Developmental Pattern Formation Controlled by *patS* in a Cyanobacterium

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INTRODUCTION. The regulation of cell fate and pattern formation is a fundamental problem in developmental biology. Cell-cell communication often plays a key role in controlling development. Diffusible molecules that directly influence cell fate determination have been found in several eukaryotic organisms (1, 2, 3). Prokaryotic development in *Bacillus*, *Streptomyces*, and *Myxococcus* is also controlled by intercellular signaling (4, 5, 6). We have investigated regulation of cell fate determination and pattern formation in a prokaryote that grows as a simple multicellular organism.

When the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 grows diazotrophically, approximately every tenth vegetative cell terminally differentiates into a heterocyst (7) (Fig. 1, A and B). This simple, one-dimensional

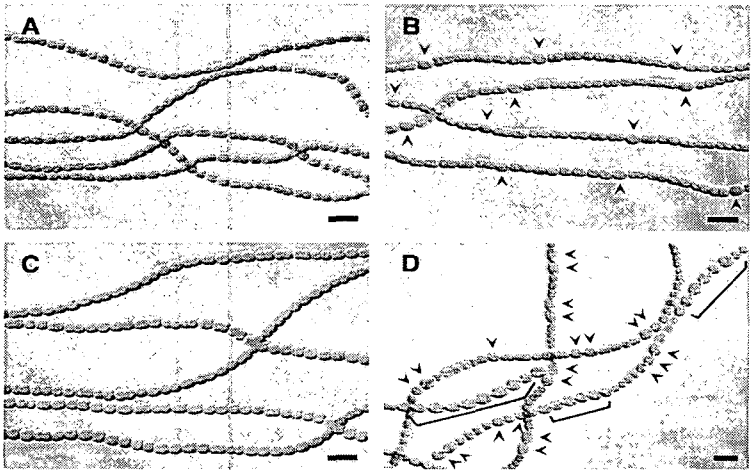

Fig. 1. *patS* controls heterocyst development in *Anabaena* PCC 7120. (**A**) Wild-type filaments grown in BG-11 medium and (**B**) after nitrogen stepdown in BG-11_0 to induce heterocysts (arrowheads). (**C**) Overexpression of *patS* from pAM1691 (Fig. 2A) prevented heterocyst formation in BG-11_0 and (**D**) deletion of *patS* (AMC451) resulted in supernumerary heterocysts with abnormal pattern in BG-11_0. Brackets indicate chains of contiguous heterocysts. *Anabaena* PCC 7120 and derived strains were grown as previously described (21). Differential interference contrast micrographs were taken before (A) and 24 hours after (B, C, and D) heterocyst induction. Scale bars, 10 μm.

developmental pattern spatially separates two incompatible processes: oxygen-evolving photosynthesis in vegetative cells and oxygen-sensitive nitrogen fixation in heterocysts. We have found that a small gene, *patS*, is crucial for the formation and maintenance of a normal heterocyst pattern.

RESULTS. The *patS* gene was identified on the conjugal cosmid 8E11 (8, 9), which suppressed heterocyst development (Fig. 2A). A 3.3-kb subclone (pAM1035) was shown to confer the heterocyst-suppression phenotype (Het^S), and its sequence was determined (accession number AF046871). The same fragment isolated from an independent cosmid, 13C12, produced the same phenotype, indicating that the dominant Het^S phenotype is a property of wild-

