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Genetic Selection Scheme for Isolation of Signal Transduction Pathway Mutants

Shivanthi Anandan* and Jennifer Uram

Department of Bioscience and Biotechnology, Drexel University, Philadelphia, Pennsylvania 19104

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Genetic characterization of a signal transduction pathway requires the isolation of mutations in the pathway. Characterization of these mutated genes and their loci enumerates the components of the pathway and leads to an understanding of the role of each gene locus in the pathway under study. We have designed and developed a strategy based on resistance to the chemical flucytosine for the identification of mutations in a given pathway. In this study, the *Escherichia coli codA* gene, which encodes the enzyme cytosine deaminase, was fused to the light-intensity-regulated gene promoter *psbDII*. Cytosine deaminase converts 5′-fluorocytosine to the toxic product 5-fluorouracil. Wild-type cells containing an intact signal transduction pathway that regulates the *psbDII* promoter will die in the presence of this chemical. Cells that carry mutations in the pathway that inactivate the *psbDII* promoter will not express the *codA* gene and, consequently, will live on 5′-fluorocytosine, allowing the isolation and subsequent characterization of mutations in this signaling pathway. Utilizing this selection method, we have successfully isolated and characterized mutations in the *psbDII* pathway. This selection scheme can be used with a tissue-specific or phase-specific promoter fused to the *codA* gene to direct the timing of expression of *codA* to obtain mutants defective in temporal or cell-specific expression of a particular pathway. This scheme also allows the isolation of mutants even when a clearly identifiable phenotype is not available. The selection scheme presented here extends the molecular tools available for the genetic dissection of signal transduction pathways.

To understand the interaction of cellular factors that regulate the expression of genes in a particular signal transduction pathway, the components of this signal transduction pathway need to be identified and characterized. In molecular genetics, this process of identification is done by isolating mutations in the pathway under study and by then deciphering the cellular role of each mutated locus (8). Many methods of creating such mutations have been described (2, 8). However, for the efficient isolation of mutations these methods need to be coupled with a selection scheme by which mutations affecting the pathway under consideration can easily be differentiated from mutations in unrelated signal transduction pathways, which is often an issue when visible or easily identifiable phenotypes are not available for selection.

The unicellular freshwater cyanobacterium *Synechococcus elongatus* has been used as a model organism for a number of genetic studies, including the identification of genes involved in photosynthetic gene expression and circadian rhythm (11, 12). This organism is amenable to molecular genetic analysis since it is naturally transformable with exogenous DNA, which allows the use of reporter gene fusions to study patterns of gene expression with the integration of such constructs into a chromosomal site (8, 9).

S. elongatus is an obligate photosynthetic prokaryote that carries out oxygenic-type photosynthesis. The photosystem II reaction center proteins, D1 and D2, are encoded by small gene families in this organism (7). Members of these gene families are differentially expressed in response to light intensity. The D2 protein is encoded by two genes, *psbDI* and *psbDII*. *psbDI* is expressed constitutively under all light intensities, while *psbDII* is induced at three- to fourfold greater levels under high-light conditions (5). Previous studies using translational and transcriptional reporter gene fusions have mapped regions of the *psbDII* untranslated leader region that are required for the expression of this gene under low- and high-light conditions (1, 5). This untranslated region (UTR) has been determined to contain three binding sites for protein factors that affect both high-light inducibility and level of expression from this promoter under low- and high-light regimes (1, 4). Binding site one (bs-1) is responsible for high-light induction of *psbDII*, while binding sites two and three (bs-2 and bs-3) are positive and negative regulatory elements, respectively (1).

We have designed a genetic selection scheme for *Synechococcus* species that facilitates the easy retrieval of mutations in a given signal transduction pathway. To test this scheme, we chose to use the high-light-regulated *psbDII* pathway in this organism. The basic premise of this scheme is that cyanobacterial strains where the *psbDII* high-light-induced signal transduction pathway is interrupted or malfunctional will not show an induction of *psbDII* expression in high light. This loss of *psbDII* induction in high light will be selected for by resistance to a toxic substrate, 5′-fluorocytosine. This chemical has been used previously as a marker in mammalian cells (10, 14). Conceptually, the cytosine deaminase selection scheme for mutant isolation should work as follows. Cells that possess an intact *psbDII* high-light-regulated pathway will induce the *psbDII* promoter under high-light conditions and express the *codA* gene and its protein product. This gene product (cytosine deaminase) will convert 5′-fluorocytosine to its toxic product,

* Corresponding author. Mailing address: Department of Bioscience and Biotechnology, Drexel University, 32nd and Chestnut Streets, Philadelphia, PA 19104. Phone: (215) 895-2631. Fax: (215) 895-1273. E-mail: anandans@drexel.edu.

