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## *patS* Minigenes Inhibit Heterocyst Development of *Anabaena* sp. Strain PCC 7120

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The *patS* gene encodes a small peptide that is required for normal heterocyst pattern formation in the cyanobacterium *Anabaena* sp. strain PCC 7120. PatS is proposed to control the heterocyst pattern by lateral inhibition. *patS* minigenes were constructed and expressed by different developmentally regulated promoters to gain further insight into PatS signaling. *patS* minigenes *patS4* to *patS8* encode PatS C-terminal 4 (GSGR) to 8 (CDERGSGR) oligopeptides. When expressed by  $P_{petE}$ ,  $P_{patS}$ , or  $P_{rbcL}$  promoters, *patS5* to *patS8* inhibited heterocyst formation but *patS4* did not. In contrast to the full-length *patS* gene,  $P_{hepA}$ -*patS5* failed to restore a wild-type pattern in a *patS* null mutant, indicating that PatS-5 cannot function in cell-to-cell signaling if it is expressed in proheterocysts. To establish the location of the PatS receptor, PatS-5 was confined within the cytoplasm as a *gfp-patS5* fusion. The green fluorescent protein GFP–PatS-5 fusion protein inhibited heterocyst formation. Similarly, full-length PatS with a C-terminal hexahistidine tag inhibited heterocyst formation. These data indicate that the PatS receptor is located in the cytoplasm, which is consistent with recently published data indicating that HetR is a PatS target. We speculated that overexpression of other *Anabaena* strain PCC 7120 RGSGR-encoding genes might show heterocyst inhibition activity. In addition to *patS* and *hetN*, open reading frame (ORF) *all3290* and an unannotated ORF, *orf77*, encode an RGSGR motif. Overexpression of *all3290* and *orf77* under the control of the *petE* promoter inhibited heterocyst formation, indicating that the RGSGR motif can inhibit heterocyst development in a variety of contexts.

The regulation of cellular differentiation and pattern formation are fundamental features of developmental biology that can be studied in heterocystous cyanobacteria. When combined nitrogen is depleted from the environment, 8 to 10% of vegetative cells in the filamentous cyanobacterium *Anabaena* (*Nostoc*) sp. strain PCC 7120 differentiate into nitrogen-fixing heterocysts, which are distributed in a semiregular pattern along filaments (12, 25, 34). It is clear that cell-to-cell communication must be involved in the control of heterocyst pattern formation (6, 11, 35, 37).

The PatS peptide is proposed to control heterocyst pattern formation by lateral inhibition, such that a diffusible PatS signal produced by differentiating cells inhibits nearby cells in the same filament from differentiating (36). In *Anabaena* strain PCC 7120, the *patS* gene contains two potential ATG start codons and could encode 13- or 17-amino-acid polypeptides (36). The closely related species *Anabaena variabilis* contains an identical *patS* open reading frame (ORF) at the nucleotide level. However, the *patS* ortholog in *Nostoc punctiforme* lacks the upstream start codon and contains only 13 codons (26). *patS* overexpression inhibits heterocyst formation in *Anabaena* strain PCC 7120, and a *patS* null mutant forms heterocysts in nitrate-containing medium and forms multiple contiguous heterocysts (Mch phenotype) after a nitrogen step-down (36). The last five carboxy-terminal amino acid residues are important

for the function of *patS*. Missense mutations affecting these residues fail to inhibit heterocyst formation, and a synthetic pentapeptide corresponding to these 5 amino acids (PatS-5, RGSGR) inhibits heterocyst development at submicromolar concentrations. A synthetic oligopeptide corresponding to the last four *patS*-encoded amino acids (PatS-4, GSGR) has much lower heterocyst inhibition activity (36).

After nitrogen step-down, wild-type filaments produce about 10% single heterocysts in a semiregular pattern, but a *patS* null mutant produces an Mch phenotype with about 30% heterocysts. From these data alone, PatS could either be directly responsible for producing a normal pattern by lateral inhibition or be required for a normal response to another, as yet unidentified, diffusible inhibitor produced by differentiating cells. This was tested by bathing filaments of a *patS* mutant with 60 nM PatS-5 pentapeptide, which reduced the number of heterocysts to about 10% but failed to restore a normal pattern (36). This experiment shows that exposure to uniform PatS concentrations cannot produce a normal pattern. However, expression of the *patS* gene from the heterocyst-specific *hepA* promoter complemented the *patS* null mutant and produced a nearly normal pattern (36). These data suggest that a gradient of PatS signal originating from differentiating cells is required to produce a normal pattern.

A *patS-gfp* transcriptional fusion showed that *patS* is expressed early during heterocyst development in differentiating cells (36, 37). The *patS-gfp* reporter strain showed that *patS* transcription was localized to individual cells or small groups of cells by 8 to 10 h after the nitrogen step-down. By 12 to 14 h, bright fluorescence was present in mostly single cells, with a pattern that resembled the wild-type heterocyst pattern. At 18 h after the nitrogen step-down, the green fluorescent pro-

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tein (GFP) fluorescence was almost exclusively from proheterocysts. The temporal and spatial pattern of *patS* expression strongly supports the lateral-inhibition model in which the *patS* product, possibly a processed C-terminal peptide, acts as an intercellular signal produced by differentiating heterocysts to inhibit the differentiation of neighboring cells. The differentiating PatS-producing cells must themselves be refractory to the PatS signal (36); however, the mechanism of this immunity to PatS inhibition is not yet known.

