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Two Functional *psbD* Genes in the Cyanobacterium *Synechococcus* sp. Strain PCC 7942

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The cyanobacterium *Synechococcus* sp. strain PCC 7942 has two copies of the *psbD* gene which encodes the D2 polypeptide of the photosystem II (PSII) reaction center. One of the genes, *psbDI*, overlaps the open reading frame of another photosystem II gene, *psbC*; the *psbDII* gene is monocistronic. Gene inactivation experiments had previously shown that *psbDII* is dispensable under normal laboratory growth conditions. However, similar experiments with *psbDI* never produced viable *psbDI*-inactivated mutants, presumably because *psbC* expression depends on transcription through *psbDI*. The experiments described here were designed to assess the need for *psbDI* independent of the need for expression of *psbC*. A strain, AMC027, was engineered in which a second copy of *psbC* was expressed from the *psbDII* locus. Northern (RNA) blot analysis confirmed that both *psbDI* and *psbDII* gave rise to dicistronic messages containing *psbC* sequences in AMC027. In this genetic background, it was possible to inactivate *psbDI*, creating strain AMC050 and indicating that the *psbDII* gene is functional. Western immunoblot analysis showed that the products of *psbD* and *psbC*, the PSII proteins D2 and CP43, respectively, were present in thylakoids of AMC050, but at reduced levels relative to the wild type, the mutant AMC027, and two *psbDII*-inactivated mutants. AMC050 consistently formed small colonies on plates and competed poorly in mixed-culture experiments. This suggested that, although not essential for viability, expression from the *psbDI* locus is required to produce sufficient D2 and CP43 for optimal growth.

The integral membrane proteins of the photosynthetic membrane complex, photosystem II (PSII), are conserved between cyanobacteria and higher plant chloroplasts (4, 26). Among these conserved proteins are D1 and D2, which act in concert to coordinate the cofactors that mediate the primary photochemical reactions of charge separation and stabilization (20, 34). The genes *psbA* and *psbD*, which encode D1 and D2, respectively, are unique genes in the chloroplast genomes of most plants (14, 21, 30, 36), but are present in more than one copy in cyanobacterial genomes (5, 7, 8, 15, 19, 35). The cyanobacterium *Synechococcus* sp. strain PCC 7942 (hereafter referred to as *Synechococcus*) has three *psbA* genes (8) and two *psbD* genes (12). The presence of multiple genes for PSII proteins in cyanobacteria is an evolutionary puzzle. Cyanobacteria lack some of the regulatory features observed in chloroplasts, such as complex transcript patterns from densely packed operons (3, 6, 16) and modulation of membrane stacking (17, 32). Multigene families may represent a strategy for regulating PSII composition and architecture, divergent from that of chloroplasts, that has evolved within the cyanobacteria. If this idea is correct, it predicts differential regulation among members of the gene families under different growth conditions.

Gene inactivation is a useful tool for assessing the function of individual genes of a multigene family. The method has been used to engineer a set of *Synechococcus* strains having each combination of two of the *psbA* genes inactivated (8). This demonstrated that the *psbA* genes are all functional and that each is capable of producing sufficient D1 protein to support photoautotrophic growth. Additional analysis has shown that two distinct forms of D1 are encoded by the *psbA* gene family and that differential gene expression results in a variable ratio of the two forms in the thylakoid (27, 28). We are currently using the same methods to follow expression of the *psbD* genes and synthesis of the D2 polypeptide.

The two *psbD* genes encode an identical D2 polypeptide, but they are arranged in different transcriptional units (12). One of the genes, *psbDI*, overlaps and is cotranscribed with another PSII gene, *psbC*, which encodes the chlorophyll *a*-binding protein called CP43 (4, 12). This is also the arrangement of the unique *psbD* and *psbC* genes of chloroplast genomes (14, 21, 30). *Synechococcus*, like other cyanobacteria (35), has an additional monocistronic *psbD* gene designated *psbDII*. Gene inactivation experiments like those used to characterize the *psbA* gene family have shown that *psbDII* is dispensable under normal laboratory growth conditions (12). A similar experiment designed to test the need for *psbDI* was complicated by dependence of the unique *psbC* gene on *psbDI* expression (12).

In this study we used transformation methods based on recombination between homologous cloned genes and the cyanobacterial chromosome to engineer a strain having an additional copy of *psbC* expressed from the *psbDII* locus. This strain provided a genetic background in which it was possible to inactivate the *psbDI* gene, indicating that *psbDII* is a functional gene and can support photoautotrophic growth. Although viable, the *psbDI*-inactivated mutant produced small colonies and competed poorly in coculturing experiments, indicating that expression from the *psbDI* locus is important for optimal fitness of the cell. This is probably the result of insufficient expression of the D2 and CP43 polypeptides from the *psbDII-psbC* locus.