TABLE 1. *Synechococcus* strains

Strain[a]	Plasmid[b]	Promoter region[c]	Source (reference)
AMC181	pAM990	None	Li and Golden, 1993 (12)
AMC378	pAM1439	−38 to +160 of *psbDII*	Anandan and Golden, 1997 (1)
DU201	pAM1573 (NSII) with *psbDII-codA* and pAM1439 (NSI)	−96 to +160 of *psbDII*	This study

[a] Carries a *lacZ* reporter gene integrated at neutral site I.
[b] Plasmid used to transform *S. elongatus* to introduce reporter gene fusions. NS, neutral site.
[c] Endpoints of DNA fragments.

5-fluorouracil, and will result in the death of all *Synechococcus* species cells that contain an intact *psbDII* high-light signal transduction pathway. Cells defective in the *psbDII* signal transduction pathway will not activate the *psbDII* promoter and hence will not express the *codA* gene and will survive on 5′-fluorocytosine, since they are unable to convert this substrate to 5-fluorouracil. These surviving colonies should carry a mutation in one component of the *psbDII* light-regulating pathway. The *psbDII* promoter was the promoter of choice for this gene fusion with which to test this strategy, since it had previously been well characterized and was known to be actively transcribed under both low- and high-light regimes (1, 4, 5). We describe here the basic design of the selection scheme together with the application of this scheme in the successful retrieval of mutations in the *psbDII* pathway.

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids used in this work are described in Table 1. Wild-type *S. elongatus* (previously known as *Synechococcus* sp. strain PCC 7942 and "*Anacystis nidulans*" R2, Pasteur Collection no. 7942) was grown in modified BG-11 medium (4) at 30°C and 150 μE m^{-2} s^{-1} irradiance in liquid culture or on solid plates containing 1.5% agar. Transformation of *S. elongatus* with plasmid constructs was carried out by the method detailed in references 8 and 9.

Escherichia coli DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used as the host for all plasmids and was propagated on Luria-Bertani broth (3) and Terrific Broth (13) media in the presence of antibiotics at standard concentrations (3).

***Synechococcus* species culture conditions.** For light-intensity experiments, cultures were grown in large petri dishes (150 × 15 mm) on a shaker at a constant temperature of 30°C in a lighted growth chamber. Light was provided by 20-W fluorescent tubes placed above the petri dishes. A light-sensitive probe (Biospherical Instruments, San Diego, Calif.) was used to measure the photosynthetic photon flux density, measured in μEm^{-2} s^{-1}, reaching the cell cultures.

Construction of the *psbDII-codA* clone. The pRTmcs-codA plasmid, which served as the starting vector for the construction of the *psbDII-codA* plasmid, was constructed as follows. The plasmid used for constructing pRTmcs-codA was pRT103, which was derived from pUC18/19 (15). In this plasmid, the cauliflower mosaic virus 35s promoter (cAMV-35s) and a plant polyadenylation site are located upstream and downstream, respectively, of the pUC18/19 multiple cloning site (15). The *codA* gene was amplified from *E. coli* by using PCR and cloned into the *Nco*I site of pRT103 to generate pRTmcs-codA (D. Morishige, personal communication).