Diffusible signal molecules influence development in several prokaryotic organisms including *Bacillus subtilis*, *Myxococcus xanthus*, and *Streptomyces* spp. (17, 20). Receptor molecules for these signals may be present either outside the cell or within the cytoplasm. For example, ComX and PhrA are two well-characterized signal peptides regulating competence and sporulation in the gram-positive bacterium *B. subtilis* (13, 27, 28). The receptor for ComX is in the plasma membrane, while the receptor for PhrA is located inside the cytoplasm. ComX is a modified peptide pheromone used by *B. subtilis* to regulate the transcription of quorum-responsive genes. A 55-amino-acid ComX precursor is cleaved by ComQ to produce a 10-amino-acid peptide, which is modified at a tryptophan residue and exported as an active intercellular signal (1). The ComX receptor, ComP, is a membrane-bound histidine protein kinase (30). ComP and the ComA transcription factor form a two-component system that regulates competence development (13, 21).

The PhrA precursor is a 44-amino-acid polypeptide that is processed to produce the C-terminal pentapeptide ARNQT, which is the active signal (31). In target cells, PhrA pentapeptide is imported by the oligopeptide permease system (Opp) (29). The PhrA receptor, RapA, is a phosphatase located in the cytoplasm. PhrA pentapeptide inhibits the phosphatase activity of RapA on Spo0F-P, which is a component of the phosphorylation controlling the initiation of sporulation (16, 27, 28).

Lazazzera et al. expressed the active PhrA pentapeptide inside cells from a minigene to establish that the PhrA receptor was in the cytoplasm (22). Expression of the *phrA* minigene was able to rescue the sporulation defect of a *phrA* mutant (22, 30). They also used minigenes to study CSF, the competence- and sporulation-stimulating factor. Expression of a *phrC* minigene encoding the mature CSF pentapeptide showed that CSF targets are intracellular (22).

In this study, we used *patS* minigenes and gene fusions in *Anabaena* strain PCC 7120 to show that the PatS receptor is likely to be located in the cytoplasm and that, unlike the wild-type PatS signal, the *patS5* minigene product cannot function in cell-to-cell signaling. Our results are consistent with recent data indicating that HetR is likely to be the PatS receptor (15). HetR is a key activator of heterocyst development (5) that was previously shown to be positively autoregulated (2) and to have autoproteolytic activity (38). Zhao's laboratory has now shown that HetR forms a homodimer with DNA-binding activity and that this DNA-binding activity is inhibited in vitro by synthetic PatS pentapeptide (15).

#### MATERIALS AND METHODS

**Strains and culture conditions.** The strains and plasmids used in this study are described in Table 1. *Anabaena* strain PCC 7120 and its derivatives were grown in BG-11 or BG-11<sub>0</sub> (which lacks sodium nitrate) medium at 30°C as previously

described (10). For strains containing shuttle plasmids, cultures were supplemented with neomycin at 25 µg/ml for both liquid and solid media. The *patS* null mutant AMC451 (36) and its derivatives were grown in media supplemented with spectinomycin and streptomycin at 1 µg/ml each. For heterocyst inductions, filaments from actively growing cultures with an optical density at 750 nm of about 0.3 were collected by centrifugation and washed twice with water before being transferred to BG-11<sub>0</sub> as previously described (37).

*Escherichia coli* strains were maintained in Lennox L broth liquid or agar-solidified medium. For plasmid preparation, strains were grown in 0.5× TB liquid medium as described previously (10). *E. coli* strain DH10B was used for plasmid maintenance, and the media were supplemented with appropriate antibiotics as required.

**Copper-inducible expression from the *petE* promoter.** *patS* minigenes, *all3290*, and *orf77* were expressed from the copper-inducible *petE* promoter ( $P_{petE}$ ) derived from plasmid pPet1 (5). BG-11 medium, with CuSO<sub>4</sub> omitted, was used for copper-deficient growth conditions. As a precaution to prevent copper contamination, disposable plasticware was used instead of glassware for medium preparation and during the assay (5). All solutions were filter sterilized instead of being autoclaved. To induce the  $P_{petE}$  promoter, dissolved CuSO<sub>4</sub> was added to copper-deficient BG-11 medium at a final concentration of 0.4 µM CuSO<sub>4</sub>.

For heterocyst induction with or without copper, rapidly growing filaments from standard BG-11 were collected and washed twice with copper-deficient BG-11<sub>0</sub> medium. The filaments were then transferred into plastic culture tubes containing 3 ml of BG-11<sub>0</sub> with or without Cu<sup>2+</sup> or into 24-well tissue culture plates (Falcon) containing 2 ml of BG-11<sub>0</sub> with or without Cu<sup>2+</sup>. The cultures were incubated for 48 h under standard growth conditions. Filaments were scored for heterocyst frequency; detached heterocysts were not scored (37).

***patS* minigenes.** *patS* minigenes designated *patS4* to *patS8* encode PatS C-terminal oligopeptides from 4 (GSGR) to 8 (CDERGSGR) amino acids, respectively. *patS* minigenes were expressed from different promoters by being fused to the native start codon downstream of the promoter sequence (Fig. 1; Table 2). Each construct was generated by PCR with a forward primer complementary to the template containing the desired promoter. Each reverse primer consisted of two parts. The 5' portion encoded the minigenes and a stop codon, and the 3' portion (approximately 17 bases) was complementary to the promoter template such that the start codon was fused to the minigene sequences. The high-fidelity Pwo DNA polymerase (Roche) was used in the PCR to generate blunt-end products.

