Introduction

The process of cellular development is too complex and beyond our present understanding. It would be fascinating to understand how we develop from one single cell into an organism comprised of masses of structurally and functionally different cells. How is the fate of cells determined? If we understand the cell differentiation mechanism in simple organisms, we may be able to take a step closer to understanding more complex cell systems. For this reason, it may be beneficial to study the mechanism of cell differentiation in heterocyst-forming cyanobacteria.

Some cyanobacteria such as *Anabaena* sp. PCC7120 are comprised of two cell types: photosynthetic vegetative cells and nitrogen-fixing heterocysts. These cells are structurally and functionally different. Heterocysts are specialized cells in which atmospheric nitrogen (N_2) is converted to nitrogen compounds such as ammonia and nitrate. They are formed with semi-regulated intervals in nitrogen-deprived condition. Heterocysts lack photosynthetic activity and carbon compounds are delivered into heterocysts from vegetative cells. Nitrogenase, the key enzyme involved in nitrogen fixation, is sensitive to oxygen; therefore protective glycolipid layer is developed around heterocysts to limit oxygen flow into the cells (1, 2, 3).

How is heterocyst development controlled by genes? One gene *hetR* has been identified as the "master regulator" of heterocyst formation. *hetR* encodes the protein HetR that is essential for heterocyst development. Nitrogen starvation triggers the transcription of the gene (1). According to the study by Buikema and Haselkorn, heterocyst formation is dependent of the level of HetR (2). HetR is a homodimeric protein that binds to DNA. Dimerization is essential to trigger other genes involved in heterocyst development such as *patS*, *hepA*, and *hetR* itself (4). However, the mechanism by which *hetR* works has not been identified.

During the study of methyltransferases in *Anabaena*, it was inadvertently discovered that interrupting one of the genes *dmtB* with antibiotic cassette resulted in the inhibition of heterocyst formation. However, when the cassette was inserted in the opposite orientation relative to *dmtB*, the ability to differentiate was restored. Further study revealed that interrupting *trpD2*, a gene downstream of *dmtB* with the cassette in the antiparallel orientation caused the same phenomenon whereas insertion of the cassette parallel to *trpD2* did not affect heterocyst formation (figure 1).

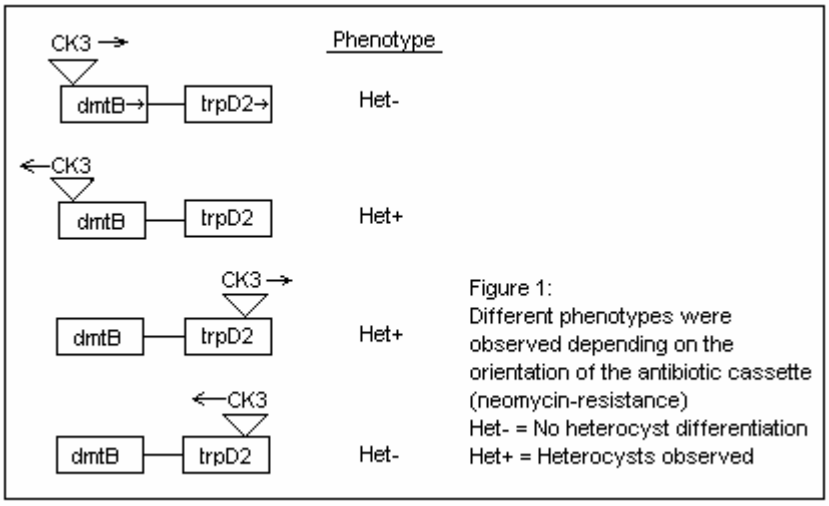

Figure 1:
Different phenotypes were observed depending on the orientation of the antibiotic cassette (neomycin-resistance)
Het- = No heterocyst differentiation
Het+ = Heterocysts observed

Overexpression of some sequence between these two genes seems to have caused the differences in phenotypes, but by neither *dmtB* nor *trpD2* itself. The phenomenon suggests the existence of non protein-coding regulator. The focus was shifted towards the intergenic region of *dmtB* and *trpD2*, a sequence of about 60 nucleotides. The intergenic DNA does not contain any open reading frame; hence it does not produce protein. Deletion of the region was attempted but was not successful, resulting in the loss of the mutant chromosome and the formation of heterocyst clusters. The existence of this region is apparently essential for the viability of the organism as well (5). Is it possible that heterocyst development is regulated by non-coding RNA? What is the mechanism of such regulation?