A 256-bp fragment containing the −96 to + 160 region of the *psbDII* gene (1) was amplified by using PCR with *Synechococcus* species genomic DNA as the template. PCRs (25 μl each) contained the following: 100 ng of DNA, 1.5 mM $MgCl_2$, approximately 0.04 to 0.07 μM (each) primer, 50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, and 0.25 units of *Taq* DNA polymerase (Promega Corporation, Madison, Wis.). Primer sequences for the amplification of the *psbDII* 256-bp fragment are as follows: forward primer, 5′-G**GAATTC**G AAGCCTGCGAGACAC-3′, and reverse primer, 5′-GACGTAAACCATCC-3′. Thirty cycles of amplification were performed as follows. Denaturation was at 92°C for 30 s, annealing of the primer to template DNA was performed at 45 to 50°C for 5 min, and elongation of the primer was at 72°C for 1 min. Reactions were carried out in a PCRExpress thermal cycler (Hybaid, Middlesex, United Kingdom). The amplified *psbDII* promoter fragment carried an *Eco*RI site on the 5′ end (see the sequence of the forward primer in bold). The fragment was digested with *Eco*RI, purified, and then cloned into the *Eco*RI-*Sma*I digested plasmid pRTmcs-codA (a gift from D.T. Morishige, Department of Biochemistry, Texas A&M University). This cloning procedure generated an in-frame translational fusion between the *psbDII* promoter and the *codA* gene to generate pRTmcs-codA#5, where the promoter, ribosome binding site, and translational start site of the gene fusion comes from the *psbDII* gene. The identity of the pRTmcs-codA#5 clone was verified by DNA sequencing.

The entire fusion construct (*psbDII-codA*) was then excised from pRTmcs-codA#5 by digestion with *Xba*I and *Xho*I and ligated into *Xba*I- and *Xho*I-digested pAM1573 (2). pAM1573 is a neutral site II chromosomal integration vector developed in the laboratory of Susan Golden of Texas A&M University. This vector contains an *E. coli* origin of replication, a chloramphenicol cassette for the selection of recombinant *E. coli* and *Synechococcus* species transformants, and a multiple cloning site. Two fragments of the *Synechococcus* species chromosome termed neutral site II flank the antibiotic cassette and multiple cloning site. DNA constructs cloned between the two neutral site fragments will recombine into the *Synechococcus* species chromosome by a homologous recombination event. pAM1573 does not replicate in *Synechococcus* species, since it does not contain a *Synechococcus* species origin of replication and does not contain any *Synechococcus* species promoters (2). Cloning of the excised *Xho*I-*Xba*I fragment from pRT-mcs-codA into pAM1573 generated pAM1573 *psbDII-codA*. The pAM1573 *psbDII-codA* plasmid was transformed into *Synechococcus* strain AMC378, which carries a *psbDII-lacZ* fusion in chromosomal neutral site I (1), to give rise to strain DU201. A diagrammatic representation of the cloning of pAM1573 *psbDII-codA* is given in Fig. 1. Successful integration of the *psbDII-codA* construct into neutral site II of AMC378 was verified by PCR. This strain (DU201) was later used for chemical mutagenesis.

To test whether the *E.coli codA* gene was actively expressed under the control of the *psbDII* promoter and whether the enzyme cytosine deaminase was active in *Synechococcus* DU201 cells, wild-type *Synechococcus* species and the DU201 strain were grown to the same optical density in 100-ml cultures, serially diluted, and plated on BG-11 agar medium containing 0.5 mg of 5′-fluorocytosine/ml. No viable colonies were formed from strain DU201 at any dilution, whereas wild-type *Synechococcus* species showed no decrease in cell viability when grown in the presence of 5′-fluorocytosine. This finding indicated that the *codA* gene was expressed under control of the *psbDII* promoter in strain DU201 and converted 5′-fluorocytosine to 5-fluorouracil, which resulted in the death of these cells.

Mutagenesis protocol. Chemical mutagenesis was carried out essentially as detailed in reference 8, with the following modifications. Cells (10^7) of strain DU201 were mixed with 1 mg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine/ml in 50 ml of BG-11 medium for 30 min at 30°C with shaking. After being incubated, the cells were washed thoroughly two to three times with basal BG-11 medium and allowed to recover in liquid BG-11 medium without antibiotics for 6 days with shaking at a light intensity of 150 μE m^{-2} s^{-1} irradiance. The surviving cells were then plated onto BG-11 agar containing 0.5 mg of 5′-fluorocytosine/ml and allowed to grow at a light intensity of 150 μE m^{-2} s^{-1} irradiance. The colonies that grew on this medium were picked and restreaked onto BG-11 agar with 0.5 mg of 5′-fluorocytosine/ml. The colonies that grew after the second streaking were then inoculated into 100 ml of liquid BG-11 and allowed to grow with shaking at 150 μE m^{-2} s^{-1} irradiance until the cultures reached high cell density. Certain strains did not do well under this growth regime and, after two restreakings and inoculation into liquid BG-11, were then allowed to grow slowly without shaking at 150 μE m^{-2} s^{-1}irradiance.

