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Growth of the cyanobacterium *Anabaena* on molecular nitrogen: NifJ is required when iron is limited

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ABSTRACT **The *nifJ* gene of *Klebsiella pneumoniae* encodes an oxidoreductase required for the transfer of electrons from pyruvate to flavodoxin, which reduces nitrogenase. The *nifJ* gene of *Anabaena* 7120, isolated from a cosmid bank, was found to contain an open reading frame encoding a 1197-aa protein. The deduced amino acid sequence shows 50% identity to the *Klebsiella* homolog. The *nifJ* gene in *Anabaena* 7120 was inactivated by chromosomal interruption. The resulting mutant was unable to grow on medium depleted of both iron and combined nitrogen but grew normally, fixing nitrogen, when iron was present. NifJ transcripts of 2.7 and 4.3 kb are induced by iron depletion irrespective of nitrogen status. One particular stretch of the *Anabaena* 7120 *nifJ* gene encodes 12 aa with no complementary matches in the *Klebsiella* protein. This insert contains five tandem repeats of the heptamer CCCCAGT. These heptamers, as well as heptamers and octamers of other related sequences, have been located in a number of cyanobacterial genomes but are usually not found within the coding region of a gene. The site of the *Anabaena* 7120 heptamers in the *nifJ* genes of other filamentous cyanobacteria contains a surprising diversity of repeated sequences, both octamers and heptamers. The corresponding protein inserts range in length from 1 to 21 aa, relative to *Klebsiella* NifJ.**

Biological nitrogen fixation is catalyzed by the nitrogenase enzyme complex. Dinitrogen is bound to the molybdenum–iron cofactor of nitrogenase, where it is reduced by three successive two-electron two-proton transfers. The electrons are donated by an iron–sulfur center in nitrogenase reductase. That center can be reduced, in turn, by a variety of redox proteins, such as flavodoxins or ferredoxins, that link nitrogen fixation to sources of reductant from glycolysis or photosynthesis (1).

The earliest complete picture of the genes required for nitrogen fixation was obtained for *Klebsiella pneumoniae* (2). Two of these genes, *nifJ* and *nifF*, encode proteins involved in electron transfer to nitrogenase. NifF is a flavodoxin and NifJ is a pyruvate:flavodoxin oxidoreductase. Mutations in either of these genes reduce nitrogenase activity ≈20-fold, sufficient to prevent growth on N_2 as the nitrogen source (3). The residual activity of nitrogenase in these mutants is probably due to a low constitutive level of ferredoxin.

In photosynthetic bacteria and cyanobacteria, the *nifF* and *nifJ* genes are dispensable because photosynthesis produces enough reduced ferredoxin to support nitrogen fixation. Heterocysts of the cyanobacterium *Anabaena* 7120 contain a unique ferredoxin, the *fdxH* gene product, that is the principal electron donor to nitrogenase in that organism (4). *Anabaena* strains contain a flavodoxin whose abundance can be increased by iron starvation (5), but an early report that this flavodoxin can donate electrons to *Anabaena* nitrogenase (6) has not been confirmed (7). To date, both biochemical and genetic approaches have failed to identify equivalents of *nifJ* or *nifF* in photosynthetic prokaryotes, with two exceptions. A homologue of *nifJ* was detected in *Rhodospirillum rubrum*; the protein has been purified, and the gene has been sequenced, but its requirement for nitrogen fixation has not been established (8). The second exception is described in this report. *Anabaena* 7120 contains a *nifJ* gene that is dispensable for growth on normal cyanobacterial medium but is essential for growth on medium depleted of iron and combined nitrogen. Growth of cyanobacteria on iron-depleted medium generally results in replacement of ferredoxin with flavodoxin (9, 10). Thus, the electron transfer path from pyruvate to flavodoxin to nitrogenase may operate in *Anabaena* as an alternative to a path through ferredoxin when iron is limiting. Additionally, the *Anabaena nifJ* gene contains short tandemly repeated sequences within the open reading frame (ORF), resulting in the apparent insertion of an exceptional peptide in the interior of the protein. Other strains of cyanobacteria with *nifJ* genes also have inserted peptides in this same site but the inserted peptides vary in their sizes and sequences.