MATERIALS AND METHODS

Synechococcus sp. strain PCC 7942 (*Anacystis nidulans* R2; Pasteur Culture Collection strain no. 7942) and transformed derivative strains were grown in liquid BG-11 medium (1) or on solid BG-11 agar (9) under constant fluorescent illumination at a photosynthetic photon flux density of approximately 100 microeinsteins m^{-2} s^{-1}. Liquid cultures

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference or source
Synechococcus strain		
PCC 7942[a]	Wild type; also called *Anacystis nidulans* R2	Lab collection
AMC015	Also called R2S2.2; *psbDII* inactivated by Ω cassette at amino acid 281	12
AMC016	*psbDII* inactivated at amino acid 93 by kanamycin resistance cassette	This study
AMC027	Second copy of *psbC* expressed from the *psbDII* locus; carries Ω cassette	This study
AMC050	*psbDI* inactivated at amino acid 93 by kanamycin resistance cassette, in AMC027 background	This study
Plasmid		
pHP45Ω	Source of Ω cassette, which encodes spectinomycin resistance	22
pSKS101	Source of kanamycin resistance cassette	29
pUC18	Cloning vector; encodes ampicillin resistance	2
pBGS18	Derivative of pUC18; encodes kanamycin resistance	31
Bluescript KS M13−	Cloning vector used for transcribing radiolabeled antisense RNA probes	Stratagene
pUR290	Expression vector for *lacZ* translational gene fusions	25
pAM011	1.2-kb b1 fragment,[b] carrying most of the *psbDI* gene, in the vector pUC18	G. Stearns and S. Golden, unpublished
pAM070	Derivative of pAM011 which carries a kanamycin resistance cassette inserted at the *Apa*I site of *psbDI*; *Bam*HI site at left end of cassette lost during cloning; recombination substrate used to produce strain AMC050	G. Stearns and S. Golden, unpublished
pAM146	2.5-kb *Pvu*II-*Pst*I DNA fragment, carrying all of *psbDII*, in the vector pBGS18	This study
pAM160	Derivative of pAM146 with the b3 fragment[b] inserted at the *Bam*HI site of *psbDII*	This study
pAM213	Derivative of pAM160 with the Ω cassette inserted at the distal *Bam*HI site of the b3 fragment[b]; recombination substrate used to produce strain AMC027	This study
pAM270	*lacZ-psbC* translational fusion in pUR290; contains a 208-bp *Hin*dIII-*Taq*I fragment of *psbC* cloned in-frame into *Hin*dIII-*Cla*I-digested pUR290	This study
pAM360	*Eco*RV-*Sau*3A fragment from untranslated region upstream of *psbDI* in the vector Bluescript KS M13−; used to transcribe a *psbDI*-specific antisense RNA probe	This study

[a] Pasteur Culture Collection number.
[b] See Fig. 1A.

were aerated by gyration or by bubbling with sterile air. All transformed cyanobacterial strains are described in Table 1. A detailed procedure for transformation and gene inactivation of *Synechococcus* has been published (9). Antibiotics were added to BG-11 plates (as 400 μl of a 100× stock) beneath the agar 4 h after inoculation (9, 11) to provide the following final concentrations: spectinomycin, 40 μg/ml; streptomycin, 5 μg/ml; kanamycin, 50 μg/ml. Transformants containing the Ω cassette (22) were initially plated on a combination of spectinomycin and streptomycin to select against spontaneous spectinomycin-resistant mutants and subsequently cultured in the presence of spectinomycin alone. DH5α (Bethesda Research Laboratories) was the *Escherichia coli* host for all plasmids except pAM270, which was propagated in a *lacI*^{q} strain, BB4 (Stratagene). LB (18) and Terrific Broth (33) media were used to propagate *E. coli* strains, and antibiotics were added to cultures at standard concentrations for maintaining the plasmids in *E. coli* (18).

Total DNA was isolated from 500-ml cultures of *Synechococcus* strains as described previously (10). A total DNA miniprep procedure was used to screen cyanobacterial transformants (9). Most restriction and modifying enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) or Boehringer Mannheim Biochemicals and used as directed by the manufacturer.