Plasmid preparation and DNA manipulations. Plasmid preparation, subcloning, and other DNA manipulations were carried out as described in reference 3. All restriction enzymes were purchased from Promega Corporation (Madison, Wis.), unless stated otherwise. Laboratory reagents and chemicals used in β-ga-

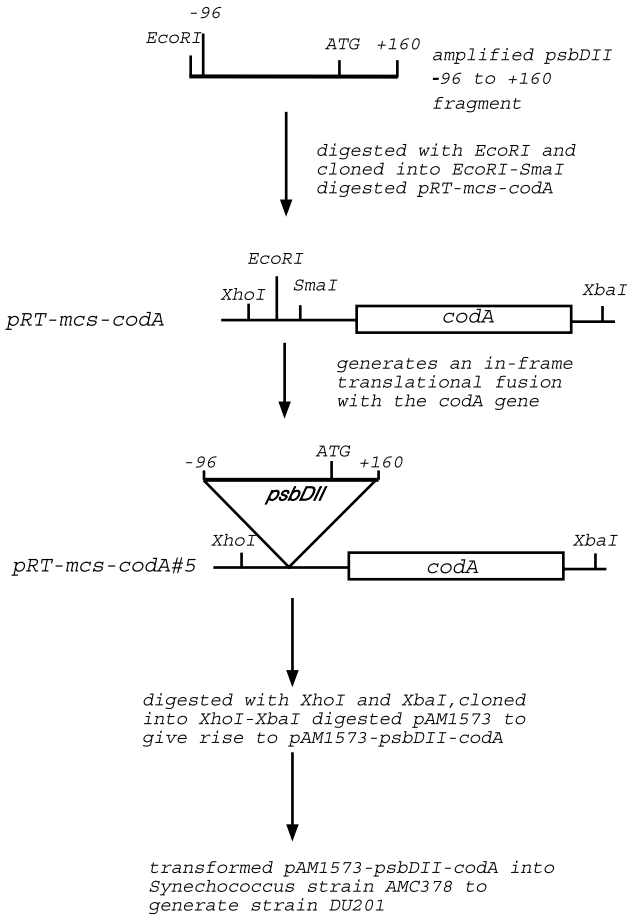

FIG. 1. Diagrammatic representation of the cloning strategy used to generate *S. elongatus* strain DU201.

lactosidase assays were purchased from Sigma Corporation. All other laboratory reagents and chemicals were purchased from Fisher Scientific Inc.

β-Galactosidase assays. β-Galactosidase assays were performed as described in reference 1.

RESULTS

We present here a scheme for the selection of mutants in a signal transduction pathway based on the toxicity of the chemical 5′-fluorocytosine. To test this selection strategy, we chose the *psbDII* high-light-regulated signal transduction pathway. The *psbDII* gene and its *cis*-acting regions have previously been well characterized (1, 4). Moreover, transcriptional and translational fusions of this gene with the reporter gene *lacZ* have previously been used to characterize promoter activity of this gene (1, 4). We constructed a chimeric fusion between the *psbDII* −86 to + 160 region and the open reading frame of the *E. coli codA* gene. In this hybrid gene, the promoter, ribosomal binding site, translational start site, and first 50 bases of the coding region were from the *psbDII* gene. This region of the *psbDII* gene had previously been used to generate translational *lacZ* fusions and had been demonstrated to show high-light induction comparable to the levels of induction shown by *psbDII* mRNA (4, 5). A *Synechococcus* strain, DU201, which contained the hybrid *psbDII-codA* gene in chromosomal neutral site II and the *psbDII-lacZ* fusion in neutral site I, was generated. Chemical mutagenesis using MMNG was chosen to generate random point mutations in the chromosome. At the time this selection method was developed, a foolproof transposon-based mutagenesis scheme had not been demonstrated for *Synechococcus*. Moreover, since we anticipated that mutations in the *psbDII* pathway could affect the viability of the mutant strain(s), we chose a mutagenesis method that would inflict the least possible alteration on gene sequences. Insertion of a transposon might prove deleterious, but a point mutation in the same locus might prove to have a less severe effect.