MATERIALS AND METHODS

Culture Conditions. *Anabaena* 7120 was grown in modified Kratz and Myers medium C (termed KM) or in medium BG-11 (11, 12). Half of the Na_2HPO_4, 1.125 mM, was replaced with K_2HPO_4 in the KM medium. Nitrogen sources were either 2.5 mM $(NH_4)_2SO_4$ or 17.6 mM $NaNO_3$. Plates contained KM or BG-11 with 17.6 mM $NaNO_3$ (if a nitrogen source was included) and 1.3% BBL purified agar. Iron-limited plates contained BG-11 without the ferric ammonium citrate component. Flame atomic absorption spectrophotometric analysis of this medium found no detectable iron. Aqueous extracts of the agar also yielded no detectable iron by atomic absorption spectrophotometry. Small cultures were grown under cool white fluorescent light at 30–40 μE per m^2 per sec (where E is einstein; 1E = 1 mol of photons) at 25–30°C in an incubator gassed with 2% CO_2/98% air. Large-scale cultures were bubbled with 2% CO_2/98% air. For *Anabaena* strains containing recombinant plasmids, selection was made with neomycin at 30 μg/ml. After chromosomal insertion of the plasmid, selection was maintained with neomycin at 100 μg/ml. For selective growth of *Escherichia coli* DH5α carrying plasmids, ampicillin at 100 μg/ml and kanamycin sulfate at 50 μg/ml were used.

Molecular Biology Techniques. All cloning, DNA manipulations, and gel electrophoresis were as described (13). Southern and Northern blot hybridizations, the preparation of nested deletions, and DNA sequencing were as described (14).

RNA Isolation. Large-scale cultures of *Anabaena* 7120 were induced to differentiate by transferring cells from KM with NH_4 to KM without combined nitrogen. For iron limitation, cells were grown to midlogarithmic phase (chlorophyll at 2–6 μg/ml; 0.7–2 × 10^7 cells per ml) in BG-11

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Abbreviation: ORF, open reading frame.

with NH_4 and then transferred to BG-11 without combined nitrogen or ferric ammonium citrate. One-liter cultures harvested at 6-hr intervals and purified heterocysts from 5 liters of 24-hr-induced cultures were used to prepare total RNA as described by Golden *et al.* (15). For the Northern blot in Fig. 2, the RNA preparation was changed as follows: aurin tricarboxylic acid was substituted for vanadyl ribonucleoside as the nuclease inhibitor and a 4 M LiCl precipitation was added to separate RNA from DNA and polysaccharides. An *Anabaena* flavodoxin gene probe was generously provided by N. Straus (University of Toronto) (16).

Heterocyst-Specific Subtracted cDNA Library. The details of construction of developmental stage-specific cDNA libraries are described elsewhere (17). The heterocyst-specific library from which a fragment of the *nifJ* gene was isolated was prepared as follows. Total heterocyst RNA was prepared as described (15) and then used as a template for first-strand cDNA synthesis with reverse transcriptase, priming with a complete set of random hexamers (Pharmacia). RNA was removed by treatment with RNase H and RNase A and then the nucleases were removed by phenol extraction. Next, a 10-fold excess of vegetative-cell RNA was annealed to the DNA, to prevent those cDNAs corresponding to vegetative-cell RNA from serving as template for second-strand cDNA synthesis. The second-strand DNA synthesis was carried out with the Klenow fragment of DNA polymerase I, using the same collection of random hexamers to prime. The resulting double-stranded DNA fragments were filled-in with T4 DNA polymerase, ligated into appropriately cut pUC19, and used to transform *E. coli* DH5α.

