Full Paper

Use of segment-based microarray in the analysis of global gene expression in response to various environmental stresses in the cyanobacterium *Anabaena* sp. PCC 7120

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We prepared microarrays that contain genomic sequences of a heterocyst-forming filamentous cyanobacterium *Anabaena* sp. PCC 7120. The complete genome of this cyanobacterium codes for about 5,368 protein-coding genes in the main chromosome of 6.4 Mbp. In total, 2,407 DNA segments were selected from the sequencing clones, and amplified by PCR, then spotted on glass slides in duplicate. These microarrays differ from the widely used commercial or custom-made ones for other microorganisms in that each DNA segment was 3–4 kbp long, and contained about 3–4 predicted genes on average. This feature, however, did not decrease the usefulness of the microarrays, since we were able to detect a number of potentially novel genes that are induced in response to nitrogen deprivation, low temperature and drought. In addition, we found some genomic regions in which dozens of contiguous genes are simultaneously regulated. These results suggest that these segment-based microarrays are useful especially for such large genomes as *Anabaena*, for which the number of genes exceeds either technical or practical limitations.

Key Words—*Anabaena*; desiccation; genome-wide gene expression; low temperature; microarray; nitrogen deficiency

Introduction

Cyanobacteria are a well-defined group of bacteria that perform oxygenic photosynthesis as do chloro-

plasts of plants and algae. The nucleotide sequences of the genomes of various species of cyanobacteria are now available for further post-genomic researches. One of the major areas of such research is the analysis of genome-wide gene expression either in response to various environmental stresses, or during the cell cycle, or caused by mutation of a known gene, through the use of microarray. This type of research is related to bioinformatic analysis of the network of gene

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regulation.

Responses to various environmental stresses such as low temperature, heat, drought, osmotic pressure, salt, high light, and nutrition have been described in cyanobacteria. In a unicellular cyanobacterium *Synechocystis* sp. PCC 6803, the expression of genes in the whole genome, including about 3,200 genes, was analyzed extensively by DNA microarray analysis to unravel responses to high light, low temperature, light-to-dark transition, salt stress and osmotic stress, and to survey the target genes for SYCRP1, a cyanobacterial cAMP receptor protein (Gill et al., 2002; Hihara et al., 2001; Kanesaki et al., 2002; Suzuki et al., 2001; Yoshimura et al., 2002). Response to change in nitrogen nutrition has also been analyzed in plants (Wang et al., 2000, 2001). Gene expression patterns during sporulation of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and the life cycle of *Drosophila melanogaster* have been reported (Arbeitman et al., 2002; Chu et al., 1998; Mata et al., 2002).

The complete genomic sequence of *Anabaena* sp. PCC 7120 (also called *Nostoc*), hereafter nicknamed 'Anabaena,' has been determined (Kaneko et al., 2001). *Anabaena* is a filamentous strain of cyanobacteria, which has many properties that the unicellular strains do not have, such as tolerance to drought, and differentiation of akinetes, heterocysts, and hormogonia. *Anabaena*, as well as its close relative *Nostoc* sp. HK01, is highly tolerant to complete desiccation for months, and is able to rapidly recover growth after rehydration (Kato et al., 2003). *Anabaena* grows as vegetative cells in the supply of combined nitrogen, such as nitrate or ammonium, while heterocyst differentiation is induced by nitrogen deprivation. One out of every 10 to 15 vegetative cells becomes morphologically distinct and differentiates into heterocysts in about 24 h. At the same time, the oxygen-evolving photosystem II complex is inactivated in heterocysts, and the thick envelope, which consists of an inner glycolipid layer and an outer polysaccharide layer, is synthesized around a cell wall to protect nitrogenase from oxygen (Wolk et al., 1994). The ability of temperature acclimation has also been studied in *Anabaena variabilis* IAM M-3, a close relative of PCC 7120, with respect to fatty acid desaturation (Sakamoto et al., 1994; Sato et al., 1986) and RNA-binding proteins (Sato, 1994, 1995).

This report describes collaborative efforts to prepare microarrays of *Anabaena*, with initial data showing the

performance of the segment-based microarray in analyzing the genome-wide expression in response to nitrogen deprivation, desiccation, and temperature shift.