TABLE 2. Phenotypes of isolated cyanobacterial mutant strains

Phenotype	No. of independent isolates
Increased *psbDII-lacZ* activity in high light	5
Decreased *psbDII-lacZ* activity in high light	24
Wild-type values for *psbDII-lacZ* activity (low and high light)	7
To be tested	36

A preliminary screening step to assay the effect of the mutations generated on *psbDII* promoter activity utilized the *psbDII-lacZ* construct in chromosomal neutral site I (1). In this step, β-galactosidase assays were used to monitor the promoter activity of the *psbDII* gene. The β-galactosidase levels would reflect the effect of a mutation that altered the *psbDII* promoter activity level in any of the isolates compared to that of the nonmutagenized control strain. This screening step separated those strains with mutations in the *codA* gene from those with mutations in the *psbDII* high-light pathway. Strains with mutations in the *codA* gene should show 5′-fluorocytosine resistance but have normal levels of *psbDII* activity. Strains that carried a mutation in the *psbDII* high-light pathway should show 5-fluorocytosine resistance and should also have altered *psbDII* promoter activity, as measured by β-galactosidase levels, compared to that of the nonmutagenized DU201 control strain. Table 2 gives the distribution of different phenotypes for *psbDII* promoter activity retrieved after mutagenesis and selection with 5′-fluorocytosine-based selection criteria. The control strain used for comparison was nonmutagenized DU201.

Of 72 5′-fluorocytosine-resistant independent isolates, 7 isolates showed wild-type levels of *psbDII* activity, 5 strains had increased *psbDII* activity, and 24 strains had decreased *psbDII* activity, as monitored by β-galactosidase assays (Table 2). Thirty-six mutants that were isolated have yet to be characterized for *psbDII* activity with this assay. Strains that had impaired growth or had different pigmentation profiles than that of the wild type were chosen for further study, and β-galactosidase assays in triplicate were carried out for these representative strains. The results of these assays are shown in Fig. 2.

These results show that mutant U has elevated *psbDII* promoter activity, as assessed by β-galactosidase assays (Fig. 2). This mutant very likely contains a defect in the protein factor that binds to the negative element mapped to the *psbDII* UTR (1). Mutants Z and JU4, however, show decreased *psbDII* promoter activity (Fig. 2), implying that the defect is in the gene encoding the factor that binds to the upregulatory element in the *psbDII* UTR (1). All three mutants do maintain

