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Excision of an 11-Kilobase-Pair DNA Element from within the *nifD* Gene in *Anabaena variabilis* Heterocysts

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The 3′ region of the *Anabaena variabilis nifD* gene contains an 11-kilobase-pair element which is excised from the chromosome during heterocyst differentiation. We have sequenced the recombination sites which border the element in vegetative cells and the rearranged heterocyst sequences. In vegetative cells, the element was flanked by 11-base-pair direct repeats which were identical to the repeats present at the ends of the *nifD* element in *Anabaena* sp. strain PCC 7120 (*Anabaena* strain 7120). Although *Anabaena* strain 7120 and *A. variabilis* are quite distinct in many ways, the overall sequence similarity between the two strains for the regions sequenced was 96%. Like the *Anabaena* strain 7120 element, the *A. variabilis* element was excised in heterocysts to produce a functional *nifD* gene and a free circularized element which was neither amplified nor degraded. The *Anabaena* strain 7120 *xisA* gene is located at the *nifK*-proximal end of the *nifD* element and is required for excision of the element in heterocysts. The *A. variabilis* element also contained an *xisA* gene which could complement a defective *Anabaena* strain 7120 *xisA* gene. *A. variabilis* did not contain the equivalent of the *Anabaena* strain 7120 *fdxN* 55-kilobase-pair element.

Anabaena variabilis is a filamentous cyanobacterium that performs oxygenic photosynthesis and can also reduce atmospheric nitrogen when deprived of fixed nitrogen. The induction of nitrogen fixation in *Anabaena* spp. culminates in the terminal differentiation of approximately every 10th vegetative cell into a heterocyst. The nitrogen fixation (*nif*) genes are expressed exclusively in heterocysts (32). The *nifH* gene codes for nitrogenase reductase, and the *nifD* and *nifK* genes code for the α and β subunits, respectively, of nitrogenase (9, 35).

Although the DNA sequences of the *nifH*, *nifD*, and *nifK* genes are remarkably similar among diazotrophs (37), at least three different organizations of the *nifH*, *nifD*, and *nifK* genes exist among procaryotes. In the slow-growing rhizobia *Parasponia rhizobium* (43) and *Bradyrhizobium japonicum* (17), the *nifH* gene is in a separate operon some distance away from the *nifDK* operon. In the fast-growing rhizobia the genes are on a large Sym plasmid and are organized into one *nifHDK* operon (2, 38). *Klebsiella* (24) and *Azotobacter* (4) spp. and unicellular cyanobacteria that fix nitrogen (22, 39) contain a contiguous *nifHDK* operon located on the chromosome.

Filamentous cyanobacteria display dichotomy in their *nif* gene arrangement. Nonheterocystous nitrogen-fixing cyanobacteria, grouped in section III by Rippka et al. (36), have a contiguous *nifHDK* arrangement. The branching heterocystous cyanobacterium *Fischerella* sp. strain ATCC 27929 (section V) also contains a contiguous *nifHDK* operon (39). In contrast, most free-living, nonbranching heterocystous cyanobacteria (section IV) have an interruption between the *nifK* gene and the bulk of the *nifD* gene in the vegetative cell genome (22, 30, 39).

In *Anabaena* sp. strain PCC 7120 (*Anabaena* strain 7120), the interruption is due to the insertion of an 11-kilobase-pair (kb) element into the 3′ region of the *nifD* open reading frame (13). This element is excised from the chromosome during heterocyst differentiation. Excision of the element joins the 3′ 129 base pairs (bp) of the *nifD* gene to the bulk of the open reading frame and forms a contiguous *nifHDK* operon.

Excision of the *nifD* element occurs by site-specific recombination between 11-bp directly repeated sequences that flank the element in vegetative cells (13). The excised element persists as a free circle in terminally differentiated heterocysts. The *nifD* element rearranges at a low and unregulated rate when cloned into an *Escherichia coli* plasmid vector (26). A region on the *nifD* element near the *nifK*-proximal end is required for excision. This region was sequenced and contains an open reading frame, named *xisA*, that could produce a 41.6-kilodalton protein. The *xisA* gene is thought to encode the site-specific recombinase involved in the excision of the *nifD* element.

Anabaena strain 7120 also contains a 55-kb DNA element within the *fdxN* gene that is excised in heterocysts (11, 12, 13, 31). The ends of this element are flanked by directly repeated sequences that differ from the terminal repeats that flank the *nifD* 11-kb element. Excision of the *fdxN* element can occur independently of the excision of the *nifD* element (11, 14).

Anabaena variabilis ATCC 29413 is, like *Anabaena* strain 7120, a filamentous nonbranching heterocystous cyanobacterium with a noncontiguous *nifHDK* operon (18). The evolutionary distance between *A. variabilis* and *Anabaena* strain 7120 is unclear. There are, however, numerous genetic and phenotypic differences between the two species, including the absence of a 55-kb element in *A. variabilis*. We have shown that the *A. variabilis nifHDK* operon is interrupted by an element similar to the *Anabaena* strain 7120 *nifD* element. The *A. variabilis* element is excised in heterocysts, is flanked by 11-bp repeats identical to those in the *Anabaena* strain 7120 *nifD* element, and contains an *xisA* gene that complements the *Anabaena* strain 7120 *xisA* gene.

