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The *Anabaena* sp. Strain PCC 7120 Gene all2874 Encodes a Diguanylate Cyclase and Is Required for Normal Heterocyst Development under High-Light Growth Conditions▽†

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The genome of the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 harbors 14 genes containing a GGDEF diguanylate cyclase domain. We found that inactivation of one of these genes, all2874, caused abnormal heterocyst development. The all2874 mutant showed a pronounced reduction in heterocyst frequency during diazotrophic growth and reduced vegetative cell size compared to the wild type. The severity of the mutant phenotype varied with light intensity; at high light intensity, the mutant phenotype was accentuated, whereas at low light intensity the phenotype was similar to wild type. Under high-light growth conditions, the initial heterocyst frequency and pattern for the all2874 mutant were normal, but within 4 days following nitrogen step-down, many intervals between heterocysts increased to as many as 200 vegetative cells, whereas in the wild type the intervals were less than 25 vegetative cells. Filaments containing these unusually long vegetative cell intervals between heterocysts also contained intervals of normal length. An all2874 mutant strain carrying a P_{patS}-*gfp* transcriptional reporter fusion failed to show normal upregulation of the reporter, which indicates that the decrease in heterocyst frequency is due to an early block in differentiation before induction of the *patS* gene, which in the wild type takes place 8 h after nitrogen step-down. Genetic epistasis experiments suggest that All2874 acts upstream of the master regulator HetR in differentiating cells. We also showed that purified All2874 functions as a diguanylate cyclase in vitro. We hypothesize that All2874 is required for the normal regulation of heterocyst frequency under high-light growth conditions.

Cyclic nucleotides are commonly used as second messengers in signal transduction networks of both prokaryotes and eukaryotes. Cyclic-di-GMP is a bacterial second messenger that has been recognized as an important regulatory molecule in diverse bacteria (14). It was discovered 20 years ago in *Acetobacter xylinum*, where it functions as a positive allosteric regulator of cellulose synthase (27). Recent studies have shown that c-di-GMP is involved in the regulation of a variety of complex physiological processes, including production of exopolysaccharides, biofilm formation, motility, virulence, and cellular differentiation (14, 29). The intracellular concentration of c-di-GMP is controlled by two classes of enzymes with opposing activities: diguanylate cyclase and phosphodiesterase, which catalyze c-di-GMP synthesis and breakdown, respectively. Diguanylate cyclase activity is associated with the conserved GGDEF domain, whereas phosphodiesterase activity is associated with the conserved EAL or HD-GYP domains, named after the conserved sequence motifs that are present in the active sites (29). These two domains are often found on the same protein, and they are typically coupled with a variety of sensory input domains (26). Some of the signals perceived by c-di-GMP signaling pathways include oxygen, blue light, red light, nutrient starvation, antibiotics, bile salts, intercellular signaling molecules, and mucin (14).

It is not yet well understood how c-di-GMP mediates its regulatory effects on downstream physiological processes. The recent in silico identification of the putative c-di-GMP-binding PilZ domain reported by Amikam and Galperin (3) has been followed by experimental evidence demonstrating c-di-GMP binding by PilZ domain proteins from several bacteria (4, 20, 25). These c-di-GMP-sensing proteins have been shown to regulate biofilm formation, motility, and virulence (29). A recent study of a crystal structure of c-di-GMP bound to the PilZ domain of VCA0042/PlzD from *Vibrio cholerae* showed that binding of the small ligand induces a conformational change of VCA0042/PlzD. It was proposed that this conformational change could favor direct interaction with downstream effector targets to allosterically activate them (4). Bioinformatics analysis of currently available genomes revealed that the majority of cyanobacteria do not harbor the PilZ domain (3). There are no PilZ domains in *Anabaena* sp. strain PCC 7120.

The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 responds to deprivation of combined nitrogen by undergoing a developmental program to produce heterocysts, nitrogen-fixing cells that are usually positioned every 10 to 15 cells along filaments of photosynthetic vegetative cells. Numerous genes have been identified that are involved in heterocyst development and/or function. Two key players in heterocyst formation are the master regulator HetR and the inhibitory peptide PatS. HetR is essential for heterocyst development (5, 7, 16). The *hetR* gene is expressed early in differentiating cells

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Anabaena sp. strains		
PCC 7120	Wild type	R. Haselkorn
AMC484	PCC 7120 carrying pAM1951; Nm^r	31
AMC1289	PCC 7120 carrying pAM3318; Nm^r	This study
AMC1572	Insertional inactivation of all2874 by single homologous recombination of pAM4108; Sp^r Sm^r	This study
AMC1574	PCC 7120 carrying pAM4114; Nm^r	This study
AMC1575	AMC1572 carrying pAM4114; Nm^r Sp^r Sm^r	This study
AMC1577	AMC1572 carrying pAM3318; Nm^r Sp^r Sm^r	This study
AMC1578	AMC1572 carrying pAM1951; Nm^r Sp^r Sm^r	This study
Plasmids		
pRL277	Conjugal suicide plasmid; Sp^r Sm^r	5
pRP89	pET11 containing C-terminal His-tagged *pleD**; Ap^r Cm^r	24
pAM1951	Shuttle plasmid pAM505 carrying a P_{patS}-*gfp* transcriptional fusion; Km^r Nm^r	31
pAM2770	Shuttle plasmid containing XhoI-P_{petE}-NdeI-*lacZ*-alpha SapI(Cys)-6His-(stop)-ClaI; Km^r Nm^r	18
pAM3318	Shuttle plasmid pAM504 carrying P_{petE}-$hetR_{R223W}$-Ω	16
pAM4097	$pET30b^+$ expression plasmid containing the C-terminal His_6-tagged all2874 ORF; Km^r	This study
pAM4108	pRL277 containing 630-bp internal fragment of all2874; Sp^r Sm^r	This study
pAM4114	pAM2770 containing the entire all2874 ORF; Nm^r	This study