RNA interference (RNAi) is a process in which small non-coding RNAs suppress gene expression. The silencing mechanism requires the RNA to have a homologous sequence to the coding-region of the target. In some plants, interfering RNA was found to have sequence homology in the promoter, inducing promoter methylation (6). Small interfering RNA (siRNA) is processed from double-stranded precursor by RNase III-like enzyme called Dicer, generating RNA of approximately 20 nucleotides. These RNAs may degrade their target mRNA or block translation. siRNA binds to a protein complex which recognizes and destroys the target mRNA endogenously. Other microRNAs block the translation by binding to their target mRNAs but not degrading them (7). In *C. elegans*, small temporal RNA (stRNA) has been found to have a role in the temporal regulation of gene expression. The product of *lin-4* gene regulates cell fate transitions through early stages of larval development whereas *let-7* influences later transitions. Studies showed that normal larval development was not achieved when these RNAs lost their functions by mutation (8)

In bacteria, small RNAs (sRNAs) have roles in regulating plasmid copy number and gene expression (9). In *E.coli*, a small RNA known as 6S RNA was found to have a regulatory role in the stationary phase. Cells respond to the depletion of nutrients and 6S RNA activity is triggered. 6S RNA mediates the expression of essential genes in the stationary phase by altering the behavior of the RNA polymerase (RNAP). Sigma subunits are essential for the binding of bacterial RNAP to specific promoters. The affinity of σ^{70}, which is dominant during exponential growth declines by the binding of

6S RNA to the RNAP-σ^{70} holoenzyme. In turn, σ^{s}, which is responsible for the expression of essential genes in the stationary phase, becomes active (9, 10, 11).

It is interesting to note that 17-nucleotide sequence of the intergenic region of *dmtB* and *trpD2* match a part of *hetR* promoter (P_{hetR}). This coincidence gives us an incentive to further investigate this intergenic region.

[A] *dmtB-trpD2*intergenic region in *Anabaena* sp. PCC7120

AAAATGGCAACAAATCAACCTGATTTACAGTTAGAACTTAATATTTCAACCTCGCTTTGA
TGACAGTCGATTTTTCGGTAGGATAACTGCCATGCCTCTCAAAGTACACAAACTTGAGTT
ATGACCAGTTCTCCTACATCTACACAAGAATCTTCTACTAGCTGGTATCTTCTACTGCAA

[B] *Anabaena* P_{hetR}

GGCAGTTATTTTGTGATTTTTCGGTAAGATACAAGCACGTCTAGTAACAACACTGTTGCT
AGTTATATGCCATAAATTACGGAGGAATGAGCATCTTGACCATTAATCGACTTTTATTTA
AAACATCATAAGCATTTTGGTGTCACAATCTCACCCCCCTATGGGTGTATACCAACTACT
TTTTTTAGACTAATAAGCGTGCTATTCAACTATTTGTAATATGAGTAACGACATCGATCT

Figure 2:
In A, orange highlight indicates *dmtB-trpD2* intergenic sequence. Blue indicates trpD2 start codon. B shows hetR promoter sequence. Blue highlight indicates hetR start codon. About 17 bp sequence is shared between the intergenic DNA and P_{hetR} (highlighted in red)

This research explores the possible role of microRNA as a mediator in heterocyst formation in *Anabaena* sp. PCC7120. Plasmids were constructed in order to control the expression of *dmtB-trpD2* intergenic DNA. The constructs were conjugated into the partial mutant strain 2S6, which contains both wild type and the mutant chromosome lacking the region between *dmtB* and *trpD2*. Segregation of chromosome was assessed by Southern hybridization. The research extends into the investigation of the minimal effective fragment that affects differentiation. Recombinants were to be constructed by progressively delete the intergenic DNA to achieve different fragments.

Materials and Methods

1. Plasmid construction and cloning

pVCU300 was constructed from pVCU245 and a shuttle vector pRL11. The plasmid contains the intergenic region and the entire *dmtB* and *trpD2* genes. pVCU245 was digested with PstI and BsaHI, and the insert bearing the interested region was ligated into pRL11 opened with ClaI and NsiI.