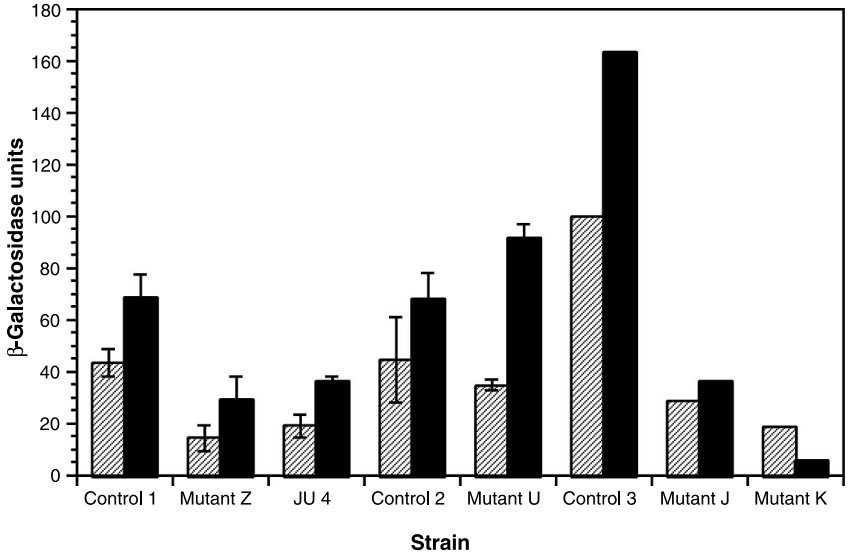

FIG. 2. Expression of *psbDII-lacZ* fusions in isolated mutant strains of *S. elongatus*. Specific activity (nanomoles of *O*-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein) was determined for each *psbDII-lacZ* reporter strain. β-Galactosidase levels were corrected by subtracting background levels from a promoterless *lacZ* strain, AMC181. The graphed values represent the means of the results of triplicate experiments, with the standard deviations indicated. The histograms show β-galactosidase levels for strain DU201 (control 1, control 2, and control 3) with mutant strains U, Z, JU4, J, and K 0 h before (hatched bars) and 2 h after (black bars) the shift to high light (800 $\mu E\ m^{-2}\ s^{-1}$). The graphed values for the data for control 1, control 2, and mutants U, Z, and JU4 are the means of the results of triplicate independent experiments, with the standard deviations indicated. The data for control 3 and mutant strains J and K were from one experiment. Due to the poor growth of strains J and K in high light, it was difficult to repeat the experiments.

high-light induction of the *psbDII* gene, indicating that binding site 1 in the *psbDII* UTR is still functional in a wild-type manner. Mutants K and J, however, lost high-light induction of the *psbDII-lacZ* reporter gene (Fig. 2), indicating that the mutations in these two strains affect the protein factor(s) that binds to the light-regulating element in the *psbDII* UTR (1).

Three mutants, mutants J, K, and Z, also showed impaired growth rates. These mutants were initially isolated without shaking in liquid culture. As seen in Fig. 3, all three mutants had lower growth rates when grown under high-light intensity in liquid culture than that of control strain DU201. In particular, mutant Z could not be grown under high-light conditions, as this strain rapidly bleached and died under these conditions. Thus, no β-galactosidase values were obtained for this strain.

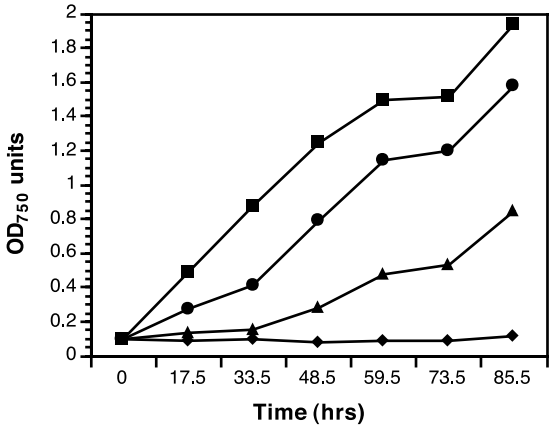

FIG. 3. Growth curve for control strain DU201 and selected mutant strains under high-light conditions. The following symbols represent the indicated strains: ■, strain DU201; ●, strain K; ▲, strain J; and ◆, strain Z. OD_{750}, optical density at 750 nm.

DISCUSSION

A genetic selection scheme was designed for the isolation of mutants in the *psbDII* high-light-regulating pathway that controls activity of the *psbDII* promoter. The *psbDII* high-light-regulating pathway in *S. elongatus* was chosen to test this scheme, since this gene had previously been well characterized in this organism and insertional mutagenesis of *psbDII* was shown to not be lethal to this organism (5). The advantage of the selection strategy proposed here is that the viable colonies that arise are all mutant in nature. The wild-type cells are killed by selection on 5′-fluorocytosine, allowing easy isolation of mutant colonies.

In this study, mutations were created by treatment of strain DU201 with the chemical agent MMNG (8). However, this scheme lends itself to use with a variety of mutagenic strategies and agents. Mutations can as easily be created by transposon or interposon mutagenesis. A scheme for these types of mutagenesis in *Synechococcus* species has recently been designed and would be appropriate for use with this selection strategy (2). In particular, this strategy is useful where a visible or easily identifiable mutant phenotype is not available for purposes of selection. This screen uses resistance to 5′-fluorocytosine as the phenotypic selection criterion for mutant identification.

It was anticipated that this selection scheme would give rise to cells with decreased *psbDII* promoter activity in high light. However, we isolated five independent mutant strains that showed elevated *psbDII* activity in low and high light, which implies that these strains also produce elevated levels of cyto-

sine deaminase. If this were indeed the case, these mutants would not have been isolated as viable colonies by this selection method. It is very likely that these mutants possess a secondary mutation in the *codA* gene that obliterates cytosine deaminase function, thereby conferring a 5′-fluorocytosine-resistant phenotype on these cells that allowed them to be isolated in this screen. Identification of strains with secondary mutations in the *codA* gene could be carried out by replacing the *lacZ* gene in neutral site I with a second copy of the *psbDII-codA* construct, which would cause these strains to be sensitive to 5′-fluorocytosine. With replica plating on medium without the selective agent, the strains could be kept viable, while at the same time the second site mutation in the *codA* gene could be confirmed. This strategy would then also allow for the isolation of mutations in the signal transduction pathway that confer elevated levels of expression of the target gene.