Southern blot analysis was performed by alkaline capillary transfer of DNA (24) from 0.7% agarose gels to Magnagraph nylon membranes (Micron Separations, Inc.). Restriction fragments were labeled with [^{32}P]dCTP by random primer labeling (2); alternatively, probes were synthesized as [^{32}P]UTP-labeled antisense RNA transcribed by T7 polymerase from Bluescript vector-based plasmids (Stratagene). DNA-DNA hybridization mixtures contained 5× SSPE (18), 1% sodium dodecyl sulfate (SDS), denatured salmon sperm DNA (100 μg/ml), and denatured probe and were incubated at 60°C. Unhybridized probe was removed from filters by washing in 0.5× SSPE–0.1% SDS at 65°C. Hybridization mixes for RNA probes were as for DNA probes except that they contained 50% (vol/vol) deionized formamide, carrier DNA was omitted, and mixtures were incubated at 50°C.

Total RNA was isolated from 1.5-liter cyanobacterial cultures as previously described by Golden et al. (9) and modified by Schaefer and Golden (28). Samples were denatured with formaldehyde (2), separated by electrophoresis in a 1.4% agarose gel (2), and transferred to Magnagraph membranes (Micron Separations, Inc.) for Northern (RNA) blot analysis. ^{32}P-labeled antisense RNA probes were prepared and hybridized with membranes as for Southern analysis. Unhybridized probes were removed by washing filters in 0.1× SSPE–0.2% SDS at 65°C; however, the 16S rRNA band was visible when this hybridization and washing regimen was used with the *psbDII*-specific probe. The ribosomal RNA artifact could be removed by hybridizing filters in 2.5× SSPE–1% SDS at 60°C and washing in 0.25× SSPE–0.1% SDS at 65°C (data not shown).

Antisense RNA probes that detect transcripts from *psbC* and *psbDII* have been described before (12). Plasmid pAM360, which was used to produce a *psbDI*-specific antisense RNA probe, contains a 64-base-pair (bp) *Eco*RV-*Sau*3A fragment from upstream of the *psbDI* gene (12) inserted into *Bam*HI- and *Eco*RV-cleaved Bluescript KS M13− (Stratagene).

Thylakoid membranes were isolated from cyanobacterial cultures as described by Schaefer and Golden (27). Samples