Cloning and Sequence Determination of the *nifJ* Gene. Fifty clones from the heterocyst-specific library were sequenced using the pUC19 forward primer. DNA and possible ORFs were compared to known sequences in GenBank Release 7.0. One cDNA from the library (clone 83) showed similarity to part of the *nifJ* gene of *K. pneumonia*. It was used to probe a cosmid bank of wild-type *Anabaena* 7120 genomic DNA fragments (18). Nine cosmids were found to have regions complementary to the cDNA. One was chosen for subcloning *nifJ*. *Ssp* I fragments of this insert were subcloned into pUC19 and pUC18 and sequenced. These fragments were 300, 500, 600, 700, and 2400 bp long. The largest fragment was sequenced by making nested deletions with the Erase-a-Base kit from Promega Biotech. The complete sequence is available from GenBank (accession no. L14925).

Construction of a *nifJ* Mutant of *Anabaena* 7120. A pUC19-derived plasmid, pCCB1002, containing the 2.4-kb *Ssp* I fragment internal to the *nifJ* gene, was fused to a shuttle vector for transfer to *Anabaena* by conjugation. Construction of the vector, which has enhanced expression of the neomycin phosphotransferase gene but cannot replicate in *Anabaena*, is described elsewhere (17). After conjugation as described (19), exconjugants were selected on plates containing KM-NO_3 and neomycin (100 μg/ml). Individual colonies were picked, grown in liquid BG-11 with NO_3 for several days, and then streaked on plates with or without iron and with and without combined nitrogen to score their phenotypes (Table 1). For each strain, interruption of the *nifJ* gene by insertion of the entire plasmid was confirmed by Southern blot hybridization.

Amplification of *nifJ* Inserts by PCR. Four primers were made flanking the heptamer repeat region in *Anabaena* 7120 *nifJ* located at positions 1150–1200 bp from the start of the ORF. These primers are shown in Table 2. Chromosomal DNAs from related *Anabaena* and *Nostoc* strains, described earlier (14), were used as templates for the PCR. The amplified bands were cloned into pUC19 and sequenced.

Table 1. Growth of wild-type and *nifJ* mutants of *Anabaena* 7120

Growth condition	Wild type	*nifJ* mutants
BG-11 + NO_3 + Fe	+	+
BG-11_0 + Fe	+	+
BG-11 + NO_3 − Fe	+	+
BG-11_0 − Fe	+	−

Cultures were incubated on agar plates for 2 weeks in an incubator under fluorescent lighting in 2% CO_2/98% air. BG-11_0 has no added NO_3 or NH_4^+. Medium with Fe contained 32 μM ferric ammonium citrate. Three independent isolates of *nifJ* insertional mutants were used.

RESULTS

Identification of a *nifJ*-Like Gene. *Anabaena* 7120 contains a gene related to *K. pneumoniae nifJ*. This gene was discovered first as a fragment in a cDNA library corresponding to the RNAs present uniquely in heterocysts, the cells specialized for nitrogen fixation in filaments of *Anabaena*. Of the 50 fragments from that library that were sequenced, 1 fragment contained a stretch of 261 nt that, when translated, is highly similar to a portion of the *Klebsiella nifJ* gene product.

The putative *nifJ* cDNA was used to screen a cosmid library of *Anabaena* 7120 chromosomal DNA fragments. One cosmid identified by the probe was reduced by deletion and then *Ssp* I fragments spanning the entire ORF were subcloned and sequenced (Fig. 1). The ORF contains 1197 aa, with a predicted molecular weight of 132,166. With the exception of a 12-aa insertion in the *Anabaena* sequence, the *Anabaena* and *Klebsiella* NifJ sequences are very similar: 50% of the residues are identical and an additional 19% are similar. The two sets of four cysteines thought to accommodate Fe_4S_4 clusters in *Klebsiella* NifJ are present in *Anabaena* 7120 NifJ (20).

