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Identification of Iron-Responsive, Differential Gene Expression in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 with a Customized Amplification Library

ABHAY K. SINGH AND LOUIS A. SHERMAN*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana

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We describe the use of a method called differential expression using customized amplification library (DECAL) to study the global changes in gene expression in iron-deficient versus iron-reconstituting cells of *Synechocystis* sp. strain PCC 6803. We identified a number of genes, such as *isiA*, *idiA*, *psbA*, *cpcG*, and *slr*0374, whose expression either increased or decreased in response to iron availability. Further analysis led to the identification of additional genes related to those identified by DECAL (e.g., *psbC*, *psbO*, *psaA*, *apcABC*, *cpcBAC1C2D*, and *nblA*) that were differentially regulated by iron availability. Expression of *cpcG*, *psbC*, *psbO*, *psaA*, *apcABC*, and *cpcBAC1C2D* increased, whereas that of *isiA*, *idiA*, *nblA*, *psbA*, and *slr*0374 decreased, in iron-reconstituting cells. S1 nuclease protection studies showed that increased transcript levels of *psbA* in iron-deficient cells was due to the increased expression of both *psbA2* and *psbA3* genes, although the steady-state level of *psbA2* remained higher than that of *psbA3*.

Subtractive cDNA cloning, differential display, and DNA microarrays have been used to analyze global gene expression under different growth and environmental conditions (22, 40, 43, 46). These procedures are easier to perform in eukaryotes, due to the presence of a poly(A) tail on mRNA that permits easy separation from rRNA (3). Unfortunately, bacterial mRNA lacks this poly(A) tail and cannot be routinely extracted from total RNA. This problem also makes it difficult to use microarrays in bacteria, because of the high background that is observed when total RNA is used as a labeled probe (17). Moreover, the cost and/or commercial unavailability of DNA chips from prokaryotic organisms make their use largely beyond the financial resources of most within the scientific community. An alternative and cost-effective process to study the differential expression in prokaryotic organisms with small genomes may be available in the form of membranes on which are spotted arrays of *Escherichia coli* cells containing a library of clones. Nonetheless, an absolute requirement when using such membranes is the removal of the abundant rRNA from total RNA before labeling to avoid high background which may interfere with signal analysis. Recently, a new method called differential expression using customized amplification library (DECAL) has been used in global comparisons of gene expression in *Mycobacterium tuberculosis* (1). DECAL accomplishes this by first creating a customized amplification library (CAL) of genomic DNA that has been manipulated for optimal performance during analysis. The success of CAL depends on three factors: (i) physical removal of abundant sequences, i.e., rRNA genes; (ii) reduction in the complexity of the sequences and conversion of all DNA sequences to fragments of smaller and similar size; and (iii) selection of sequences that amplify efficiently.

In this study, we developed a DECAL for analysis of the global gene expression in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803, an oxygenic photosynthetic organism. We chose *Synechocystis* sp. strain PCC 6803 as a model organism because it is transformable with external DNA, the frequency of homologous recombination is very high, thus facilitating genetic analysis, and it is capable of both photoheterotrophic and mixotrophic growth (45). Moreover, the complete sequence of *Synechocystis* sp. strain PCC 6803 has recently been determined (23). These characteristics have made *Synechocystis* sp. strain PCC 6803 a model organism for studies of photosynthetic processes and of modifications in response to external and internal stimuli. Such studies have wide application, because cyanobacteria are considered the progenitor of chloroplasts and because a number of biotic and abiotic stresses are known to directly affect photosynthesis and cyanobacterial growth (6, 14, 35, 37, 42).

To demonstrate proof of concept, we used iron-deficient growth of *Synechocystis* sp. strain PCC 6803 as a growth-limiting condition to analyze gene expression using DECAL. Iron deficiency is known to cause a variety of physiological and morphological changes in cyanobacteria, including loss of light-harvesting phycobilisomes (20), changes in the spectral characteristics of chlorophyll (Chl) within the thylakoids (20, 21, 31, 32), reduction in the number of thylakoids (41), and replacement of cofactors containing iron with noniron cofactors, such as ferredoxin with flavodoxin (24). A new Chl-binding protein, CPVI-4, which is similar to CP43 and encoded by *isiA*, associates with PSII (and possibly even with PSI) under iron-deficient conditions (31, 32). Similarly, synthesis of IdiA associated with the cytoplasmic side of the thylakoid membrane is greatly enhanced in iron-deficient cells (27). Despite these massive changes under iron-deficient conditions, cells continue to grow to high densities, although the growth rate is somewhat lower and the cells are smaller (41). Furthermore, these changes are reversible; after 24 h of iron addition, cells return to normal (41). In this work, we describe the information provided by the DECAL that can help us understand transcriptional regulation during reconstitution from iron starvation in *Synechocystis* sp. strain PCC 6803.

* Corresponding author. Mailing address: Department of Biological Sciences, Purdue University, 1392 Lilly Hall of Life Sciences, West Lafayette, IN 47907-1392. Phone: (765) 494-8106. Fax: (765) 496-1495. E-mail: lsherman@bilbo.bio.purdue.edu.

MATERIALS AND METHODS

Cyanobacterial strain and growth conditions. Cultures of *Synechocystis* sp. strain PCC 6803 were grown phototrophically in liquid BG-11 medium at 30°C under a light intensity of 40 to 50 microeinsteins m^{-2} s^{-1} in the presence or absence of iron as described previously (41). Cells were subcultured in iron-deficient media at least two to three times prior to experimental use. Recovery of iron-deficient cultures was accomplished by addition of 6 mg of ferric ammonium citrate per liter of medium. Cells were harvested during recovery at the 0 h (iron-deficient culture) or at 3, 12, and 24 h after the addition of iron (reconstituting cultures).

Construction of a cosmid library. A cosmid library of *Synechocystis* sp. strain PCC 6803 was constructed in pYUB328 (a kind gift of W. R. Jacobs) by using the double cosmid vector strategy as described previously (44). Genomic DNA from *Synechocystis* sp. strain PCC 6803 was isolated as described by Williams (45). Genomic DNA was partially restricted with *Sau*3AI, and fragments of 35 to 45 kb were isolated on a sucrose gradient as described previously (2). Arms of pYUB328 were prepared by digestion with *Xba*I, alkaline phosphatase, and then *Bam*HI (4). These arms were ligated with genomic fragments at 1:10 molar ratio (insert to arms). The ligated DNA was in vitro packaged with GigaPack II Gold packaging mix (Stratagene); the resulting recombinant cosmids were transduced into *E. coli* XL1-Blue MRF′ and selected for ampicillin resistance on solid Luria-Bertani (LB) plates.