MATERIALS AND METHODS

Strains and plasmids. *A. variabilis* ATCC 29413 and cosmid 33D12 (18) were graciously supplied by the laboratory of C. Peter Wolk (Plant Research Laboratory, Michigan State University). The cosmid 33D12 was originally in *Esch-*

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erichia coli DH1 and was later transferred into *E. coli* ED8767 (28). Plasmids were maintained in *E. coli* DH5α (Bethesda Research Laboratories). Complementation experiments were carried out in *E. coli* M8820TR (26). For routine growth and maintenance, *E. coli* was grown in LB broth or on LB agar (Lennox L; Gibco Laboratories); for plasmid preparations, *E. coli* was grown in 0.5× TB liquid medium, a variation of Terrific Broth (41) containing (per liter) 100 ml of KH_2PO_4 (0.17 M)–K_2HPO_4 (0.72 M), 6 g of peptone, 12 g of yeast extract, and 2 ml of glycerol.

Plasmid pUC1819RI was constructed by ligation of the small *Sca*I-*Eco*RI fragment of pUC18 (46) to the large *Sca*I-*Eco*RI fragment of pUC19 (46). This plasmid has an inverted multiple cloning site with a single *Eco*RI site in the middle and can be used to place different restriction sites at the ends of *Eco*RI fragments. pUC1819RI without an insert produces light blue colonies when grown on agar medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Cyanobacterial growth conditions. Eight-liter cultures of *A. variabilis* vegetative cells were grown in the liquid medium of Allen and Arnon (1) diluted eightfold (AA/8) with the following modifications: the K_2HPO_4 concentration was doubled to 0.5 mM; nickel, chromium, tungsten, and titanium salts were omitted; and the medium was supplemented with MOPS (3-[*N*-morpholino]propanesulfonic acid) (5 mM), pH 8.0, and NH_4NO_3 (2.5 mM). Cells were grown at 30°C with illumination at approximately 100 $\mu E\ s^{-1}\ m^{-2}$ and bubbled with 1% CO_2 in air. Heterocyst differentiation was induced by resuspending pelleted vegetative cells in AA/8 medium lacking ammonium nitrate and MOPS. Cells were incubated under these conditions for 48 h before heterocysts were harvested.

Nucleic acid isolation and hybridization. Plasmids were prepared from *E. coli* by the boiling miniprep method (28). In cases of low yield, particularly for cosmid minipreps, the DNA was further purified by binding to glass fines in the presence of NaI (15). Chromosomal DNA from vegetative cells and heterocysts of the *Anabaena* spp. were prepared as described previously (13). Horizontal agarose electrophoresis of DNA fragments was performed in 0.5× TBE buffer (28). Southern blotting, with GeneScreen Plus (New England Nuclear), was performed by the alkaline blotting procedure of Reed and Mann (34). DNA probes were prepared by using α-^{32}P-labeled nucleotides and a random-primer labeling kit supplied by Bethesda Research Laboratories. Hybridization conditions were 5× SSPE (28)–1% sodium dodecyl sulfate (SDS) at 65°C. The blots were washed in 0.5× SSPE–0.1% SDS at 65°C.

DNA sequencing. Double-stranded DNA was prepared for sequencing by an alkaline lysis-polyethylene glycol precipitation procedure (25). DNA sequencing was performed with a Sequenase kit (United States Biochemical) and [α-^{32}P]dATP according to the vendor's instructions.

Cloning the heterocyst recombination sites. The two heterocyst recombination sites were isolated from a genomic library of *Eco*RI-digested *A. variabilis* heterocyst DNA cloned into λgt7-*ara6* (8). This vector allows efficient in vitro packaging of inserts between 10 and 15 kb and is therefore biased towards the 11-kb excised circle linearized at its single *Eco*RI site and the 11.5-kb *Eco*RI heterocyst chromosomal fragment produced by the rearrangement. Clones containing these *Eco*RI fragments were detected by hybridization with the vegetative cell recombination site *Cla*I fragment in pAM288. The two heterocyst recombination sites were subcloned as *Cla*I and *Rsa*I fragments in Bluescript (Stratagene).

Complementation of the *xisA* gene in *E. coli*. *E. coli* M8820TR cells containing the rearrangement substrate plasmid MX32 (26) were made competent by a $CaCl_2$ procedure (28) and were frozen in 100-μl portions after addition of glycerol to a final concentration of 10%. Samples of competent MX32 cells were transformed with excess complementing plasmid by standard techniques (28). The cells were then plated on LB agar plates containing kanamycin (50 μg/ml), X-gal (50 μg/ml), and either chloramphenicol (25 μg/ml) for pAn207.62, pAM434, and pAM341 or spectinomycin (50 μg/ml) for pAM388. These conditions prevent excision of the *nifD* element in MX32 and select for the complementing plasmid. A blue colony was selected and grown overnight at 37°C in 0.5× TB containing ampicillin (100 μg/ml) and either chloramphenicol or spectinomycin as above. The absence of kanamycin allows rearrangement of MX32 to take place. Plasmid DNA was isolated from 1.5 ml of the overnight culture and used to transform competent *E. coli* DH5α cells. The transformed cells were plated and grown on LB agar containing ampicillin (100 μg/ml) and X-gal (50 μg/ml). These conditions allow cells containing either unrearranged or rearranged MX32 to grow. The transformation assay enriches for rearranged MX32 plasmids due to their smaller size (26).