**Plasmid constructions.** DNA fragments (~500 bp) containing  $P_{petE}$ -*patS4* to  $P_{petE}$ -*patS8* minigenes (Fig. 1A) were inserted into pBluescript II SK(+) at the EcoRV site, and the BamHI-SmaI fragments from the resulting plasmids were inserted into conjugal shuttle vector pAM504 (32) at the same sites to produce plasmids pAM2525, pAM2424, pAM2526, pAM2529, and pAM2527, respectively. The BamHI-digested PCR product  $P_{patS5}$ -*patS5* (~750 bp) (Fig. 1B) was inserted into pAM504 between BamHI and SmaI sites, resulting in plasmid pAM2528. The blunt-ended PCR product  $P_{rbcL}$ -*patS5* (~400 bp) (Fig. 1C) was inserted into the pBluescript II SK(+) EcoRV site, resulting in pAM2506. The PvuII fragment containing the  $P_{rbcL}$ -*patS5* construct from pAM2506 was inserted into pAM504 at the SmaI site, resulting in plasmid pAM2537. The BamHI-digested PCR product  $P_{hepA}$ -*patS5* (~800 bp) (Fig. 1D) was inserted into pBluescript II SK(+) between BamHI and EcoRV sites, resulting in plasmid pAM2811. The SalI DNA fragment containing  $P_{hepA}$ -*patS5* from pAM2811 was inserted into the SalI sites of shuttle vectors pAM504 and pAM1956, resulting in plasmids pAM2814 ( $P_{hepA}$ -*patS5*) and pAM2816 ( $P_{hepA}$ -*patS5*-*gfp*), respectively. In pAM2816, *gfp* is downstream of *patS5* as a transcriptional fusion.

$P_{rbcL}$ -*gfp*-*patS5* was generated by PCR with primers AMO-367 and AMO-617 and pAM1954 as the template to produce a translational fusion between *gfp* and *patS5*. The 1.7-kb PCR fragment containing  $P_{rbcL}$ -*gfp*-*patS5* was inserted into pAM504 at the SmaI site, resulting in pAM2873.

A fusion of full-length PatS with a C-terminal His<sub>6</sub> tag was constructed in two cloning steps. First, PCR primers AMO-569 and AMO-583 were used to produce *patS* and its upstream sequences with flanking XhoI and SapI sites. The resulting XhoI-SapI fragment was inserted into pAM2600, resulting in pAM2821, such that *patS* is translationally fused to six histidine codons. Second, an XhoI-ClaI fragment carrying *patS*-6His from pAM2821 was inserted into shuttle vector pAM2770 (23), resulting in plasmid pAM2826.

A DNA fragment containing ORF *all3290* was produced by PCR with primers AMO-659 and AMO-660, digested with NdeI and XmaI, and then inserted into the same sites of pAM2770, resulting in pAM2918, such that *all3290* is expressed by the *petE* promoter.

A DNA fragment containing ORF *orf77* was produced by PCR with primers AMO-661 and AMO-662, digested with NdeI and XmaI, and then inserted into

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Derivation and/or relevant feature(s)	Source or reference
<i>Anabaena</i> strains		
PCC 7120	Wild type	R. Haselkorn
AMC451	$\Delta patS$	36
<i>E. coli</i> strains		
DH10B	F <sup>-</sup> <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80dlacZ\Delta M15$ $\Delta lacX74$ <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> $\Delta(ara, leu)7697$ <i>galU</i> <i>galK</i> $\lambda^-$ <i>rpsL</i> <i>nupG</i> Sm <sup>r</sup>	GIBCO BRL Life Technologies
AM1359	DH10B containing pRL623 and pRL443; conjugal donor strain	8, 36
Plasmids		
pAM504	pDU1-based shuttle vector; Km <sup>r</sup> Nm <sup>r</sup>	32
pAM505	pDU1-based shuttle vector; Km <sup>r</sup> Nm <sup>r</sup>	36
pAM1630	0.9-kb EcoRV-SalI fragment containing P <sub>hepA</sub> from pRL1905 cloned in pBluescript II KS(+); Ap <sup>r</sup>	This study
pAM1690	P <sub>rbcL</sub> - <i>patS</i> cloned in pAM504	23
pAM1715	P <sub>hepA</sub> - <i>patS</i> cloned in pAM504	36
pAM1951	P <sub>patS</sub> - <i>gfp</i> cloned in pAM505	36
pAM1954	P <sub>rbcL</sub> - <i>gfp</i> cloned in pAM504	36
pAM1956	Promoterless <i>gfp</i> in pAM505	36
pAM2269	<i>petE</i> promoter in pAM504	This study
pAM2424	P <sub>petE</sub> - <i>patS5</i> cloned in pAM504	This study
pAM2506	P <sub>rbcL</sub> - <i>patS5</i> cloned in pBluescript II SK(+)	This study
pAM2525	P <sub>petE</sub> - <i>patS4</i> cloned in pAM504	This study
pAM2526	P <sub>petE</sub> - <i>patS6</i> cloned in pAM504	This study
pAM2527	P <sub>petE</sub> - <i>patS8</i> cloned in pAM504	This study
pAM2528	P <sub>patS</sub> - <i>patS5</i> cloned in pAM504	This study
pAM2529	P <sub>petE</sub> - <i>patS7</i> cloned in pAM504	This study
pAM2532	P <sub>patS</sub> - <i>patS5</i> cloned in pBluescript II SK(+)	This study
pAM2537	P <sub>rbcL</sub> - <i>patS5</i> from pAM2506 cloned in pAM504	This study
pAM2600	SapI-6His-ClaI sequence cloned in pUC18 downstream of <i>lacZ</i>	23
pAM2770	Shuttle vector containing P <sub>petE</sub> ; Km <sup>r</sup> Nm <sup>r</sup>	23
pAM2811	P <sub>hepA</sub> - <i>patS5</i> cloned in pBluescript II SK(+)	This study
pAM2814	0.8-kb SalI fragment containing P <sub>hepA</sub> - <i>patS5</i> from pAM2811 cloned in pAM504	This study
pAM2816	P <sub>hepA</sub> - <i>patS5</i> from pAM2811 cloned in pAM1956 to form P <sub>hepA</sub> - <i>patS5-gfp</i> transcriptional fusion	This study
pAM2821	668-bp XhoI-SapI PCR fragment containing <i>patS</i> cloned in pAM2600 to form <i>patS</i> -6His	This study
pAM2826	688-bp XhoI-ClaI fragment carrying <i>patS</i> -6His from pAM2821 cloned in shuttle vector pAM2770	This study
pAM2873	1.7-kb PCR fragment containing P <sub>rbcL</sub> - <i>gfp-patS5</i> translational fusion cloned in pAM504	This study
pAM2898	P <sub>petE</sub> - <i>orf77</i> cloned in shuttle vector pAM2770	This study
pAM2918	P <sub>petE</sub> - <i>all3290</i> cloned in shuttle vector pAM2770	This study
pRL1905	<i>hepA</i> upstream region containing P <sub>hepA</sub> in pRL487	39

the same sites of pAM2770, resulting in pAM2898, such that *orf77* is driven by the *petE* promoter.