after nitrogen step-down. HetR has two known activities, autoproteolysis and specific DNA binding, that require formation of a homodimer (12). Increased HetR levels or activity is sufficient to force heterocyst differentiation. Overexpression of *hetR* on a multicopy plasmid either from its native promoter or from the copper-regulated *petE* promoter leads to an increased heterocyst frequency regardless of the presence of nitrate or ammonium (7). Overexpression of a mutant allele of *hetR* ($hetR_{R223W}$) is able to bypass the main inhibitory signals of heterocyst pattern formation and results in a conditionally lethal phenotype caused by complete differentiation of nearly all cells under nitrogen-limiting conditions (16).

The initial heterocyst pattern and its maintenance are controlled by two negative regulators, PatS and HetN, respectively. The *patS* gene is predicted to encode a small peptide involved in establishing the initial heterocyst pattern by lateral inhibition (31). A P_{patS}-*gfp* reporter strain showed that *patS* expression is localized to individual cells or small groups of cells by 8 to 10 h after nitrogen step-down (32). Overexpression of *patS* blocks heterocyst development, whereas a *patS* null mutant forms heterocysts even in the presence of nitrate (31). The *hetN* gene encodes a protein similar to ketoacyl reductases and plays a role in the maintenance of heterocyst pattern (6). When *hetN* is overexpressed, heterocyst development is completely blocked. In the absence of *hetN* expression, filaments develop a normal heterocyst pattern in the first 24 h after nitrogen step-down, but by 48 h, excessive differentiation produces a multiple contiguous heterocyst phenotype (8).

The role of c-di-GMP in heterocyst development has not been explored. Because c-di-GMP is an important signaling molecule involved in cellular functions such as cellular differentiation, exopolysaccharide synthesis, and light sensing, we hypothesized that c-di-GMP may be important for some aspects of heterocyst development in *Anabaena* sp. strain PCC 7120. There are 14 genes containing the GGDEF domain in *Anabaena* sp. strain PCC 7120 (30). In an effort to elucidate the role of c-di-GMP in heterocyst development, we have inactivated each of these 14 genes and screened for heterocyst-related phenotypes. Under our conditions, only 1 of the 14 mutant strains produced altered heterocyst development. Inactivation of all2874 caused a pronounced decrease in heterocyst frequency as well as a reduction in vegetative cell size. The heterocyst-related defects were accentuated under high-light growth conditions. We also showed that All2874 has diguanylate cyclase activity in vitro.

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this study are presented in Table 1. Genetic locus designations for *Anabaena* sp. strain PCC 7120 are from CyanoBase (http://bacteria.kazusa.or.jp/cyanobase/). *Anabaena* sp. strain PCC 7120 and its derivatives were grown in BG-11 (containing sodium nitrate) or $BG\text{-}11_0$ (BG-11 lacking sodium nitrate) medium at 30°C as previously described (10). Cultures were grown under fluorescent white light illumination at 25 μM photons m^{-2} s^{-1} (low), 50 μM photons m^{-2} s^{-1} (medium, normal), and 150 μM photons m^{-2} s^{-1} (high), as measured with a Biospherical Instruments model QSL100 sensor, which collects light from all directions. For derivatives of *Anabaena* sp. strain PCC 7120 carrying plasmids, neomycin (Nm; 25 μg/ml) and/or streptomycin (Sm) and spectinomycin (Sp) (2 μg/ml each) were used when strains were grown on BG-11 or $BG\text{-}11_0$ agar plates. For liquid media, the antibiotic concentration was reduced by half. Heterocyst development was induced by nitrogen step-down; filaments from an actively growing BG-11 culture with an optical density at 750 nm of 0.1 to 0.3 were collected by centrifugation and washed three times with sterile water before being transferred to the original volume of $BG\text{-}11_0$. For inducing multiple samples, cultures were grown under different illumination intensities in 2 ml of $BG\text{-}11_0$ in 16- by 150-mm culture tubes. Strains carrying the copper-inducible *petE* promoter were initially grown in medium lacking copper; filaments from standard medium were washed twice by centrifugation and resuspension of the pellet in copper-deficient BG-11 medium before resuspending it in the final growth medium. Activity of the *petE* promoter was induced by transferring filaments to medium supplemented with 0.6 μM $CuSO_4$. Copper-deficient medium was prepared in plasticware and filter sterilized as previously described (7).

Plasmids were transferred to *Anabaena* by conjugation from *Escherichia coli* following published protocols (9, 11) with some minor modifications (19).

E. coli strains were maintained in LB (Lennox L) liquid or agar-solidified medium supplemented with appropriate antibiotics.