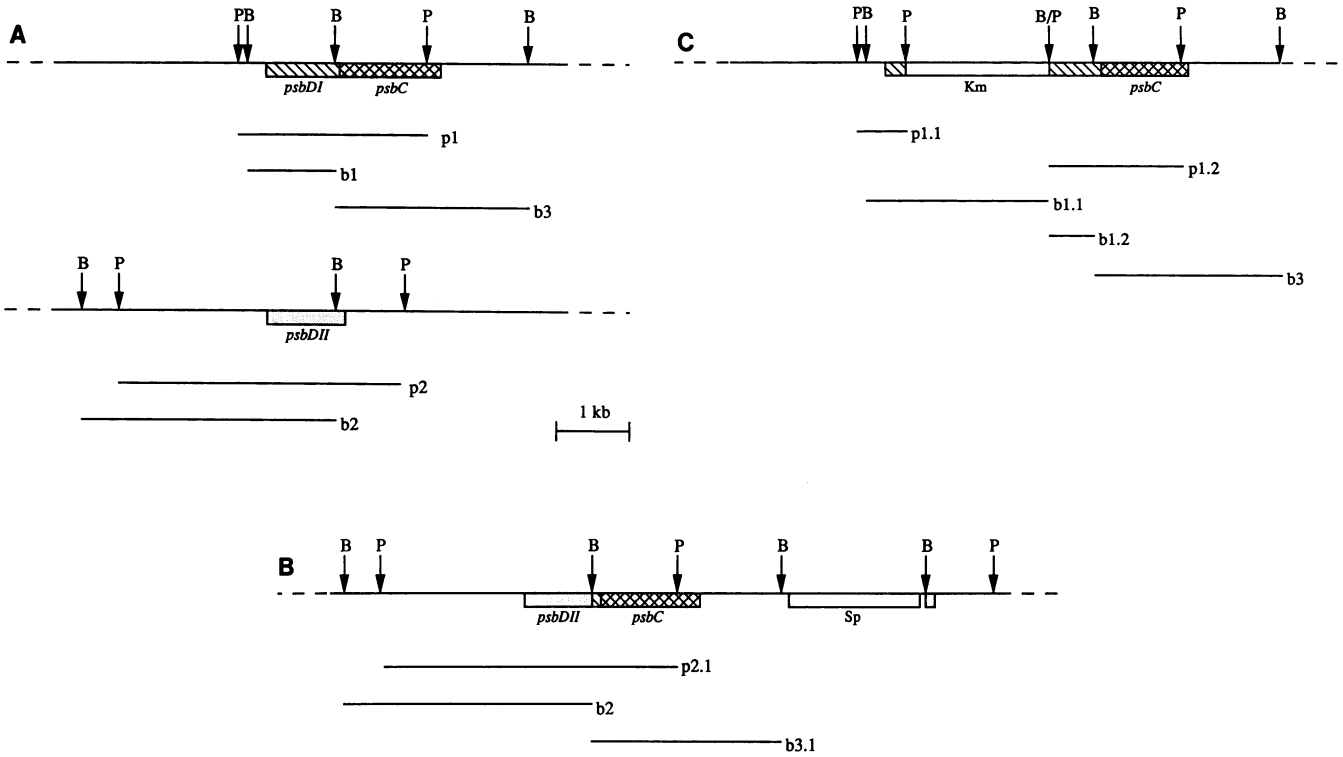

FIG. 1. Restriction maps of the *psbDI* and *psbDII* loci in wild-type *Synechococcus*, AMC027, and AMC050. The *Synechococcus* chromosome is depicted as a solid horizontal line that is dashed at either end to indicate that the DNA extends in each direction, and no linkage is suggested between the two *psbD* loci. The positions of open reading frames are indicated by boxes, which represent the following genes: *psbDI*, slanted stripes; *psbC*, cross-hatching; *psbDII*, stippled gray; Ω cassette, open box marked Sp; kanamycin resistance cassette, open box marked Km. Restriction sites are designated by the following letters: B, *Bam*HI; P, *Pst*I. The sizes and positions of restriction fragments are shown beneath the chromosome line for *Bam*HI and *Pst*I fragments, identified by b and p, respectively, followed by a numeral. The scale is the same for all panels. (A) Map of the *psbD* loci in wild-type *Synechococcus*. (B) Map of the *psbDII* locus in AMC027; *psbDI* is identical to that of the wild type. (C) Map of the *psbDI* locus in AMC050; the *psbDII* locus is identical to that of AMC027.

were solubilized and subjected to lithium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Guikema and Sherman (13). Separated membrane proteins were transferred electrophoretically to nitrocellulose membranes (2), and Western immunoblot analysis was performed as described by Schaefer and Golden (27), except that antibody-antigen reactions were visualized by alkaline phosphatase-conjugated anti-rabbit immunoglobulin G secondary antibodies (Bio-Rad Laboratories).

Antiserum directed against the spinach D2 polypeptide was a gift from W. Vermaas. Antiserum recognizing the *Synechococcus* CP43 polypeptide was raised by injecting rabbits with a β-galactosidase-CP43 hybrid protein produced from a translational gene fusion between the *lacZ* gene of *E. coli* and a portion of the *Synechococcus psbC* gene. The gene fusion plasmid pAM270 was constructed by inserting a 208-bp *Hin*dIII-*Taq*I fragment of the *psbC* gene (nucleotides 1928 to 2136 of the *psbDI-psbC* sequence [12]) into the *lacZ* expression vector pUR290 (25), which had been cleaved with *Hin*dIII and *Cla*I. The *lacZ* gene was induced with isopropyl-β-D-thiogalactopyranoside for production of the hybrid protein, which was purified by anti-β-galactosidase affinity chromatography as described by Schaefer and Golden (27).

RESULTS

Our lab has shown that the *psbDII* gene can be inactivated in the *Synechococcus* chromosome without noticeably impairing growth (12). Attempts to inactivate the *psbDI* gene by a similar method consistently resulted in recombination of the inactivation cassette at the *psbDII* locus, suggesting that the *psbDI-psbC* operon is essential. Because the unique *psbC* gene depends on transcription through *psbDI*, the apparently lethal phenotype could be the result of a polar effect on *psbC* expression rather than an indication that *psbDI* itself is necessary. In order to test this hypothesis, a strain was constructed that carried a second copy of *psbC* downstream of the *psbDII* gene. This strain provided a genetic background in which we could attempt to inactivate *psbDI* while still providing *psbC* expression.

Figure 1A represents the wild-type organization and restriction map of the *psbDI-psbC* and *psbDII* loci. Addition of a second copy of *psbC* downstream of *psbDII* is depicted in Fig. 1B. The recombination substrate used to engineer this strain was constructed in two steps with *E. coli* as the plasmid host. A plasmid carrying the entire *psbDII* gene was opened at a *Bam*HI site, near the end of the *psbDII* gene, that is conserved in the nearly identical *psbDI* gene (Fig. 1A). A *Bam*HI fragment from the *psbDI-psbC* operon that carries the carboxy terminus of the *psbDI* coding sequence and the entire *psbC* open reading frame was inserted into the *psbDII* gene (Fig. 1A and B). The conservation between *psbDI* and *psbDII* resulted in restoration of the *psbDII* open reading frame. Because the *Bam*HI fragment includes the two nucleotide differences between *psbDI* and *psbDII*, the presumptive GTG start codon for the *psbC* gene, missing in

