and lower limits (cut-off values). Closed circles represent up-regulation, open circles represent down-regulation. Dashed line corresponds to the assigned 'false positive rate' of 0.005.

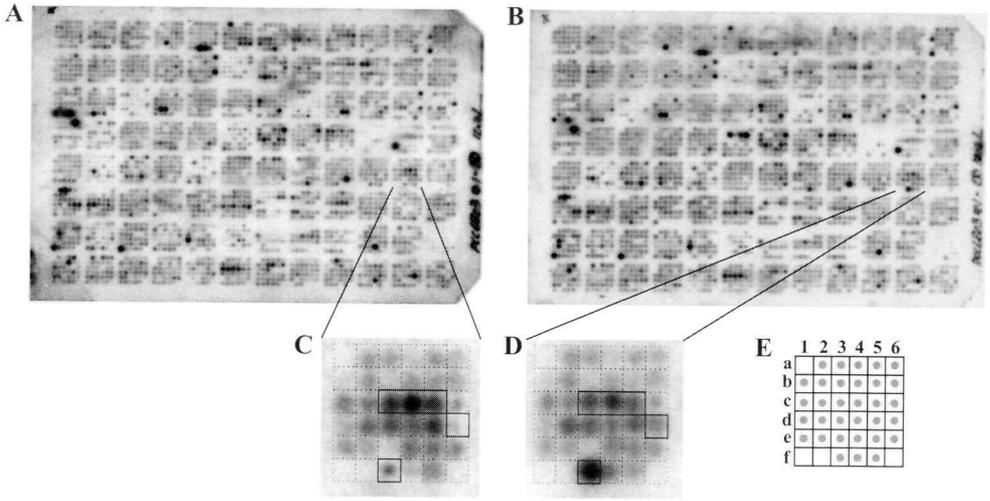


Figure 4. Autoradiograph of the DNA arrays. Arrays were probed with cDNA derived from control (A) or treatment (B) cells. Magnification of selected grids of the DNA array from control (C) and treatment (D) growth conditions; (E), a square grid example of spot organisation (grey circles represent the actual position of spots within the grid). Rectangles in C and D represent spots with significantly up- or down-regulated signal: c3, c4 and c5 contain ORF *slr0876*, d6 contains *slr1308*, and f3 contains *slr2135*.

regulated, than the number of regulated spots is determined and the confidence, which directly correlates with the number of regulated spots and the fpr, is assigned for the prediction. The selectable fpr parameter was set at 0.005 as a trade off between reasonable borderline ‘noise’ limits (with a cut-off of 0.5 for down regulation and 2.0 for up-regulation; see dashed line in Fig.2) and acceptable confidence of the prediction. A fpr of 0.005 means that on average 27.7 of differentially regulated spots are falsely predicted. With this fpr 358 spots were predicted as up-regulated with a statistical confidence of 93%, and 450 spots were predicted as down-regulated with a confidence of 97%.

All spots with altered relative intensity thus identified by the PaGE method were also identified by the ratio method. The PaGE method with selectable statistical restraints appeared to be more stringent than 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. 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 12 % of all predicted data were excluded from conversion because of interference of more genes and occurrence of simultaneous up- and down-regulation coinciding in a single spot. An example of the analysis is shown in Fig. 4. Fig. 4A and B illustrate overall blots probed with cDNA from control and treatment experiments; frames C and D show the hybridisation signals in a selected grid. Fig. 4E 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 bearing *sll1308* that encodes a probable oxidoreductase was strongest in samples isolated from the treatment experiment with salt stress (cf. Fig. 4C, D). From full analysis of all three replicates expression of *sll1308* was judged as induced with a factor of about 3 in cells that were exposed to salt stress. Similarly, the signal from *slr2135* on spot f3 encoding a hydrogenase accessory protein was also significantly stronger in samples isolated from the salt treatment. Finally, in this quadrant, the signal in spots bearing *slr0876* encoding a hypothetical protein was significantly lower in the treated cells. Statistical analysis of data revealed slight down-regulation of expression of this gene in cells that were exposed to salt stress.

Concluding remarks

The genome array blots, composed of overlapping M13 clones directly acquired from the *Synechocystis* genome-sequencing project, were probed with labelled cDNA from control and treated cells to monitor differential gene expression. An extraction method of cDNA representing messenger RNA was optimized. Application of the method results in the removal of more than 90% of 23S, 16S and 5S ribosomal RNA, and highly increases the sensitivity of array analyses for prokaryotes. A recently introduced approach for statistical data evaluation, which allows confident prediction of differentially expressed genes from sets of data with a small number of replicates, was applicable to the array data thus obtained. Our findings suggest that DNA arrays acquired from genome-sequencing projects may be successfully applied for initial determination of gene expression patterns and may attract attention of researchers prior to an appearance of commercially available microarrays.

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