Materials and Methods

Strains and growth conditions. *Anabaena* sp. PCC 7120 was grown at 32°C in the light ($50 \mu\text{E m}^{-2} \text{s}^{-1}$) in BG-11 medium (Rippka et al., 1979) buffered with 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.5) unless otherwise stated. Liquid culture was bubbled with air containing 1.0% (v/v) CO_2 . For experiments of changing nitrogen sources, a 0.6-L culture in the mid-log phase (A_{750} was about 0.5) grown in BG-11 medium (containing 17.6 mM NaNO_3 as a nitrogen source) was supplemented with 1 mM ammonium chloride and grown for a further 12 h. The amount of ammonium was sufficient to repress the genes that are otherwise expressed in the absence of ammonium, such as the *nir* operon. Then the cells in a 100-ml culture were collected by centrifugation as described below (zero time). The remaining culture was divided into portions, and the cells were collected by centrifugation at room temperature. The cells in each portion were washed with BG-11₀ medium (BG-11 medium lacking nitrate), and resuspended in the original volume of BG-11 or BG-11₀ medium. For each time point, cells in a 100-ml culture were collected by centrifugation. In each cell harvest, cells in the culture medium were mixed with an equal volume of ice-cold ethanol containing 5% phenol to kill them rapidly and to eliminate additional effects during centrifugation (Sato, 1995). Then, the cells were collected by centrifugation at 4°C, rapidly frozen in liquid nitrogen, and stored at -80°C until isolation of RNA.

For desiccation experiments, the *Anabaena* cells grown in liquid culture at 30°C were placed on filter paper and were allowed to dry for 1 h at 30°C and 30% relative humidity. The light intensity was $30 \mu\text{E m}^{-2} \text{s}^{-1}$, which was similar to the light condition during the growth. After designated time, the cells were harvested and RNA was isolated as described above.

Anabaena variabilis strain IAM M-3 was grown in the medium C of Kratz and Myers (1955) at 38°C. The cells were transferred to 22°C and grown for further 2.5 h as described previously (Sato, 1994).

RNA isolation. RNA was isolated from the cells of *Anabaena* as described previously (Sato, 1995). Briefly, the cells were mixed with 90% phenol and bro-

ken with glass beads. The total nucleic acids were recovered from the aqueous phase, and precipitated by ethanol. RNA was isolated by centrifugation through 5.7 M CsCl at 32,000 rpm for 20 h at 20°C. RNA was dissolved in 400 µl of TE containing 0.1% sodium lauroyl sarcosinate, and then precipitated with ethanol. Purified RNA was finally dissolved in TE, and stored at -80°C.

Preparation of DNA microarrays. To construct microarrays that include the whole genomic sequence, we selected 2,407 clones from the sequencing clones that had been used for the assembly of genomic sequence at Kazusa DNA Research Institute (Kaneko et al., 2001). The insert of each selected clone was amplified by polymerase chain reaction (PCR) directly from the phage suspension using Ex Taq (TaKaRa Bio, Otsu, Japan) with a pair of universal primers: M13-M (5'-GGGTTTCCAGTCACGAC-3') and M13-RV (5'-TTATGCTTCCGCTCGTATGTTGTG-3'). PCR was performed for 30 cycles, each cycle consisting of denaturation at 98°C for 10 s and annealing/elongation at 68°C for 6 min. After the final cycle, the reaction mixture was incubated at 70°C for 10 min and then maintained at 4°C. The amplified fragments were precipitated with an equal volume of 2-propanol and then resuspended in H₂O. The DNA fragments were blotted in duplicate onto glass slides coated with poly-L-lysine with SPBIO-E (Hitachi Software Engineering, Yokohama, Japan). The DNA was then crosslinked to the glass surface with UV light with a total radiance of 2,500 µJ. The surface of the glass slides was blocked for 15 min with 140 mM succinic anhydride dissolved in 100 mM boric acid (pH 8.0), 90% (v/v) 1-methyl-2-pyrrolidone. Then, the glass slides were washed three times in distilled water, once in boiling distilled water for 3 min, and then immersed in ice-cold 100% ethanol. The microarrays were then air-dried and stored in a desiccator until use.

Preparation of fluorescent probes. Single-stranded cDNA was prepared in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Biosciences, NJ, USA). The mixture of RNA and random primer (Invitrogen, CA, USA) was denatured and annealed, and then subjected to reverse transcription. A 20-µl reaction contained 20 µg RNA, 50 µM random primer, 10 mM DTT, 500 µM each dATP, dCTP and dGTP, 200 µM dTTP, 100 µM Cy3-dUTP or Cy5-dUTP, 25 U of RNAGuard (Amersham Biosciences), and 200 U of Superscript II (Invitrogen) in 1× first-strand buffer (50 mM Tris-HCl,

pH 8.3, 75 mM KCl, 3 mM MgCl₂). The mixture was incubated at 42°C for 1 h. Then, 200 U of Superscript II was added and the mixture was incubated for another hour. The enzyme was finally inactivated at 70°C for 10 min. The reaction products of two reactions (one with Cy3 labeling and the other with Cy5 labeling) were combined. Unincorporated fluorescent nucleotides were removed by a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and then the labeled cDNA was recovered in 29 µl of 10 mM Tris-HCl (pH 8.5). The cDNA solution (29 µl) was added to 10 µl of 20× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), and then denatured at 95°C for 2 min. The mixture was allowed to cool at room temperature, and then mixed with 1 µl of 20% SDS.