Requirement for Nitrogen Fixation. The wild-type *nifJ* gene in *Anabaena* 7120 was interrupted by chromosomal insertion of a plasmid carrying an internal fragment of the *nifJ* gene. The plasmid was transferred to *Anabaena* by conjugation with *E. coli*, as described (19). After conjugation, selection for neomycin-resistance yielded strains in which the entire plasmid is inserted into the chromosome within the *nifJ* coding sequence, causing a tandem duplication in which neither copy of the *nifJ* gene is complete. Southern blot hybridization was used to verify that every copy of the *nifJ* gene was interrupted in the neomycin-resistant strain (data not shown). The tandem duplication is unstable: relaxation of antibiotic selection allows the survival of cells in which recombination has removed the interrupting plasmid. Restoration of the wild-type *nifJ* gene organization in such revertants was confirmed by Southern blot hybridization (data not shown).

The ability of strains with interrupted *nifJ* genes to grow on plates containing various media is summarized in Table 1. *nifJ* is dispensable for growth on N_2 in the usual BG-11

Table 2. Sequences of PCR primers used to amplify the polymorphic region of *nifJ*

```
5'-C G   TT    A G    G    G
   TTT IGC IAG ATT  ATC  AAA  IAC  IGC-3'
5'-                  C              A    A
   ATI GCI GTI CTI GAT CGI ACI AAG GAG CCI GG-3'
5'-G ATC GCT GTC CTC GAC CGC AC-3'
5'-G TTA CAT CAT CAT TAA TCC CG-3'
```

Degenerate primers were used for *Anabaena* 7120, *Nostoc* MACR1, *Nostoc* 77S15, *Anabaena* L31, and *Anabaena azollae*. Nondegenerate primers were used for *Anabaena* 7118, *Anabaena* 7119, *Anabaena* 7120, *Nostoc* 29103, *Anabaena variabilis*, and *Nostoc* 840215.

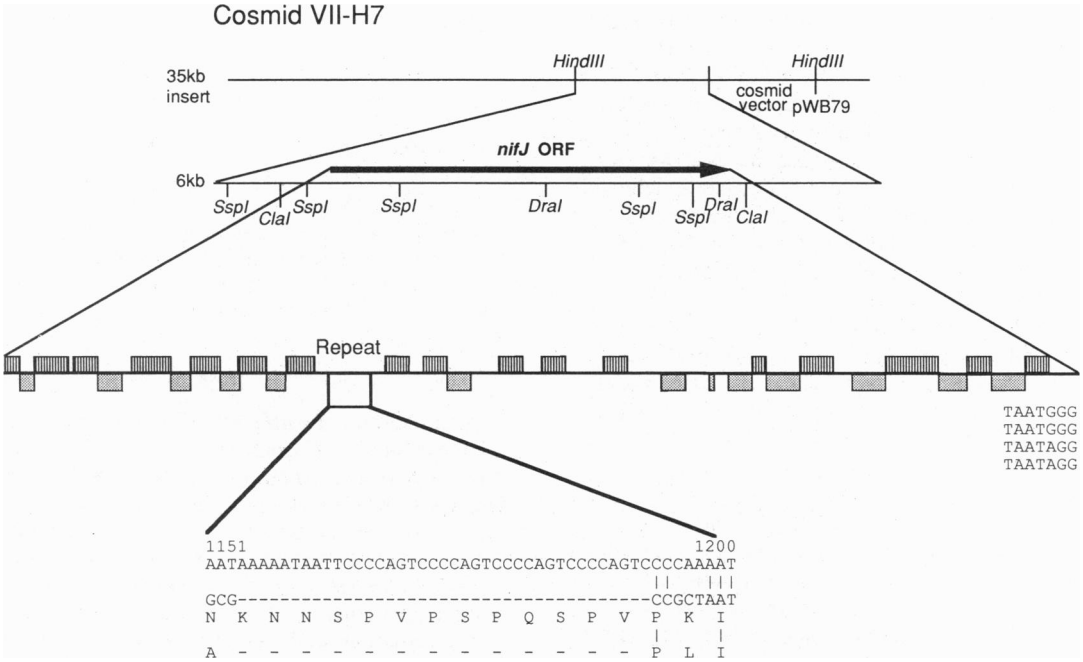

FIG. 1. Location of the *nifJ* gene on cosmid clone VII-H7. Representation of the homology between NifJ of *Anabaena* 7120 and *K. pneumoniae*. Vertically hatched box, area of high similarity; stippled box, area of low similarity. The DNA and protein sequences for the region of repeated DNA in *Anabaena* are shown matched to the corresponding region in *Klebsiella*. A repeated DNA motif located at the end of the *nifJ* gene is also shown.