Second, two vectors that allow the controlled expression of the intergenic DNA were constructed. The insert containing *dmtB-trpD2* intergenic region was excised from pVCU238 with BstBI and BglII and ligated into BamHI /ClaI site of pVCU251, creating pVCU301. The insert is oriented antiparallel to the promoter (P_{trc}). pVCU302 was constructed with the same insert cut out of pVCU238 with XhoI and BamHI, which were ligated into XhoI/BamHI site of pVCU251 in the orientation parallel to the promoter. These constructs also carry *LacI*^{q} gene that encodes Lac repressor so the promoter can be induced by IPTG (isopropyl ß-D-thiogalactopyranoside). All constructs were transformed into E.coli strain DH10B.

Strain or Plasmid	Relevant Characteristics
Strains of *Anabaena* sp.	
PCC7120	Wild Type
2S6	Mutant with Nm casette. Nmr
Plasmids	
pRL11	pDU1-based shuttle Vector, Cmr/Emr/Smr
pVCU238	Km cassette replacing half of 5'-end dmtB gene, Kmr/Spr/Smr
pVCU245	dmtB-trpD2 region cloned into pGEM T-easy vector, Apr
pVCU251	Contains lacIq and trc promoter, pDU1-based shuttle vector, Cmr/Emr
pVCU300	dmtB-trpD2 region cloned into pRL11, Cmr/Emr
pVCU301	dmtB-trpD2 region from pVCU238 cloned into pVCU251 downstream of trc promoter, insert antiparallel to the promoter, Cmr/Emr
pVCU302	dmtB-trpD2 region from pVCU238 cloned into pVCU251 downstream of trc promoter, insert parallel to the promoter, Cmr/Emr
pRL443	RP4 derivative conjugal plasmid, Km-sensitive, Apr/Tcr
pRL1124	Helper plasmid, Km^r
pDS4101	Helper plasmid, Ap^r

Table 1: Bacterial strains and plasmids

2. Conjugation of parents and *Anabaena* growth condition

The constructs were transferred into wild type *Anabaena* and its semi-mutant 2S6. Neomycin at 10ug/ml was added to 2S6 culture to select for the mutant. Conjugation of parents was performed according to the method described by Elhai et al (12). The constructs were electroporated into *E.coli* 4102EC bearing helper plasmids pDS4101 and pRL1124 then mixed with the conjugal plasmid pRL443. Spot mating method was employed in order to mate the parents. Mutant recombinant strains were grown with or without IPTG. Filters were incubated without antibiotic on BG11 solid media for two days at 30^{o}C, and then transferred to BG11 media with following antibiotics: erythromycin at 1ug/ml for the wild type and erythromycin at 1ug/ml and neomycin at 10ug/ml for the mutants. The plates were incubated for one week to allow for antibiotic selection. A single colony was obtained for each recombinant and grew in BG11 liquid media for one week to ten days.

3. Chromosomal DNA extraction and Southern hybridization

Cells were harvested during log-phase growth and washed in $BG11_0$ medium. Small amount of each culture was introduced to nitrogen-deprived condition ($BG11_0$) and incubated for 48 hours to observe heterocyst formation.

Total chromosomal DNA was extracted using phenol/chloroform extraction method. Approximately 5µg of extracted DNA from seven recombinant strains, wild type and mutant 2S6 were digested with BsaA1 to create template DNA for Southern blot analysis. The fragments were separated by electrophoresis on 1.0% agarose and transferred onto Zeta-Probe membrane (Bio-Rad) and the membrane was baked for two hours at 80^{o}C. Hybridization was performed using DIG-labeled probe derived from pVCU245 digested with BsaHI and BstXI, following the manufacturer's protocol (13). The membrane was exposed to an X-ray film.

4. Progressive deletion of the intergenic region

In order to identify the minimal effective fragment of the intergenic region that affects heterocyst formation, different DNA fragments need to be generated. Progressive deletion of the intergenic region was performed utilizing exonuclease activity. Vector pVCU238 was opened with BamHI. Six reaction tubes were prepared to digest DNA with exonuclease III. Exonuclease III digests approximately 60 bps per minute at 22oC. The tubes were incubated in 20^{o}C and the enzyme was heat inactivated at one-minute interval. Fragments were repaired with T4 DNA polymerase and digested with XhoI to excise the insert fragments. The fragments were ligated into EcoRV/XhoI site of pBluescript (KS+). The constructs were transformed into *E.coli* strain DH10B, and incubated on LB media and selected for ampicillin resistance. The transformants were identified by blue-white screening. Extracted plasmid recombinants were digested with PvuII to confirm whether the inserts were ligated.