The two vegetative-cell *Eco*RI fragments containing the *A. variabilis nifD* element were subcloned from the cosmid 33D12 to test their ability to rearrange MX32. The 7.5-kb *Eco*RI fragment that contains the *nifK* gene was cloned into the *Eco*RI site of pUC1819RI and then transferred as a *Bam*HI fragment into pACYC184 (7) to produce pAM434. The 15-kb *Eco*RI fragment that contains *nifD*, *nifH*, *nifS*, and the bulk of the *nifD* element was inserted into the *Eco*RI site of pACYC184. Because MX32 has a functional tetracycline resistance gene, it was necessary to provide an additional selectable marker. This was accomplished by inserting a spectinomycin resistance cartridge (33) into the *Bam*HI site of pACYC184, thereby inactivating the tetracycline resistance gene and providing spectinomycin resistance. The resulting plasmid was named pAM388.

The excised *nifD* element was subcloned into the *Eco*RI site of pUC1819RI. The *nifD* element was then removed from the pUC1819RI vector with *Bam*HI and inserted into the *Bam*HI site of pACYC184 (7). The resulting plasmid, named pAM341, has an inactivated tetracycline resistance gene and a functional chloramphenicol resistance gene and is compatible with the pBR322-based plasmid MX32.

RESULTS

Detection of the *A. variabilis nifD* element. The map of the *nifHDK* region of *A. variabilis* developed by Herrero and Wolk shows an approximately 11-kb gap between the *nifD* and *nifK* genes (18). Clones containing the *nifK* gene (pAn207.8), the *nifD* gene (pAn256), and the *nifD* element (pAn207) of *Anabaena* strain 7120 (35) were used to probe genomic Southern blots of *A. variabilis* vegetative-cell and heterocyst DNA digested with *Eco*RI, *Cla*I, and *Kpn*I. The *Anabaena* strain 7120 probes detected the expected *Eco*RI and *Cla*I fragments in vegetative-cell DNA reported in the map of Herrero and Wolk (18). In addition, these blots revealed that a rearrangement had occurred in *A. variabilis* heterocyst DNA (data not shown). The differences in the vegetative-cell and heterocyst DNA patterns were consistent with the excision of an 11-kb element from the *A. variabilis nifD* gene.