Growth rate determination. Cultures were grown on a shaking platform at 30°C and at light intensities of 25, 50, and 150 μM photons m^{-2} s^{-1}. Growth was monitored spectrophotometrically as the optical density at 750 nm. At 12-h intervals, 1-ml samples were removed from 100-ml cultures for optical density measurements. To avoid sampling errors caused by filament clumping in $BG\text{-}11_0$ medium, the filaments were dispersed by repeated pipetting before optical density measurements. Antibiotics were not added to medium during growth rate experiments.

DNA manipulations and plasmid constructions. Standard protocols were used for cloning, *E. coli* transformation, and PCR. Total DNA from cyanobacterial strains was isolated as previously described (10). DNA sequencing of plasmid inserts was performed by the Gene Technologies Laboratory (Texas A&M University) following the Big Dye sequencing protocol (Applied Biosystems).

Plasmids used in this study are listed in Table 1. The suicide plasmids used to inactivate the 13 GGDEF domain genes other than all2874 are described in Table S1 of the supplemental material. For the construction of each suicide plasmid, an internal fragment of the open reading frame (ORF) extending into the GGDEF motif (ranging from 580 to 1,277 bp) was amplified from genomic DNA by PCR using forward and reverse primers that contained XhoI and SacI sites, respectively, at their 5′ ends (see Table S2 in the supplemental material); the PCR-generated fragments were cloned into the conjugal suicide plasmid pRL277. For all2874, which is the focus of this study, the suicide plasmid pAM4108 was constructed by cloning a 630-bp internal fragment of all2874, PCR amplified with primers AMO-1069 and AMO-1070, into pRL277. Plasmid pAM4114 carrying P_{petE}-all2874 was made by subcloning a 993-bp NdeI-XmaI fragment containing the all2874 ORF into the respective sites of plasmid pAM2770. All plasmid constructions were verified by DNA sequence analysis.

For each targeted gene, six exconjugants were picked and subcultured repeatedly to ensure complete segregation of the mutant phenotype. For the all2874 mutant strain, insertion of the pAM4108 plasmid at the desired locus and segregation of the mutation were confirmed by PCR. The pAM4114 plasmid carrying the entire all2874 ORF under the control of the *petE* promoter was able to complement the mutant when grown in standard BG-11_0 medium, which contains 0.3 μM $CuSO_4$ and induces half-maximal expression from the *petE* promoter (7).

To express and purify the All2874 protein, we constructed pAM4097. The all2874 ORF was amplified by PCR with primers AMO-1927 and AMO-1928, which contained NdeI and XhoI restriction sites at their 5′ ends, respectively. The PCR product was then cloned into the pET-30b(+) (Novagen) expression plasmid at its NdeI and XhoI restriction sites, which generated plasmid pAM4097 carrying an in-frame fusion of all2874 with a carboxy-terminal His_6 tag (All2874-6His). DNA sequencing was used to verify the insert and tag region of pAM4097.

Microscopy. Fluorescence and bright-field images were captured using an Olympus IX70 inverted microscope with a CoolSNAP HQ2 camera (Photometrics) and Simple PCI software version 6.0 (Compix Inc.). The Simple PCI software controlled the camera, light path shutters (ProScan; Prior Scientific), and automated focus. A Piston green fluorescent protein (GFP) band-pass filter set (no. 41025; Chroma Technology Corp.) was used for fluorescence images. Photomicrographs were minimally processed with Adobe Photoshop 8.0 to improve brightness and contrast.

Scoring of heterocyst patterns along the filaments was performed as previously described (32). Detached single heterocysts and aggregates of heterocysts were not scored. At least three biological replicate samples were scored for each experimental condition. Statistical analyses were performed using StatView software. Vegetative cell size measurements were obtained using Image J software and micrographs of wild-type and all2874 mutant filaments at 96 h after nitrogen step-down under high-light growth conditions; a total of 200 cells were measured for each of the two strains. Alcian blue staining was used to detect the heterocyst-specific polysaccharide layer of the proheterocyst envelope (19). Briefly, a solution of 0.5% Alcian blue (Sigma) in 50% ethanol–water was mixed with an equal volume of an *Anabaena* culture and incubated for 10 min before microscopic examination.

Expression and purification of All2874. The pAM4097 plasmid carrying the all2874-6His fusion was transformed into *E. coli* strain BL21(DE3), producing strain AM4097. An overnight culture of AM4097 grown at 37°C in LB (Lennox L) medium supplemented with kanamycin (50 μg/ml) was diluted 1:100 into fresh medium and grown at 37°C with vigorous shaking. At an optical density at 600 nm of 0.5, expression of the fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM and incubation at 28°C for 8 h. Cells were then harvested by centrifugation at 4,000 × *g* for 10 min, resuspended in binding buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, and 20 mM imidazole) containing phenylmethylsulfonyl fluoride (0.1 mg/ml) and lysed by passage through a French press twice (18,000 lb/in^2). The lysate was cleared by centrifugation at 35,000 × *g* for 25 min at 4°C. To reduce viscosity, the supernatant was briefly sonicated with a microprobe tip to shear genomic DNA and the centrifugation was repeated for 10 min. The supernatant was loaded onto a 1-ml HisTrap HP column (GE Healthcare) with a syringe pump and washed with 10 ml of binding buffer. A linear gradient of imidazole was used to elute the fusion protein using an Äkta fast-performance liquid chromatography system (GE Healthcare). Fractions containing All2874-6His were pooled and then dialyzed against 25 mM Tris-HCl pH 8.0, 250 mM NaCl, and 2.5 mM β-mercaptoethanol for 12 h at 4°C. Fractions containing >95% purified protein, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see Fig. S5 in the supplemental material), were used for enzyme activity assays. Protein concentrations were determined using the Bradford assay (Bio-Rad). The strain carrying plasmid pRP89, which expresses a C-terminal His-tagged fusion of PleD*, was generously provided by Urs Jenal (University of Basel). PleD* purification was performed as previously described (24).