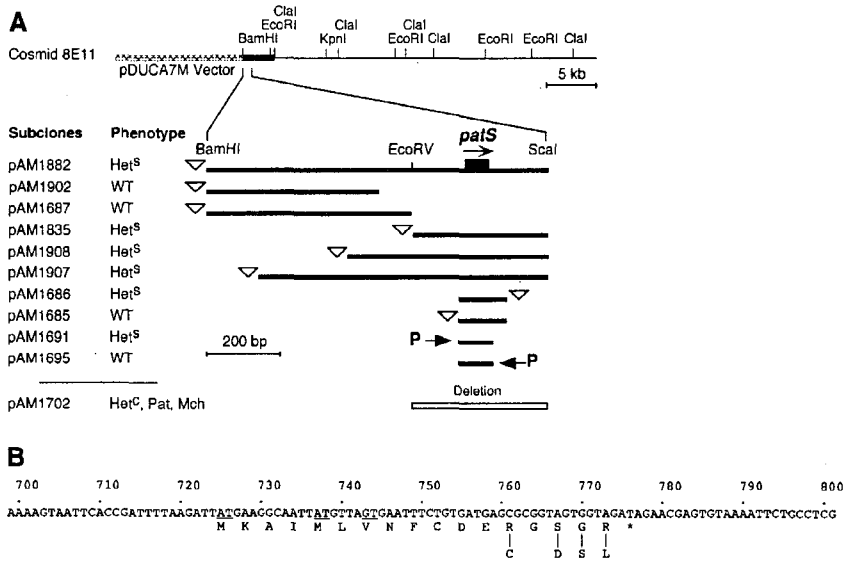

Fig. 2. (**A**) Identification of the *patS* gene on cosmid 8E11. Subclones were generated by cloning the indicated DNA fragments into shuttle vectors (22, 23). The Bam HI–Cla I fragment in pAM1035 is indicated as a black bar on the 8E11 map. Cloning and molecular techniques were performed as previously described (24). Plasmids were transferred into *Anabaena* PCC 7120 by conjugation from *Escherichia coli* (25, 26). WT, wild type; Het^S, heterocyst suppression; Het^C, heterocyst formation on nitrate-containing medium; Pat, abnormal heterocyst pattern; Mch, multiple contiguous heterocysts; inverted triangle, transcription terminator; P with arrow, external promoter. (**B**) Nucleotide sequence of the smallest tested DNA fragment sufficient to suppress heterocyst development, and the deduced amino acid sequence of PatS. Potential start codons are underlined. The amino acids encoded by four missense mutants are shown below the wild-type sequence. Nucleotide numbering begins with the first nucleotide in pAM1882.

type sequences. Analysis of various subcloned fragments in shuttle vectors (Fig. 2A) prompted us to investigate a small, 51-bp, open reading frame (ORF), named *patS* (Fig. 2B).

A 140-bp polymerase chain reaction (PCR) fragment containing the *patS* ORF (pAM1686) conferred the Het^s phenotype (Fig. 2A). However, if a transcription terminator was upstream of *patS* (pAM1685), the Het^S phenotype was not produced. Overexpression of *patS* by the *Anabaena* PCC 7120 *glnA* promoter (pAM1691) also conferred the Het^s phenotype (Fig. 1C and 2A). The antisense orientation (pAM1695) produced no noticeable phenotype. *patS* blocks development at an early stage because even the cryptic pattern of non-fluorescent cells produced by some developmental mutants (10) was not observed.

To test if different levels of transcription correlate with the degree of heterocyst inhibition, we placed *patS* under control of the copper-inducible *petE* promoter (11, 12) (Fig. 3A). Without added $CuSO_4$, the strain containing P_{petE}-*patS* was wild type. With 15 nM $CuSO_4$, there was a distinct decrease in the frequency of heterocysts, and heterocyst development was completely blocked above 135 nM $CuSO_4$. We observed no influence of $CuSO_4$ on heterocyst development when *patS* was cloned in the reverse orientation (Fig. 3A).

Mutations in *patS* resulted in loss of ability to suppress heterocysts. pAM1882 (Fig. 2A) was mutagenized and screened for plasmids that failed to suppress heterocysts. Four plasmids were identified, each with a missense mutation within *patS* (13) (Fig. 2B).

patS potentially encodes a 17 amino-acid peptide, starting at the first available ATG; however, other inframe ATG and GTG codons are present. PatS has no homologs or sequence motifs in the databases. It contains a stretch of five hydrophobic amino acids in its NH_2-terminal half, and its COOH-terminal half is mostly hydrophilic.

Because many cell-cell signaling molecules in Gram-positive bacteria are peptides (14, 15), and because a long-standing model for the control of heterocyst pattern involves a diffusible inhibitor produced by proheterocysts (7), we suspected that *patS* might encode an exported signaling molecule. We were intrigued with the phosphate regulator (*phr*) genes from *Bacillus*