Hybridization and scanning of microarray. The fluorescent cDNA solution was applied to a microarray glass slide and the glass slide was covered with a cover slip. The glass slide was placed in an ArrayIt hybridization cassette (TeleChem International, CA, USA), which was subsequently incubated for 12 to 15 h in a water bath at 65°C. After hybridization, the glass slides were washed three times in 2× SSC, 0.2% SDS at room temperature for 10 min each, three times in 0.2× SSC, 0.2% SDS at room temperature for 10 min each, and three times in 0.2× SSC, 0.2% SDS at 60°C for 10 min each. To eliminate SDS, the glass slides were washed two times in 0.2× SSC at room temperature for 5 min each and finally dried by gentle centrifugation.

Microarray glass slides were scanned with a scanning laser microscope model ScanArray 4000 (GSI Lumonics, Ottawa, Canada) at a resolution of 10 µm per pixel. To avoid saturation of signals, the photomultiplier gain was adjusted to an appropriate value.

Data analysis was performed using the ArrayVision software version 6.0 (Imaging Research, Ont, Canada). Expression data were plotted along the genome in a circular form with the GenoMap software version 1.021204 (Sato and Ehira, 2003), which has been written in Tcl/Tk and is available at the URL (<http://www.molbiol.saitama-u.ac.jp/~naoki/GenoMap/>).

Results and Discussion

Construction of DNA microarrays for the genome of Anabaena sp. PCC 7120

The basic principle of constructing the Anabaena microarray was that all the genomic regions are present

Fig. 1. A microarray image showing fluorescent hybridization signals.

The total RNA was isolated from *Anabaena variabilis* M3 and reverse transcribed to produce fluorescently labeled probes. Red (Cy3), cells grown at 38°C; blue (Cy5), cells grown at 38°C and then transferred to 22°C and grown at 22°C for 2.5 h. For visibility, about 1/4 of a microarray is shown. This is an overlay of two pseudocolor images scanned with two laser beams at different wavelengths. Note that identical sets of DNA segments were spotted in duplicate in two neighboring 7×14 blocks.

in a single microarray. The upper limit of number of spots was about 6,000 in the arrayer that we planned to use (about 2 years ago). The total number of protein-coding genes in *Anabaena* was estimated to be 5,368 in the main chromosome and 763 in the seven plasmids. It was difficult to use all the open reading frames (ORF) for a single microarray. We also preferred to load two sets of spots on a single microarray to get reliable results. We therefore decided to use only the sequence in the main chromosome, and to use DNA segments of the sizes of 3–4 kbp. This strategy was expected to have various benefits: first, the DNA was easily amplified from the sequencing clones used in the Kazusa DNA Research Institute. Second, all the chromosomal regions were expected to be loaded in duplicate on a single microarray. However, we had persistent doubt that we could get enough hybridization intensity with such large DNA segments. We also doubted that the hybridization signal faithfully reflects expression of individual genes. The latter problem was expected to be alleviated by the fact that most *Anabaena* genes are organized in polycistronic transcription units.

We first selected 2,407 sequencing clones that had

Fig. 2. Expression profile after switching nitrogen source.

Anabaena sp. PCC 7120 cells were grown in the presence of nitrate supplemented with ammonium chloride at 32°C. A part of the cells were washed with BG11 medium without combined nitrogen (BG11₀). The cells were then grown in BG11₀ medium for 3 h. Another part of the cells were washed with normal BG11 medium (without ammonium), and allowed to grow for a further 3-h time period. This is a GenoMap representation of expression data along the circular genome. The outermost two narrow rings indicate the position of open reading frames in the forward (outer ring) and the reverse (the second ring) directions. The next two narrow rings indicate the position of RNA genes in the forward (the third ring) and the reverse (the fourth ring) directions. The inner area represents expression data in logarithmic scale along the genomic position. The larger two rings indicate the expression pattern after a shift from ammonium to nitrate. The signals representing induction are shown in red, while the signals representing repression are separately shown in dark blue. The innermost two rings indicate the expression pattern after nitrogen deprivation. Induction is shown in magenta, while repression is shown in cyan. The full scale is 2.0 in log₁₀. Only the clusters of DNA segments that showed significant changes in expression are indicated.

been used for the assembly of genomic sequence at Kazusa DNA Research Institute (Kaneko et al., 2001). Each of the selected clones bore an insert of approximately 3 kbp, containing one to eight ORF of various sizes. The selection was made in such a way that each clone overlapped another on each end. The microarray used in the present study contained about 90% of the whole genomic sequence. Figure 1 shows

Fig. 3. Expression profile after desiccation.