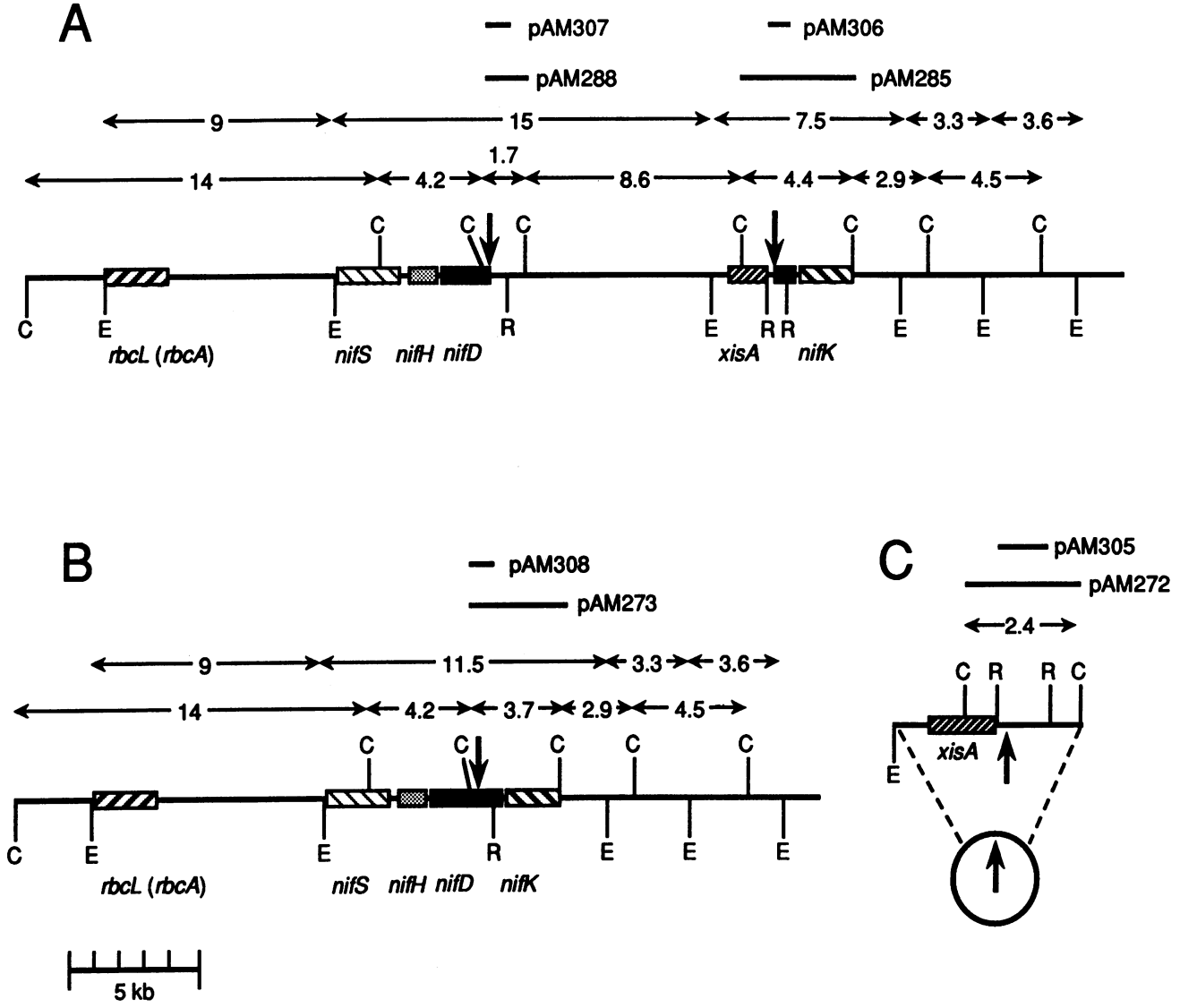

FIG. 1. Restriction map of *A. variabilis nifHDK* region in vegetative cells and heterocysts. The restriction maps are based on the map of Herrero and Wolk (18) and further analysis reported here. Gene sizes and positions were estimated by comparison with *Anabaena* strain 7120. The recognition sites for restriction endonucleases *Eco*RI (E), *Cla*I (C), and *Rsa*I (R) are shown. Only the *Rsa*I sites nearest the borders of the *nifD* element are shown. The large vertical arrows denote the recombination sites of the *nifD* element. Fragment sizes are given in kilobases. (A) *nifHDK* region of vegetative cells. (B) Chromosomal *nifHDK* region of heterocysts. (C) Excised *nifD* element of heterocysts. A 2× magnification of the junction region is shown above the circle. The sizes of the *Rsa*I junction fragments are: pAM305, 1.1 kb; pAM306, 0.93 kb; pAM307, 1.0 kb; and pAM308, 0.79 kb.

***A. variabilis* lacks an *fdxN* element.** The presence of the *fdxN* element in the *Anabaena* strain 7120 vegetative-cell chromosome places the *nifS* gene about 65 kb from the *rbcLS* operon (11, 31). After excision of the *fdxN* element, this distance is reduced to about 10 kb. In *A. variabilis* vegetative cells, the *nifS* gene is approximately 9 kb from the *rbcLS* operon (18), indicating the absence of an element similar to the *Anabaena* strain 7120 *fdxN* element. Vegetative-cell and heterocyst DNAs from *A. variabilis* produced identical patterns when probed with pAn154.2, which contains the *nifS*-proximal border of the *Anabaena* strain 7120 *fdxN* element. When the blots were probed with cosmid T3D4 (11), which contains regions internal to the 55-kb *fdxN* element, no hybridization was detected (data not shown). This confirms that an element similar to the *Anabaena* strain 7120 *fdxN* element is absent in *A. variabilis*.

Cloning the *A. variabilis nifD* element recombination sites. A map of the cosmid 33D12 insert (18) which contains the *nifHDK* region of *A. variabilis* is shown in Fig. 1A. The *Cla*I fragments that contained the left and right borders of the rearrangement (determined with *Anabaena* strain 7120 probes) were subcloned from 33D12 into the *Cla*I site of pBR328 (40) to produce pAM288 and pAM285 (Fig. 1A). The two recombination sites were further subcloned as *Rsa*I fragments in pBluescript II KS+ to produce pAM307 and pAM306 (Fig. 1A).

The map of the *A. variabilis* rearranged heterocyst chromosome and excised *nifD* element are shown in Fig. 1B and C, respectively. The *Cla*I and *Rsa*I fragments that contain the heterocyst chromosome recombination site were cloned into plasmids pAM273 and pAM308, respectively (Fig. 1B). Similarly, the fragments that contain the excised *nifD* ele-

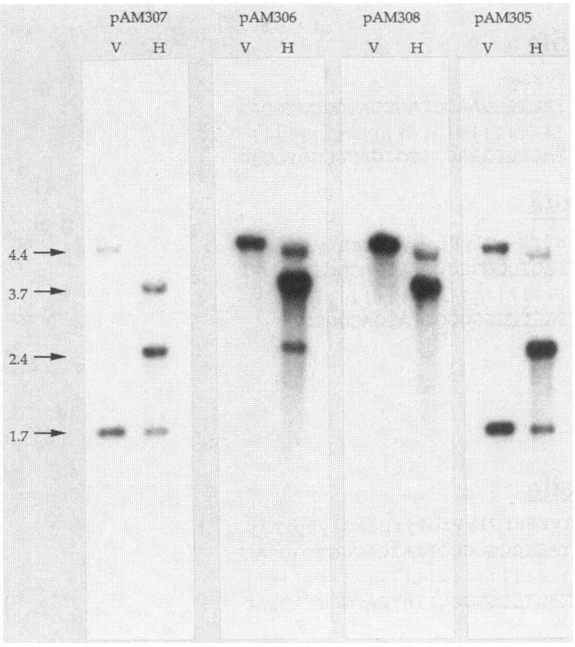

FIG. 2. *A. variabilis* genomic DNA probed with *A. variabilis* recombination site fragments. Each Southern blot contains DNA from *A. variabilis* vegetative cells (V) and heterocysts (H) digested with *Cla*I and electrophoresed in a 1% agarose–0.5× TBE-buffered gel. The blots were hybridized and washed as described in Materials and Methods. The probes were prepared from gel-purified *Rsa*I inserts from the indicated plasmid. See Fig. 1 for location of probe fragments and hybridizing bands. Fragment sizes are shown in kilobases.

ment recombination site were cloned into plasmids pAM272 and pAM305 (Fig. 1C).

