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 PpatS, PpatSs, or PrbcL, promoters, patSS to patS8 inhibited heterocyst formation but patS4 did not. In contrast to the full-length patS gene, PhepA-patSS failed to restore a wild-type pattern in a patS null mutant, indicating that PatS 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 (Nostoc) 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 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 ARNQI, 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 phosphate activity of RapA on SpoOF-P, which is a component of the phosphorelay controlling the initiation of sporulation (16, 27, 28).

Lazzazera et al. expressed the active PhrA pentapeptide inside cells from a minigenie 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-110 (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 induction, filaments were cultured actively with 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-11G, 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, allL290, and orf77 were expressed from the copper-inducible petE promoter (PetE) derived from plasmid pPet1 (5). BG-11 medium, with CuSO4 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 PetE promoter, dissolved CuSO4 was added to copper-deficient BG-11 medium at a final concentration of 0.4 μM CuSO4.

For heterocyst induction with or without copper, rapidly growing filaments from standard BG-11 were collected and washed twice with copper-deficient BG-11G medium. The filaments were then transferred into plastic culture tubes containing 3 ml of BG-11G with or without CuSO4 or into 24-well tissue culture plates (Falcon) containing 2 ml of BG-11G with or without CuSO4. 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. patS5 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 PpetE-patS4 to PpatE-patS8 minigenes (Fig. 1A) were inserted into pBlueScript II SK(+) at the EcoRV site, and the BamHI-Smal fragments from the resulting plasmids were inserted into conjugal shuttle vector pAM504 (32) at the same sites to produce plasmids pAM2525, pAM2524, pAM2526, pAM2529, and pAM2527, respectively. The BamHI-digested PCR product PpetE-patS5 (−750 bp) (Fig. 1B) was inserted into pAM504 between BamHI and Smal sites, resulting in plasmid pAM2528. The blunt-ended PCR product PpetE-patS5 (−400 bp) (Fig. 1C) was inserted into the pBlueScript II SK(+) EcoRV site, resulting in pAM2506. The PuvI1 fragment containing the PpetE-patS5 construct from pAM2506 was inserted into pAM504 at the SmaI site, resulting in plasmid pAM2537. The BamHI-digested PCR product PpetE-patS5 (−800 bp) (Fig. 1D) was inserted into pBlueScript II SK(+) between BamHI and EcoRV sites, resulting in plasmid pAM2811. The Sall DNA fragment containing PpetE-patS5 from pAM2811 was inserted into the Sall sites of shuttle vectors pAM504 and pAM1956, resulting in plasmids pAM2814 (PpetE-patS4) and pAM2816 (PpetE-patS5-gfp), respectively. In pAM2816, gfp is downstream of patS5 as a transcriptional fusion. PpetE-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 PpetE-gfp-patS5 was inserted into pAM504 at the SmaI site, resulting in pAM2873.

A fusion of full-length PatS with a C-terminal His6 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 Xhol and Sapl sites. The resulting XhoI-SapI fragment was inserted into pAM2600, resulting in pAM2621, such that patS is translationally fused to six histidine codons. Second, an Xhol-ClaI fragment carrying patS-His6 from pAM2821 was inserted into shuttle vector pAM2770 (23), resulting in plasmid pAM2826.

A DNA fragment containing ORF allL290 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 allL290 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
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 patS minigene. A strain carrying P

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**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...
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 PpetE, suppressed heterocyst development (36). The PpetE promoter is expressed in vegetative cells (5) and is down-regulated in heterocysts (7). We found that a PpetE-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 PpetE 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 PpetE-patS4 to PpetE-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 PpetE-patS4 to PpetE-patS8 minigenes (Fig. 1A), respectively, were transferred into wild-type Anabaena strain PCC 7120 containing PpetE-patS5, PpetE-patS6, and PpetE-patS8 formed less than 1% heterocysts (Table 3) and a strain containing PpetE-patS7 formed 4% heterocysts. However, PpetE-patS4 failed to inhibit heterocyst formation or alter the heterocyst pattern. Heterocyst formation in the patS null mutant AMC451 was partially inhibited by minigenes PpetE-patS5, PpetE-patS6, PpetE-patS7, and PpetE-patS8, with PpetE-patS5 showing the strongest effect. PpetE-patS4 had no effect on heterocyst development in AMC451. Even under copper-deficient conditions, PpetE-patS5 to PpetE-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-

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**TABLE 2. PCR primer sequences used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
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</thead>
<tbody>
<tr>
<td>GTL-T7</td>
<td>aatacgactcactatag</td>
</tr>
<tr>
<td>AMO-367</td>
<td>accggtacctagctgctgt</td>
</tr>
<tr>
<td>AMO-398</td>
<td>gatccctggaaagagctcga</td>
</tr>
<tr>
<td>AMO-389</td>
<td>ATG translational start codon followed by sequences encoding</td>
</tr>
<tr>
<td>AMO-421</td>
<td>MRGSGR*</td>
</tr>
<tr>
<td>AMO-432</td>
<td>CTATCTACCACTACCGCGCATaatcttaaaatcggtg</td>
</tr>
<tr>
<td>AMO-431</td>
<td>CTATCTACCACTACCGCGCTCATCACACATggc</td>
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<tr>
<td>AMO-430</td>
<td>CTATCTACCACTACCGCGCTCATCCATggcgttct</td>
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<tr>
<td>AMO-429</td>
<td>CTATCTACCACTACCGCGCTCCATggcgttctccta</td>
</tr>
<tr>
<td>AMO-428</td>
<td>CTATCTACCACTACCGCGCATatgtatatct</td>
</tr>
<tr>
<td>AMO-557</td>
<td>MRGSGR*</td>
</tr>
</tbody>
</table>