Diguanylate cyclase assays. The protocol used for enzyme assays was based on methods described by Paul et al. (24) and modified by Kazmierczak et al. (15). Briefly, purified proteins were added to reaction buffer (25 mM Tris-HCl [pH 8.0], 250 mM NaCl, 2.5 mM β-mercaptoethanol, and 10 mM $MgCl_2$) in a total reaction volume of 9 μl. Reactions were started by addition of 1 μl of α-labeled [^{32}P]GTP (3.33 pmol; 3,000 Ci mmol^{-1}; Perkin-Elmer). Reaction mixtures were incubated at 25°C for 60 min before termination by addition of 2 μl of 0.5 M EDTA pH 8.0. The product of the PleD* reaction served as a control for the production of c-di-GMP. HetL, a protein from *Anabaena* sp. strain PCC 7120 that does not contain the GGDEF domain, was assayed as a negative control (data not shown).

Reaction products from diguanylate cyclase assays were separated by thin-layer chromatography on 20- by 20-cm polyethyleneimine-cellulose plates (Macherey-Nagel), using 1:1.5 (vol/vol) saturated $(NH_4)_2SO_4$ and 1.5 M KH_2PO_4, pH 3.6, as the running buffer. From each reaction mixture, a 2-μl volume was spotted and dried onto the polyethyleneimine-cellulose thin-layer chromatography plates. Plates were developed in running buffer, air dried for 3 h under a fume hood, and visualized by autoradiography.

RESULTS

Identification of putative GGDEF and EAL proteins in *Anabaena* sp. strain PCC 7120. The *Anabaena* sp. strain PCC 7120 genome harbors eight genes encoding a GGDEF domain but not the EAL domain, one gene encoding only the EAL domain, and six genes encoding both domains; all these genes are present on the chromosome. A schematic representation of the domain architecture of all the proteins predicted to have GGDEF domains is shown in Fig. S4A of the supplemental material. The majority of these proteins contain additional domains predicted to be involved in signal transduction, including PAS, GAF, CBS, HAMP, CHASE, FHA, and receptor domains.

To determine the evolutionary relationship between the GGDEF domains, we constructed a phylogenetic tree based on the alignment of the predicted amino acid sequences of all the GGDEF domains present in *Anabaena* sp. strain PCC 7120 (see Fig. S1 in the supplemental material); in the alignment we included the GGDEF domain from the PleD protein of *Caulobacter crescentus*, whose diguanylate cyclase activity has been previously confirmed (28). The phylogenetic tree shows that the GGDEF domains can be grouped in two clusters. Cluster I contains almost exclusively GGDEF domains that are not coupled with EAL domains, and the majority display the variant GGEEF motif. All domains in cluster II have a conserved GGDEF motif and most of them are coupled with the EAL domain.

Inactivation of all2874 caused a decrease in heterocyst frequency. To determine if c-di-GMP signaling plays a role in heterocyst development, we inactivated all 14 genes predicted to encode diguanylate cyclases by single homologous recombination with suicide plasmids carrying an internal fragment of the targeted gene. We screened all the mutants for heterocyst defects, including heterocyst differentiation and morphology, pattern formation, heterocyst frequency, and diazotrophic growth.