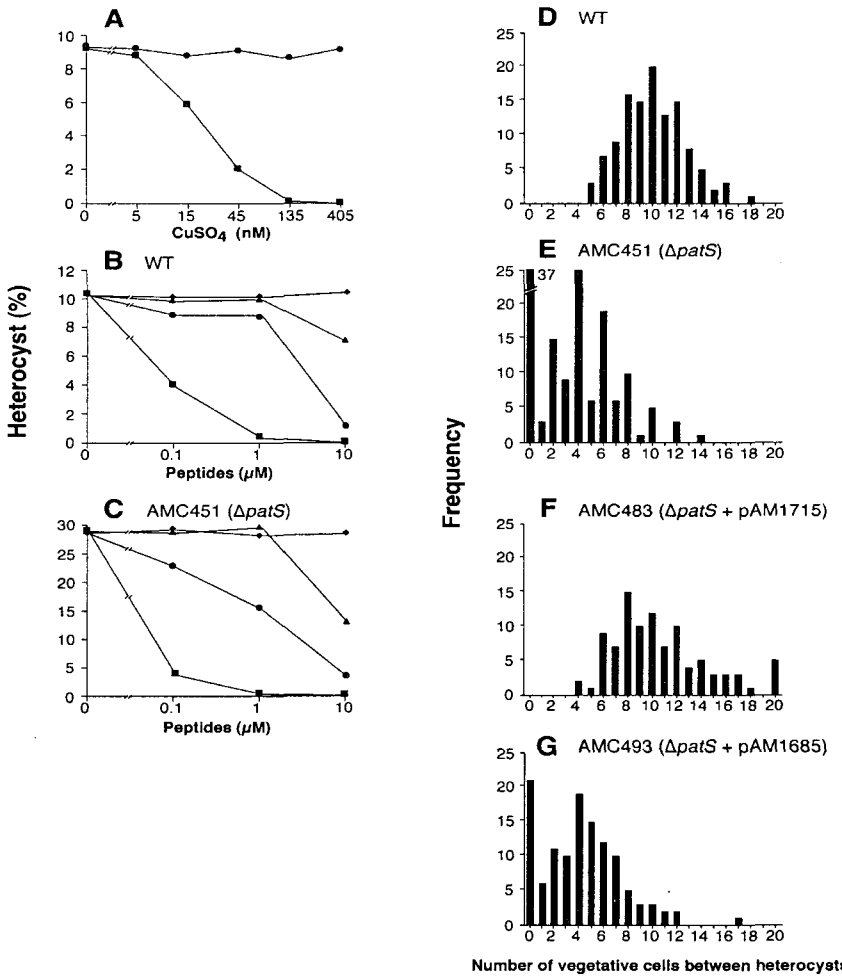

Fig. 3. (**A**) Transcription of *patS* suppressed heterocyst formation. Strains containing pAM1714 (■), which carries P_{petE}-*patS*, or the control plasmid pAM1716 (●) (11) were washed and grown for several generations in copper-free BG-11 liquid medium to deplete residual copper. At OD_{750} = 0.4, cultures were induced by transferring the filaments to 2 ml $BG\text{-}11_0$ containing different concentrations of $CuSO_4$. The percentage of heterocysts was determined microscopically 24 hours after induction. At least 1000 cells were scored for each sample. Values are shown for one representative of four independent experiments. (**B** and **C**) Inhibition of heterocyst differentiation by synthetic peptides. Peptides (Genosys Biotechnologies, Inc.) were added to wild-type (B) and *patS* deletion strain AMC451 (C) immediately after transfer to 2 ml $BG\text{-}11_0$. One representative of three independent experiments is shown. PatS-5 (RGSGR) (■); PatS-4 (GSGR) (▲); PatS-G4S (RGSSR) (●); mixture of amino acids (R, G, and S) (◆). (**D** to **G**) Abnormal heterocyst pattern in AMC451 and complementation by heterocyst-specific expression of *patS*. Filaments were grown in liquid BG-11 to OD_{750} = 0.2, induced in $BG\text{-}11_0$ 24 hours, and scored microscopically. (D) wild type; (E) AMC451; (F) AMC483 (AMC451 containing pAM1715, which carries *patS* driven by the *hepA* promoter); and (G) AMC493 [AMC451 containing pAM1685 (Fig. 2A)].

subtilis (16, 17), and *phrC* in particular, which encodes the quorum sensing pheromone CSF (5). CSF is an unmodified, exported pentapeptide processed from the COOH-terminal end of a 40 amino acid precursor. These precedents, and the fact that the four *patS* missense mutations happened to be in the last five codons

(Fig. 2B), led us to test a synthetic pentapeptide corresponding to the COOH-terminal end of PatS.

