Characterization of a nitrogen-fixation (*nif*) gene cluster from *Anabaena azollae* 1a shows that closely related cyanobacteria have highly variable but structured intergenic regions

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The exact identity of cyanobacteria that have been cultured from symbiotic associations with the water fern *Azolla* spp., whether they are required in the symbiotic process, and their relationship to the symbiotic species, is a matter of some debate. We have characterized a 6 kb region containing the *nifB* operon and the *nifH* gene from cyanobacterium *Anabaena azollae* 1a, a putative symbiont of *Azolla caroliniana*. Five complete open reading frames have been sequenced. All are very highly conserved when compared with the corresponding regions of *Anabaena* sp. PCC 7120, with 93% to 97% identity at the nucleotide level and 93% to almost 100% at the amino-acid level. The intergenic regions, however, are not highly conserved (53–89% identity) when compared to the corresponding regions of *Anabaena* 7120: the *A. azollae* genome contains both more copies and more types of short tandemly repeated repetitive sequences than *Anabaena* 7120. The startpoints of transcription for both the *nifB* and *nifH* operons were mapped and found to be the same as those in *Anabaena* 7120. It was not possible to discern an improved consensus *nif* promoter sequence, but it was possible to define the likely extent of the promoter to within 40 bases upstream of the transcription start-point.

Keywords: *Anabaena azollae*, cyanobacteria, intergenic region, nitrogen-fixation genes, short tandemly repetitive sequences

INTRODUCTION

The fixation of atmospheric nitrogen is restricted to select micro-organisms, among which are the heterocyst-forming cyanobacteria. A number of symbiotic relationships have evolved to take advantage of the nitrogen-fixing ability of micro-organisms, including cyanobacteria (Evans & Burris, 1992). One such association, which has been used in traditional agriculture, is the association between species of the water fern *Azolla* and heterocyst-forming cyanobacteria. In *Azolla*, cyanobacterial filaments are found inside a specialized leaf cavity. The fern stimulates differentiation of a higher-than-normal

frequency of heterocysts and the cyanobacterium provides combined nitrogen for the fern.

The cyanobacterium *Anabaena azollae* 1a was originally isolated from and was believed to be the endosymbiont of the water fern *Azolla caroliniana* (Newton & Herman, 1979). However, there is considerable doubt as to whether this, or any other, cultured isolate is the true symbiont of *Azolla* spp. (Peters & Meeks, 1989). It has not been possible to reconstitute the symbiosis between *Azolla* spp. cured of cyanobacteria and any cultured isolate; and restriction maps of nitrogen fixation (*nif*) genes in cultured isolates are not the same as those from freshly-isolated symbiotic cyanobacteria (Frache & Cohen-Bazire, 1985, 1987; Meeks *et al.*, 1988). The identity of the cultured isolates, whether they are required in the symbiotic process, and their relationship to the symbiotic species are of interest.

Abbreviations: IHF, integration host factor; STRR, short tandemly repeated repetitive (sequence).

The GenBank accession number for the sequence reported in this paper is L34879.

The identification of different cyanobacterial species or strains and their relationship to one another has been difficult to resolve using traditional taxonomic morphological and physiological methods. Since *nif* genes are highly conserved, the nucleotide sequence of *nif* genes from different strains of cyanobacteria can be used as a taxonomic tool (Ben-Porath *et al.*, 1993; Ben-Porath & Zehr, 1994; Mathur & Tuli, 1990). Cyanobacterial *nif* genes have been most extensively characterized in *Anabaena* sp. PCC 7120 (*Anabaena* 7120) (for a review see Haselkorn & Buikema, 1992). *nif* genes have also been characterized from *Anabaena* sp. ATCC 29413 (*Anabaena* 29413) (Hirschberg *et al.*, 1985; Thiel, 1993; Lyons & Thiel, 1995), *Anabaena* sp. L31 (*Anabaena* L31) (Murphy *et al.*, 1993), *Nostoc* sp. PCC 6720 (*Nostoc* 6720) (Beesley *et al.*, 1994), *Plectonema boryanum* (Fujita *et al.*, 1991) and *Nostoc commune* (Defrancesco & Potts, 1988).

In this work, we show that related strains of cyanobacteria have distinct and different repeating sequence structure in their genomes. This observation is based on the nucleotide sequence of the *nifB*-*fdxN*-*nifS*-*nifU* operon (*nifB* operon), the *nifH* gene and the 5' part of *nifD* from *A. azollae* 1a. Our results also indicate that *A. azollae* 1a is very closely related to the naturally occurring free-living strain *Anabaena* 29413.

METHODS

Enzymes and chemicals. All chemicals were reagent grade or better. Restriction endonucleases and other enzymes were purchased from Boehringer Mannheim, GIBCO-Bethesda Research Laboratories, New England Biolabs or Pharmacia and used in accordance with standard protocols (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). [³²S]dATPαS (> 1000 Ci mmol⁻¹; > 37 TBq mmol⁻¹) was purchased from NEN-Dupont or Amersham. [³³P]ATP (3000 Ci mmol⁻¹; 111 TBq mmol⁻¹) was purchased from NEN-Dupont. Sequenase 2.0 was obtained from US Biochemical Corp. DNA and RNA probes for nucleic acid hybridization were prepared using digoxigenin non-radioactive labelling and detection kits (Boehringer Mannheim). Oligonucleotides used in nucleotide sequencing were the same as those used previously (Mulligan & Haselkorn, 1989) or were purchased from the Queen's University Oligonucleotide Synthesis Laboratory (Kingston, Ontario, Canada).