The mutants isolated by this approach that have presently been characterized had impaired function of the target gene, *psbDII*. Mutants J, K, Z, JU4, and U all had *psbDII* promoter activities that differed significantly from those of the wild type. Previous studies with the *psbDII* promoter have shown that a mutation in binding site 1 negates high-light induction of *psbDII*, while mutations in binding sites 2 and 3 affect positive and negative regulatory elements, respectively (1). Thus, mutants J and K should have mutations in the protein factor that binds to binding site 1, while mutants Z and JU4 should have abnormal protein factors that bind to binding site 2 (1). It is anticipated that mutant U is aberrant for the protein factor binding to binding site 3 or to the direct repeat region within the *psbDII* open reading frame (1).

In addition, mutants J and K, which showed no high-light induction of the *psbDII* promoter, also showed aberrant growth rates in high light. It has been demonstrated that loss of *psbDII* function, while not lethal in *Synechococcus* species, causes the organism to be less competitive than the wild type in mixed-culture experiments (5). Thus, these mutants show phenotypes similar to that of the previously reported *psbDII*-null mutant. It has been demonstrated by gel shift analysis that the upstream regions of the *psbDII*, *psbAII*, and *psbAIII* genes bind the same *trans*-acting factors (4). It is possible that mutations in one or more of these protein factors could have a pleiotropic effect, affecting not only expression of the *psbDII* gene but also expression of other high-light-inducible genes like *psbAII* and *psbAIII* in *Synechococcus* species. This effect could account for the low growth rate and defects in pigmentation seen in isolates J and K.

Rescue of these mutations with genomic DNA allows us to precisely pinpoint the site of the mutations and to identify those genes that are part of the *psbDII* high-light pathway. Rescue of the mutations, however, confers wild-type regulation on the *psbDII* promoter, which in turn leads to expression of the *codA* gene and death of the rescued mutant on 5′-fluorocytosine. To overcome this problem, rescued mutants should be replica-plated on medium with and without 5′-fluorocytosine after transformation with genomic DNA to obtain viable rescued cells. Indeed, the sensitivity of the rescued strains to 5′-fluorocytosine can be used as an indication that a successful rescue of the mutant phenotype has taken place with the consequent reconstitution of the wild-type signal transduction pathway.

The *psbDII* promoter used in this selection method is active under both conditions tested, i.e., low- and high-light intensities. Since our goal was to isolate mutants that were affected by both the low- and high-light expression of this gene, this characteristic of the promoter was crucial for our outcome. The choice of promoter to be used in this selection scheme can greatly influence the outcome of the mutants isolated. A promoter that is tightly regulated in a temporal or cell-specific manner can also be used with this selection scheme, and the expression window of the promoter can be utilized to select for mutants that are aberrant in either temporal or cell-specific regulation of the signal transduction pathway under analysis. Indeed, the utility of this selection method can be extended by using an inducible promoter-operator system like *lacI-lacZ* to control the expression of the *codA* gene in a time- or cell-specific manner for the isolation of mutants defective in either temporal or cell-specific expression of the signaling pathway under study.

The ability to generate and screen random mutations is a crucial step in isolating the components and understanding the functioning of a signal transduction pathway (6, 11). We have described a genetic selection scheme that lends itself to the isolation of mutants defective in a cellular signal transduction pathway where the resulting viable colonies are mutant, thus allowing for easy selection of mutant phenotypes. Identification of the mutated loci could be done by transformation of the mutant with a genomic library and scoring for rescue of the mutant phenotype (8). If mutagenesis were by a transposon, then isolation of the transposon would indicate the mutated locus of the gene (2). This selection scheme is easy to carry out once the *codA* construct is generated and can be used in systems where an intrinsic *codA* gene is absent or where the native *codA* gene has been deleted. The genetic selection scheme described here extends the tools available for the isolation and characterization of mutations in a given signal transduction pathway.

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