This pentapeptide (PatS-5, RGSGR) inhibited heterocyst formation when added at micromolar concentrations to induced filaments (Fig. 3B). Two other peptides had greatly reduced heterocyst-suppression activity: PatS-4 (GSGR), which contains the last four amino acids, and PatS-G4S (RGSSR), which contains a glycine to serine substitution corresponding to a mutation that reduced *patS* activity (Fig. 2B). A mixture of each amino acid (R, G, and S) present in PatS-5 had no effect on heterocyst formation (Fig. 3B).

A *patS* deletion strain, AMC451 (18), formed heterocysts on nitrate-containing medium, conditions in which the wild type does not produce heterocysts. Twenty-four hours after nitrogen stepdown, wild-type filaments form a pattern of single heterocysts separated by 8 to 14 vegetative cells (Fig. 1B and 3D). After nitrogen stepdown, AMC451 formed multiple contiguous heterocysts and strikingly short vegetative-cell intervals (Fig. 1D and 3E). In the experiment shown in Fig. 3D and 3E, the wild type formed 100% single heterocysts, whereas AMC451 formed 39% single, 55% double, and 3% each quadruple and sextuple heterocysts. Longer chains of up to 10 contiguous heterocysts were occasionally formed (Fig. 1D).

AMC451 was complemented by *patS* introduced on a plasmid. AMC451 containing pAM1882 formed no heterocysts, and pAM1835 and pAM1686 restored an approximately wild-type heterocyst pattern. The AMC451 phenotype was also suppressed by exogenously added synthetic PatS-5 pentapeptide (Fig. 3C).

Expressing *patS* in only proheterocysts suppressed the pattern defects of AMC451, and indicates that PatS functions cell-nonautonomously. The *hepA* promoter is induced in proheterocysts between 4.5 and 7 hours after nitrogen stepdown (19). A *patS* deletion mutant containing a plasmid-borne P_{hepA}-*patS* fusion (AMC483) had a nearly wild-type pattern (Fig. 3F). A control strain, AMC493, containing a promoterless *patS*, retained the mutant phenotype (Fig. 3G).

DISCUSSION. PatS appears to play a key role in heterocyst pattern formation by inhibiting the formation of adjacent heterocysts and maintaining a minimum

number of vegetative cells between heterocysts. Inhibition of neighboring cells by selected differentiating cells (lateral inhibition) is an important mechanism of pattern formation in eukaryotic organisms (20). Because it takes approximately 20 hours for heterocysts to mature and begin supplying fixed nitrogen to the filament, a specialized early inhibitory signal is required to allow only a fraction of starving cells to terminally differentiate. It appears that the first cells to differentiate increase production of PatS to inhibit neighboring cells from also forming heterocysts. The differentiating cells must themselves be refractory to the PatS signal.

The PatS signal is likely to be a processed COOH-terminal peptide that is confined to the periplasm of this Gram-negative cyanobacterium. There is no evidence of heterocyst inhibition between filaments in mixed cultures or by conditioned medium from a strain overexpressing *patS*. The PatS peptide, originating from differentiating proheterocysts, would diffuse along the filament's contiguous periplasmic space and be taken up by neighboring cells, creating a gradient of the inhibitory signal.

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11. pAM1697 contains *patS* amplified by PCR and cloned into pPet1 (W. J. Buikema and R. Haselkorn, personal communication), which contains the *petE* promoter. pAM1714 contains the P_{petE}-*patS* fragment from pAM1697 in

pAM504 (25). pAM1716 contains *patS* in the reverse orientation. Two independent clones for each construct were tested and produced similar results.

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22. Details of plasmid constructions are available from the authors. Subclones were from pAM1035, which contains *patS* on a 3.3-kb Bam HI–Cla I fragment cloned into the same sites of pBluescript II KS(-). pAM1882, pAM1687, and pAM1835 contain restriction fragments in the conjugal shuttle vector pAM504 (25). pAM1902, pAM1908, and pAM1907 contain inserts amplified by PCR from pAM1035 and cloned into pAM504. pAM1685 contains *patS* amplified by PCR and cloned into pAM504. pAM1686 contains the same insert cloned into pAM505 (identical to pAM504 except the Bam HI to Sac I sites are reversed). The PCR fragment was also used to construct pAM1691 and pAM1695. pAM1691 contains *patS* cloned into pAM743, which contains the *Anabaena* PCC 7120 *glnA* promoter from pAM658 (23) in pAM504. pAM1695 contains *patS* in the reverse orientation. The sequences of all inserts generated by PCR were confirmed after subcloning.
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