Culture and preparation of nucleic acids from cyanobacteria. *A. azollae* 1a (obtained in the laboratory of R. Haselkorn, University of Chicago, IL, USA) was maintained in Chu 10 medium (Luftig & Haselkorn, 1967) supplemented with 2.5 mM NaNO₃. Small-scale cultures (100 ml) were passaged approximately every eight weeks by inoculating 1–5 ml into fresh medium. Large-scale cultures of *Anabaena* sp. PCC 7120 (*Anabaena* 7120) were grown essentially as described by Belknap & Haselkorn (1987).

DNA and RNA was prepared from large-scale cultures of cyanobacteria as described by Golden *et al.* (1987). DNA from small-scale stationary cultures (100 ml) was prepared as follows. Filaments were harvested by centrifugation, washed in TEN (50 mM Tris/HCl, pH 8; 50 mM Na₂EDTA; 0.5 M NaCl), and resuspended in 5 ml TEN. Strains with mucous polysaccharide sheaths were incubated for 30 min at 4 °C with constant mixing. Occasionally, 25 µg lysozyme was added during this incubation. Equal volumes of sterile glass beads (150–200 µm; Sigma) and phenol/CHCl₃ (1:1, v/v) were added and suspensions were

Fig. 1. Map of the *A. azollae* 1a *nifB* operon, *nifH* gene and flanking sequence. A partial restriction map of the 6061 bp region is shown. The major restriction sites that were used in cloning and sequencing are indicated: A, *AccI*; D, *DraI*; H2, *HincII*; H3, *HindIII*; P, *SspI*; R1, *EcoRI*; R5, *EcoRV*; S, *SalI*; X, *XbaI*. Only one of the *HincII* sites in this region is shown. The *nifB*, *fdxN*, *nifS*, *nifU* and *nifH* genes and the 5' end of *nifD* are indicated by the shaded boxes.

vortex mixed three times for 3 min. Suspensions were kept on ice between vortexing. Following centrifugation at 10000 r.p.m. for 10 min, the supernatant was removed and re-extracted once with phenol and once with phenol/CHCl₃ (1:1, v/v). Nucleic acids were then precipitated with ethanol or propanol and resuspended in TE (10 mM Tris/HCl, pH 8; 0.1 mM Na₂EDTA). Solutions were extracted twice with phenol and once with CHCl₃. Ethanol was added to precipitate the nucleic acids; where possible, DNA was spooled using a sealed Pasteur pipette and resuspended in TE.

Nucleic acid hybridizations. Southern blots of restriction digests of DNA, fractionated by agarose gel electrophoresis, were transferred to Hybond N+ (Amersham) nylon membranes by alkaline transfer (Reed & Mann, 1985). Recombinant clones were identified from size-directed libraries by screening colonies using Colony-Plus (Dupont) nylon membranes. RNA:RNA blots were prepared as described previously (Mulligan & Haselkorn, 1989) using total RNA (50 µg per lane) prepared from cells grown under either nitrogen-replete (N+) or nitrogen-limited (N-) conditions. All membranes were hybridized for 40 h at 60 °C with digoxigenin-labelled DNA or RNA probes.

Following hybridization, membranes were washed twice for 5 min at room temperature with 1 × SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7) plus 0.5% SDS; then washed three times for 20 min at 60 °C with 0.5 × SSC plus 0.5% SDS; and finally washed twice for 10 min at room temperature with 0.5 × SSC. Bound probe was detected using an alkaline-phosphatase-conjugated anti-digoxigenin antibody followed by colorimetric detection according to the manufacturer's directions.

DNA cloning and sequencing. We isolated a 4.1 kb *HindIII* fragment of *A. azollae* 1a from a size-directed library of *HindIII* fragments cloned in the plasmid pUC19 by using a 1.8 kb *HindIII* fragment, An154.3 (Rice *et al.*, 1982), containing the *Anabaena* 7120 *nifH* gene, as a probe. Subsequent restriction and nucleotide sequence analysis showed that the 4.1 kb fragment is equivalent to both of the *Anabaena* 7120 clones AnH20.1 (Golden *et al.*, 1987) and An154.3 and that it contains the 3' end of *nifB*, the *fdxN*, *nifS*, *nifU* and *nifH* genes as well as the 5' end of *nifD*. An adjacent 6.4 kb *HindIII* fragment (Fig. 1) containing the remainder of the *nifB* gene was subsequently cloned using a 700 bp *EcoRI*-*HindIII* fragment from *Anabaena* 7120 as a probe. These clones were mapped using selected restriction endonucleases to identify suitable enzymes for subcloning and sequencing (Fig. 1).

The complete nucleotide sequence of a 6061 bp region of *A. azollae* 1a containing the *nifB* operon as well as the *nifH* gene and

the 5' part of the *nifD* gene was determined after subcloning appropriate fragments in M13mp18/19 or in the plasmids pUC18, pUC19, pBR322, pSP73 (Promega), or pK184 (Jobling & Holmes, 1990). Sequence was determined on both strands using the dideoxynucleotide sequencing method with Sequenase (US Biochemical Corp.), following the recommended protocol.

Analysis of gene expression. Primer extension analysis was performed as described previously (Mulligan & Haselkorn, 1989) using total RNA (50 µg per reaction) prepared from cultures of *A. azollae* 1a grown under either N+ or N- conditions. Either a *nifH*-specific or a *nifB*-specific oligonucleotide (see Fig. 2) was annealed to the RNA and extended using avian myeloblastosis virus reverse transcriptase (Pharmacia).