Construction of CAL. A CAL from *Synechocystis* sp. strain PCC 6803 was constructed essentially as described by Alland et al. (1), with certain modifications. A total of 1,080 cosmid clones were streaked on several solid LB plates containing 50 μg of ampicillin ml^{-1} using sterile toothpicks, and the toothpicks were subsequently transferred into liquid LB with 50 μg of ampicillin ml^{-1}. LB plates were grown overnight at 37°C, and colonies were transferred onto a positively charged nylon membrane (Schleicher & Schuell). The lysis of colonies and DNA binding to membrane were performed as described previously (39). The liquid cultures were used to isolate cosmid DNA by the sodium dodecyl sulfate (SDS)-alkaline method. Identification of the clones containing rRNA genes was performed by colony hybridization and dot blotting. Cosmid DNA was mixed in a set of 10 and spotted onto the positively charged nylon membrane using a minifold apparatus (Schleicher & Schuell). PCR-amplified rRNA genes (5S, 16S, and 23S) were gel purified, radiolabeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using a random primer Ready-to-Go labeling kit (Amersham Pharmacia Biotech); unincorporated radiolabeled nucleotides were removed by passing the solution through Probe Quant G-50 columns (Amersham Pharmacia Biotech) and used to probe the blots. The DNA from negative clones was pooled and digested individually with *Not*I and *Pac*I. Insert DNA from *Not*I and *Pac*I digest was purified from agarose gels with a QiaExII gel extraction kit (Qiagen). Approximately 1-μg fragments from *Not*I and *Pac*I digest were mixed and separately digested with *Alu*I and *Hae*III and were then mixed and fractionated on a 2% NuSieve GTG low-melting-point agarose gel (FMC). Gel slices containing DNA fragments in the range of 400 to 1,500 bp were excised and stored at −20°C. Five microliters of gel slice solution was ligated with 1 μl of *Xho*I adapters (2 pmol μl^{-1}). Ten microliters of the ligation mix was PCR amplified with 2 μl of 10 μM primers using *Taq*I DNA polymerase (Promega). The amplification cycle consisted of 3-min hot start followed by 10 cycles of PCR with 1-min segments of 94, 65, and 72°C. After the end of the 10th cycle, 4 U of fresh *Taq*I DNA polymerase was added and 27 additional cycles at 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min were performed.

Total RNA extraction. Total RNA from *Synechocystis* sp. strain PCC 6803 was isolated using the procedure described by Reddy et al. (34), with modifications (15). RNA was isolated using cells harvested from 1-liter cultures collected at 0, 3, 12, and 24 h after the addition of iron to the iron-deficient cells. At each time point, cells were mixed with 1/20 volume of stop solution (200 mM Tris-HCl [pH 8.0], 20 mM EDTA, 20 mM sodium azide) and 20 mM aurintricarboxylic acid pelleted, and stored at −80°C. Aurintricarboxylic acid was omitted from cells when the total RNA was used for enzymatic reactions.

Identification of differentially regulated genes. One microgram of total RNA was treated with RQ1 RNase-free DNase (Promega) and reverse transcribed with 7.7 μg of biotin-labeled random hexamers and biotin-dATP (one-seventh of total dATP) using Superscript II (GIBCO-BRL) at 45°C for 1 h. Synthesis was terminated by heating the mix at 70°C for 15 min. The complementary RNA was removed by the addition of RNase H with incubation for 30 min at 37°C. Subsequently, 300 ng of CAL, 20 μg of salmon sperm DNA, and 20 μg of tRNA were added to the cDNA for a final volume of 150 μl. The mix was extracted with phenol-chloroform and ethanol precipitated overnight. The pellet was resuspended in 6 μl of 30 mM EPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; Sigma], pH 8.0–3 mM EDTA, overlaid with oil, and denatured by heating at 99°C for 5 min followed by addition of 1.5 μl of 5 M NaCl preheated to 69°C. The sample was incubated at 69°C for 4 days followed by addition of 150 μl of incubation buffer (1× Tris-EDTA [pH 7.6], 1 M NaCl, 0.5% Tween 20) that had been preheated to 69°C. Fifty microliters of washed streptavidin-coated magnetic beads (Boehringer Mannheim) that had been preheated to 69°C was added to the mix and incubated at 55°C with occasional mixing for 60 min; the solution was then washed three times for 30 min each at room temperature and three times at 69°C with 0.1% SDS–0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with slow continuous shaking. The sample was then washed with 2.5 mM EDTA and eluted by boiling in 50 μl of water. PCR was performed as for the CAL preparation, using 20 μl of eluted fragments in each sample.

Colony array hybridization. The cosmid library arrays were prepared using a Bio-grid (BioRobotics) by double-spotting 1,080 cosmid clones into a 22- by 22-cm nylon membranes that rested on solid LB plates containing 50 μg of ampicillin ml^{-1}. The biobank containing 1,080 clones was prepared manually by transferring 1,080 clones into three 384-well microtiter plates in LB containing 10% glycerol and 50 μg of ampicillin ml^{-1}. The cells were stored at −80°C and regularly used for gridding. The colonies were spotted using a three-by-three double-offset pattern. The colonies were grown at 37°C to the optimum size. The lysis of colonies and DNA binding to membrane was done as described elsewhere (39). This yielded six identical replica blots containing 1,080 clones. The PCR-amplified fragments were labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) for at least 6 h, using a random primer Ready-to-Go labeling kit (Amersham Pharmacia Biotech); unincorporated radiolabeled nucleotides were removed by passing the solution through Probe Quant G-50 columns (Amersham Pharmacia Biotech) and hybridized to cosmid library arrays for 16 to 18 h essentially as described elsewhere (39). The blots were washed twice at room temperature for 15 min each in 2× SSC–0.1% SDS and twice at 68°C for 30 min each in 0.1× SSC–0.1% SDS. Double-spotted colonies that showed different intensities with PCR probes were selected for further analysis.