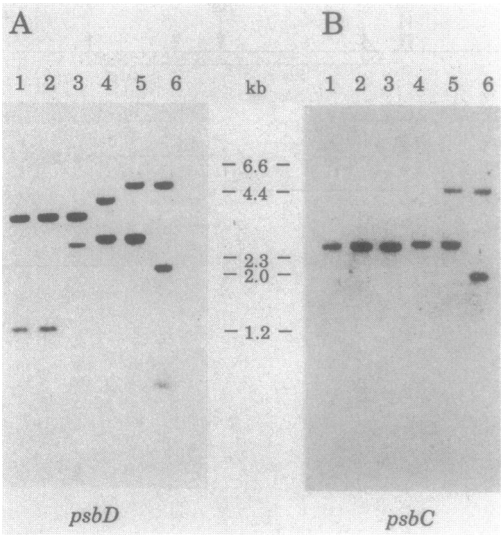

FIG. 2. Southern blot analysis of the *psbD* and *psbC* loci of wild-type *Synechococcus*, AMC027, and AMC050. DNA from wild-type *Synechococcus* (lanes 1 and 4), AMC027 (lanes 2 and 5), and AMC050 (lanes 3 and 6) was cleaved with *Bam*HI (lanes 1 to 3) or *Pst*I (lanes 4 to 6) and separated by agarose gel electrophoresis as described previously (9). Southern analysis was performed as described in Materials and Methods. (A) Blot probed with fragment b1 (Fig. 1A), which was labeled by a random primer reaction. The *psbD* probe was removed, and the blot was probed with an antisense RNA complementary to the *psbC* message to produce panel B. Migration positions of fragment size standards are shown in the center of the figure.

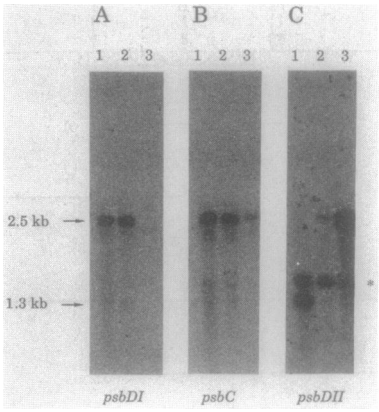

FIG. 3. Northern blot analysis of *psbD* and *psbC* transcripts from wild-type *Synechococcus*, AMC027, and AMC050. Triplicate samples of RNA (4 μg per lane) from the wild-type (lane 1), AMC027 (lane 2), and AMC050 (lane 3) were treated with formaldehyde, separated on agarose gels, and transferred to nylon membranes. One set of lanes was hybridized with an antisense RNA probe specific for each of the following transcripts: (A) *psbDI*; (B) *psbC*; (C) *psbDII*. The sizes of the wild-type *psbD* messages are indicated. An asterisk marks the position of the 16S rRNA band, which bound the *psbDII*-specific probe.

psbDII (12), was imported with the *psbC* open reading frame.

To provide a selection for moving the *psbDII-psbC* artificial operon into *Synechococcus*, a spectinomycin resistance cassette was inserted at the distal *Bam*HI site (Fig. 1B). Because the marker is flanked by homology to the *Synechococcus* chromosome, the predicted recombination event following transformation of *Synechococcus* to spectinomycin resistance is a reciprocal recombination or a gene conversion that would replace an endogenous *psbD* locus with the cloned construct (9). Other organizations of a recombination substrate are possible that would result in gene duplication events at the target locus (9). The most likely locus for recombination would be the *psbDII* locus, since DNA from upstream and downstream of *psbDII* surrounds the *psbDII-psbC* operon and the spectinomycin resistance marker. All five of the spectinomycin-resistant *Synechococcus* transformants that were analyzed had this arrangement. One of these, AMC027, was chosen for further analysis.

Figures 1A and B describe the alterations in patterns of *Bam*HI and *Pst*I restriction fragments, labeled b or p, respectively, followed by a numeral, that allowed mapping the chromosome of transformants. Southern blot analysis of these fragments is shown in Fig. 2. A probe that recognizes both *psbD* genes hybridized to *Bam*HI fragments of 1.2 (b1) and 3.4 kilobases (kb) (b2) in the DNA of wild-type cells (Fig. 2A, lane 1). In transformant AMC027 (Fig. 2A, lane 2), both of these fragments were unchanged, but an alteration in the *Pst*I fragment p2 was evident (Fig. 2A, lane 5). The new fragment, p2.1, resulted from the insertion of the *psbC*-containing *Bam*HI fragment at the carboxy terminus of *psbDII*, which brought in a *Pst*I site that is 0.5 kb further from the left end of p2 than the native *Pst*I site downstream of *psbDII* (Fig. 1B). The p2 fragment was also recognized by a probe from the interior of *psbC* (Fig. 2B, lane 5), consistent with the map shown in Fig. 1B.