RNA:RNA hybridization analysis was performed using either a *nifH*-specific or a *nifB*-specific digoxigenin-labelled RNA probe. Both probes were prepared by transcribing cloned fragments of the appropriate *A. azollae* 1a genes with T7 RNA polymerase. The *nifH*-specific probe was prepared from a 449 bp *HincII* fragment cloned in pSPT18 (Boehringer-Mannheim); the *nifB*-specific probe was prepared from a 436 bp *Bst*UI fragment cloned in pSPT19.

Computer analysis. Routine analysis of nucleic acid sequences was performed using the DNAnalysis program of Dr Bill Buikema (University of Chicago, IL, USA). Comparisons of nucleic acid and deduced amino acid sequences were calculated using the ALIGN program of the FASTA suite of programs for the Apple Macintosh (Pearson & Lipman, 1988). Sequence entries were retrieved from the GenBank database using the Indiana University (IUBIO) Gopher Server (Gilbert, 1993; Parker, 1993). Analysis of integration host factor binding sites was performed using the MacTargSearch program (Goodrich *et al.*, 1990). The MulFold program was used to calculate free-energies of stem-loop formation (Jaeger *et al.*, 1989; Zuker, 1989).

RESULTS

nif gene organization

We have characterized a 6061 bp region of *A. azollae* 1a (Fig. 2) that contains the *nifB* operon as well as the *nifH* gene and the 5' part of *nifD* gene in a contiguous arrangement. The *A. azollae* 1a vegetative cell genome does not contain a 55 kb element interrupting the *fdxN* gene and has a gene arrangement identical to that of the *Anabaena* 7120 heterocyst genome in this region. The *nifH* gene follows the *nifB* operon with a gap of 265 bp which is similar in length to that in many other species of cyanobacteria. There are no open reading frames between the end of *nifU* and the transcription start-point of the *nifH* operon – *A. azollae* 1a does not contain a cyanoglobulin gene between *nifU* and *nifH* as in *Nostoc commune* (Potts *et al.*, 1992) and *Nostoc* sp. MUN 8820 (*Nostoc* 8820) (T. J. Belbin & M. E. Mulligan, unpublished results).

Coding region sequences

The nucleotide and inferred amino acid sequences of the six coding regions that are reported here are all highly similar to the corresponding regions of *Anabaena* 7120 (Table 1). Identities range from 97.4% (*nifB*) to 93.4% (*fdxN*) at the nucleotide level, and from 99.6% (*NifB*) to 93.1% (*FdxN*) at the amino-acid level. Table 2 shows the

degree of identity between the *A. azollae* 1a *nifH* sequence and that of four cyanobacteria for which the complete sequence of the *nifH* gene has been determined. At the amino-acid level, values range from 99.7% identity for the *Nostoc* 6720 gene product to 84.5% identity for that of *P. boryanum*, which does not form heterocysts. Our values are consistent with those obtained in a study of partial *nifH* sequences for four heterocyst-forming and four non-heterocyst-forming cyanobacteria using PCR amplification (Ben-Porath & Zehr, 1994). In that study, values ranged from 94 to 98% for the heterocyst-forming cyanobacteria and from 85 to 94% for the non-heterocyst-forming cyanobacteria. The *Anabaena* 7120 gene product is longer than other *NifH* gene products, with five additional amino acids at the carboxy-terminal end, and all of the other cyanobacterial sequences contain a single amino-acid insertion (Ile 251 in the *A. azollae* 1a sequence) that is not found in *Anabaena* 7120.

Intergenic regions

In general, the level of similarity between the intergenic regions of *A. azollae* 1a and those of *Anabaena* 7120 is not very high. In the interval between *nifB* and *fdxN*, *A. azollae* 1a and *Anabaena* 7120 are only 52.6% identical. For the other intergenic regions the figures are: between *nifS* and *nifU*, 59.3%; between *nifU* and *nifH*, 73.9%; and between *nifH* and *nifD*, 72.3%. The highest overall level of identity occurs upstream of *nifB*, where 88.5% identity was found. Since this region contains the *nifB* promoter, it is not surprising that this nucleotide sequence is conserved. Most of the difference upstream of *nifB* is due to the presence of a 40 bp insertion in *A. azollae* 1a that is not present in *Anabaena* 7120 (Fig. 2).

The intergenic regions, which are defined here as the nucleotide sequence between one coding region and the adjacent coding region and include the stop codon, contain some very interesting features. Most striking is the occurrence and diversity of short tandemly repeated repetitive (STRR) sequences. These sequences are a prominent feature in heterocyst-forming cyanobacteria (Mazel *et al.*, 1990). They have been noted in the *nifB* operon of *Anabaena* 7120 (Mulligan & Haselkorn, 1989) and three STRR sequences were described in *Calothrix* sp. PCC 7601 (Mazel *et al.*, 1990).

Here, we describe six new STRR sequences which we have tentatively named following the convention of Mazel *et al.* (1990). Fig. 3 is an alignment of the intergenic regions of *A. azollae* 1a, *Anabaena* 7120 and *Anabaena* L31 in which the STRR sequences are shown. In some cases, the STRR sequences include the stop codon of the preceding gene; hence its inclusion in our definition of an intergenic region.