Densitometry analysis. Density measurements of the autoradiograms were performed with IPLab (Signal Analytics Corporation, Vienna, Va.) on a Macintosh G3 computer. A scanned image of the autoradiogram was segmented into 384 identical squares, and the density of each square was determined. The density value was inversely proportional to the intensity of spots and was divided into 256 segments. We standardized the autoradiograms by analyzing a series of spots with low density that did not change after the addition of iron. This led us to conclude that changes within ±10 density units should be considered identical. We observed 71 spots that differed by ±20 units and 36 spots that differed by more than ±50 units. We chose to analyze further a total of 14 spots: 8 with a density difference of ±50, 3 with a density difference of ~±20, and 3 with a density difference of ~±10.

Northern blots. Five- to fifteen-microgram aliquots of total RNA isolated from cells taken at various time points were electrophoresed on 1% denaturing agarose gels. Each gel was soaked with 20 mM NaOH for 20 min and then transferred in 10× SSC for 45 min with slow shaking. RNA was capillary transferred onto positively charged nylon membranes (Schleicher & Schuell) for 2 to 6 h, using the procedure described in reference 11. RNA was fixed to the membrane by baking at 80°C for 1 to 2 h in a vacuum oven and stained with methylene blue to mark the position of the standard RNA markers and also to check the transfer. Prehybridization and hybridization were performed as described elsewhere (39). The membranes were washed twice at room temperature for 15 min each in 2× SSC–0.1% SDS and twice at 68°C for 15 min each in 0.1× SSC–0.1% SDS. Labeling and purification of various probes were done as described above.

S1 nuclease protection assay. The S1 nuclease protection assay was performed as described previously (2), with certain modifications. Sufficient primers specific to *psbA2* and *psbA3* were labeled with [γ-^{33}P]ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase (New England Biolabs). The reaction was terminated by heating at 75°C for 10 min and ethanol precipitated twice following the addition of tRNA and ammonium acetate. Twenty micrograms of total RNA was hybridized with excess primers at 51°C for 16 h. Nonhybridized nucleic acid was enzymatically removed by the addition of S1 nuclease (GIBCO-BRL). The mix was ethanol precipitated and loaded on an 8% polyacrylamide gel containing 6 M urea. After completion of the run, the gel was dried and exposed to the X-ray film.

DNA probes and primers. The following probes were used: for *psbA*, a 0.6-kb *Bst*EII fragment from *Synechococcus* sp. strain PCC 7942 (plasmid pSG200 [18]); for *psbC*, a 0.9-kb *Bsu*36I-*Eco*RI fragment from *Synechocystis* sp. strain PCC 6803 (plasmid pKW1344 [10]); for *psbO*, a 0.7-kb *Stu*I-*Bst*EII fragment from *Synechocystis* sp. strain PCC 6803 (plasmid pRB1 [7]); for *psaA*, a 2.8-kb *Eco*RI-*Bgl*II fragment from *Synechococcus* sp. strain PCC 7002 (plasmid pAQPR80 [9]); and for *cpcBA*, a 1.2-kb *Sma*I-*Xho*I fragment from *Synechococcus* sp. strain PCC 7002 (plasmid pAQPRI [9]).

The primers used were based on sequences available in Cyanobase and were synthesized at IDT (Coralville, Iowa). Primers used for rRNA were 5′-ACTTGGCATCGGACTATTGTGCCG-3′, 5′-ACGAGTGACCGTGTGCCTGTTGAA-3′, 5′-ATCGAGCTCCCATTGCTTGTAGGC-3′, and 5′-TTGATCCTGGCTCAGGATGAACGC-3′; for *Xho*I adapter sequence, 5′-CCTCTGAAGGTTCCAGAATCGATAGCTCGAGT-3′ (top strand) and 5′-P-ACTCGAGCTATCGATTCTGGAACCTTCAGAGGTTT-3′ (bottom strand); *Xho*I primer sequence, 5′-CCTCTGAAGGTTCCAGAATCGATAG-3′; for *cpcG*, 5′-GGCTCTGAAGAGAAGCCTGTTGTT-3′ and 5′-GGGCACAGAAGCTTCGATGTTGAT-3′; for *slr*0374, 5′-CAAGAAGAGCTGAGTGTACTGCTG-3′ and 5′-GAACTCCAGTCGCTGATATTCAGC-3′; for *idiA*, 5′-ATGACAACTAAGATTTCCCGGCGG-3′ and 5′-TGAATCGGGTTGGTAACGTCCCAA-3′; for T3 primer, 5′-AATTAACCCTCACTAAAGG-3′; for T7 primer, 5′-TAATACGACTCACTATAGGG-3′; for *psbA2*, 5′-CGCTGTTGGAGAGTCGTTGTCATTTGGTTATAATTCCTTATGTATTTGTCGATGTTCAGATTGGAACTGACTAAACTTAGTC-3′; for *psbA3*, 5′-CGCTGTTGGAGAGTCGTTGTCATTTGGTTATAATTCCTTATGTATTTGTCAATGTTCAAAGGATTTGGCCTCAAGCTC-3′, for *apc*, 5′-TTACGGGGGCAGTGTAATCAGG-3′ and 5′-TGGA

GCAAAACG-GTTGGACG-3′; and for *nblA*, 5′-CCCAGAGCAACAACAAG AGTTACTG-3′ and 5′-CAGGTAAGATCAAGTTTGCGGC-3′.

RESULTS

Construction of CAL from *Synechocystis* sp. strain PCC 6803. A CAL from *Synechocystis* sp. strain PCC 6803 was constructed as described by Alland et al. (1) (Fig. 1). Two restriction enzymes, *Not*I and *Pac*I, were used to excise the genomic fragment from the pYUB328 vector. Both enzymes restrict *Synechocystis* sp. strain PCC 6803 DNA (12), and it was expected that this approach would minimize the loss of DNA fragments due to any internal site. The uniform size of fragments from the larger genomic fragments were generated following digestion with *Dra*I and *Hae*III. These selected fragments were PCR amplified a number of times to select the fragments, which are efficiently amplified. This step was necessary for maintaining the proportional amplification of mRNA in two different population of cells.