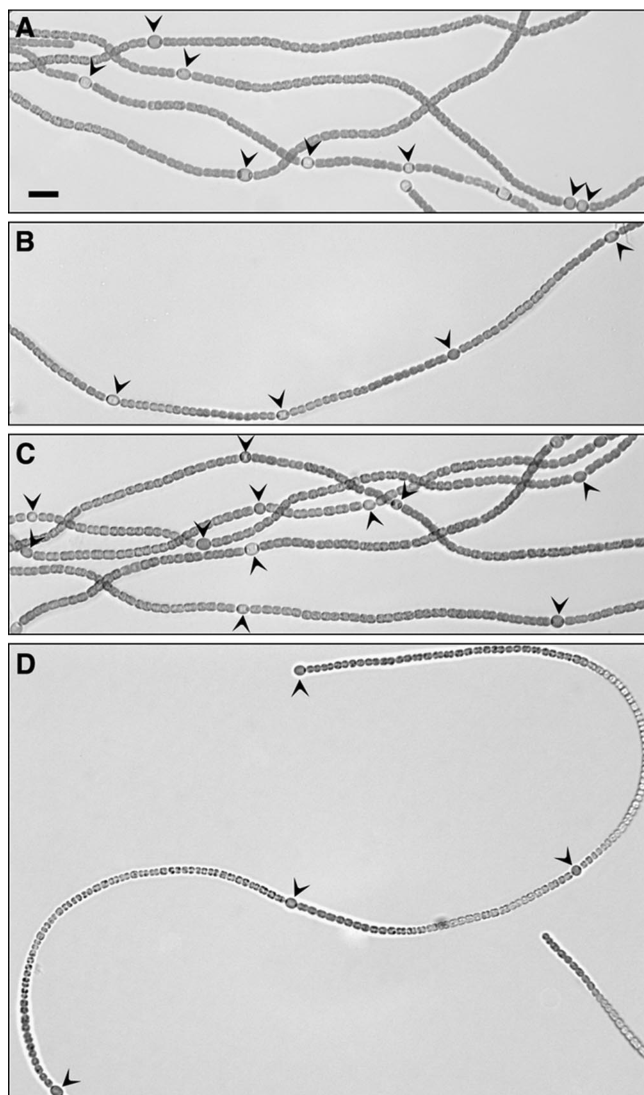

FIG. 1. Inactivation of all2874 caused a reduced heterocyst frequency. Filaments grown in BG-11 liquid medium under normal light intensity were transferred to BG-11_0 medium to induce heterocyst development and were grown under high light. At 24 h after nitrogen step-down, the all2874 mutant (B) formed heterocysts with a frequency similar to that of the wild type (A), but by 96 h the heterocyst frequency of the all2874 mutant (D) was considerably lower than that of the wild type (C). Arrowheads indicate heterocysts. Bar, 10 μm.

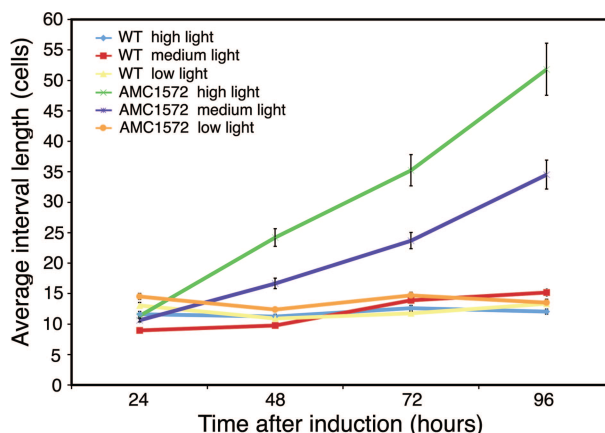

FIG. 2. The all2874 mutant showed a light intensity-dependent defect in heterocyst frequency. Vegetative cell intervals between heterocysts of the wild type (WT) and all2874 mutant (AMC1572) grown at three different light intensities (low, medium, and high) for 4 days following nitrogen step-down are shown. The average numbers of vegetative cells in the interval between heterocysts remained similar in the wild type grown under all three light conditions, whereas the all2874 mutant showed increased interval lengths over time in a light intensity-dependent manner. Error bars indicate standard errors.

Only one of the mutants, strain AMC1572, in which gene all2874 was disrupted, displayed a heterocyst-related phenotype. Under our normal growth conditions on medium lacking a source of combined nitrogen, the initial heterocyst frequency was essentially wild type, but over the next 4 days, the mutant strain showed a pronounced decrease in heterocyst frequency (Fig. 1; see also Fig. S2 in the supplemental material). In addition, the vegetative cell size of the mutant was diminished compared to that of the wild-type strain; at 96 h after nitrogen step-down under high-light growth conditions, wild-type vegetative cell length was 3.6 ± 0.56 μm (mean ± standard deviation), whereas vegetative cell length for the all2874 mutant was 1.89 ± 0.33 μm.

Heterocyst frequency phenotype of the all2874 mutant is light and/or growth rate dependent. We noticed that older (denser) nitrogen-deprived cultures of the mutant strain showed a less severe phenotype. This prompted us to investigate if light intensity plays a role in the mutant phenotype, because all2874 is potentially cotranscribed with the upstream gene all2875, which contains several PAS domains, including one that has been shown to be involved in sensing light (23). The all2875 open reading frame overlaps all2874 by 4 nucleotides. To determine whether light intensity affects heterocyst frequency of the mutant strain, filaments of the wild-type and mutant strains grown in nitrate-containing medium under normal light conditions were washed and transferred to medium lacking fixed nitrogen and grown at three different light intensities: low, medium, and high. At 24 h after nitrogen step-down, the heterocyst frequency of the all2874 mutant was similar to that of the wild-type strain under all three light conditions (Fig. 2; see also Fig. S2 in the supplemental material). When grown at medium or high light intensity, the average number of vegetative cells between heterocysts (vegetative cell interval) exceeded that of the wild type and continued to increase over time. At 96 h after nitrogen step-down, the last time point recorded, the average vegetative cell interval increased to 34.5 ± 24.8 when filaments were grown under medium light and 51.8 ± 35.5 under high light (Fig. 2; see also Fig. S2 in the supplemental material). In addition, the interval between heterocysts in the mutant filaments became more variable, ranging from normal intervals to abnormally long intervals of up to 200 cells. The variability of heterocyst spacing is reflected in the large standard deviations of the average vegetative cell intervals of the mutant filaments grown at high light. Similar results were produced by nitrogen step-down of strains that were grown in high light (instead of medium light) prior to nitrogen deprivation. No significant differences in heterocyst frequency and spacing were observed in the wild-type

strain grown under the different light conditions (Fig. 2; see also Fig. S2 in the supplemental material).