A. azollae 1a has a greater abundance and variety of STRR sequences in the *nifB* operon than does *Anabaena* 7120. In the intergenic region between *nifB* and *fdxN*, the *A. azollae* 1a genome contains two sets of STRR sequences even though this interval is 40 nucleotides shorter than the corresponding interval of *Anabaena* 7120. Five copies

/Hinc2
1 AACAAATAGCCCAACAGCCCTTGAATACTTGTGTAGTTGGGTTACAAATGAGTACAATCAGCTCCTGAACCCCTTCCATAAATACTCGTACCCCAACTAGAAAATACTAGAACCCTTA 120

/Dra1
121 CCTGGAAAAATTTTAAAAATTTCTCAAAACGTCCTTGAAATCATCATTTGAGATTTTGAGTATAACTGTAAATAAATCCAATAAAATTTCTATTCCAGAGATACAAACTGTAAATAGTCGAAGA 240

241 CGACTGCTATAAATCTTATATCGCCAATCACTTAAACGCTTCTACATAAAACGCAAGCAAAAGTGAGTGGAGGGATGAGATAGCACCATGCCATCTGGAATAGAAGTCGCGCCAGTTG 360

/Hinc2
361 CTACAGTTCGGGAACCCGCCAACGCCCTGGCTTGGCTGTAAACACATTCTACCAAGACACCAAGTTGACACACACAGTCCAAAACCAAGGACTTTATAGCTGCCCTACTCAGCGAA 480

481 GCCGCTACAGGCAATGTGGCAGACGCGACTTCAATTCTTCGGGCAACACAATCAGCAACGGCATCTCCACAGTGGCAGCTAGTCCACGACATTTCGGAATTCATGACACCACAG 600
nifB> M T P P V

601 TTACAGGCTCTTCCGTTACTGAATCGACACCTACCAAAGCAAAATCAGGTGGTTCGGGATGCGACACACGACACCACCGTGGAAATGGACGAAAAGCTCCAAAGACGTATTGCTAAACATC 720
T G S S V T E S T P T K A K S G G C G C D T S T T V E M D E K L Q E R I A K H P

721 CCTGCTACAGCGAAGAAGCTCACCACCTTACGCGAGAATGCACATTTGCTGTGACCTGCTTGCAACATTCAATGCAACTACTGCAACGAAAATATGACTGCGCTAACGAAAGCGTC 840
C Y S E E A H H H Y A R M H V A V A P A C N I Q C N Y C N R K Y D C A N E S R P

841 CTGGCGTAGTTAGTGAATTACTCACACGAAGAAGCAGCACACAAGTCTTAGTAATGCAGGCAAAATCCCCAATGACAGTTCTGGGAATGTGCGCTGGCGATCCTTGCGGA 960
G V V S E L L T P E E A A H K V L V I A G K I P Q M T V L G I A G P G D P L A N

961 ACCCAGAAAAGACTTTCGCGACCTTTGAATTGATTGCAGACAAAGCCCGAGATTAAGCTGTGCTATCAACCAATGGTTTGTGCTGCGGAAATATGCGATCGCATTAAACAACTAA 1080
P E K T F R T F E L I A D K A P D I K L C L S T N G L M L P E Y V D R I K Q L N

/EcoR1
1081 ATATCGACCAGCTTACTATTACCTAAATACTATTGATCCAGAAATCGGCGACAGATTTATTCTTGGGTTCACTACAAACGACAGCTTATAGAGGTGCGGAAGGCGCGAGAATTTCT 1200
I D H V T I T L N T I D P E I G A Q I Y S W V H Y K R R R Y R G A E G A R I L L

/EcoRV
1201 TGGAAAAACAGATGGAAGGCTTACAAGCTCTCAGAGAAGCTGATATCTTGTGTAAAGTCAATCTGTGATGATTTCCGGCATTAAACGACCAACATCTAGTTGAAGTCAACAAGATGATTC 1320
E K Q M E G L Q A L R E A D I L C K V N S V M I P G I N D Q H L V E V N K M I R

/Hinc2
1321 GGAACAAGTGCAATTTCTGCAACATTATGCCCTTGATTTCTGCACCAAGACCGGTACACACTTTGGCTTAACGTGCAACGGGACCTTCAACAAAAGAACTGAAGTCAGTGAAG 1440
E Q G A F L H N I M P L I S A P E H G T H F G L T G Q R G P S Q K E L K S V Q D

1441 ACCAATGTTCTGGCAACATGAAATGATGCGCCATTGCGCCAGTGTGCTGCTGATGCTGTAGGCTTGTAGGAGAAGACCGCAGCAGGAATTTACCAAGATAAATTCCTCGAAATGG 1560
Q C S G N M K M M R H C R Q C R A D A V G L L G E D R S Q E P T K D K F L E M A

/Sca1
1561 CTCCAGAAATGACTTTGACAAACGTCAGAAGTCCACGAAGGTATTGAGAAATTTAGAGTAGAACTAAAGTAGCCAAAGAAAAGTACTAGTGGCAAGAAAACAGCTAGCAATC 1680
P E Y D F D K R Q E V H E G I E K F R V E L K V A K E K V L A G K E K T A S N P

/Hinc2
1681 CTAAATCTTAGTTGCAATAGCCACCAAGGCGCGGATAGTTAAACCAACTTCGGCCATGCAAGGAATTTAGGTTTACGAAGTAGACGGTAGTGAAGTTGCTTCGTCAGTCACC 1800
K I L V A I A T K G G G L V N Q H F G H A K E F Q V Y E V D G S E V R F V S H R

/Ssp1
1801 GCAAGGTTGATCATTATTGTCAAGGTGATACGCGAAGAAGCCACATTTGACAATATTGTTAAACTATCGCAGATTGTAAAGCAGTTTGGTTTCCAAGATTGGCGAATCTCCCAAAG 1920
K V D H Y C Q G G Y G E E A T F D N I V K T I A D C K A V L V S K I G E S P K E