For all constructs, the ribosome-binding site and start codon were derived from the promoters used. In all constructs, the inserts were downstream of a transcription terminator on shuttle vectors pAM504, pAM505, and pAM2770 (23, 32, 36). All plasmid constructs were confirmed by sequencing.

**Plasmid conjugations.** Shuttle plasmids were transferred into *E. coli* conjugal donor strain AM1359 (36) by electroporation and then transferred into *Anabaena* strain PCC 7120 by conjugation, using standard methods (8).

**Heterocyst inhibition bioassay.** A bioassay, similar to that used in previous work (36), was used in an attempt to detect the presence of heterocyst inhibition activity in the culture supernatant of a strain overexpressing the *patS5* minigenes. A strain carrying P<sub>rbcL</sub>-*patS5* on pAM2537 was grown in BG-11<sub>0</sub> medium to an optical density of 0.5 at 750 nm, and then the culture supernatant was collected after centrifugation at 2,000 × g for 7 min at room temperature. The culture supernatant was filtered through a sterile 0.45- $\mu$ m-pore-size filter. The assay was performed with 100  $\mu$ l of medium in a 96-well flat-bottom microtiter plate (Thermo LabSystems). Wild-type *Anabaena* strain PCC 7120 was used as the test strain for the heterocyst inhibition assay. Vegetative-cell filaments were grown in BG-11 medium to an optical density of about 0.3 at 750 nm, harvested by centrifugation, and washed twice with BG-11<sub>0</sub> medium. Heterocyst development

was induced by resuspending the filaments in either 100  $\mu$ l of BG-11<sub>0</sub> medium, 100  $\mu$ l of conditioned culture supernatant, or a 0.5× dilution containing 50  $\mu$ l of conditioned culture supernatant and 50  $\mu$ l of BG-11<sub>0</sub> medium. As a positive control for inhibition, synthetic PatS-5 peptide at 0.1, 0.5, and 1  $\mu$ M was added to 100  $\mu$ l of both BG-11<sub>0</sub> medium and conditioned culture supernatant. The assay plates were incubated under standard conditions for 24 to 48 h, and then samples were scored microscopically for the frequency of heterocysts as described previously (36, 37).

**Microscopy and scoring of the heterocyst pattern.** Microscopy, image processing, and scoring of the heterocyst pattern were performed as described previously (36, 37). Three or more samples were scored for each experimental condition. Images of GFP fluorescence with excitation wavelengths of 450 to 490 nm and emission wavelengths of 505 to 531 nm were recorded with a Hamamatsu color charge-coupled device camera.

## RESULTS

**Suppression of heterocyst development by *patS* minigenes driven by a copper-inducible promoter.** The *patS* gene product is thought to function as an intercellular signal produced by



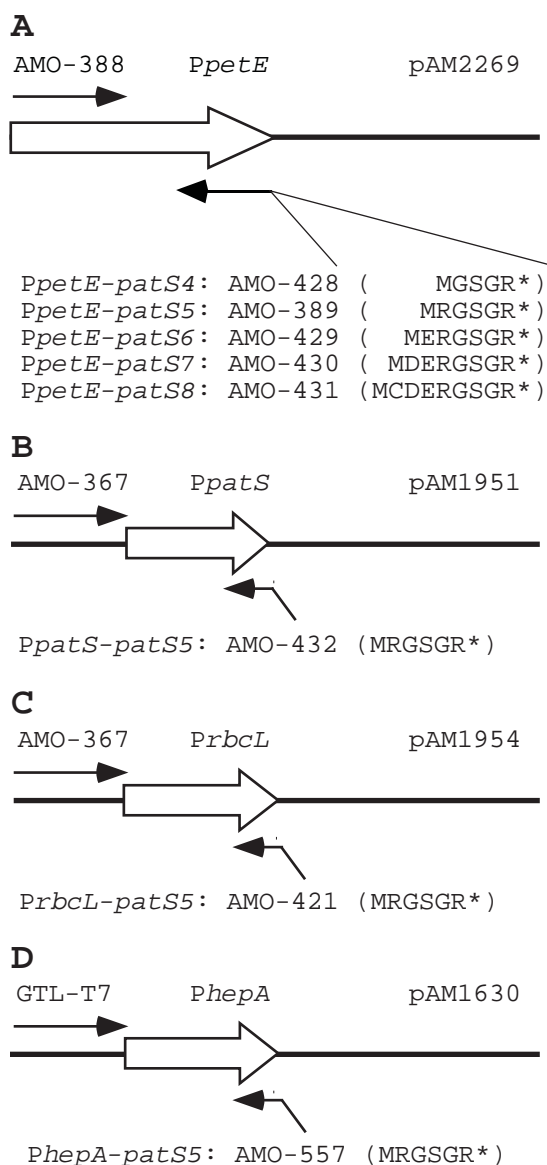


FIG. 1. Diagram of *patS* minigene constructs. Each PCR product results in a promoter upstream region (open arrows) fused at the start codon to a *patS* minigene sequence, ending with a UAG stop codon encoded by the 5' end of the reverse primer. The 3' ends of the reverse primers are complementary to the promoter template. (A) Construction of  $P_{petE}$ -*patS4* to  $P_{petE}$ -*patS8*. pAM2269 was used as the PCR template for  $P_{petE}$ . The forward primer was AMO-388 for all five constructions. The PatS peptides encoded by the reverse primers are shown. (B) To generate  $P_{patS}$ -*patS5*, pAM1951 was used as the PCR template for  $P_{patS}$  and the *patS5* sequence was encoded on the reverse primer AMO-432. AMO-367 was the forward primer. (C) To generate  $P_{rbcL}$ -*patS5*, pAM1954 was used as the PCR template for  $P_{rbcL}$  and the *patS5* sequence was encoded on the reverse primer AMO-421. AMO-367 was the forward primer. (D) To generate  $P_{hepA}$ -*patS5*, pAM1630 was used as the PCR template for  $P_{hepA}$  and the *patS5* sequence was encoded on the reverse primer AMO-557. GTL-T7 was the forward primer. The closed arrows indicate PCR primers. \*, stop codon. Diagrams are not to scale.