We also compared the growth rates of the all2874 mutant and wild-type strains grown at low, medium, and high light intensities in the presence or absence of a source of combined nitrogen. In the presence of nitrate, growth of the mutant strain was similar to that of the wild type under all three light conditions (data not shown). In the absence of a source of combined nitrogen, growth of the mutant strain was slower than that of the wild type at medium and high light intensities but remained similar to the wild type under low light, suggesting that the growth defect of the mutant was caused by the decreased heterocyst frequency at higher light levels (see Fig. S3 in the supplemental material).

The all2874 mutant phenotype raised the possibility that expression of the all2874 gene is induced by high light and/or that its expression is cell type specific. However, Northern RNA blot experiments showed no significant change in expression of all2874 grown at different light levels, and a reporter strain containing a *gfp* transcriptional fusion to the upstream region of the putative all2875-all2874 operon showed GFP fluorescence in both vegetative cells and heterocysts (data not shown).

Heterocyst development is blocked before upregulation of P_{patS}-*gfp* in the all2874 mutant. To determine if some cells within the long vegetative cell intervals between heterocysts of the all2874 mutant strain had initiated differentiation but were blocked before morphogenesis, the shuttle plasmid pAM1951 (18, 32), which carries a *gfp* transcriptional fusion to the *patS* promoter, was introduced into the all2874 mutant, producing strain AMC1578. Filaments were then induced to form heterocysts by nitrogen step-down and incubated at low or high light intensity. The results for low and high light were similar; Fig. 3 shows the high-light data. Surprisingly, we found that heterocyst differentiation was blocked for the first 3 to 4 days following nitrogen step-down in the all2874 mutant carrying the P_{patS}-*gfp* transcriptional fusion. During this period of time, the GFP fluorescence intensity from the *patS* reporter remained at a low level in all vegetative cells and there was no apparent pattern of GFP-fluorescent cells. This was in contrast to the wild-type reporter strain, which showed an initial pattern of GFP-bright cells at 6 to 8 h after nitrogen step-down. After 4 days, some AMC1578 filaments differentiated at a low frequency (2.5% ± 1.56% under high light) of heterocysts. Under high light, some filaments did not differentiate to any heterocysts even after 7 days following nitrogen step-down.

Once the first round of heterocysts was formed, we wanted to know whether subsequent rounds of differentiation were occurring normally. We examined more than 100 vegetative cell intervals containing 30 to 200 cells between heterocysts. In the majority of filaments, intense GFP fluorescence was limited to only proheterocysts and heterocysts, indicating that the abnormally low frequency of heterocysts was due to an early block in differentiation before the upregulation of the *patS* gene. A few filaments contained some cells that were GFP bright, but these occurred at a low frequency with no apparent pattern. Similar results were obtained with wild-type and all2874 mutant strains carrying a P_{hetR}-*gfp* transcriptional fusion on a shuttle plasmid (data not shown); heterocyst development was delayed, and the *hetR* reporter was upregulated in

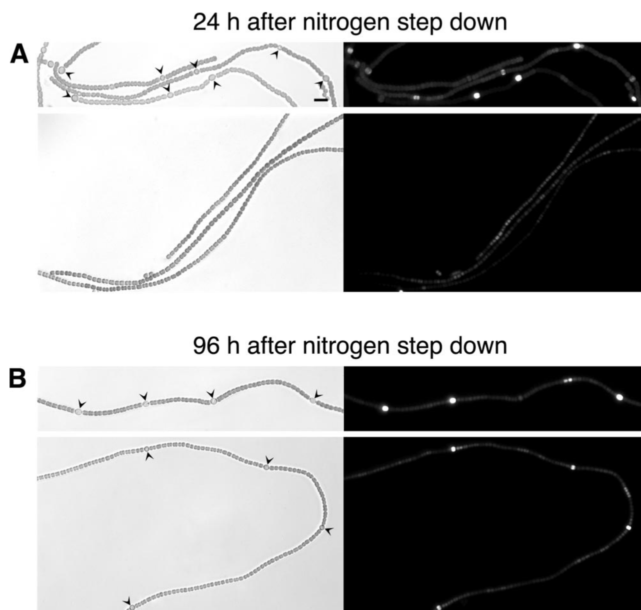

FIG. 3. A P_{patS}-*gfp* reporter is upregulated in only the low-frequency differentiating cells in the all2874 mutant background, and the presence of the P_{patS}-*gfp* reporter plasmid delayed heterocyst development. Images show GFP reporter fluorescence produced by the wild type and all2874 mutant containing a P_{patS}-*gfp* reporter on plasmid pAM1951 at 24 h (A) and 96 h (B) after nitrogen step-down under high-light growth conditions. In both panels A and B, the top two panels show the wild type carrying the P_{patS}-*gfp* reporter (AMC484) and the bottom two panels show the all2874 mutant carrying the P_{patS}-*gfp* reporter (AMC1578). (Left panels) Bright-field images. (Right panels) Corresponding GFP fluorescence images. Arrowheads indicate heterocysts. Bar, 10 μm.

only the low percentage of cells that differentiated into heterocysts. These results indicate that the low frequency of heterocysts in the all2874 mutant is due to a decreased tendency to initiate heterocyst differentiation. In addition, the delayed development caused by the presence of the *patS* and *hetR* promoters on a multicopy plasmid suggests that the all2874 mutant is deficient for active transcription factors required to trigger differentiation.