Rearrangement of the *A. variabilis* heterocyst genome was confirmed by hybridization with cloned homologous probes. Four Southern blots containing *A. variabilis* DNA from vegetative cells and heterocysts digested with *Cla*I were probed separately with the four *Rsa*I recombination site fragments (Fig. 2). The two recombination site probes from vegetative cells, pAM307 and pAM306, hybridized to the *Cla*I fragments from which they were derived and to the two *Cla*I fragments that contained rearranged DNA. Similarly, the two probes containing heterocyst recombination sites, pAM308 and pAM305, hybridized to the *Cla*I fragments from which they were derived and to the two *Cla*I vegetative-cell fragments, each of which contained a border of the *nifD* element. Probe pAM308 from heterocysts weakly detected the 1.7-kb *Cla*I fragment containing the *nifD*-proximal vegetative-cell recombination site because it shared only 170 bp of similarity with that target fragment. A faint 4.4-kb band was detected with the pAM307 probe in vegetative-cell DNA; this band may represent a gene homologous to *nifD*, such as the *nifE* gene (5). Our heterocyst DNA preparations were routinely contaminated by minor amounts of vegetative-cell DNA. This resulted in all four probes detecting vegetative-cell recombination site fragments in the heterocyst DNA lanes. *Anabaena* strain 7120 and *A. variabilis* probes detected recombination site fragments of identical sizes in *A. variabilis* DNA.

Sequence of *A. variabilis* recombination sites. About 200 bp of DNA surrounding the recombination sites in vegetative cells before rearrangement and in heterocysts after rearrangement were sequenced. The sequences of the recombination sites and flanking DNA from *A. variabilis* are compared with the corresponding sequences from *Anabaena* strain 7120 (13, 27, 29) in Fig. 3A. The overall similarity was 96%, and the 11-bp direct repeats that flank the two elements were perfectly conserved. Our mapping and sequencing data show that the *A. variabilis* heterocyst rearrangement was very similar to that found in *Anabaena* strain 7120. The rearrangement was a conservative site-specific recombination between directly repeated 11-bp sequences that flank an 11-kb element. The heterocyst-specific rearrangement excised the element from the vegetative-cell chromosome and resulted in a rejoined chromosome and a circular element, each containing one copy of the 11-bp recombination site.

The *A. variabilis nifD* element, including the *xisA* open reading frame, was in the same position and orientation as the *Anabaena* strain 7120 element. In the region we sequenced, the only difference found in the NifD proteins predicted for the two strains was a change from glycine in *Anabaena* strain 7120 to alanine in *A. variabilis* at position 442. There were also four silent third-base changes between the two *nifD* open reading frames. The region between the *nifK*-proximal border of vegetative-cell DNA through the second of two possible start codons of the *xisA* open reading frame contained only two base differences, indicating the presence of an *xisA* gene on the *A. variabilis* element (Fig. 3B). It is not known which of the two start codons in the *xisA* gene is used in vivo (26).

***xisA* complementation.** Cosmid 33D12 was unstable and consistently produced a single deleted variant after overnight growth in *E. coli* strains DH1 and ED8767. The *Cla*I restriction pattern of the rearranged cosmid was identical to the heterocyst *Cla*I restriction pattern seen on genomic Southern blots. It is likely that 33D12 properly excises the *nifD* element when grown in *E. coli*, as seen previously with plasmid clones containing the *Anabaena* strain 7120 *nifD* element (26).

The plasmid MX32 (26) was used as a rearrangement substrate for an *xisA* complementation assay in *E. coli*. The plasmid MX32 contains the complete *nifD* element of *Anabaena* strain 7120 cloned into pBR322. Spontaneous excision of the *nifD* element from MX32 is extremely rare due to the transposition of a mini-Mu *d*I1734 into the 5′ region of the *xisA* gene. The mini-Mu *d*I1734 contains a kanamycin resistance gene as well as a β-galactosidase gene. When the *nifD* element is excised from a plasmid clone, it produces a circle that lacks an origin of replication. Therefore, cells that contain rearranged plasmid DNA lose both β-galactosidase activity and kanamycin resistance, but retain the ampicillin resistance from pBR322. The defective excision of the *nifD* element from MX32 can be complemented in *trans* by the plasmid pAn207.62, which contains a functional *Anabaena* strain 7120 *xisA* gene (26).

We tested the ability of *A. variabilis* clones to complement the defective *xisA* gene of MX32 in *E. coli*. A complementing plasmid must contain a functional *xisA* gene and a selectable marker other than resistance to kanamycin or ampicillin. To produce such a plasmid, the excised *A. variabilis nifD* element was subcloned into pACYC184. This plasmid, pAM341, complemented the defective *xisA* gene in MX32 (Fig. 4).