medium or for growth on ammonia in low iron medium but is essential for growth on N_2 in low iron medium. When the interruption in *nifJ* is removed (by growth without neomycin), the ability to fix N_2 in a low concentration of iron is restored. These results suggest that the NifJ–flavodoxin pathway for electron transfer to nitrogenase operates in *Anabaena* 7120 under low iron conditions.

Expression of the *nifJ* and Flavodoxin Genes. Northern blot analysis utilizing RNA taken from cultures in the process of differentiating heterocysts indicated that the *nifJ* gene was not expressed in cultures containing iron. Fig. 2 shows a blot of vegetative-cell RNA and RNA from cultures induced for 24 hr, with or without ferric ammonium citrate added to the medium. The two transcripts of 4.3 and 2.7 kb are seen only in the iron-limited cultures, independent of the nitrogen status of the cells. Only the 4.3-kb RNA is large enough to cover the complete 3.6-kb coding region for NifJ. Similar results (data not shown) were obtained for a Northern blot probed with a fragment of the *Anabaena* 7120 flavodoxin gene, except that the 3.0-kb flavodoxin mRNA was faintly visible in the iron replete cultures as well.

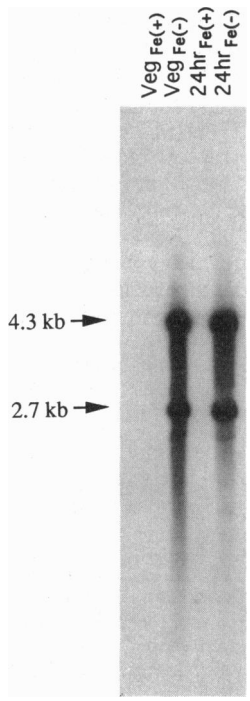

FIG. 2. Northern blot of RNA extracted from vegetative cells and from cultures induced to differentiate for 24 hr, with (+) and without (−) added iron. RNA (60 μg) was used per lane. Hybridization was done at 65°C overnight. The probe was the 2.4-kb *Ssp* I fragment internal to *nifJ* (Fig. 1).

Unusual Structure of the *nifJ* Gene. The *Anabaena* NifJ reading frame contains an insert of 12 aa not found in the *Klebsiella* NifJ sequence (Fig. 1). This insert corresponds to five tandemly repeated copies of the heptanucleotide sequence CCCCATG found in the *Anabaena* DNA. Noted previously, this heptamer is repeated between 4 and 30 times in many locations of the *Anabaena* 7120 chromosome (21–23). In the cases where the chromosomal locations were determined, the tandemly repeated elements are downstream of an ORF and are correlated with sites of transcription termination (22). In NifJ, the extra 12 aa are encoded by rarely used codons (C. Halling and R.H., unpublished data). Either the insertion is a recent event or there is very weak selection for frequently used codons in NifJ.