All2874 is upstream of HetR in the heterocyst differentiation pathway. To determine whether All2874 is upstream or downstream of HetR in the regulatory cascade controlling heterocyst differentiation, we overexpressed the mutant allele $hetR_{R223W}$ in wild-type and the all2874 mutant background and compared heterocyst frequencies over time under low light and high light. The shuttle plasmid pAM3318 carrying the $hetR_{R223W}$ allele under the copper-inducible *petE* promoter was used for overexpression. The wild-type and the all2874 mutant strain carrying pAM3318 were grown in copper-free medium containing nitrate at low light to minimize the tendency to form heterocysts prior to induction. Heterocyst differentiation was induced by transferring the two strains to medium lacking combined nitrogen and containing 0.6 μM $CuSO_4$. At 24 h after nitrogen step-down, both strains displayed multiple contiguous heterocysts and a similar heterocyst frequency under both light conditions (Fig. 4). Over the following days, heterocyst frequency remained abnormally elevated in both strains at both light intensities (Fig. 4). Even after

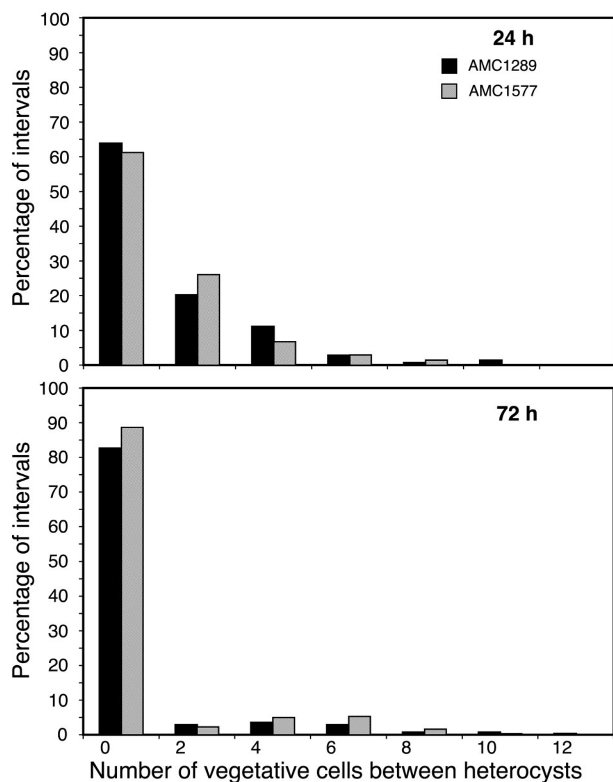

FIG. 4. Overexpression of the $hetR_{R223W}$ mutant allele is epistatic to the all2874 mutation. The bar graphs show the percentages of intervals that contained different numbers of vegetative cells in the interval between heterocysts at 24 h and 72 h after nitrogen step-down under high-light growth conditions for a wild-type strain containing the pAM3318 plasmid carrying the $hetR_{R223W}$ allele expressed from the copper-inducible *petE* promoter (AMC1289) and the all2874 mutant containing the same plasmid (AMC1577).

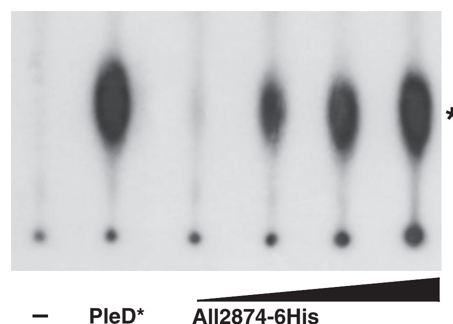

FIG. 5. Purified All2874-6His exhibited diguanylate cyclase activity in a concentration-dependent manner. In vitro synthesis of c-di-GMP by All2874-6His was determined by thin-layer chromatography. PleD* (3 μM) served as a positive control for the synthesis of c-di-GMP. Reactions were performed with increasing concentrations of All2874-6His (2 μM, 6.3 μM, 12.6 μM, and 18.3 μM). No protein was added to the reaction mixture spotted in the left-most lane. The position of c-di-GMP is marked with an asterisk.

several days of growth under high light, the all2874 mutant overexpressing the $hetR_{R223W}$ allele never showed a reduction in heterocyst frequency. Therefore, the $hetR_{R223W}$ allele is epistatic to the all2874 mutation, which suggests that All2874 is upstream of HetR in the heterocyst differentiation pathway.

All2874 has diguanylate cyclase activity in vitro. To directly evaluate the ability of All2874 to synthesize c-di-GMP from [α-^{32}P]GTP, we performed an in vitro diguanylate cyclase assay (15, 24). As a positive control, we included the constitutively active allele of the PleD protein (PleD*), a well-characterized diguanylate cyclase. All2874-6His produced c-di-GMP in a concentration-dependent manner (Fig. 5). Purified HetL protein was used in the assay as a negative control and it produced no c-di-GMP product (data not shown). These data show that purified All2874-6His functions as a diguanylate cyclase in vitro.

DISCUSSION

In many cases, cellular levels of c-di-GMP lead to changes in cell surface properties which, in turn, affect motility and biofilm formation (13). Thus far, there has been only one example in which inactivation of a gene encoding a diguanylate cyclase had an effect on cellular differentiation: PleD, from *Caulobacter crescentus*, was shown to be required for polar development (2). In this paper, we have shown that a diguanylate cyclase encoded by the all2874 gene is required for normal heterocyst development.