The *nifD* element in pAM341 was originally cloned in a bacteriophage λ genomic library by digesting the excised and circularized *nifD* element at its single *Eco*RI site. The termini of the *Eco*RI insert of pAM341 are therefore circularly permuted with respect to the normal ends of the *nifD* element in the vegetative-cell chromosome. Because the

A
Vegetative Cell

nifD proximal recombination site

```
        nifD→ArgGlnMetHisSerTrpAspTyrSer
variabilis TCCGTCAAATGCACTCTTGGGATTACTCCGAACCTAGCGATGGGGTGCAA
           |||||||||||||||||||||||||||||| | |||| ||||||||
      7120 TCCGTCAAATGCACTCTTGGGATTACTCCGAACTTGGCGACGGGGTGCAG
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nifK proximal recombination site

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                                    nifD→GlyProTyrHisGly
variabilis AGCTATTAAACCACAAAAAGGATTACTCCGGCCCTTATCACGGTTA
           ||| ||||||||||||||||||||||||||||||||||||||||
      7120 AGCCATTAAACCACAAAAAGGATTACTCCGGCCCTTATCACGGTTA
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Heterocyst

Chromosomal recombination site

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        nifD→ArgGlnMetHisSerTrpAspTyrSerGlyProTyrHisGlyTyrAsp
variabilis TCCGTCAAATGCACTCTTGGGATTACTCCGGCCCTTATCACGGTTACGAC
           |||||||||||||||||||||||||||||||||||||||||||||||||
      7120 TCCGTCAAATGCACTCTTGGGATTACTCCGGCCCTTATCACGGTTACGAC
```

Excised circle recombination site

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variabilis AGCTATTAAACCACAAAAAGGATTACTCCGAACCTAGCGATGGGGTGCAA
           ||| |||||||||||||||||||||||||| | |||| ||||||||
      7120 AGCCATTAAACCACAAAAAGGATTACTCCGAACTTGGCGACGGGGTGCAG
```

B
xisA 5' region

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variabilis CGGAGTAATCCTTTTTGTGGTTTAATAGCTTTCAACAGCTTAATGTTTGC
           ||||||||||||||||||||||| ||||||||||||||||||||||||
      7120 CGGAGTAATCCTTTTTGTGGTTTAATGGCTTTCAACAGCTTAATGTTTGC

                .         .         .         .         .
variabilis ACTGAGCAGTGTTAGTGATGAACATTGTTTGCTGATAACACATTAATTTT
           |||||||||||||||||||||||||||||||||||||||||||||||||
      7120 ACTGAGCAGTGTTAGTGATGAACATTGTTTGCTGATAACACATTAATTTT

                                    xisA→MetGlnAsnGlnGlyGlnAsp
                .         .         .         .         .
variabilis TAGGCTAAATATAGGCTAATACCACAGCGATGCAAAATCAGGGTCAAGAC
           |||||||||||||||||||||||||||||||||||||||||||||||||
      7120 TAGGCTAAATATAGGCTAATACCACAGCGATGCAAAATCAGGGTCAAGAC

           LysTyrGlnGlnAlaPheAlaAspLeuGluProLeuSerSerThrAspGly
                .         .         .         .         .
variabilis AAATATCAACAAGCCTTTGCAGACTTGGAGCCACTTTCATCTACCGACGG
           ||||||||||||||||||||||| |||||||||||||||||||||||||
      7120 AAATATCAACAAGCCTTTGCAGACTTAGAGCCACTTTCATCTACCGACGG
                                    Leu

            SerPheLeuGlySerSerLeuGlnAlaGlnGlnGlnArgGluHisMet
                .         .         .         .         .
variabilis CAGTTTTCTCGGCTCAAGTCTGCAAGCACAGCAGCAAAGAGAACACATGA
           |||||||||||||||||||||||||||||||||||||||||||||||||
      7120 CAGTTTTCTCGGCTCAAGTCTGCAAGCACAGCAGCAAAGAGAACACATGA
```

FIG. 3. DNA sequence of *A. variabilis* recombination sites and the 5′ region of the *xisA* gene. (A) DNA sequence of the *nifD* element recombination sites before (vegetative cell) and after (heterocyst) rearrangement. The coding strand for the *nifD* gene is shown in the 5′ to 3′ direction for each recombination site. The equivalent *Anabaena* strain 7120 sequence is shown below each *A. variabilis* sequence. The 11-bp repeat is underlined in each sequence. (B) 5′ region of the *xisA* gene from the 11-bp recombination site to the second in-frame start codon. The *xisA* open reading frame starts at the first of two in-frame start codons. The *xisA* and the *nifD* genes are transcribed in opposite directions.