Iron-starved cells. Cyanobacteria, such as *Synechococcus* sp. strain PCC 7942, respond to iron deficiency with distinct morphological and physiological changes (36, 37, 41). We used iron-deficient growth and subsequent iron reconstitution to demonstrate proof of concept for DECAL, because iron deficiency leads to robust physiological alterations with continued cell growth. Major changes under iron-deficient growth include pigment changes, such as lowered Chl and phycobilisome composition, which result in a very yellowish culture. Such growth also leads to a shift in the Chl absorption peak to about 671 nm; this is due to the presence of a new Chl-binding protein (CP43′) which is encoded by the *isiA* gene (7, 25, 31–33). The readdition of iron leads to a reconstitution of normal cellular physiology and morphology within 12 to 24 h, a process which is easily identified by the regreening of the culture (41). Thus, analysis of iron deficiency and iron reconstitution provides specific well-characterized landmarks as well as an opportunity to discover the identity of many other genes that are involved in the significant cellular perturbations caused by iron-deficient growth.

Therefore, we used DECAL to examine transcriptional changes during the first 24 h of reconstitution from iron deficiency. The pigment analysis of iron-starved *Synechocystis* sp. strain PCC 6803 cells showed decreased Chl and phycocyanin, and the PC/Chl ratio decreased as reported earlier for *Synechococcus* sp. strain PCC 7942 (36, 37). Addition of iron led to gradual accumulation of pigments, and by 24 h, the pigments levels were very similar to that found in normal cells (data not shown). Figure 2 shows the room temperature absorption spectra of *Synechocystis* sp. strain PCC 6803 cells that were collected at different time periods after the addition of iron to iron-starved cells. As reported earlier for *Synechococcus* sp. strain PCC 7942 and shown in Fig. 2 for *Synechocystis* sp. strain PCC 6803, the Chl absorption peak was blue shifted by 8 nm in iron-deficient cells relative to the peak in iron-sufficient cells. Addition of iron led to a gradual shift of the Chl absorption peak, which was restored to 679 nm by 24 h.

Identification of differentially regulated genes. The differentially regulated genes in *Synechocystis* sp. strain PCC 6803 during iron starvation and iron reconstitution were identified by examining the differential hybridization patterns of cosmid library arrays with PCR probes prepared by the CAL library (Fig. 3). As indicated in Materials and Methods, we chose 11 spots which showed significant changes in iron-starving cells versus iron-reconstituting cells, plus 3 which showed virtually no change. Because of our interest in the physiological changes that occur during iron-deficient growth, this first group emphasized spots that decreased in density after the addition of iron. Cosmid clones representing these spots were further analyzed by dot blot and Northern blot analysis. Northern blot analysis revealed that six of the eight spots with density differences of ~±50 units (D21, F11, F12, O14, P13, and P14) demonstrated differential expression, whereas only 1 of the 6 spots within ±20 units demonstrated differential expression. However, one of these (B15) included a cosmid containing *cpcG*. A1 is an example of a control spot with a density difference of ±10 that showed no change in transcription upon further analysis. These results provide some idea of the sensitivity of the *Synechocystis* sp. strain PCC 6803 DECAL library. We have an additional ~25 spots with density differences in excess of 50 density units and an additional ~40 spots with density differences of ±20 from this iron deficiency/iron reconstitution DECAL.

The genes in the seven clones that showed differential regulation were identified by sequencing the DNA from both ends using T3 and T7 primers. These sequences were then used to search for homologous regions in Cyanobase. The specific homologous region was mapped, and all of the genes present in this region were identified. The possible open reading frames (ORFs) were selected based on transcript size showing differential regulation on Northern blots. The primers specific to various ORFs were designed based on sequences available in Cyanobase, and the amplified fragment was used to hybridize the blots containing total RNA isolated from iron-deficient cultures and those at 3, 12, and 24 h during recovery. Using this strategy, we identified two clones containing the *isiA* gene and two clones with the *psbA* gene; the other three clones contained *cpcG*, *idiA*, and *slr*0374 (Fig. 4). Regulation of *isiA* and *idiA* in response to iron deficiency has been previously characterized (7, 25, 27). As shown in Fig. 4, the steady-state levels of both genes were high in iron-deficient cells and the addition of iron led to rapid loss of transcripts.

Two clones showing differential regulation contained the *psbA* gene. As shown in Fig. 4, the steady-state level of the *psbA* transcript gradually decreased with increasing time periods after the addition of iron. The high *psbA* transcript level in iron-starved cells was surprising, since it has been shown that *Synechocystis* sp. strain PCC 6803 cells growing in iron-limiting conditions have decreased levels of PSII and PSI centers (36, 37). Therefore, we expected that steady-state levels of transcripts coding for photosystem proteins would be minimal in iron-deficient cells, with rapid accumulation following addition of iron. Indeed, the transcript levels of *psaA*, *psbC*, and *psbO*, coding for the reaction center protein of PSI, a Chl-binding protein of PSII, and the Mn-stabilizing protein of the oxygen-evolving complex, respectively, were low in iron-starved cells and increased following the addition of iron (Fig. 5).

In *Synechocystis* sp. strain PCC 6803, there are three genes for *psbA*: *psbA1*, *psbA2*, and *psbA3* (28). *psbA1* is a cryptic gene and does not code for a functional protein, whereas *psbA2* and *psbA3* are highly homologous and both code for a functional D1 protein (38). Recently, Mate et al. (26) showed the differential regulation of *psbA* genes in *Synechocystis* sp. strain PCC 6803 in response to UV-B. Thus, it is possible that the increase in the *psbA* transcript levels in iron-deficient cells was due to differential expression from the two *psbA* genes. To decipher the gene-specific regulation by iron, we performed an S1 nuclease protection assay using a synthetic oligonucleotide specific to either *psbA2* or *psbA3*. As shown in Fig. 6, iron starvation led to the accumulation of transcripts originating from both *psbA2* and *psbA3*. Addition of iron to the iron-starved cells had little effect during the first 3 h after iron addition (Fig. 6). However, steady-state transcript levels of the *psbA* genes decreased after further recovery toward iron sufficiency. It is