Northern blot analysis of transcripts from *Synechococcus* strain AMC027 probed with radiolabeled antisense RNAs specific for *psbDI*, *psbC*, or *psbDII* is shown in Fig. 3. These blots showed that the *psbDII* message in AMC027 migrated as a 2.5-kb species, the expected size for a *psbDII-psbC* dicistronic message (Fig. 3C, lane 2). Messages that hybridized to *psbDI* and *psbC* probes migrated at the same positions in RNA from AMC027 and from wild-type cells (Fig. 3A and B, lanes 1 and 2); this result was expected, since the *psbDII-psbC* dicistronic message should be only 50 nucleotides longer than the *psbDI-psbC* message (12) and not detectable over the more abundant wild-type dicistronic RNA. These data confirmed that *psbC* was being expressed in AMC027 from the *psbDII* locus as well as from the *psbDI* locus, suggesting that if *psbDI* is dispensable, it should be possible to inactivate that gene in AMC027 without jeopardizing *psbC* function.

Synechococcus strain AMC027 was used as a transformation host for a plasmid in which the *psbDI* gene had been interrupted by a kanamycin resistance cassette (Fig. 1C). The recombination substrate plasmid pAM070 is the same plasmid that failed to produce *psbDI*-inactivated mutants from wild-type cells. Of six kanamycin-resistant transformants of AMC027 that were characterized by Southern blot analysis, recombination between the inactivation cassette and the chromosome occurred at the *psbDII* locus in four clones and at the *psbDI* locus in two clones (data not shown). The viability of two clones in which the *psbDI* gene was interrupted indicated that the gene is dispensable, suggesting that *psbDII* can produce sufficient D2 polypeptide to sustain photoautotrophic growth. However, only two of six transformants showed this recombination event, despite the fact that the inactivation construction had slightly more sequence identity to *psbDI* than to *psbDII*; this suggested that there might be a selective advantage to maintaining an active *psbDI-psbC* operon.

One of the *psbDI*-interrupted transformants, AMC050, was characterized further to determine whether the *psbDI* gene was fully inactivated. Figure 1C shows the expected changes in *Bam*HI and *Pst*I restriction fragments following recombination between the inactivation cassette and the *psbDI* locus. These fragments were observed in the Southern blot analysis shown in Fig. 2. The fragments associated with *psbDI* to which a *psbD* probe hybridized in wild-type DNA, b1 (Fig. 2A, lane 1) and p1 (Fig. 2A, lane 4), were altered in DNA from AMC050 (lanes 3 and 6). Fragment b1 was replaced by two new fragments, b1.1 and b1.2, that resulted from the introduction of a *Bam*HI site at the right end of the kanamycin resistance cassette between the two native *Bam*HI sites (Fig. 1C). Fragment b1.1 contained the first half of the *psbDI* gene and the entire kanamycin resistance cassette and was recognized by a probe from the antibiotic cassette as well as the *psbD* probe (data not shown). Fragment b1.2 contained the second half of the *psbDI* gene, from the *Bam*HI site at the right end of the kanamycin resistance cassette to the native end of b1. Restriction digests with *Pst*I also supported the inactivation of *psbDI*. Fragment p1, which included all of *psbDI* and most of *psbC* in wild-type cells (Fig. 1A), was replaced by two new fragments, p1.1 and p1.2, in the transformant (Fig. 1C and 2A and B, lane 6). Fragments b1 and p1 were undetectable in AMC050 DNA (Fig. 2A, lanes 3 and 6), indicating that all copies of the chromosome carried the inactivated *psbDI* locus. The *Bam*HI fragment b3 was unchanged in the mutant (Fig. 2B, lane 3); this result was expected because the alterations at the *psbDI* locus occurred upstream of the *Bam*HI site at the left end of b3 (Fig. 1C).