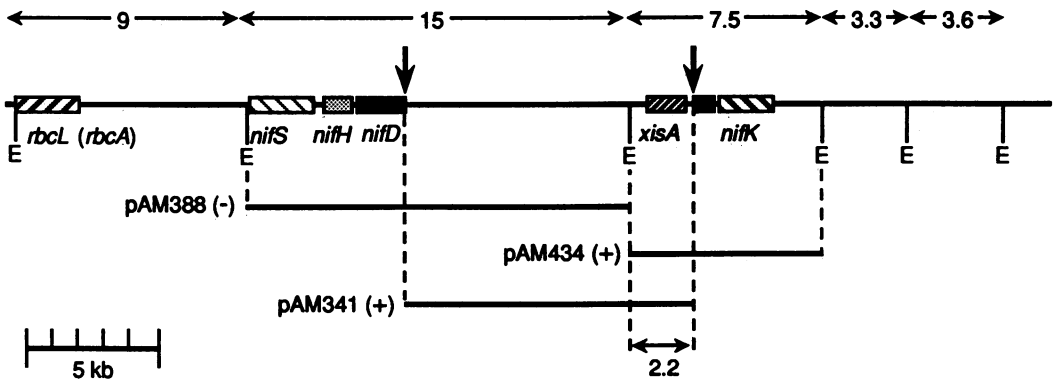

FIG. 4. Complementation of a defective *Anabaena* strain 7120 *xisA* gene by *A. variabilis* DNA. The recognition sites for restriction endonuclease *Eco*RI (E) are shown. The large vertical arrows denote the recombination sites of the *nifD* element. The ability of an *A. variabilis* plasmid clone (shown below the restriction map) to complement the defective *Anabaena* strain 7120 *xisA* gene is indicated by a + or −.

xisA gene is expressed both in the chromosome of *A. variabilis* and in pAM341, the *Eco*RI site is not within the *xisA* gene. To establish that the *xisA* gene lay between the internal *Eco*RI site and the *nifK*-proximal end of the element, as in *Anabaena* strain 7120, the two vegetative-cell *Eco*RI fragments containing the *A. variabilis nifD* element were subcloned from the cosmid 33D12 into pACYC184. This generated plasmids pAM388 and pAM434 (Fig. 4).

The results of the complementation experiments are presented in Table 1. The *nifK*-proximal 7.5-kb *Eco*RI fragment in pAM434 complemented MX32. The low number of viable colonies that grew when these cultures were plated on selective medium is presumably due to the previously observed instability of plasmids that contain the *nifD* element recombination sites in the presence of an active *xisA* gene (26). The mechanism of this instability is not known. The *nifD*-proximal 15-kb fragment in pAM388 did not complement MX32. DNA from several of the white colonies obtained with each complementing plasmid was digested with *Hin*dIII and analyzed by agarose electrophoresis. For the complementing plasmids pAn207.62, pAM341, and pAM434, all but one white colony gave DNA with the *Hin*dIII pattern expected from a properly rearranged MX32 plasmid (26). For the noncomplementing plasmids pAM388 and pACYC184, all but one of the rare white colonies screened contained randomly rearranged MX32 plasmids. One white colony from the pAM388 complementation contained plasmid DNA that had apparently excised the *nifD* element properly, although the rearrangement junction was not sequenced. This colony may have been produced by a low level of *xisA* activity in pAM388, but is more likely the result of a low level of spontaneous MX32 rearrangement. The *A. variabilis xisA* activity mapped to a 2.2-kb region near the *nifK* end of the element (Fig. 4); this corresponds to the location of the *Anabaena* strain 7120 *xisA* gene.

TABLE 1. Complementation of the MX32 rearrangement with *A. variabilis* clones

Complementing plasmid	Total no. of colonies screened	% White colonies
pACYC184	481	1.25
pAn207.62	1,956	26.5
pAM341	4,541	41.9
pAM434	68	38.2
pAM388	1,115	0.63

DISCUSSION

We have shown that *A. variabilis* contains an 11-kb element in the 3′ region of the *nifD* open reading frame that closely resembles the *nifD* element of *Anabaena* strain 7120. The element is excised from the chromosome in heterocysts of both strains. Both elements are bordered by identical, directly repeated 11-bp sequences that are involved in the excision of the element by site-specific recombination. The *A. variabilis* element contains an *xisA* gene in the same location as the *Anabaena* strain 7120 *xisA* gene, and the *A. variabilis xisA* gene product will rearrange an *Anabaena* strain 7120 *nifD* element substrate.

The *nifD* element in the *A. variabilis* cosmid clone 33D12 was excised at a significantly higher frequency in *E. coli* than were similar *Anabaena* strain 7120 clones. The reason for this is not clear. It may result from increased expression of the *xisA* gene from a fortuitous promoter in the upstream *A. variabilis* sequence. Alternatively, the *A. variabilis xisA* gene product may function better in *E. coli* than does the *Anabaena* strain 7120 *xisA* gene product.