/Acc1
1921 AAAAAGTCTCAAGCGGTATACAGACTGTTGAAGCTTACGACGTGATTGAGAAGGTTGCTTTAGAGTTTACGAGCAATGGAATAAGGGCTAATGAGTAATAGGTAATGGGTAATAGG 2040
K L L Q A G I Q T V E A Y D V I E K V A L E F Y E Q W N K G * *

2041 TAATGGAGAGAAAATCATCAATTACCAATTACCAATTACCAATCCCACTCATCCAATCAACAAGGAGAATAATCATGGCTTACACAATTACTAGCCAATGTATTTCTCTG 2160
fdx> M A Y T I T S Q C I S C

2161 CAAGCTCTGTTGCTGTATGCCCCACTGGTGCAATTAAGGTGCGCGAAGACGACGACCTGATGACCAAGCACTGTGTACAAATTCGGTTGATAGCGTTACACCGTACCTCAATG 2280
K L C S S V C P T G A I K V A E D G Q H W I D Q A L C T N C V D S V H T V P Q C

2281 TAAAGCTGGCTGCTCCACTTGGCATGGTGGCTTAAAGTACCTAGCGATTATTTGGGAAGGCTGGTTTGTCAACTACAACGAGTTATAGCGAAATGACAAAAACAGACTATTGGGA 2400
K A G C N T A L V T I M Y A N N E T G T V F P I E E I G K R V K E R G A I F H V

2401 ACGTTGGTTAATGTTATCTCAGAAATTTCCGAACAAATTCAAAGCATCAGGGTGAATCTTAGGGGTATAAAGATGAGTGTATTATCTCGATAAATAGCTACCCTAAGGTA 2520
R W F N C Y S Q K F S E Q I Q K H Q G E I L G V *
nifS> M S V I Y L D N N A T T K V

2521 GATCCAGAGGTTGTAGAGGCAATCATGCTTACCTGACCGATTATTACGGCAATCCTTCCAGTATGCACACCTTTGGTGGACAACCTGGGAAAGGAGTGAAGACAGCGAGAGAACAGTT 2640
D P E V V E A I M P Y L T D Y Y G N P S S M H T F G G Q L G K A V R T A R E Q V

2641 GCTGCCCTGTTGGGAGCCGATGAATCAGAAATGTTTTCAGAGTTGCGGAACCGAGGGTGATAATGCTGCCATTCGTGCGCATTTGTTAGCCCAACCGGAAAAACGCCACATCATTACA 2760
A A L L G A D E S E I V F T S C G T E G D N / A A I R A A L L A Q P E K R H I I T

/Xba1
2761 ACCCAGGTTGAACACCCCGCAGCTTTAAATGTCTGCAAAATAGAAAACCAAGGCTACACTGTTACCTATCTTTCCGGTAAATGGTCACGGGCAATTGGATCTAGATGAACAGAACCC 2880
T Q V E H P A V L N V C K Q L E T Q G Y T V T Y L S V N G H G Q L D L D E L E A

2881 TCGCTGACAGGTAACACCGCCCTGGTGACAATTATGATGCGAACAACGAAACCGGGAGCTGATTCCCAATCGAAGAGATTGGGAAACGAGTTAAGGAACGTCGGCGCAATTTTCATGTG 3000
S L T G N P T A L V T I M Y A N N E T G T V F P I E E I G K R V K E R G A I F H V

/Cla1
3001 GATCGGGTGCAAGCAGTAGTGAAGTACCTCTGAATATGAAGACGACACCATCTGATATGTTAACCATTCTGGTCAACAAATCCACGACCCCAAGGTTATGGCGCTTTGTATGTGCGCT 3120
D A V Q A V G K I P L N M K T S T I D M L T I S G H K I H A P K G I G A L Y V R

3121 CGCGGTGTGAGATTCCGTCCTTGTGATTTGGTGGACACCAAGACGTGTCGCGTGCAGGGACAGAGAATGTACCCGGAATTTGTCGGCTTAGGTAAGCCGCGAGATTAGAATTAATT 3240
R G V R F R P L L I G G H Q E R G R R A G T E N V P G I V G L G K A A E L E L I

/Hinc2
3241 CATATCGAAACAGCGAATTAAGAAAGAAACAGGCTGCGCGATCGCCTAGAACAAACCTTACTCGCTAAATCCCTGACTGCGAAGTTAACGGTGACGTTACGAGAGATTGCCAACACC 3360
H I E T A I K K E T R L R D R L E Q T L L A K I P D C E V N G D V T Q R L P N T

3361 ACCAATCGGCTTCAAATACATTGAAGGCGAAGCGATTCTGCTCTCTTAAACAAATATGGCATCTGTGCGTGTGCTGGTTCGGCTTGTACCTCTGGTTCGCTGGAACCATCCACGTC 3480
T N I G F K Y I E G E A I L L S L N K Y G I C A S S G S A C T S G S L E P S H V

/Cla1
3481 CTCGGGCAATGGGTTTACCATACACCACCTCCACGGTTCGATTTCGCTTCACTCTTGTGCTACACTACAGAAGCTCAATTCGATCGCGTCATCGAAGTCATCGCCGAAATGTGCGAA 3600
L R A M G L P Y T T L H G S I R F S L C R Y T T E A Q I D R V I E V M P E I V E

Fig. 2. For legend see facing page.

of the sequence TAAT(^A/_G)GG (which we will name STRR4) are followed by eight copies of the sequence CAAT(^C/_T)AC (STRR5) (Fig. 3). Counterparts to the STRR4 sequences are not identifiable in *Anabaena* 7120

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Table 1. Comparison of *A. azollae* 1a *nif* genes with those of the corresponding *nif* genes of *Anabaena* 7120

Gene	Length (bp)	Nucleotide identity (%)	Amino acid identity (%)*	No. of amino acid changes†
<i>nifB</i>	1425	97.4	99.6	2 (0)
<i>fdxN</i>	348	93.4	93.1	7 (5)
<i>nifS</i>	1200	95.8	99.0	4 (2)
<i>nifU</i>	900	94.9	98.3	5 (3)
<i>nifH</i>	885	93.9	95.9	10 (4)

* Pairwise identity with the corresponding gene product from *Anabaena* 7120.