Northern blot analysis confirmed that AMC050 did not produce any detectable *psbDI* message (Fig. 3A, lane 3). However, in this strain, a *psbC* probe recognized a 2.5-kb RNA which should correspond to the *psbDII-psbC* dicistronic message (Fig. 3B, lane 3). A *psbDII*-specific probe confirmed that the 2.5-kb band was produced from the *psbDII* locus (Fig. 3C, lane 3). Since all of the lanes shown in Fig. 3 were loaded with a constant amount of RNA, the Northern blot shows the relative abundance of messages among the three strains. Comparison of the amount of 2.5-kb message in lanes 2 and 3 of panel C indicates that the steady-state level of message from the *psbDII* locus was greatly increased in AMC050. Since the *psbDII-psbC* operon is identical between AMC027 and AMC050, this suggests that transcription of the *psbDII* gene is upregulated by loss of function of *psbDI*. Lanes 2 and 3 of panel B provide a comparison of the total amount of *psbC* message in AMC027 and AMC050. It is important to note that the *psbC* probe detected the messages from both the *psbDI-psbC* and *psbDII-psbC* loci in AMC027 (lane 2). These data indicate that the dicistronic message arising from *psbDII* (lane 3) is significantly less abundant than wild-type *psbD-psbC* RNA (lanes 1 and 2). Thus, the increased level of dicistronic transcript from the *psbDII* locus in AMC050 fails to reach the level of *psbD-psbC* message that is present in cells containing a functional *psbDI-psbC* operon. This suggests that AMC050, although photosynthetically competent, might be somewhat impaired in production of the *psbD* and *psbC* products, D2 and CP43.

Western blot analysis of thylakoid membranes for D2 and CP43 indicated that AMC050 had a lower steady-state level of each of these proteins than did wild-type cells of other mutants in which the *psbDI-psbC* operon was active (Fig. 4). The amount of D2 (panel A) and CP43 (panel B) was constant among thylakoids of the wild type (lanes 1),

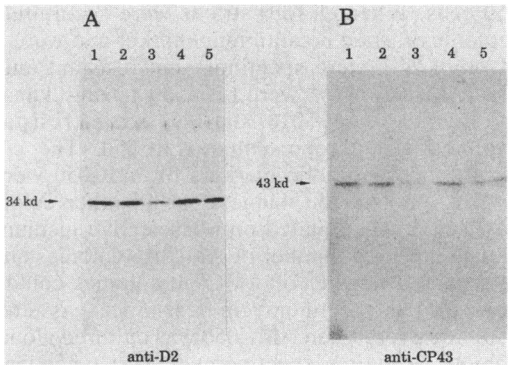

FIG. 4. Western blot analysis of thylakoid membrane proteins from wild-type *Synechococcus* and four mutant derivative strains. Duplicate thylakoid membrane samples containing 80 μg of total protein from wild-type *Synechococcus* (lane 1), AMC027 (lane 2), AMC050 (lane 3), AMC015 (lane 4), and AMC016 (lane 5) were solubilized and loaded onto lanes of a 10 to 20% polyacrylamide gradient gel. Following electrophoresis, the proteins were transferred to nitrocellulose and incubated with antisera directed against the spinach D2 polypeptide (A) or the *Synechococcus* CP43 polypeptide (B). Antibody reactions were visualized with alkaline phosphatase-conjugated secondary antibodies and photographed. The approximate size of each band relative to prestained molecular mass standards is shown in kilodaltons (kd).

AMC027 (lanes 2), and two *psbDII*-interrupted mutants, AMC015 and AMC016 (lanes 4 and 5). However, both bands were lighter in the AMC050 lane. Strain AMC015 carries an interrupted *psbDII* gene that is missing 71 amino acids from the carboxy terminus of the coding region, and its truncated *psbDII* mRNA is detectable by Northern blot analysis (12). Since no truncated polypeptide was detectable in Fig. 4A, lane 4, the mutant protein does not appear to accumulate in thylakoids and is probably nonfunctional. The *psbDII* gene of AMC016 is interrupted by the same construction used to inactivate *psbDI* in AMC050; it could produce a peptide of only 91 amino acids.

If the levels of D2 and CP43 are depressed in AMC050, it is likely that the cells would show some growth impairment relative to wild-type *Synechococcus*. Liquid cultures of each of the strains in the absence of selective antibiotics showed indistinguishable growth rates as measured by changes in optical density at 750 nm. However, the information obtained from these measurements is limited. *Synechococcus* growth rate is light dependent and slowed by slight increases in culture density which cause shading. This results in a very short period of logarithmic growth; only gross differences in rates would be detected. As a more sensitive measure of subtle changes in fitness of mutant cells, coculturing experiments were carried out in which equal numbers of mutant and wild-type cells were mixed and grown for several days without antibiotic selection. The advantage of this method is that it tests the relative growth rates over a longer period of time and through a range of light intensities able to penetrate the developing culture over the course of the experiment. After the coculturing period, the samples were diluted and plated to assay the makeup of the resulting population by the distribution of characteristic antibiotic resistance markers. When the culture contained wild-type cells mixed with either AMC016 or AMC027, mutant and wild-type colonies were recovered in approximately equal numbers. However, only 27 to 34% of the colonies were resistant to spectinomycin and kanamycin following coculture of wild-type and