differentiating cells and possibly mature heterocysts to inhibit heterocyst formation of neighboring cells by lateral inhibition. Previous work showed that a full-length *patS* gene, driven by the copper-inducible promoter  $P_{petE}$ , suppressed heterocyst

TABLE 2. PCR primer sequences used in this study

Primer	Sequence <sup>a</sup>
GTL-T7.....	aatacagactcactatag
AMO-367.....	accgcggaactgcgcgcta
AMO-369.....	cgctctgctgaagccag
AMO-388.....	gatccccgggtaccgagctcga
AMO-389.....	CTATCTACCACTACCGCGCATggcgtttccta
AMO-421.....	CTATCTACCACTACCGCGCATatgtatatct
AMO-428.....	CTATCTACCACTACCCATggcgtttccta
AMO-429.....	CTATCTACCACTACCGCGCTCCATggcgtttccta
AMO-430.....	CTATCTACCACTACCGCGCTCATCCATggcgtttccta
AMO-431.....	CTATCTACCACTACCGCGCTCATCACACATggcgtttccta
AMO-432.....	CTATCTACCACTACCGCGCATaatcttaaaatcggtg
AMO-557.....	CTATCTACCACTACCGCGCATgtcaaccagcta
AMO-569.....	aggacctcgaggaaaagtagggttaagtaaacggcg
AMO-583.....	gtctgctcttcgcatcaccactaccgctc
AMO-617.....	CTATCTACCACTACCGCGTttgtatagttc
AMO-659.....	aaccaacatattggtgattcgtcttgaatc
AMO-660.....	aaccaaccgggtcatgcttgactacctc
AMO-661.....	aaccaacatattggtgaccattatcaaacacag
AMO-662.....	aaccaaccgggttaccaagaatcagttcttaagc

<sup>a</sup> Capital letters indicate *patS* minigene sequences.

development (36). The  $P_{petE}$  promoter is expressed in vegetative cells (5) and is down-regulated in heterocysts (7). We found that a  $P_{petE}$ -*gfp* transcriptional reporter showed green fluorescence in both vegetative cells and heterocysts (data not shown), suggesting that the *petE* promoter is at least partially active in heterocysts. In an effort to determine the localization of the PatS receptor and the characteristics of its interaction with different PatS C-terminal peptides, we expressed *patS* minigenes from the  $P_{petE}$  promoter and measured the heterocyst inhibition activity of the gene products produced in the cytoplasm of vegetative cells. The *patS* minigenes contain an ATG translational start codon followed by sequences encoding PatS C-terminal oligopeptides from 4 (GSGR) to 8 (CDERGSGR) amino acids.

Plasmids pAM2525, pAM2424, pAM2526, pAM2529, and pAM2527 (Table 1), containing the  $P_{petE}$ -*patS4* to  $P_{petE}$ -*patS8* minigenes (Fig. 1A), respectively, were transferred into wild-type *Anabaena* strain PCC 7120 and the *patS* null mutant strain AMC451 by conjugation. The resulting strains were induced for heterocyst formation in copper-replete and copper-deficient conditions. Under copper-replete conditions, wild-type *Anabaena* strain PCC 7120 containing  $P_{petE}$ -*patS5*,  $P_{petE}$ -*patS6*, and  $P_{petE}$ -*patS8* formed less than 1% heterocysts (Table 3) and a strain containing  $P_{petE}$ -*patS7* formed 4% heterocysts. However,  $P_{petE}$ -*patS4* failed to inhibit heterocyst formation or alter the heterocyst pattern. Heterocyst formation in the *patS* null mutant AMC451 was partially inhibited by minigenes  $P_{petE}$ -*patS5*,  $P_{petE}$ -*patS6*,  $P_{petE}$ -*patS7*, and  $P_{petE}$ -*patS8*, with  $P_{petE}$ -*patS5* showing the strongest effect.  $P_{petE}$ -*patS4* had no effect on heterocyst development in AMC451. Even under copper-deficient conditions,  $P_{petE}$ -*patS5* to  $P_{petE}$ -*patS8* were able to partially inhibit heterocyst formation, indicating that some expression from the *petE* promoter was occurring under our experimental conditions (Table 3). In an attempt to further deplete copper in the cells, we grew filaments in copper-free BG-11 medium for several days before induction, but we ob-

TABLE 3. Suppression of heterocyst formation by  $P_{petE}$ - $patS$  minigenes in wild-type *Anabaena* strain PCC 7120 and the  $patS$  mutant AMC451

<i>patS</i> minigene (plasmid)	Heterocyst frequency <sup>a</sup> (%)			
	Wild type		AMC451	
	-Cu <sup>2+</sup>	+Cu <sup>2+</sup>	-Cu <sup>2+</sup>	+Cu <sup>2+</sup>
None (pAM504)	10.9 ± 0.5	10.6 ± 0.5	18.1 ± 0.2	17.2 ± 0.9
$P_{petE}$ - $patS4$ (pAM2525)	11 ± 0.2	10.6 ± 0.5	16.4 ± 1.7	16.9 ± 0.7
$P_{petE}$ - $patS5$ (pAM2424)	4.3 ± 4.9	0.3 ± 0.6	8.3 ± 3.5	0.5 ± 0.5
$P_{petE}$ - $patS6$ (pAM2526)	1.8 ± 1.3	0 ± 0	12 ± 3.6	6.7 ± 4.7
$P_{petE}$ - $patS7$ (pAM2529)	8.3 ± 2.9	4.0 ± 5.2	12 ± 2.5	8.2 ± 4.1
$P_{petE}$ - $patS8$ (pAM2527)	4.0 ± 5.2	0.2 ± 0.3	12 ± 2.5	2.2 ± 1.8