Repeated elements are widely distributed among filamentous cyanobacteria, sufficiently to be useful in taxonomic and ecological studies (24). Fig. 3 shows the results of Southern blot hybridization to DNA digests from several *Anabaena* and *Nostoc* strains, using either *nifJ* DNA or an oligonucleotide containing the tandemly repeated sequence as probe. The principal conclusions from these blots are that many strains contain a *nifJ*-related sequence, and most strains contain many sites for the tandemly repeated heptamer, but most fragments containing these repeats are of different sizes in the different strains. *Anabaena torulosa* DNA has the heptamer repeats but no detectable *nifJ*-related sequences, whereas *Nostoc* 77S15 and two other *Nostocs* have *nifJ*-related sequences but no CCCCAGT repeats. The only other strains that appear to contain *nifJ* and the heptamer repeat in the same fragment are the closely related strains *Anabaena*

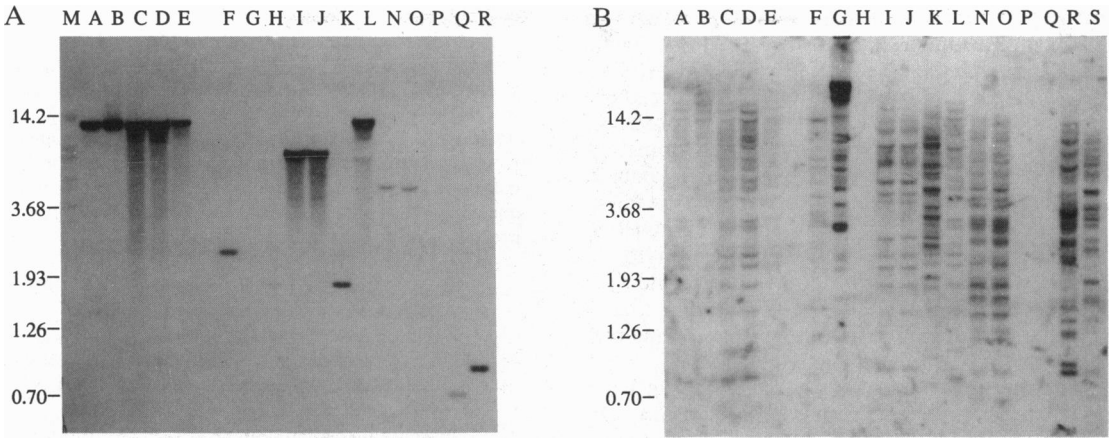

FIG. 3. Distribution of the *nifJ* gene and the CCCCAGT repeat in various cyanobacterial strains. All genomic DNAs were digested with *Hin*dIII. Hybridization was done at 65°C overnight. Lanes: M, λ *Bst*EII markers; A, *Anabaena* sp. PCC 7120; B, *Anabaena* sp. PCC 7120 strain 216 (mutated in the *hetR* gene); C, *Anabaena* sp. PCC 7118; D, *Anabaena* sp. PCC 7118 Rev (a Het+ revertant of strain 7118); E, *Anabaena* sp. M-131; F, *Anabaena* sp. L31; G, *Anabaena torulosa*; H, *Nostoc* sp. 77S15; I, *Anabaena variabilis* ATCC 29413; J, *Anabaena azollae* A1; K, *Anabaena* sp. CA ATCC 33047; L, *Anabaena* sp. PCC 7119; N, *Nostoc* sp. Mac 7911 R1; O, *Nostoc* sp. Mac 7911 R2; P, *Nostoc* sp. 7801; Q, *Nostoc* sp. ATCC 29107; R, *Nostoc* sp. 840215; S, *Calothrix* sp. PCC 7601. (*A*) Probed with the internal 2.4-kb *Ssp* I fragment of the *nifJ* gene. Note that the S lane is missing due to a mistake in trimming the figure. No band was visible in this lane. (*B*) Probed with an oligonucleotide based on the heptamer repeat internal to the *nifJ* gene: 5′-GGGGACTGGGGACTGGGGACTGGGG-3′.

7118, 7119, and M131. At the resolution of the Southern blot, it was not possible to determine whether there are variations in the number of repeats in closely related strains, so a higher-resolution examination of this region of the *nifJ* gene in other cyanobacteria was undertaken.