AMC050 cells. When all four strains were cocultured, 23% of the colonies carried no antibiotic marker and were scored as wild type, 27% were spectinomycin resistant and were scored as AMC027, 36% were resistant to only kanamycin and were scored as AMC016, and 14% carried resistance to both antibiotics and represented AMC050. The colonies carrying the two antibiotic markers of AMC050 were only one-third to one-half the diameter of all other colonies in each case, even when plated on nonselective medium. The higher-than-expected number of AMC016 colonies and lower-than-expected number of AMC050 colonies could result from loss of the spectinomycin resistance cassette from AMC050; however, when AMC050 was cultured alone without antibiotic selection, spectinomycin resistance was very stable, and its loss would account for no more than a 0.5% change in the representation of AMC050 in the population. These data indicate that loss of function of the *psbDI-psbC* operon, although not lethal, impaired the fitness of *Synechococcus*, as predicted by the reduced levels of *psbD-psbC* message and their products, the D2 and CP43 polypeptides.

DISCUSSION

These experiments demonstrated that the monocistronic *psbDII* gene is functional and suggest that the essential nature of the *psbDI-psbC* operon is explained by the need for CP43 produced from the unique *psbC* gene. Although dispensable, function of the *psbDI* locus appears to be important for optimal growth under standard laboratory conditions, since AMC050 competed poorly in mixed culture with other strains and consistently formed small colonies. Comparison of *psbD* and *psbC* transcripts and D2 and CP43 polypeptides among AMC050 and strains having an active *psbDI-psbC* locus indicated that the *psbDII* locus cannot produce wild-type levels of the two proteins.

The message from the *psbDII-psbC* artificial operon in AMC027 was present at a lower steady-state level than the monocistronic *psbDII* message from wild-type cells; this may be due to a difference in transcriptional efficiency caused by the structural alteration of the transcription unit, or it may reflect a decreased stability of the dicistronic message relative to the monocistronic species. However, inactivation of *psbDI* greatly increased the abundance of this transcript. Since the *psbDII-psbC* dicistron is structurally identical between AMC027 and AMC050, this can only be interpreted as upregulation of *psbDII* expression in response to loss of function of the *psbDI* gene; a sharp decline in the steady-state level of D2 following *psbDI* inactivation is a possible signal for increased expression from *psbDII*.

No growth impairment was noted in either of the two mutants that lack function of *psbDII*. The gene may, however, have an important function in the cell under growth conditions that were not tested in our experiments. Differential expression of other PSII genes has been reported (28). The *psbAII* and *psbAIII* genes of *Synechococcus* are expressed at a very low level under typical laboratory growth conditions, contributing only 5 to 6% of the total *psbA* message population (8, 28). However, expression of these genes is elevated at light intensities that exceed standard photon flux (28), resulting in a higher proportion of a minor form of D1 in the thylakoid membrane (27). The *psbDII* gene might likewise be induced under conditions that have not yet been identified. Preliminary experiments suggest that the abundance of the *psbDII* transcript in wild-type cells is affected by changes in light intensity (S. A. Bustos and S. S. Golden, unpublished data).

The differential expression of the three *psbA* genes results in an altered ratio of two forms of D1 in the thylakoid membrane (27). The two *psbD* genes of *Synechococcus* encode an identical polypeptide; thus, no qualitative change in PSII composition would be effected by modulating expression of *psbDII*. Evidence that the monocistronic *psbDII* locus is regulated is provided by the increased steady-state level of *psbDII-psbC* message in AMC050 relative to that in AMC027. This suggests that *psbDII* is able to compensate partially for loss of *psbDI* by increased expression. Consistent with this suggestion is the lack of a typical procaryotic −35 element in the promoter region of the *psbDII* gene, a characteristic of *E. coli* promoters that are under positive regulation (23). The primary result of regulated expression of the monocistronic *psbD* locus would be to alter the ratio of messages that encode *psbD* or *psbC*. In plant chloroplasts there is a single overlapping *psbD-psbC* operon, but a complex pattern of transcripts is produced from this locus, including some monocistronic species (6). Changes in the transcript pattern occur during chloroplast development in barley, which correlate with increased expression of *psbD* relative to *psbC* (6). No monocistronic messages have been detected from the *psbDI-psbC* locus of *Synechococcus* (12). Gene duplication may be an evolutionarily divergent mechanism for coordinating the synthesis of PSII components that has evolved in the cyanobacteria.

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