<sup>a</sup> Frequencies measured 48 h after the nitrogen step-down. Results are presented as mean ± standard deviation.

tained similar results (data not shown). Although the minigene results fail to clearly identify the most likely candidate for the genuine PatS signal, it is interesting that  $patS5$  produced the strongest heterocyst inhibition activity. Because the minigene peptide products all contain at least one polar and one charged amino acid and should be confined to the cytoplasm, these data support a cytoplasmic location for the PatS receptor.

**Expression of the  $patS5$  minigene in proheterocysts failed to inhibit multiple contiguous heterocysts in a  $patS$  null mutant.** To test the hypothesis that PatS-5 is confined to the cytoplasm, we determined if the minigene product could function as a cell-to-cell signal. The  $patS5$  minigene was expressed from a proheterocyst-specific promoter in the  $patS$  null mutant AMC451 to determine if it could suppress the multiple-contiguous-heterocyst (Mch) phenotype. Failure to suppress the Mch phenotype would indicate that a cell-to-cell signal was not being produced. The  $patS5$  minigene was used in these experiments because it produced the strongest heterocyst inhibition (Table 3).

Expression of full-length  $patS$  and the  $patS5$  minigene from the  $rbcL$  promoter in constructs  $P_{rbcL}$ - $patS17$  (pAM1690) (23) and  $P_{rbcL}$ - $patS5$  (pAM2537) completely inhibited heterocyst formation in both the wild-type strain and the  $patS$  null mutant AMC451 (Table 4).  $P_{rbcL}$  is strongly expressed in vegetative cells (7, 9). In BG-11<sub>0</sub> liquid medium, cultures of both strains turned yellow within a few days and no heterocyst formation

TABLE 4. Suppression of heterocyst formation by  $patS5$  and  $patS17$  in wild-type *Anabaena* strain PCC 7120 and the  $patS$  mutant AMC451

<i>patS</i> minigene (plasmid)	Heterocyst frequency <sup>a</sup> (%)		% of Mch in AMC451 <sup>b</sup>
	Wild type	AMC451	
None (pAM504)	12.2 ± 2.0	19.9 ± 2.1	11.5
$P_{rbcL}$ - $patS5$ (pAM2537)	0.0 ± 0.0	0.0 ± 0.0	0
$P_{rbcL}$ - $patS17$ (pAM1690)	0.0 ± 0.0	0.0 ± 0.0	0
$P_{patS}$ - $patS5$ (pAM2528)	6.4 ± 4.4	4.0 ± 3.5	ND <sup>c</sup>
$P_{hepA}$ - $patS5$ (pAM2814)	12.1 ± 1.9	18.8 ± 2.1	11.2
$P_{hepA}$ - $patS17$ (pAM1715)	0.0 ± 0.0	9.8 ± 0.2	0
$P_{hepA}$ - $patS5$ - $gfp$ (pAM2816)	0.7 ± 1.0	18.8 ± 1.9	12.3

<sup>a</sup> Frequencies measured 24 h after the nitrogen step-down. Results are presented as mean ± standard deviation.

<sup>b</sup> The percentage of heterocysts that are multiple constitutive heterocysts (Mch).

<sup>c</sup> ND, not determined.

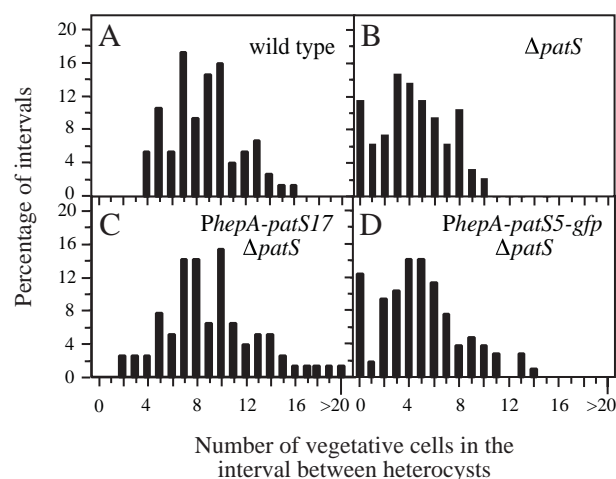


FIG. 2. PatS-5 does not function as a cell-to-cell signal. Plasmids containing  $patS17$  and  $patS5$  expressed from the proheterocyst-specific promoter  $P_{hepA}$  were used to complement the  $patS$  deletion strain AMC451. The heterocyst pattern was determined 24 h after nitrogen step-down for the wild type (A), strain AMC451 (B), AMC451 containing pAM1715 ( $P_{hepA}$ - $patS17$ ) (C), and AMC451 containing pAM2816 ( $P_{hepA}$ - $patS5$ - $gfp$ ) (D).

was observed. These results show that PatS-5 pentapeptide can completely block heterocyst differentiation when it is produced in cells before they have become immune to PatS inhibition.  $P_{patS}$ - $patS5$  (pAM2528) only partially inhibited heterocyst formation in both strains (Table 4), reflecting the weak expression of the  $patS$  promoter in vegetative cells (36, 37).

$P_{hepA}$  is expressed in developing proheterocysts and has much lower expression in vegetative cells (14, 33, 39). Consistent with previous results (36),  $P_{hepA}$ - $patS17$  (pAM1715), which contains the full-length  $patS$  gene, complemented the  $patS$  deletion strain AMC451, suppressed the Mch phenotype, and restored a nearly wild-type heterocyst pattern (Fig. 2; Table 4). This shows that the  $hepA$  promoter is apparently turned on strongly only after differentiating cells have become immune to self-inhibition and shows that the wild-type PatS product can function in cell-to-cell signaling to prevent the formation of multiple contiguous heterocysts.