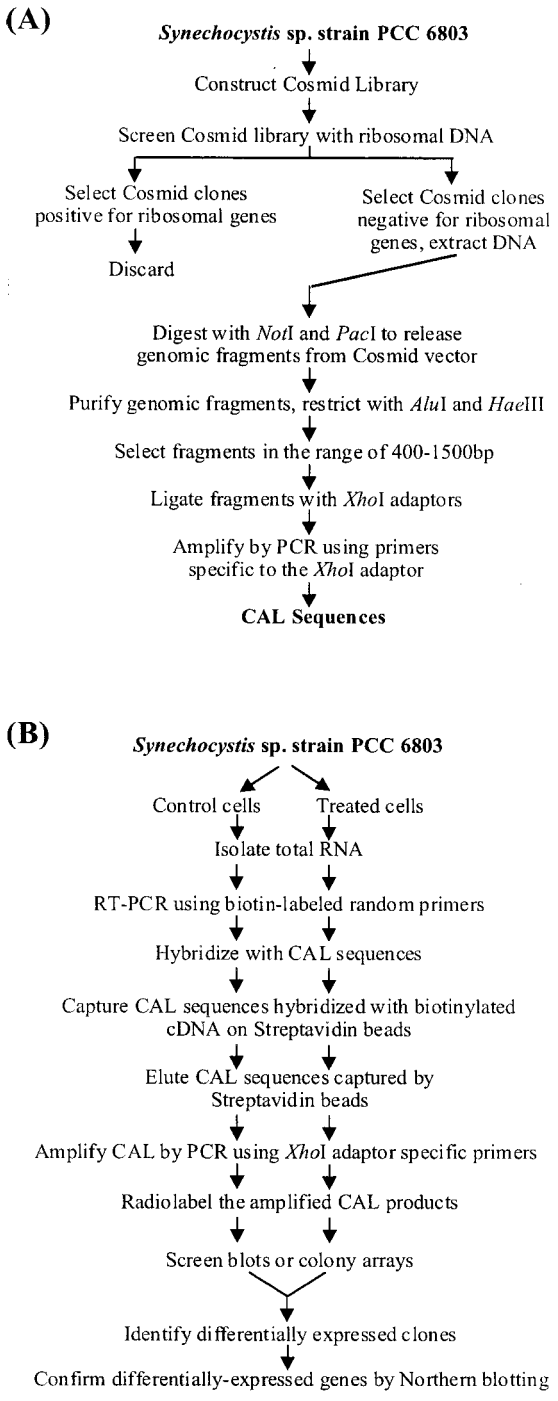

FIG. 1. Flow chart showing various steps involved in construction of a DE-CAL for *Synechocystis* sp. strain PCC 6803. (A) Construction of CAL. A cosmid library carrying 35- to 45-kb genomic fragments of *Synechocystis* sp. strain PCC 6803 was constructed in pYUB328 and screened for clones containing ribosomal DNA sequences. Nonribosomal cosmids were pooled, restricted with suitable enzymes, and gel purified to generate smaller and similar-sized fragments. These fragments were ligated with adapters and PCR amplified to generate CAL sequences. (B) Identification of differentially regulated genes. Total RNA was isolated from *Synechocystis* sp. strain PCC 6803 cells that were treated under different conditions, reverse transcribed using biotin-labeled random hexamers, and hybridized to CAL sequences. CAL sequences representing cDNA in total RNA was eluted and amplified to generate PCR probes. These probes were radiolabeled and hybridized to replicate colony arrays of the cosmid library. Colonies showing differences in signal in the two arrays were selected, and differential gene expression was confirmed by Northern blot analysis.

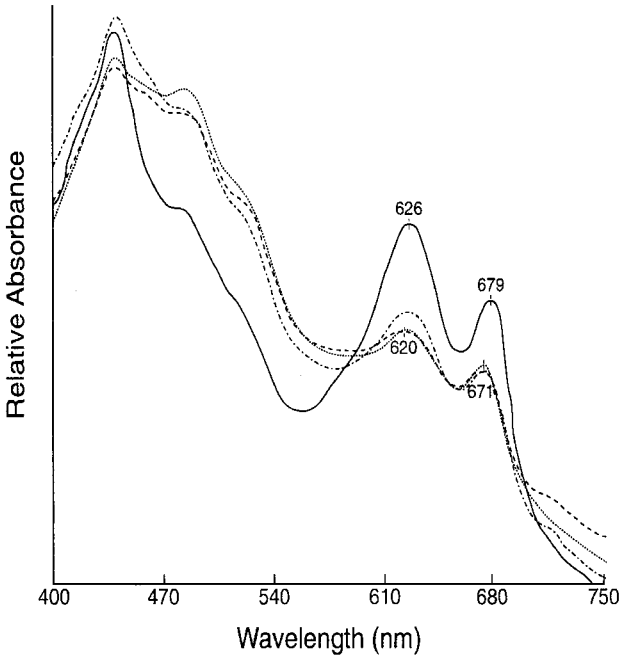

FIG. 2. Absorption spectra of the iron-deficient *Synechocystis* sp. strain PCC 6803 cultures (····), and those collected at 3 (– – – –), 12 (—·—·—·—), and 24 (————) h after the addition of iron.

interesting that whereas transcription of *psbA3* continued to decrease until 24 h, the transcription of *psbA2* increased between 12 and 24 h after iron addition.

Another gene differentially regulated in response to iron availability was identified as *cpcG* (Fig. 4B). *cpcG* encodes for a 30-kDa linker polypeptide that serves in the attachment of phycocyanin hexamers to the phycobilisome core (19). The steady-state level of the *cpcG* transcript was very low in iron-deficient cells, and iron addition led to the accumulation of transcript. Following this observation, Northern blot analysis was performed to determine the expression of genes coding for allophycocyanin and phycocyanin subunits. Analysis of transcript originating from allophycocyanin operon (*slr*2067, *slr*1986, and *ssr*3383) revealed two transcripts of 1.4 and 1.8 kb, both of which were differentially regulated by iron starvation (Fig. 7A). Similarly, analysis of the phycocyanin operon (*sll*1577, *sll*1578, *sll*1579, *sll*1580, and *ssl*3093) revealed three transcripts of 3.7, 3.3, and 1.6 kb which were differentially regulated in response to iron availability (Fig. 7B). The steady-state level of the transcripts originating from phycocyanin and allophycocyanin operons followed kinetics similar to that of *cpcG*.