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* Capital letters indicate patS minigene sequences.
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 a cell-to-cell signal. The patS5 minigene was expressed from a proheterocyst-specific promoter in the 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_{o} liquid medium, cultures of both strains turned yellow within a few days and no heterocyst formation was observed. These results show that PatS-5 pentapeptide can completely block heterocyst differentiation when it is produced in cells before they become immune to PatS inhibition. P_{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).

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

| TABLE 3. Suppression of heterocyst formation by P_{petE-patS} minigenes in wild-type Anabaena strain PCC 7120 and the patS mutant AMC451. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| patS minigene (plasmid) | Heterocyst frequency (%) | | | |
| patS (plasmid) | Wild type | AMC451 | | |
| None (pAM504) | 10.9±0.5 | 10.6±0.5 | 18.1±0.2 | 17.2±0.9 |
| P_{petE-patS4} (pAM2525) | 11.2±0.2 | 10.6±0.5 | 16.4±1.7 | 16.9±0.7 |
| P_{petE-patS5} (pAM2424) | 4.3±0.9 | 0.3±0.6 | 8.3±3.5 | 0.5±0.5 |
| P_{petE-patS6} (pAM2526) | 1.8±1.3 | 0.0±0.0 | 12.3±6.0 | 6.7±4.7 |
| P_{petE-patS7} (pAM2529) | 8.3±2.9 | 4.0±5.2 | 12.2±5.2 | 8.2±4.1 |
| P_{petE-patS8} (pAM2527) | 4.0±5.3 | 0.2±0.3 | 12.2±5.2 | 2.2±1.8 |

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

| TABLE 4. Suppression of heterocyst formation by patS5 and patS17 in wild-type Anabaena strain PCC 7120 and the patS mutant AMC451. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| patS minigene (plasmid) | Heterocyst frequency (%) | % of Mch | | |
| patS (plasmid) | Wild type | AMC451 | 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_{rbcL-patS5} (pAM2528) | 6.4±4.4 | 4.0±3.5 | ND |
| 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 |

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

b The percentage of heterocysts that are multiple constitutive heterocysts (Mch).
c ND, not determined.
enough PatS heterocyst inhibition activity to overcome the
immunity to self-inhibition and blocked heterocyst differenti-
ation.

However, unlike the wild-type PatS produced from P_{patS4}/
patS17, the PatS-5 pentapeptide produced from P_{patS4}/patS5-gfp
failed to complement the patS deletion strain. AMC451
carrying P_{patS4}/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 me-
dium.* 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 condi-
tioned culture supernatant from a strain containing P_{patS5} (pAM2537)
for heterocyst inhibition activity. The supernatant from a
culture of the strain grown in BG-11, medium failed to
inhibit heterocyst formation. In a reconstruction experiment to
verify that our assay could detect PatS-5 pentapeptide in condi-
tioned medium, we added synthetic PatS-5 to conditioned
culture supernatant and BG-11, medium at 0.1, 0.5, and 1 μM.
Heterocyst formation was inhibited at each peptide concentra-
tion in both media. These results indicate that the patS5 mini-
gene product is not exported or does not leak from filaments at
concentrations capable of inhibiting heterocysts and are con-
sistent 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 sig-
naling 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_{pct} promoter. The fusion of GFP to the N terminus
of PatS-5 should confine the RGSGR motif to the cytoplasm.
P_{pct}/gfp-patS5 on pAM2873 completely inhibited heterocyst
formation after the nitrogen step-down and, as expected, pro-
duced 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 hetero-
cyst 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 dif-
ferentiation.** We searched the *Anabaena* strain PCC 7120 ge-
nome 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 heter-
cyst 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 ade-
nylate 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 actu-
ally 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, plates,
stains 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 pheno-
type 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 for-
mation. However, unlike the full-length patS gene, the patS5
minigene expressed from the heterocyst-specific promoter
P_{petE} 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 hetero-
cyst 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 ei-
ther 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_psc. 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_hetD promoter in pAM2816 failed to complement the patS deletion mutant AMC451 to produce a normal heterocyst pattern. pAM2816, which carries P_hetD-patS5 and a downstream gfp ORF, suppressed heterocysts in the wild-type background, which indicates that this construct produces levels of PatS-S pentapeptide sufficient to inhibit heterocysts. Since synthetic Pat-S pentapeptide can inhibit heterocysts when added exogenously to filaments, the PatS5 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 Pat-S 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, patS6-His, and the patS minigenes P_psc-patS5 to P_psc-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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