Anabaena sp. PCC 7120 cells were grown in liquid medium and then spread over a sheet of filter paper. The cells were allowed to dry for 1 h. The outer four narrow rings are identical to those in Fig. 2. The main part shows the induction (red) and repression (dark blue). The full scale is 2.0 in $\log_{10}$. Gene names are indicated for those DNA segments whose expression is either increased more than 10-fold or decreased below 1/3-fold.

Fig. 4. Expression profile of *Anabaena variabilis* M3 after temperature shift from 38°C to 22°C.

The outer four narrow rings are identical to those in Figs. 2 and 3. The main part shows the induction (red) and repression (dark blue). The full scale is 2.0 in $\log_{10}$. Gene names are indicated for those DNA segments that have been known to be regulated by temperature and showed, in fact, significant changes in expression.

Fig. 5. Basic nucleotide profile of the *Anabaena* genome.

The data were calculated by the SISEQ package using the published *Anabaena* genome sequence (GenBank BA000019). The compositional data were calculated with an interval of 2,000 bp, and presented as a GenoMap. The outer four narrow rings are identical to those in Fig. 2. The main part indicate compositional data in linear scale. Outer circle (green), G+C content (25–52%); middle circle (blue), GC skew (–0.25–0.30); inner circle (red), AT skew (–0.25–0.30).

representative view of hybridization signals on a microarray. Fortunately, the background was very low because of the tight binding of the DNA segments on the glass slide, which tolerated stringent washing. This made it possible to detect small differences in the intensity of hybridization, and thus to increase the sensitivity.

Almost all (2,380) of the 2,407 DNA segments showed a significant level of fluorescence. The normalization of fluorescence intensity was made in such a way that the average of fluorescence intensity of all the DNA segments was defined as a unity and the intensity of each DNA segment was expressed as a multiple of the unity. The normalized fluorescence intensity of each DNA segment was greater than 0.04, while the average of fluorescence intensity of the background was 0.009. The data points showed little deviation, namely within 2-fold difference, when an identical preparation of RNA was labeled with Cy3 and Cy5 and hybridized (data not shown). The spots that showed

significant deviation or very low intensity of signals (27 spots) were not included in the further analysis.

Global changes in gene expression in response to various environmental stresses

Figures 2–4 show GenoMap representation of the expression analysis of various environmental stresses, namely, nitrogen availability (Fig. 2), desiccation (Fig. 3), and temperature shift (Fig. 4). The data are plotted in logarithmic scale according to the position of DNA segments within the genome. The original numerical data used to draw GenoMap and the GenoMap software (viewer) are available at the author's web site (<http://www.molbiol.saitama-u.ac.jp/~naoki/ArrayData/> and <http://www.molbiol.saitama-u.ac.jp/~naoki/GenoMap/>, respectively). We found a number of DNA segments, in which fluorescence intensity (i.e., hybridization signal) changed drastically after the changes in growth conditions. One of the advantages of GenoMap representation is to visually recognize the regions, in which many segments are expressed as a cluster. We are, therefore, interested in the fact that many, though not all, of the highly expressed segments are clustered (we call them “expressed islands”). We will focus on such clustered expressed segments in the following discussion. The genes that are included in the respective segments are found in the data distributed on the web site.

In the nitrogen stress experiment (Fig. 2), we compared the effects of a switch of nutrition from ammonium to nitrate (Fig. 2, inner part), and nitrogen depletion (Fig. 2, outer part). The results were similar during the first 3 h. In both cases, the expression increased more than 10-fold in a cluster of DNA segments in a region 0.70–0.72 Mbp. This cluster contains the *nir* operon including *nirA-nrtABCD-narB*, which encodes subunits of nitrite reductase, nitrate transporter, and nitrate reductase. This indicates that the *Anabaena* cells try to incorporate as much nitrate as possible if ammonium is not available. Another DNA segment at 0.50 Mbp (*all0426–all0428*) also showed increased expression. The region 3.15–3.22 Mbp was expressed as a cluster under nitrogen depletion. We must admit that the expression in this large region was not very reproducible, but the expression in this region is always seen as a cluster under various conditions such as desiccation as well (Fig. 3). This region (3.15–3.22 Mbp) is peculiar in that the genes are oriented in a counter-clockwise direction and the G+C content as well as

the GC skew and AT skew are different from the flanking regions (Fig. 5). Various genes that are possibly involved in the synthesis of polyketide are found in this region, although no such compound is known to be produced by this strain of *Anabaena*. In the nitrogen stress experiments, the extent of decrease in the expression was small until the third hour. A comprehensive analysis of the genes that are regulated by nitrogen stress or involved in heterocyst differentiation has been published elsewhere (Ehira et al., 2003).