By using the PCR, DNAs from some of the strains shown in Fig. 3 were amplified around the locus of the insert in the *Anabaena* 7120 *nifJ* gene. The PCR primers were based on conserved sequences in the *Anabaena* and *Klebsiella nifJ* genes (see Table 2). Each of the amplified regions was sequenced, with the results shown in Fig. 4. Each cyanobacterium contains an inserted element at the same location as in *Anabaena* 7120, but each inserted element is different, except for the closely related strains 7120, 7118, and 7119, which have identical inserts. Three strains have tandem repeats of either heptamers or octamers. One strain, *Nostoc* MACR1, has two sets of repeats, one an octamer and one a heptamer. None of the repeated heptamers or octamers can be related in a simple way to CCCCATG or its complement. The insert sizes, relative to *Klebsiella*, range from 3 nt for strain *Anabaena* L31 to 63 nt for *Nostoc* MACR1. In every case, the insert is a multiple of 3 nt, suggesting that preservation of the reading frame is important and that the insert is translated. However, since each sequence is different, neither the precise sequence nor the length of the peptide insert seems to be critical.

DISCUSSION

Control of Expression of *nifJ* by Iron. Iron regulation of gene expression has been studied in several cyanobacterial systems in addition to the widely known translational control of transferrin and the iron storage protein ferritin in animal cells. In the latter cases, interaction of iron-binding proteins with the relevant mRNA determines efficiency of translation and the stability of the RNA (25–27). In cyanobacteria, there are two sets of data: one deals with induction of proteins of photosynthetic membranes by iron starvation and the other deals with flavodoxin and ferredoxin mRNA levels controlled by iron. In the first case, a *Synechococcus* gene encoding a membrane protein induced by iron starvation was sequenced and found to have a typical bacterial iron-response element upstream of the coding region (28). The second case is more complicated. Flavodoxin mRNA increases when iron is removed, but the ferredoxin mRNA level does not change. Ferredoxin protein, however, turns over rapidly in low iron medium (29).