† The number of conservative changes is indicated in parentheses.

sequences are similar to STRR1 sequences but can be distinguished from them. STRR4 sequences have three consecutive cytidine or guanine nucleotides but differ in the sequence which follows; STRR5 sequences lack three consecutive cytidine nucleotides.

The *nifS*–*nifU* intergenic region is similar in length in both *A. azollae* 1a and *Anabaena* 7120. *A. azollae* 1a contains three sets of STRR sequences in this region (Fig. 3b). Five copies of the sequence (A/G)GGGACT are followed by five copies of A(A/G)T(C/T)CCC. Both of these are STRR1 sequences (Mazel *et al.*, 1990), which could form a stem-loop structure with a free energy of -36.6 kcal mol $^{-1}$ (-153.1 kJ mol $^{-1}$) in transcribed RNA. The third set of STRR sequences consists of four repeats of the sequence C(C/T)AAAAAT (STRR6). By contrast, *Anabaena* 7120 has six STRR1 sequences in the *nifS*–*nifU* intergenic region corresponding to the second set in *A. azollae* 1a. In *Anabaena* 7120, the region corresponding to the first set of *A. azollae* 1a STRR1 sequences is best characterized as a purine-rich region with five repeats of the general sequence R₆Y, where R stands for a purine and Y for a pyrimidine. This feature was not described previously (Mulligan & Haselkorn, 1989). There are no

counterparts to the STRR6 sequences in *Anabaena* 7120 (Fig. 3b).

In contrast to the above intergenic regions, the *nifU*–*nifH* region in *A. azollae* 1a is longer than that in *Anabaena* 7120 (Fig. 3c). It contains three sets of STRR sequences: seven copies of TAGTCA(A/T) (STRR3); three copies of ATGGACT (STRR7); and two copies of CA(T/G)CACT (STRR8). The *nifU*–*nifH* intergenic regions of *A. azollae* 1a and *Nostoc* 6720 are identical. Our analysis of the STRR structure of this region differs from that of Beesley *et al.* (1994). STRR sequences have not been described in the *nifU*–*nifH* intergenic region of *Anabaena* 7120 before, but four copies of the sequence CAGTTAA (STRR9) occur (Fig. 3c). Six copies of STRR9 occur in *Anabaena* L31 (Murphy *et al.*, 1993). The STRR3 and STRR9 sequences are similar.

There are no tandemly repeating sequences in the intergenic region between *nifH* and *nifD* in either *A. azollae* 1a or *Anabaena* 7120, although a poorly conserved repeat was noted in *Anabaena* L31 (Murphy *et al.*, 1993). Instead, this region is distinctive for the presence of a purine-rich tract followed immediately by a pyrimidine-rich tract of nucleotides. In *A. azollae* 1a, a 33 bp 85 % purine-rich region is followed by a 71 bp 90 % pyrimidine-rich region. In *Anabaena* 7120, a 31 bp 85 % purine-rich tract is followed by an 82 % pyrimidine-rich tract. In *Anabaena* L31 a 32 bp 81 % purine-rich tract is followed by a 71 bp 83 % pyrimidine-rich region (Murphy *et al.*, 1993). *Nostoc* 6720 has similar regions (Beesley *et al.*, 1994).

Other sequences

In *Anabaena* 7120, the *fdxN* gene in the vegetative cell chromosome is interrupted by a 55 kb excisable element (Golden *et al.*, 1987, 1988; Mulligan *et al.*, 1988; Mulligan & Haselkorn, 1989). The *fdxN* gene of *A. azollae* 1a is intact in vegetative cell DNA and it contains a sequence that is identical to one of the *Anabaena* 7120 rearrangement breakpoints (Golden *et al.*, 1987). The nucleotide sequence of the 5 bp core sequence as well as 18 bp on each side are completely conserved. All of the conserved bases that

Table 2. Pairwise comparisons of *nifH* gene sequences to *nifH* of *A. azollae* 1a

Organism	Nucleotide identity (%)*	Amino acid identity (%)*	No. of amino acid changes†	Reference
<i>Anabaena</i> 7120	93.9	95.9	10 (4)	Mevarech <i>et al.</i> (1980)
<i>Anabaena</i> L31	94.0	98.3	4 (2)	Murphy <i>et al.</i> (1993)
<i>Nostoc</i> 6720	99.7	99.7	1 (0)	Beesley <i>et al.</i> (1994)
<i>Plectonema boryanum</i>	77.7	84.5	41 (13)	Fujita <i>et al.</i> (1991)

* Pairwise identity with the *nifH* gene or gene product of *A. azollae* 1a.

† The number of conservative changes is indicated in parentheses.