In the desiccation experiment (Fig. 3), the expression in various DNA segments was regulated, and the details are currently being analyzed. Here, we explain some interesting genes out of those whose expression is modified significantly. The signal for the cluster of DNA segments at 0.16–0.18 Mbp containing *all0167* (encoding maltotoligosyltrehalose synthase) and *all0168* (encoding α -amylase) increased 13-fold after 1 h desiccation. The expression in the DNA segment at 3.82 Mbp containing *all3153* and *all3154* (encoding potassium-transporting ATPase B and A chains, respectively) increased 29-fold. These genes are likely to be involved in the regulation of osmotic pressure within the cells, which is a part of acclimation to desiccation. The expression in the cluster of DNA segments at 0.54–0.55 Mbp containing *all0457*, *all0458*, and *all0459* (encoding proteins of unknown function) increased 24-fold at the same time. In contrast, the expression of the RNA polymerase gene cluster (*alr1594*, *alr1595*, and *alr1596*, encoding *rpoB*, *rpoC1*, and *rpoC2*, respectively) was lowered to 43%. This down-regulation of *rpo* genes under desiccation is consistent with the results in *Nostoc commune* UTEX 584 (Xie et al., 1995). The large gene cluster, *all2635–alr2680*, was downregulated. A comprehensive analysis of the genes that are regulated by desiccation stress will be published elsewhere (Katoh et al., 2004).

The temperature shift experiment was done in strain IAM M-3, which has been used for the study of cold acclimation. The strain IAM M-3 was derived from the original strain used by Myers' group, which is identical to the present day *Anabaena* sp. PCC 7118. The strain IAM M-3 is able to start differentiation in the absence of combined nitrogen, but is unable to form mature heterocysts, presumably due to a point mutation in an uncharacterized locus. Despite this defect, the DNA sequence of IAM M-3 is highly similar to that of PCC 7120. As far as the *rbp* genes encoding RNA-binding proteins are concerned, the sequences of the two

strains are more than 99% identical. However, PCC 7120 lacks the *rbpA3* gene that was found in IAM M-3. Therefore, these two strains are essentially identical with respect to DNA sequence but are different in insertion, deletion or rearrangement of a small number of genes. We took advantage of this situation to test if the microarray can be used to analyze gene expression after temperature shift in M-3. Figure 1 clearly shows signals of hybridization with the IAM M-3 probes. Figure 4 shows the GenoMap representation of the results. The DNA segments that showed enhanced expression after a temperature shift from 38°C to 22°C included a cluster *all0408–alr0428* encoding enzymes of tryptophane synthesis plus a number of proteins of unknown function, and *all5195* encoding a glycosyltransferase, as well as various genes that were previously known as cold-regulated, such as *Iti2* (*Iti* for low-temperature-induced, *all0875*, Sato, 1992), *Iti46* (*all0457–all0459*), and *desBAC* (*all1597–all1599*). In addition, various genes involved in photosynthesis, such as *pec* and *cpc*, *isiA*, and *psbC*, as well as *groESL*, were down-regulated by low temperature as expected. These results are in general agreement with the previous studies on the cold-regulation in *Anabaena variabilis* IAM M-3. Therefore, this microarray constructed based on *Anabaena* sp. PCC 7120 sequences are useful in analyzing gene expression in a closely related strain IAM M-3. We expect to use this microarray for the analysis of a wider range of cyanobacterial species, such as wild isolates of *Nostoc*.

Concluding remarks

The present paper reports an initial attempt to identify genes that are regulated during the course of application of various environmental stresses. The data indicated clearly that the expression of various genes that have been known to be regulated by various environmental stresses was in fact regulated in a manner exactly as expected. In addition, the same data suggest that a number of hitherto unknown genes are also regulated by the same treatment. One of the main consequences from this study is that the segment-based microarray is effective in identifying various genes that are induced by environmental stresses. The effectiveness of such a microarray in detecting induced genes may be limited to some extent as described in INTRODUCTION, but it is useful as a tool for primary screening of induced genes. Another significant point is that the

microarray is useful in analyzing global gene expression in closely related strains. However, we still need to know how this type of microarray can be used for various other distant strains.

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