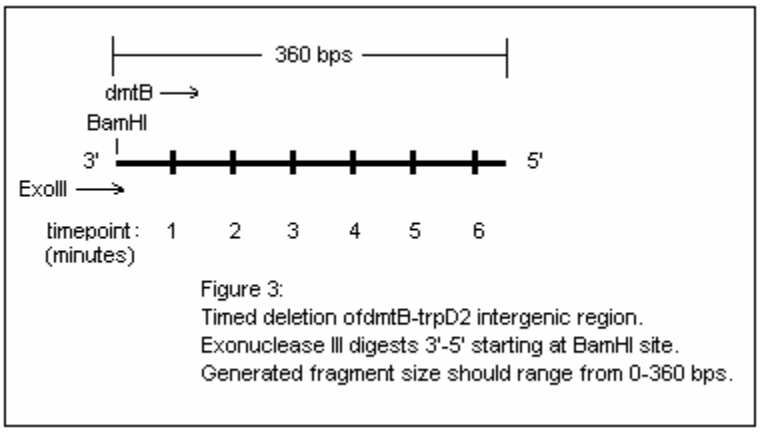

Figure 3:
Timed deletion ofdmtB-trpD2 intergenic region.
Exonuclease III digests 3'-5' starting at BamHI site.
Generated fragment size should range from 0-360 bps.

Results and Discussion

Phenotype observation and plasmid construction

The observed characteristics of the phenotypes are summarized in table 2. After 2-day incubation, no heterocyst was observed in any of the recombinant strain. In addition, all the strains showed poor growth under microscope. Colonies appeared unhealthy and cells were crowded together in the media. Absence of heterocysts in -IPTG condition suggests that the absence of the inducer did not turn off the promoter. The promoter was strong enough to initiate the transcription of the intergenic region. Copy numbers of the plasmids also need to be taken into account. For future investigation, weaker promoter may be used but the result is unpredictable. More reasonable approach is to perform a mutagenesis on trc promoter in order to completely inactivate it. Also, the growth conditions of the strains can be modified to closely monitor the cell behavior. They can be grown in +IPTG condition initially then transferred to –IPTG condition, or vice versa.

Strains	Plasmid	Observed characteristics
Wild Type	None (control)	Healthy growth, long filaments, Het+
	pVCU300	Small cells, short filaments, Het-
	pVCU301	Small cells, short filaments, Het-
	pVCU302	Small cells, short filaments, Het-
2S6 (+IPTG)	pVCU301	Short filaments, clumped up colonies, Het-
	pVCU302	Dying cells, Het-
2S6 (-IPTG)	pVCU301	Short filaments, clumped up colonies, Het-
	pVCU302	Short filaments, clumped up colonies, Het-

Table 2: Observation of phenotypes under light microscope (x40). [Het-: no heterocyst differentiation. Het+: heterocysts observed]

Progressive deletion of the intergenic region

Gel electrophoresis failed to show the isolated gene fragments after exonuclease III treatment of pVCU238 due to small fragment sizes and RNA contamination. Ligation of the fragments into pBluescript was still attempted, but there was no evidence that the fragments were contained in the transformants. Clearly, the procedure must be reviewed in order to accurately assess and make sure each step. Without being able to see the isolated fragment on the gel, it is difficult to say whether exonuclease digestion was successful.

Hybridization of chromosomal and plasmid DNA

All lanes appeared to have both wild type chromosome and recombinants (data not shown). pVCU300 strain (wild type) showed the expected fragment at 14000 bps. pVCU301 strains (wild type, +IPTG, -IPTG) showed the expected fragments at approximately 4000 bps. Two of the pVCU302 strains (+IPTG, -IPTG) showed fragments at 5000 bps, which were not expected. Ladder DNA was unexpectedly seen on the film. This may be due to the fact that the probe contained some sequences from the plasmid DNA, which coincidentally matched the ladder DNA. Overall, it was difficult to distinguish the larger bands. It is possible that DNA was not completely digested prior to separation. Also, the choice of restriction enzyme might have been unsuitable, generating bands too close for comparison. Different restriction enzyme(s) may be considered to digest the template DNA to create fragments that are compared more easily. Complete digestion of the DNA must be achieved to avoid confusion. DNA probe may have been unreliable because of the contamination. The construction of the probe will also be reconsidered so only the interested region will be detected. Also, the strains can be grown for a longer period of time so the segregation would be more apparent.

As the results indicated, the expression of the intergenic region has a strong effect on the inhibition of heterocyst formation. I encountered some issues and unexpected results that need to be reviewed. Hopefully, further investigation will answer the proposed question.

Reference

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