An earlier report by Hirschberg et al. (19) contains a restriction map of the *nifH* and *nifD* region of *A. variabilis* that differs from our own and that reported by Herrero and Wolk (18). The Hirschberg et al. restriction map does not correspond to either the vegetative-cell or heterocyst arrangement of the *nifHDK* operon described in this paper. We believe the clones reported by Hirschberg et al. may represent a second region of the *A. variabilis* genome similar to the *nifH* and *nifD* genes for the following reasons. First, the heterologous probes they used were derived from *Klebsiella pneumoniae* and are presumably less similar to the *A. variabilis* genes than the *Anabaena* strain 7120 probes used in this study. The low-stringency hybridizations and washes used to detect the *nif* genes might have allowed detection of cross-hybridizing fragments. Second, they did detect as minor bands the 7.5-kb *Eco*RI *nifK* fragment and the 15-kb *Eco*RI *nifHD* fragment that we detected by using *Anabaena* strain 7120 probes. Third, we detected additional weak bands with the *Anabaena* strain 7120 *nifD* probe pAn256, including a 1.9-kb *Cla*I fragment seen by Hirschberg et al. in their genomic Southern blots and in their *nifHD* genomic clone. Finally, there is precedent for other genes that cross-hybridize to *nif* gene probes. In *Azotobacter* spp., the *nifE* and *nifN* genes are similar to the *nifD* and *nifK* genes, respectively, at the level of protein sequence and may have

a common ancestral origin (5). Rice et al. (35) have reported a genomic clone of *Anabaena* strain 7120 that contains a second region similar to the *Klebsiella pneumoniae nifH* gene. This clone, however, does not contain sequences similar to *nifD*. If the clone of Hirschberg et al. represents a second copy of the *nifH* and *nifD* genes, they have diverged from the *nif* genes described in this paper, since our *A. variabilis* probes did not detect additional copies of *nif* genes at moderate hybridization stringencies.

A. variabilis and *Anabaena* strain 7120 share many characteristics. They are both nonbranching filamentous heterocystous cyanobacteria in section IV of the classification scheme of Rippka et al. (36). Herrero and Wolk (18) have previously shown that the organization of the *A. variabilis nifHDK* region is similar to the organization of the *Anabaena* strain 7120 genes. In this paper we have shown that *A. variabilis* contains a *nifD* element nearly identical to the *Anabaena* strain 7120 *nifD* element and that the surrounding *nifD* gene sequences are very similar.

Although *A. variabilis* and *Anabaena* strain 7120 are closely related, they are not simply separate isolates of the same organism. There are a number of significant differences between the two strains. Their morphology under the light microscope differs, and *A. variabilis* forms akinetes (spores) (3), whereas *Anabaena* strain 7120 does not (36). *A. variabilis* is a heterotroph and is capable of growth in the dark on fructose (44), while *Anabaena* strain 7120 is an obligate phototroph (36). *A. variabilis* and *Anabaena* strain 7120 have different susceptibilities to cyanophages (20). Shuttle vectors containing an origin of replication derived from a *Nostoc* sp. strain PCC 7524 plasmid can be conjugated to and selected for in *Anabaena* strain 7120 (45) and a number of facultative *Nostoc* species (10), but not in *A. variabilis* (M. Murry, personal communication). The restriction maps of the *nifHDK* regions in the two strains show little similarity to each other (18, 35). Comparison of partial DNA sequence data of the two phycocyanin genes of *A. variabilis* with those for the *Anabaena* strain 7120 genes show 95% similarity for *cpcA* and 90% similarity for *cpcB* (21). These genes are slightly more diverged from those in *Anabaena* strain 7120 than are the sequences reported here. Finally, the *fdxN* 55-kb element near the *nifS* gene of the *Anabaena* strain 7120 vegetative-cell chromosome (11–13, 31) is absent in *A. variabilis*.

A noncontiguous *nifHDK* operon appears to be a common feature of free-living nonbranching heterocystous cyanobacteria (22, 30, 39). It is not known whether all cyanobacteria of this group have sequences similar to the *Anabaena* strain 7120 *nifD* element. The similarity of the elements in *Anabaena* strain 7120 and *A. variabilis* suggests a common evolutionary origin. The presence of the *nifD* element in most nonbranching heterocystous cyanobacteria may represent an insertion event in an ancestral cell. Alternatively, the element may have spread horizontally by independent insertion into the 11-bp target sequence that should be conserved among nonbranching heterocystous cyanobacteria because of its location within the open reading frame of a highly conserved gene.

The excision of the *nifD* element resembles the excision of a lysogenic phage. The e14 element, present in some strains of *E. coli*, may be an example of a defective lysogenic phage. The e14 element is excised upon UV irradiation (16) and appears to code for genes needed for lysogeny and rearrangement at the e14 attachment sites (6, 42). The e14 element also contains the *pin* recombinase gene, a member of the *hin*, *gin*, and *pin* family of recombinases, which catalyzes the inversion of the adjacent 1.8-kb P region (42). If the *nifD* element is a defective lysogenic phage, it might offer an advantage to the host vegetative cell in the form of immunity to infection by the same or related phage or by conferring a restriction-modification system. Alternatively, the *nifD* element might protect against infection by a phage unrelated to the element. The e14 element contains the *lit* gene, which, if activated by a promoter-up mutation, blocks bacteriophage T4 infection (23).

The ability to excise the *nifD* element in heterocysts is essential for growth of *Anabaena* strain 7120 on atmospheric nitrogen. A derivative of *Anabaena* strain 7120 with an inactivated *xisA* gene does not excise the *nifD* element in heterocysts, produce functional nitrogenase, or grow on atmospheric nitrogen (14). A functional *xisA* gene is therefore required for strains containing a *nifD* element if they are to fix nitrogen. It is likely that all strains containing a *nifD* element will also retain an active, developmentally regulated *xisA* gene that allows excision of the element in heterocysts.

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