Recently, *nblA* has been identified in *Synechococcus* sp. strain PCC 7942 as a gene involved in phycobilisome degradation (13). It has been shown that the *nblA* transcript is quite abundant during sulfur and nitrogen starvation in *Synechococcus* sp. strain PCC 7942 (13), conditions which led to the rapid loss of phycobilisomes. Since iron deficiency leads to the loss of phycobilisomes, we were interested in determining the iron regulation of *nblA*. Northern blot analysis revealed that *nblA* transcripts were present at very high levels in iron-starved cells and at low levels 3 h into reconstitution and were virtually absent at 12 and 24 h after the addition of iron (Fig. 7C). Similar to the case for *Synechococcus* sp. strain PCC 7942, transcripts originating from *nblA* in *Synechocystis* sp. strain

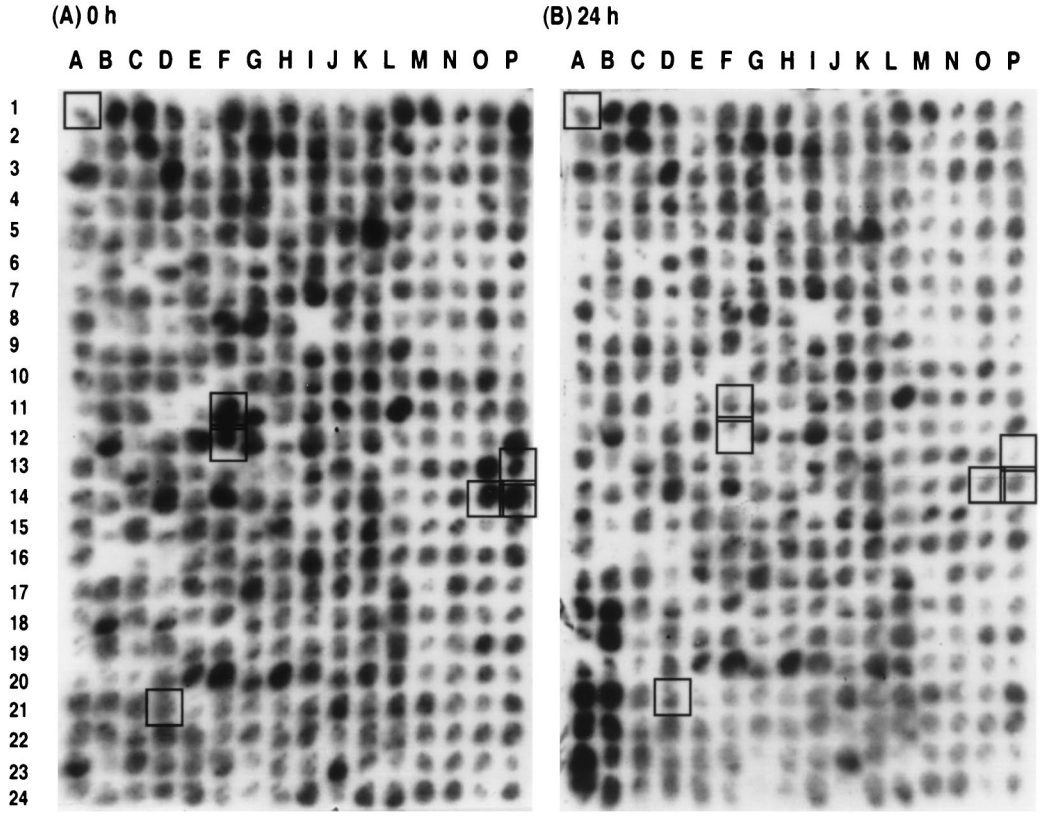

FIG. 3. Cosmid arrays hybridized with radioactively labeled CAL sequences selected after hybridization with single-stranded cDNA prepared from iron-deficient cells (A) and 24 h after the addition of iron (B). The boxed spots represent cosmids that demonstrated major (D21, F11, F12, O14, P13, and P14) or minor (A1) density differences between the two conditions and that were analyzed further.

PCC 6803 revealed a smear of transcripts ranging from 0.25 to 1.0 kb.

Another gene found to be differentially regulated during iron starvation was *slr*0374. This ORF, which is classified as a cell division cycle protein in Cyanobase, has homology with cell division proteins CDC48 and FtsH from a number of organisms (23). It also demonstrated homology with hypothetical chloroplast protein RF46 from *Guillardia theta* and hypothetical protein ycf46 from *Odontella sinensis* and *Porphyra purpurea*. Although its function in *Synechocystis* sp. strain PCC 6803 is not known, iron starvation led to an increase in the steady-state level of the *slr*0374 transcript. Reconstitution of iron-starved cells led to a decrease in the transcript; however, unlike transcripts of iron-regulated *isiA* and *idiA* genes, its transcript was present even after 24 h of iron addition. In addition, the blot probed with *slr*0374 showed a large smear indicative of multiple transcripts.

DISCUSSION

Cyanobacteria are found in virtually all terrestrial niches and face fluctuating chemical and physical parameters such as nutrient availability, light quality and quantity, and temperature. Like other bacteria, they have a plethora of regulatory systems that enable them to respond quickly to such environmental alterations (5), something they have done successfully for billions of years. These adaptive processes involve global changes in gene expression. This study had two objectives: to determine if DECAL was useful for the study of global gene expression in cyanobacteria, and to learn more about genes regulated by iron availability in this unicellular strain. We have demonstrated that our DECAL identified two genes, *isiA* and *idiA*, that had previously been shown to be regulated by iron availability (7, 25, 27). In addition, we identified a series of genes involved in photosynthesis or pigment synthesis that had not previously been identified as under iron regulation (*psbA*, *cpcG*, and *slr*0374). We conclude that DECAL can be used successfully to detect genes that are differentially regulated by environmental fluctuations, but that the sensitivity of the current library must be improved.

The differential regulation of the *cpcG* gene was consistent with the pigment and photosynthetic alterations found in iron-deficient cyanobacteria (20, 21, 31, 32, 41). *cpcG* is not the only gene which encodes a phycobilisome component that is regulated by iron, since transcripts originating from the *apc* and *cpc* operons were also differentially regulated by iron availability. Significantly, the kinetics of transcript accumulation of *cpcG*, *apcABC*, and *cpcBAC1C2D* during reconstitution were similar. In addition, the transcript level of *nblA* increased in the iron-starved cells. *nblA* has recently been identified as a gene whose product is involved in the degradation of phycobilisomes (13).