(a) *nifB-fdxN* (b) *nifS-nifU* (c) *nifU-nifH*

Fig. 3. Alignment of the nucleotide sequence of the intergenic regions. Each of the intergenic regions from *A. azollae* 1a (*azollae*) was aligned with the corresponding region from *Anabaena* 7120 (7120) or *Anabaena* L31 (L31) using the ALIGN program of the FASTA program package. Dashes indicate gaps that were introduced during alignment. STRR sequences are indicated by arrows above or below the nucleotide sequence; different STRR sequences are denoted by the number within each arrow. The sequences are numbered beginning with the start of the sequence that is listed. (a) Alignment of the complete nucleotide sequence of the *nifB-fdxN* intergenic region (nucleotides 2013–2125 in Fig. 2). (b) Alignment of the complete nucleotide sequence of the *nifS-nifU* intergenic region (3679–3800). (c) Alignment of a partial nucleotide sequence of the *nifU-nifH* intergenic region (4701–4846). The location of the probable *nifH* transcription start-point is also indicated (●).

surround the central 5 bp core in *Anabaena* 7120 (Golden *et al.*, 1987) are conserved in the *A. azollae* 1a sequence.

We searched the *A. azollae* 1a nucleotide sequence for the presence of integration host factor (IHF) binding sites. Two moderately high scoring sites were found. One is located near the *nifB* promoter and is centred approximately 65 bp upstream of the putative *nifB* transcription startpoint (Fig. 2). This site has a similarity score of 56.5. A second site, which is in the opposite orientation, located near the end of the *nifU* gene (Fig. 2) is centred 470 bp upstream of the *nifH* promoter. This site has a similarity score of 53.8. The similarity scores of known IHF sites range from 46 to 77 (Goodrich *et al.*, 1990) with, for example, the bacteriophage lambda *attP* H2 site having a similarity score of 56.3.

Expression in *A. azollae* 1a

Expression of the *nifB* and *nifH* operons in *A. azollae* 1a was examined first by blot hybridization analysis. A *nifH*-specific RNA probe was used to probe membrane blots of RNA isolated from non-induced (N-replete) and induced (N-depleted) cultures of *A. azollae* 1a. *nifH*-specific RNA was not present in non-induced cultures but at least two specific transcripts were detectable from induced cultures (Fig. 4a). The estimated sizes of these transcripts (1.2 kb and 2.6 kb) could correspond to RNA species that contain

nifH and *nifHD*, respectively. A larger transcript that could correspond to a *nifHDK* transcript was observed on some other blots (not shown). Similar patterns and sizes of transcript have been reported for *nifH* transcription in *Anabaena* 7120 (Haselkorn *et al.*, 1983, 1986). We were unable to detect *nifB*-specific transcripts by blot hybridization analysis.

The startpoint of transcription for both the *nifB* and *nifH* operons was determined by primer extension analysis. When a *nifH*-specific oligonucleotide primer was extended, three major products that differed in size by only a few bases were observed (Fig. 4b). All three products map within a region of five consecutive thymine nucleotides. Since this region potentially forms an unstable RNA:DNA hybrid, it is likely that the true startpoint is elsewhere, perhaps the guanine nucleotide at position 4836 (Fig. 2). Similar results were obtained previously for S1 nuclease mapping of the *Anabaena* 7120 *nifH* transcription startpoint (Haselkorn *et al.*, 1983).

The results of primer extension mapping of the *nifB* transcription startpoint were not as clear cut. When a *nifB*-specific oligonucleotide primer was used, extension products were observed using RNA isolated from both uninduced and induced filaments (Fig. 4c). One of the products, however, was more abundant when RNA from induced filaments was used. If this product is due to a

Fig. 4. Transcription of the *nifH* and *nifB* operons. RNA isolated from cultures of *A. azollae* 1a grown under nitrogen replete (+) or nitrogen starved (–) conditions was used for RNA:RNA hybridization analysis and primer extension experiments. (a) RNA:RNA analysis of RNA probed with a *nifH*-specific RNA probe. The estimated sizes (in kb) of the two major hybridizing bands are shown. The locations of rRNA bands are shown by the dots. (b) Primer extension analysis using the *nifH1* primer (Fig. 2). Arrows indicate the positions of extended products. A DNA sequencing ladder was used to calibrate the length of the extended products; sizes of the sequencing bands are indicated. (c) Primer extension analysis using the *nifB4* primer (Fig. 2). Arrows indicate the positions of extended products. A DNA sequencing ladder was generated using the same primer; the sequence of the region surrounding the major extended product is shown and the putative startpoint is indicated by a dot.

genuine induced transcript, then the startpoint mapped by it is the same as that previously observed in *Anabaena* 7120 (Mulligan & Haselkorn, 1989).

DISCUSSION

STRR sequences in cyanobacteria

A major feature of interest in cyanobacterial genomes may well be the variety of STRR sequences that are found mostly in the non-coding regions rather than any variation of the coding regions. The function of the STRR sequences is not known. It has been suggested that they may regulate transcription termination (Haselkorn & Buikema, 1992) or be the target of DNA-binding proteins responsible for chromosome maintenance in the cell (Mazel *et al.*, 1990). Alternatively, it is possible that these sequences serve as binding sites for proteins involved in mRNA stability or processing.

The prevalence and conservation of STRR sequences in heterocyst-forming cyanobacteria suggests that these sequences have some important function. And yet, the number and sequence of STRR sequences at comparable locations in closely related strains, such as the *nifU*–*nifH* intergenic region of *Anabaena* 7120, *A. azollae* 1a and *Anabaena* L31, are quite different. It is unlikely that these sequences play a direct role in the regulation of gene expression. Otherwise, it would be necessary to propose that different regulatory mechanisms exist in each strain of heterocyst-forming cyanobacteria depending on its exact complement of STRR sequences. In any case, the high conservation of STRR sequences suggests that they may be valuable taxonomic tools for the study of heterocyst-forming cyanobacteria. It may even be more informative

to analyse and compare the intergenic regions (using, for example, PCR techniques) than the coding regions.