The *nifJ* sequence from *Anabaena* is uninformative with respect to possible iron-response elements: there are no similarities between the 5′ flanking sequence and any bacterial iron-response elements. The appearance of the *nifJ* cDNA in the heterocyst-specific library is paradoxical. As seen in Fig. 2, there should be little or no nifJ mRNA in heterocysts from a culture induced in complete medium. Nevertheless, at least one molecule of mRNA initially had to have been present and was randomly selected in the sample of cDNAs to be sequenced from the heterocyst-specific library. One possibility, suggested by Dennis Dean (Virginia Polytechnic Institute, Blacksburg, VA), is that appearance of the iron-rich and abundant nitrogenase in heterocysts creates a transient condition of iron starvation sufficient to induce *nifJ* expression in these cells.

```
An7118/19/20  GCGATTCATGAAGGATGGGT----------AAATAA------AAATAATTCCCCAGTCCCCAGTCCCCAGTCCCCAGTCCCCAAAATTATTGG
Nostoc 840215 GCTATTCATGAAGTATGGGGAATGGGGCA-TGGGGCATTGGGCATTGGGCATGGAGAGAATGAA----AAATTA-CCTCT----TGCCCCTCTGCCTAAAATTATTGG
NostocR1      GCCATCCATGAAGCATGGGAGCAGGGGAGCAAGGGAGCAGAGGAGAAATTAGAGTTAAATTCCCCCCTGCGCCT-CTGCTCCTCTGCCCCTCTGCCTAAAATTGTTGG
Nostoc77      GCTATTCACGAAG----------TTAA----------TTCCAAATCTCAAATCCCAAATCTCCAATGC------ATTGTTGG
Klebsiella    GCCTTCTACCACCACGACGATGCGCCGCT----------AATCGTCGG
AnL31         GCTTTGTATGA----------AGCAGGGGAGA----------CGATACCCAAAATTGTCGG
AnVar         GCTATCCATGAGGAATGGGAA--------GATAAGAGAGCAGGGGAGAAATTAAAGTTAGATTCCCC----------TCTGCCTAAAGTCGTTGG
Azolla        GCTTTGTATGAAAAATGGGGTG----------TAGG----------ATCTCTTCCTACGGTT----------ATTGG
Nostoc29103   GCGATTCATGAAAGGATGGG----------GTGTAGGGGGCAAGT----------TACTACG----------GTTGTGGG
              ** * *..*                                                                  .* .* **
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FIG. 4. Comparison of the heptamer and octamer repeats found within the *nifJ* gene in various strains of *Anabaena* and *Nostoc*. Heptamer repeats and octamer repeats are displayed using alternating boldface type and underlining. Asterisks indicate positions of complete agreement and periods indicate positions of six or seven matches. *Anabaena* 7118, 7119, and 7120 are closely related strains from North America; *Nostoc* MAC R1 is a variant of *Nostoc* MAC isolated from a Macrozamia plant in New Zealand; *Nostoc* 77 (Fig. 3, lane H) was isolated in Senegal; *Anabaena* L31 is from India; the remaining strains are from the United States.

Function of the *nifJ* Gene in *Anabaena*. Based on the known electron transfer path to nitrogenase in *Klebsiella*, it seems reasonable to assume that the NifJ–flavodoxin pathway functions in *Anabaena* under low iron conditions. However, there are some experimental observations that are inconsistent with this pathway. *Anabaena* flavodoxin purified from vegetative cells will replace the vegetative-cell ferredoxin in electron transport from photosystem I to $NADP^+$ via ferredoxin nucleotide reductase but does not support glucose-6-phosphate- or isocitrate-dependent nitrogenase activity in *Anabaena* extracts (7). Although no equivalent of the *nifF* gene has been found in *Anabaena*, it is possible that a different flavodoxin is made in heterocysts and that this hitherto undescribed protein is the NifJ substrate.

Distribution of the *nifJ* Gene. Only *Klebsiella* has been shown previously to contain a *nifJ* gene required for nitrogen fixation. *R. rubrum* contains a NifJ protein and the corresponding gene, but it has not yet been possible to show that it is needed for nitrogen fixation under any conditions (8). Fig. 3 shows that all the *Anabaena* and *Nostoc* strains tested except *A. torulosa* (a salt-tolerant strain isolated from brackish water in India) have *nifJ*-related sequences.

The *nifJ* Gene Structure. The amino acid sequence of *Anabaena* NifJ is very similar to that of *Klebsiella* and *R. rubrum*, except for the region between nt 1150 and 1200. The inserted heptamers introduce a region of very low codon usage, suggesting that the insertion event was recent and that translation efficiency of this region is not important. Splicing to remove the inserts at the RNA level is very unlikely because the sequences are so different in the various *Anabaena* strains. There is no sequence consensus at the borders of the inserted elements. While the repeated sequences inserted in the four strains that contain them appear unrelated to each other, the four strains at the bottom of Fig. 4 contain inserts that can be derived from the insert in *Nostoc* MACR1. This can be seen by scanning down the middle of the figure, centering on the sequence AGGGG.

We have no clue to the origin of these sequences or the reason for their persistence inside the *nifJ* gene. The heptamer repeat of *Anabaena* 7120 is found between genes of the *nifBSU* operon (21) and downstream of the coding region of the *psbB* gene (22). One suggestion is that the chromosome has a hot spot for breakage at that position in *nifJ* and that the repair process uses repeated sequences (which are frequent in the chromosome) as part of the glue. Alternatively, this site could be a target for insertion of a transposable element that moved out and left behind a short tandem duplication. Slippage during replication or recombination could then expand the number of repeats. The latter possibility encouraged the PCR experiment, which, however, failed to show any strain with fewer or more copies of the CCCCAGT repeat in their *nifJ* genes. We suspect that the CCCCAGT element is mobile in some fashion; recently isolated strains of *Anabaena* and *Nostoc* from a lake in Finland were found to contain tandem repeats of this element of various lengths and in various chromosomal locations, ranging in number from 1 to >100 (K. Sivonen, L. Rouhiainen, and R.H., unpublished data).

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