Although it was anticipated that *psbA* transcription would modulate during transition from growth in iron-deficient to iron-sufficient conditions, the exact expression pattern of the *psbA* genes was surprising. An increased transcript levels of *psbA* was observed in iron-deficient cells, whereas iron reconstitution led to a gradual decrease in the transcript level. Pre-

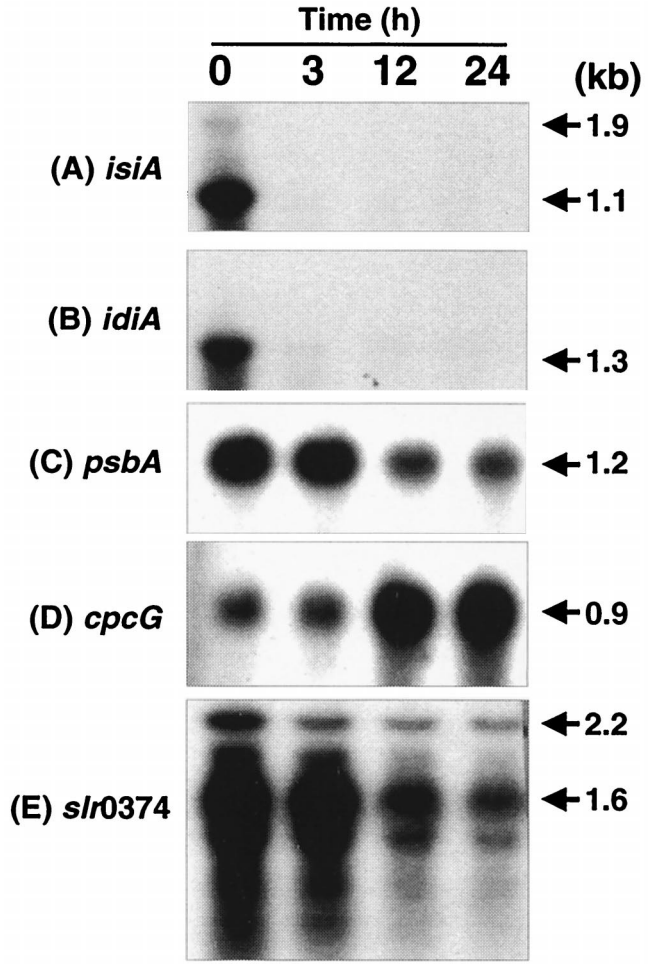

FIG. 4. Northern blot confirmation of genes that were differentially expressed in iron-deficient versus iron-sufficient conditions. Total RNA was isolated from iron-starved cells (0 h) and from cells harvested at 3, 12, and 24 h after the addition of iron. Total RNA (10 μg/lane) was separated on a 1% denaturing agarose gel, capillary transferred, and hybridized with corresponding radiolabeled probes (see Materials and Methods). (A) *isiA*; (B) *idiA*; (C) *psbA*; (D) *cpcG*; (E) *slr*0374.

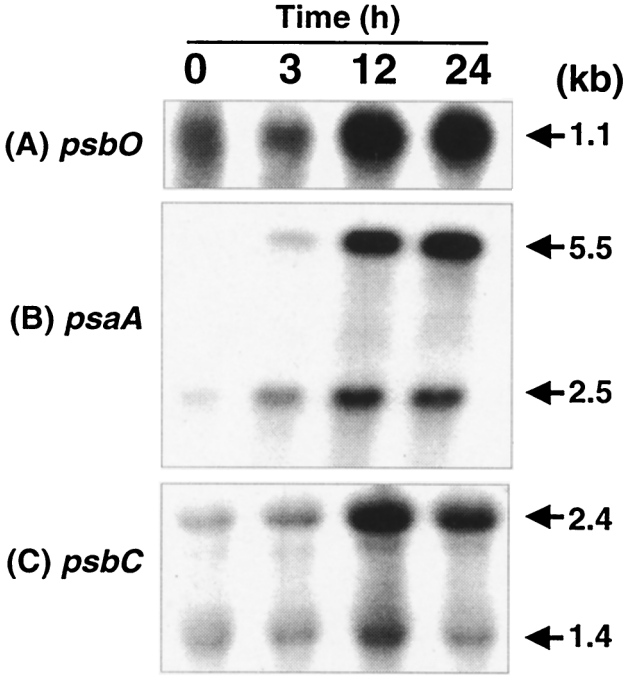

FIG. 5. Steady-state transcript levels of *psbO* (A), *psaA* (B), and *psbC* (C) genes in iron-starved and iron-reconstituted cells. Experimental conditions are as described in the legend to Fig. 3; sources of gene probes are detailed in Materials and Methods.

vious studies have shown that iron-deficient growth leads to a reduced PSI/PSII ratio and to induction of the *isiA* gene, which encodes a modified Chl-binding protein, CP43′ (7, 36, 37). Expression analysis of *psbC*, *psbO*, and *psaA* showed decreased transcript levels in iron-deficient cells, compared to an increase in the transcript level of all the three genes during iron reconstitution. The reason for such high levels of *psbA* transcription is not known, but the overall transcriptional regulation of *psbA* genes in cyanobacteria is complex. Several studies have suggested that the increase in the steady-state level of *psbA* transcripts under adverse conditions is due to increased transcription of the *psbA3* gene (8, 26, 28, 29). Under normal growth conditions, *psbA2* constitutes more than 90% of the *psbA* transcripts (29). The results with the S1 nuclease protection assay suggested that iron deficiency led to an increased transcription level of both genes in similar patterns. This was in contrast to the regulation of these genes by UV-B, which led to a 20- to 25-fold increase in transcript level of *psbA3* but only a 2- to 3-fold increase in the level of *psbA2* (26). The addition of iron to iron-deficient cells initially led to the loss of both transcripts at the same rate; however, the accumulation of *psbA2* transcript increased between 12 to 24 h during reconstitution, whereas the accumulation of the *psbA3* transcript continued to decrease. Finally, it is important to note that in iron-deficient cells, the steady-state level of the *psbA2* transcripts was higher than that of *psbA3*.