One clue to the function and/or the origin of STRR sequences may come from *Nostoc commune* and related cyanobacteria. *N. commune* UTEX 584 is notable for the presence of a cyanoglobin gene between *nifU* and *nifH* (Potts *et al.*, 1992). A second cyanobacterial strain, *Nostoc* 8820, that contains a *glbN* gene has been sequenced in our laboratory (T. J. Belbin & M. E. Mulligan, unpublished results). There are no STRR sequences in either the *nifU*–*glbN* or the *glbN*–*nifH* intergenic regions of *Nostoc* 8820. The presence of STRR sequences in many cyanobacteria (at this location, at least) might have arisen as a result of the loss of *glbN* from an ancestral strain or they may have been lost as a result of the gain of *glbN*. In either of these cases, STRR sequences would reflect unexpected genome plasticity. A similar observation was made in the case of the variation in STRR sequences found in the *nifJ* gene (Bauer *et al.*, 1993).

nif gene expression in cyanobacteria

Our motivation for commencing this work was the desire to collect nucleotide sequence data for *nif* promoters in different heterocyst-forming cyanobacteria. It is not possible to define a *nif* promoter consensus sequence from the results reported in this paper with any more certainty than has been reported previously (Borthakur *et al.*, 1990; Mulligan & Haselkorn, 1989). However, it is possible to delimit the extent of nucleotide sequence that must contain the core *nifH* promoter. Fig. 3(c) shows that the alignment of nucleotide sequence in the vicinity of the *nifH* promoter extends only 38 bp upstream of the transcription startpoint before the presence of STRR

sequences in the *A. azollae* 1a genome precludes all further alignment. All sequence elements that are required to define the *nifH* promoter should occur within these 38 bp. Although there are no STRR sequences upstream of the *nifB* promoter that can be used in the same way, it is interesting that the distance between the *nifB* transcription startpoint and the adjacent putative IHF binding site is also 40 bp.

If the IHF binding sites near the *nifB* and *nifH* promoters are functional sites in cyanobacteria, they may have interesting implications for cyanobacterial *nif* transcription. IHF is required for *nif*-specific transcription in many purple bacteria, where it functions to bend DNA, permitting close contact between an activator protein (e.g. NifA) and RNA polymerase containing the σ^{54} subunit (Weiss *et al.*, 1992). The biochemical components required for *nif*-specific transcription in cyanobacteria, however, have not yet been characterized.

Identity of *A. azollae* 1a

The suspicion that isolates of cyanobacteria cultured from the *Azolla* symbiosis might not represent the true symbiotic cyanobacterium first arose when the *nif*-specific Southern hybridization patterns of freshly isolated symbionts from four geographically diverse *Enaazolla* species were examined (Franché & Cohen-Bazire, 1985). All four were closely related to one another but were not related to a cultured isolate from *Azolla filiculoides*. Every study of cultured isolates has since obtained the same result: cultured isolates do not resemble the symbiont (Franché & Cohen-Bazire, 1987; Gebhardt & Nierzwicki-Bauer, 1991; Meeks *et al.*, 1988). Gebhardt & Nierzwicki-Bauer (1991) posed the question: what is actually being cultured? On the basis of morphology, lectin binding and the characterization of allozymes, Zimmerman *et al.* (1989) concluded that five independent cultured isolates were identical to each other and to *Anabaena* 29413. A similar conclusion was reached from RFLP analysis of three independent isolates (Gebhardt & Nierzwicki-Bauer, 1991). Restriction digests of *A. azollae* 1a and *Anabaena* 29413 DNA are indistinguishable (not shown) and Southern blots of these digests always show identical patterns. We have observed this using both *nifH* and *nifB* probes (not shown). Others have observed the same result, for example with a *betR* probe (Buikema & Haselkorn, 1991), with a *nifJ* probe (Bauer *et al.*, 1993), and with an STRR probe (Bauer *et al.*, 1993). Finally, the nucleotide sequence of the *A. azollae* 1a *nif* genes is more than 99% identical with that of *Anabaena* 29413 (Lyons & Thiel, 1995).

It is not clear, however, where the cultured isolates come from. Are they minor symbionts or are they surface contaminants? It has been argued that it is unlikely that the cultured isolates are due to contaminating epiphytic organisms associated with the surface of the ferns precisely because the same strain can be cultured from geographically distinct *Azolla* spp. (Gebhardt & Nierzwicki-Bauer, 1991). However, a tenacious *Anabaena* epiphyte, which resisted surface sterilization procedures, was frequently

found on the surfaces of root nodules from the cycad *Zamia integrifolia* (Zimmerman & Rosen, 1992), a different example of a cyanobacterial symbiosis. The very high sequence similarity between the naturally free-living species *Anabaena* 29413 and cultured isolates such as *Anabaena azollae* 1a suggests that further study is needed to resolve the issue.

NOTE ADDED IN PROOF

The cyanobacterium studied by Beesley *et al.* (1994) as *Nostoc* sp. PCC 6720 should be considered to be *Anabaena variabilis* PCC 7937 (R. J. Smith, personal communication). All references to and comparisons with *Nostoc* 6720 in the paper should therefore be interpreted as being with *Anabaena variabilis* PCC 7937.

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