Expression of the  $patS5$  minigene in differentiating cells from  $P_{hepA}$ - $patS5$  (pAM2814) did not suppress the Mch phenotype of AMC451 (Table 4). This result is consistent with the pentapeptide failing to function in cell-to-cell signaling when produced in proheterocysts. However, this construct may be producing less PatS heterocyst inhibition activity because it did not suppress heterocysts in the wild type. In an effort to monitor the expression of  $P_{hepA}$ - $patS5$ , a promoterless  $gfp$  reporter gene was placed downstream of the  $patS5$  minigene such that the  $gfp$  gene contained its own start codon and ribosome-binding site. The  $P_{hepA}$ - $patS5$ - $gfp$  construct (pAM2816) produced dim GFP fluorescence in differentiating cells, as expected, and had stronger heterocyst inhibition activity in the wild-type background, which reduced the heterocyst frequency to less than 1% (Table 4). The mechanism causing the stronger heterocyst inhibition activity is unknown, but it might be due to increased stability of the mRNA. In the presence of a wild-type copy of  $patS$ , both  $P_{hepA}$ - $patS17$  and  $P_{hepA}$ - $patS5$ - $gfp$  produced

enough PatS heterocyst inhibition activity to overcome the immunity to self-inhibition and blocked heterocyst differentiation.

However, unlike the wild-type PatS produced from  $P_{hepA}$ -*patS17*, the PatS-5 pentapeptide produced from  $P_{hepA}$ -*patS5-gfp* failed to complement the *patS* deletion strain. AMC451 carrying  $P_{hepA}$ -*patS5-gfp* had short vegetative-cell intervals and formed multiple contiguous heterocysts (Fig. 2; Table 4). These data show that the pentapeptide produced by the *patS5* minigene expressed in proheterocysts cannot function in cell-to-cell signaling and therefore is apparently confined to the cytoplasm.

**PatS-5 activity is not detectable in conditioned culture medium.** To determine if the *patS5* minigene product was either leaking or being transported out of filaments at concentrations sufficient to inhibit heterocyst formation, we tested conditioned culture supernatant from a strain containing  $P_{rbcL}$ -*patS5* (pAM2537) for heterocyst inhibition activity. The supernatant from a culture of the strain grown in BG-11<sub>0</sub> medium failed to inhibit heterocyst formation. In a reconstruction experiment to verify that our assay could detect PatS-5 pentapeptide in conditioned medium, we added synthetic PatS-5 to conditioned culture supernatant and BG-11<sub>0</sub> medium at 0.1, 0.5, and 1  $\mu$ M. Heterocyst formation was inhibited at each peptide concentration in both media. These results indicate that the *patS5* minigene product is not exported or does not leak from filaments at concentrations capable of inhibiting heterocysts and are consistent with the PatS signal interacting with a receptor in the cytoplasm.

**RGSGR fusion proteins inhibit heterocyst differentiation.** To further characterize the interaction between the PatS signaling molecule and the PatS receptor, we tested PatS fusion proteins for their ability to inhibit heterocyst formation. A *gfp-patS5* translational fusion was constructed and expressed from the  $P_{rbcL}$  promoter. The fusion of GFP to the N terminus of PatS-5 should confine the RGSGR motif to the cytoplasm.  $P_{rbcL}$ -*gfp-patS5* on pAM2873 completely inhibited heterocyst formation after the nitrogen step-down and, as expected, produced strong GFP fluorescence in the cytoplasm of vegetative cells (data not shown).

A six-histidine fusion at the C terminus of PatS produced similar results. A translational fusion was constructed in pAM2826 in which six histidine codons were added to the 3' end of the full-length *patS* ORF. When expressed from the native *patS* promoter, *patS*-6His completely suppressed heterocyst development. These results show that neither the N-terminal GFP fusion nor the C-terminal His fusion interferes with the ability of the RGSGR motif to interact with its receptor and inhibit heterocyst differentiation. These results also are consistent with the hypothesis that the PatS receptor is located inside the cytoplasm of vegetative cells.

**Proteins containing an RGSGR motif inhibit heterocyst differentiation.** We searched the *Anabaena* strain PCC 7120 genome for ORFs other than *patS* containing an RGSGR motif to determine if overexpression of any of these ORFs was able to inhibit heterocyst formation. The GeneMark program (4), set to the lowest stringency, was used to identify all possible ORFs in the *Anabaena* strain PCC 7120 genome (18). These ORF sequences were then searched with a text editor for those containing the RGSGR motif.

Four ORFs containing RGSGR were identified. One was *patS* itself. Another was *hetN*, which is known to inhibit heterocyst development when overexpressed (3, 6). However, HetN must have an independent heterocyst inhibition activity, because site-directed mutation of the RGSGR motif in HetN does not alter its heterocyst inhibition activity (24). The third gene encoding a RGSGR motif was *all3290*, which encodes an ortholog of an *N. punctiforme* protein annotated as the ATPase component of an ABC-type sugar transport system. The fourth RGSGR-encoding ORF was not documented in the annotated *Anabaena* strain PCC 7120 genome database (18). This ORF has only 77 codons and is located on the opposite strand from the much larger ORF *cyaC* (*all4963*), which encodes an adenylate cyclase (19). CyaC is a complex protein with motifs similar to those of adenylate cyclases, response regulators, and sensor kinases, and a *cyaC* mutant had much lower levels of cyclic AMP than the wild type did. Thus, it seemed unlikely that this short RGSGR-encoding ORF (termed *orf77*) is actually expressed; however, we tested both *orf77* and *all3290* for heterocyst-inhibition activity.