The chromosome of *Anabaena* sp. strain PCC 7120 harbors 14 genes containing the GGDEF domain (30), usually coupled with various sensor and/or response modules. The most prevalent of all these signal transduction domains is the PAS domain. The tendency to associate with PAS domains could be due to the functional versatility of these domains; PAS domains are important for monitoring changes in light, redox potential, overall energy level of a cell, oxygen, and small ligands (22).

A phylogenetic tree of the GGDEF domains from *Anabaena* sp. strain PCC 7120 shows the presence of two clusters that correlate with whether or not the GGDEF domain is coupled with an EAL domain. This phylogenetic clustering suggests that proteins containing only the GGDEF domain have evolved separately from those containing both GGDEF and EAL domains. A recent study showed a similar phylogenetic division of the putative diguanylate cyclases in *Pseudomonas aeruginosa* strain PA14, and the same study found that several proteins belonging to either cluster were enzymatically active (17). We tested the in vitro diguanylate cyclase activity of All2874, which belongs to cluster I and harbors the GGEEF motif, and found that this protein was indeed able to synthesize c-di-GMP in vitro.

We have inactivated all of the genes containing GGDEF domains in *Anabaena* sp. strain PCC 7120 to determine the effect on heterocyst development. Only the all2874 mutant showed a distinct mutant phenotype. However, mutation of some genes may not produce an altered phenotype if there is functional redundancy with other genes or if they produce a phenotype that was not tested for.

After nitrogen step-down, the all2874 mutant initially formed heterocysts with a normal pattern and frequency at 24 h, but over the next few days, the frequency was severely diminished. We hypothesize that this phenotype is caused by a decreased tendency to trigger heterocyst differentiation that is clearly manifested during steady-state diazotrophic growth but

is masked during the first round of heterocyst differentiation, which is initiated by a sudden and extreme nitrogen step-down. The all2874 mutant also showed reduced vegetative cell size compared to the wild type. It is possible that the change in vegetative cell size alone is sufficient to disturb the regulation of heterocyst development by influencing the intracellular concentration of regulatory molecules or through an unknown mechanism.

We used a reporter strain containing a P_{patS}-*gfp* transcriptional fusion in the mutant background to examine whether some cells within the long vegetative cell interval between heterocysts had initiated differentiation but were blocked before morphogenesis. Our results showed that the all2874 mutant failed to upregulate the *patS* reporter and therefore was affected in early regulatory events required to initiate cellular differentiation. Surprisingly, the reporter strain showed delayed heterocyst formation. The first round of heterocysts did not differentiate until about 4 days after nitrogen step-down, and then at only a reduced frequency. Since a shuttle plasmid carrying the *patS* promoter in the all2874 mutant background was sufficient to cause a long delay in heterocyst differentiation, we speculate that at least one protein that is important for heterocyst development and that binds to the *patS* promoter, such as HetR, is present at a lower effective concentration in the all2874 mutant than in the wild type. Therefore, the *patS* promoter region carried on a multicopy plasmid would further reduce the pool of the transcription factor by titrating out the protein, resulting in a failure to trigger normal levels of heterocyst differentiation in the all2874 mutant.

In *Anabaena cylindrica*, high light was shown to stimulate heterocyst differentiation (1), but in the all2874 mutant strain, high light intensity caused the opposite effect. The all2874 gene is predicted to be cotranscribed with all2875, which encodes a two-component hybrid sensor response regulator that possesses multiple domains, including several PAS domains and a GAF domain. The second PAS domain belongs to a subfamily that functions as blue-light sensors due to the presence of the LOV (light, oxygen, and voltage) domain, which binds flavin mononucleotide (21, 23). Many GAF domains in cyanobacteria can sense red light; however, the GAF domain present in all2875 is not predicted to have this property because of the absence of the conserved residues required for bilin binding (21). Thus, we speculate that all2875 and all2874 are involved in sensing and conveying information about light intensity. However, it is possible that light levels may only indirectly impact heterocyst development through an effect on growth rate. Inactivation of all2874 could reduce the tendency to differentiate heterocysts equally at different light intensities, but the increased growth rate under high-light growth conditions would accentuate the heterocyst frequency phenotype because differentiation would lag behind the rate of vegetative cell doubling and result in longer vegetative cell intervals. However, this latter hypothesis is not consistent with the very long 200-vegetative-cell intervals produced by the all2874 mutant.

To determine the epistatic relationship between *hetR* and all2874, we overexpressed $hetR_{R223W}$, which fails to respond to inhibitory signals, from the copper-inducible *petE* promoter in the all2874 mutant strain and analyzed the heterocyst frequency in filaments grown at high or low light intensity. Under both light conditions, the $hetR_{R223W}$ phenotype was produced; heterocyst frequency was abnormally high after 24 h, and after several days of growth, the frequency did not decrease in the all2874 mutant background. On the contrary, the multiple contiguous heterocyst phenotype produced by the $hetR_{R223W}$ allele in the all2874 mutant was slightly more pronounced than in the wild-type background. Therefore, the $hetR_{R223W}$ allele is epistatic to the all2874 mutation, which suggests that All2874 functions upstream of HetR activity in the regulatory pathway controlling heterocyst development. It is also possible that All2874 acts independently of HetR and that the overexpression of the $hetR_{R223W}$ allele overshadows the all2874 mutant phenotype.

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