This study also resulted in the identification of a previously uncharacterized gene, *slr*0374, as iron regulated. It is important to note that the transcript originating from *slr*0374 was abundant in iron-starved cells, suggesting that the gene product might be of greater importance during growth-limiting conditions. Another interesting finding related to *slr*0374 was the identification of multiple transcripts. Our preliminary data suggest that *slr*0374 may be part of an operon consisting of at least two additional genes, *slr*0373 and *slr*0376 (A. K. Singh and L. A. Sherman, unpublished data). Although the function of *slr*0374 is not known, analysis of its primary sequence based on amino acid sequence homology shows that it belongs to class of proteins with important cellular functions. A preliminary sequence analysis indicated that *slr*0374 may contain a leucine zipper and a Walker motif found in members of the AAA protein family, including a conserved second region of homology. AAA modules function as regulatory subunits in many complexes, including the 26S proteasome, in the assembly of various membrane-targeting protein complexes during membrane fusion, peroxisome biogenesis, assembly of mitochondrial membrane proteins, cell cycle control, mitotic spindle formation, cytoskeleton interactions, vesicle secretion, signal transduction, and transcription factors (30). We will determine if *slr*0374 is induced during alterations in other environmental parameters and whether it is a specific or a general stress response protein.

In summary, this work has enabled us to determine the sensitivity and utility of a DECAL for analyses of differential gene expression in the cyanobacterium *Synechocystis* sp. strain

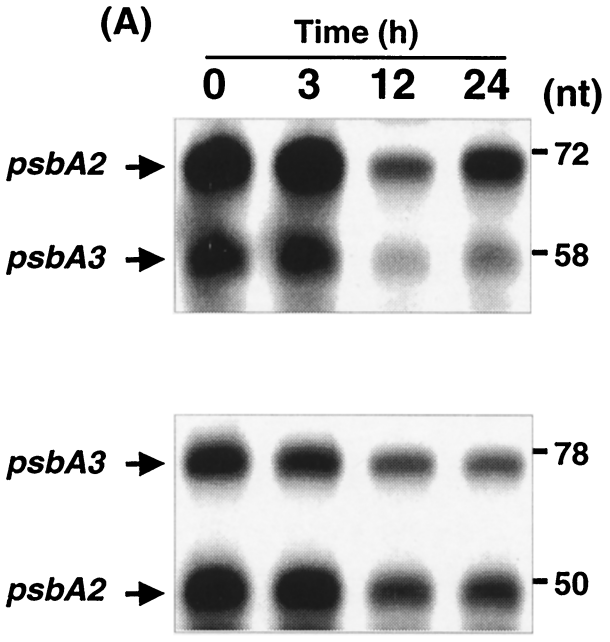

FIG. 6. S1 nuclease protection assays of *psbA2* (A) and *psbA3* (B) genes in iron-starved and iron-reconstituted *Synechocystis* sp. strain PCC 6803 cells. Primers specific to *psbA2* and *psbA3* were hybridized (at 51°C for 16 h) to 20 μg of total RNA from iron-starved cells or cells collected at 3, 12, and 24 h after the addition of iron. Nonhybridized primers were digested with S1 nuclease, and remaining sample was fractionated on an 8% polyacrylamide gel in the presence of 8 M urea, vacuum dried, and exposed to X-ray films. Lengths of the gene-specific protected fragments, 78 nucleotides (nt) for *psbA3* and 50 nt for *psbA2*, were obtained using the primer homologous to *psbA3*. In the case of the primer specific to *psbA2*, gene-specific protected fragments of 72 nt for *psbA2* and 58 nt for *psbA3* were obtained.

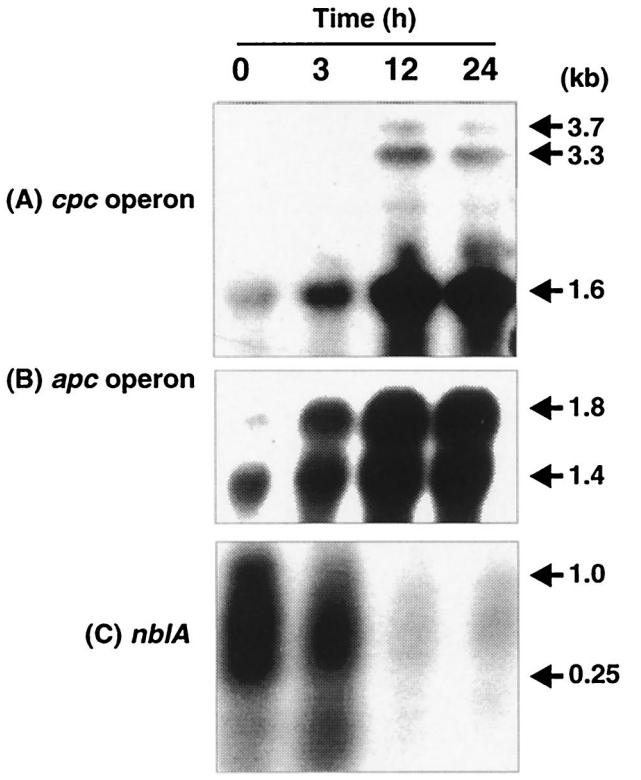

FIG. 7. Northern blot analysis of total RNA from *Synechocystis* sp. strain PCC 6803 cells probed with DNA from the phycocyanin operon *cpcBAC1C2D* (A), the allophycocyanin operon *apcABC* (B), and the *nblA* gene (C). Experimental conditions are same as for Fig. 3; sources of gene probes are detailed in Materials and Methods.

PCC 6803. The results indicated that spots demonstrating the largest density difference had a high probability of containing clones with genes that are transcriptionally regulated by iron concentration. We have an additional ~25 spots which reproducibly show density difference of ±50 units plus another 40 with medium density differences. Nonetheless, some of these spots, as well as those we chose as controls, indicated no change in expression upon further study. This could be for a number of reasons, the most important of which is the use of cosmids carrying large fragments (35 to 45 kb) of DNA. Thus, it is possible that a spot contains a clone with some genes that were up-regulated and some that were down-regulated. It is also possible that some clones may contain genes with highly abundant transcripts, which might mask the difference in the signal pattern caused by differentially regulated genes present in the same fragment. Nonetheless, once the DECAL has been constructed, it represents a relatively simple method that can generate important information on differential gene expression. We will extend this work by constructing a plasmid library array containing 6,000 clones with ~2-kb fragments, and this will be used in conjunction with the DECAL. We are also involved with the production of a complete genome microarray containing all 3,168 genes of *Synechocystis* sp. strain PCC 6803. Nonetheless, these microarrays will require a great deal of fine tuning and many duplicates before they are considered reliable. The use of a rapid and inexpensive DECAL library will still remain of use as an adjunct to such high-resolution microarrays.

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