ORFs *all3290* and *orf77* were cloned into the expression vector pAM2770 downstream of the *petE* promoter, resulting in pAM2918 and pAM2898, respectively. On BG-11<sub>0</sub> plates, strains containing these constructs were completely suppressed for heterocyst development. Although we assume that the RGSGR motif is responsible for the inhibition, we cannot exclude the possibility that these proteins inhibit heterocyst differentiation by an unknown mechanism. Note that *all3290* and *orf77* in the wild-type chromosome normally must not inhibit heterocyst formation, or they would mask the phenotype of *patS* mutants. From these experiments and those with the *patS* fusion constructs, we conclude that the PatS receptor is located in the cytoplasm and can interact with the RGSGR motif embedded in different contexts. These conclusions are consistent with the recent data indicating that the cytoplasmic HetR protein may be the PatS receptor (15).

## DISCUSSION

*patS* minigenes  $P_{petE}$ -*patS5* to  $P_{petE}$ -*patS8*, but not  $P_{petE}$ -*patS4*, on shuttle plasmids were able to inhibit heterocyst formation. However, unlike the full-length *patS* gene, the *patS5* minigene expressed from the heterocyst-specific promoter  $P_{hepA}$  did not complement the pattern formation defect of a *patS* mutant. This result indicates that the PatS-5 pentapeptide produced within differentiating cells by the minigene cannot function in cell-to-cell signaling. Overall, overexpression of a variety of genes encoding a RGSGR motif suppressed heterocyst development, including *patS* itself, *all3290*, and *orf77* and *patS* fusion protein genes *gfp-patS5* and *patS*-6His. Taken together, these results strongly support the hypothesis that the PatS receptor is in the cytoplasm of cells and that the RGSGR motif is capable of inhibiting heterocyst differentiation even when embedded in different contexts.

$P_{petE}$ -*patS4* did not suppress heterocyst development in either the wild type or the *patS* null mutant AMC451. Previous work showed that the synthetic oligopeptide PatS-4 (GSGR) had much less heterocyst inhibition activity than did PatS-5 when added to the growth medium (36). These results show that PatS-4 cannot function as the PatS signal.



When expressed from the copper-inducible *petE* promoter, *patS* minigenes from *patS5* to *patS8* all inhibited heterocyst development. The *patS5* minigene appeared to produce the strongest heterocyst inhibition, followed by *patS6* and *patS8*; *patS7* showed the weakest inhibition activity in both the wild type and the *patS* deletion strain AMC451. The basis for the differences in biological activities among the *patS* minigenes is unknown. Both full-length *patS* and the *patS5* minigene strongly suppressed heterocyst development when expressed in vegetative cells under the control of  $P_{rbcL}$ . The results of these minigene experiments are consistent with the possibility that the RGSGR pentapeptide may be the active signal molecule controlling the heterocyst pattern. Our attempts to detect the actual PatS signal molecule(s) by matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry in wild-type and *patS* overexpression strains compared to the *patS* deletion mutant AMC451 have been unsuccessful thus far.

The *patS5* minigene product cannot function in cell-to-cell signaling. Unlike full-length *patS*, expression of the *patS5* minigene from the proheterocyst-specific  $P_{hepA}$  promoter in pAM2816 failed to complement the *patS* deletion mutant AMC451 to produce a normal heterocyst pattern. pAM2816, which carries  $P_{hepA}$ -*patS5* and a downstream *gfp* ORF, suppressed heterocysts in the wild-type background, which indicates that this construct produces levels of PatS-5 pentapeptide sufficient to inhibit heterocysts. Since synthetic PatS-5 pentapeptide can inhibit heterocysts when added exogenously to filaments, the PatS-5 produced in proheterocysts from a minigene must not be able to get out of the differentiating cells to inhibit the neighboring cells. Because the full-length *patS* gene functions cell nonautonomously and the *patS5* minigene does not, the C-terminal PatS-5 pentapeptide apparently lacks sequences required for cell-to-cell signaling.

The ability of ORF *all3290* overexpression to inhibit heterocyst development might be due to only the overexpression of the RGSGR motif, or *all3290* might be normally involved in the regulation of heterocyst development. However, an *all3290* knockout mutant grew normally and showed normal heterocyst development and pattern formation (data not shown), indicating that it is not normally involved in heterocyst development. Although RNA blot analysis showed that *all3290* was expressed in filaments grown in nitrate-containing medium, transcripts were undetectable after nitrogen step-down for 6, 12, and 18 h (data not shown), when its expression might otherwise have inhibited heterocyst differentiation. We conclude that the RGSGR motif in *all3290* does not normally regulate heterocyst development but inhibits differentiation only when it is overexpressed.

Because overexpression of a wide variety of RGSGR-encoding genes suppressed heterocyst development, including *patS*, *all3290*, *orf77*, *gfp-patS5*, *patS-6His*, and the *patS* minigenes  $P_{petE}$ -*patS5* to  $P_{petE}$ -*patS8*, the PatS receptor must be able to interact with the RGSGR motif flanked by a variety of amino acid sequences. This suggests that the PatS binding site on the receptor is relatively open and accessible to the various RGSGR-containing polypeptides. It is possible that some of the PatS fusion proteins are degraded such that various smaller peptides containing the RGSGR motif are produced. However, these putative peptides must still interact with the receptor to inhibit the differentiation of heterocysts. Because all of

the RGSGR-fusion constructs and the ORFs containing an RGSGR motif strongly inhibit heterocyst differentiation and should be confined to the cytoplasm, the PatS receptor must be located in the cytoplasm of vegetative cells, which is consistent with PatS directly inhibiting HetR function. Together, the PatS inhibitor and the HetR activator could satisfy the primary requirements for regulation of the heterocyst pattern by lateral inhibition, as recently proposed by Huang et al. (15). However, other, more complex models of the regulation are possible, and the true mechanism cannot be fully understood until the actual molecule(s) that carries the inhibitory signal between cells has been identified.

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