Two heterocyst-specific DNA rearrangements of *nif* operons in *Anabaena cylindrica* and *Nostoc* sp. strain Mac

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Two site-specific DNA rearrangements occur during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120: the deletion of an 11 kb element from within the *nifD* gene and the deletion of a 55 kb element from within the *fdxN* gene. Three *Nostoc* and six *Anabaena* strains were screened for the presence of the *nifD* and *fdxN* elements by Southern hybridization with *Anabaena* PCC 7120 DNA probes. Eight of the nine strains contained DNA sequences that were similar to the *nifD* element. Three strains, *Nostoc* sp. strain Mac, *Anabaena cylindrica* and *Anabaena* sp. strain M131, also showed significant similarity to portions of the 55 kb *fdxN* element. *Anabaena* sp. strain CA lacked both the *nifD* and *fdxN* elements. Southern analysis of vegetative cell and heterocyst DNA from *A. cylindrica* and a Fox^+ revertant of *Nostoc* Mac (isolate R2) showed rearrangement of the *nifD* and *fdxN* elements in heterocysts. We found no RFLPs between *Anabaena* M131 and *Anabaena* PCC 7120 suggesting that strain M131 is a Het^- derivative of strain PCC 7120.

Keywords: nitrogen fixation (*nif*) genes, cyanobacteria, DNA rearrangement, heterocyst, recombinase genes

INTRODUCTION

Many filamentous cyanobacteria form heterocysts, which are highly specialized, terminally differentiated cells that reduce atmospheric dinitrogen to ammonia (Haselkorn, 1978; Wolk, 1982). The fixed nitrogen is exported to neighbouring vegetative cells as glutamine and in return heterocysts are provided with photosynthate. Heterocysts are formed about every tenth cell along the filament of strains such as *Anabaena* sp. strain PCC 7120 and produce a semiregular one-dimensional pattern (Adams & Carr, 1981; Wolk, 1989). Heterocyst formation is repressed by the presence of an external source of combined nitrogen, such as ammonia, or by the proximity of other heterocysts in the filament. Global changes in gene expression occur during heterocyst differentiation (Fleming & Haselkorn, 1974; Lynn *et al.*, 1986). For example, the genes responsible for nitrogen fixation are turned on while several genes necessary for carbon fixation and the oxygen-evolving reactions of photosynthesis are turned off (Wolk, 1982).

Heterocyst differentiation in *Anabaena* PCC 7120 is accompanied by two developmentally regulated genome rearrangements that occur within the *nifHDK* and the *nifB-fdxN-nifS-nifU* operons (Golden *et al.*, 1985, 1988;

Golden, J. W. *et al.*, 1987; Mulligan *et al.*, 1988; Mulligan & Haselkorn, 1989). The *nifD* rearrangement involves the deletion of an 11 kb element from within the 3' end of the *nifD* gene. The *nifD* element is bordered by 11 bp direct repeats and site-specific recombination between these sequences results in deletion of the 11 kb element from the chromosome (Golden *et al.*, 1985). The *nifD* rearrangement results in the formation of the complete ORF for the *nifD* gene and allows for the expression of the *nifHDK* operon from a single promoter (Golden *et al.*, 1991; Haselkorn *et al.*, 1986). The *xisA* gene is located on the *nifD* element and encodes a site-specific recombinase that is required for excision of the element (Brusca *et al.*, 1990; Golden & Wiest, 1988; Lammers *et al.*, 1986).

The *fdxN* rearrangement is the result of the excision of a 55 kb element from within the *fdxN* gene, which is part of the *nifB-fdxN-nifS-nifU* operon (Golden *et al.*, 1988; Mulligan *et al.*, 1988; Mulligan & Haselkorn, 1989). A site-specific recombination between directly repeated DNA sequences also occurs, but these repeated sequences are different from those involved in the *nifD* rearrangement (Golden, J. W. *et al.*, 1987). The deletion of the 55 kb element results in the formation of the *fdxN* ORF and presumably allows for the expression of the downstream genes in the *nifB-fdxN-nifS-nifU* operon by the

nifB promoter (Mulligan *et al.*, 1988). The *xisF* gene is located on the *fdxN* element and encodes a site-specific recombinase that is required for excision of the element (Carrasco *et al.*, 1994). Excision of the *fdxN* element can occur independently from excision of the *nifD* element (Carrasco *et al.*, 1994; Golden *et al.*, 1988; Golden & Wiest, 1988).

Southern analysis and mapping of nitrogen fixation genes in several cyanobacterial strains with *Anabaena* PCC 7120 probes show that the *nifHDK* operon is not contiguous in most heterocystous strains (Apte & Thomas, 1987; Franche & Cohen-Bazire, 1985, 1987; Herrero & Wolk, 1986; Kallas *et al.*, 1983, 1985; Meeks *et al.*, 1988). However, a branching heterocystous species, *Fischerella* sp. strain ATCC 27929, contains a contiguous *nifHDK* organization (Saville *et al.*, 1987). *Anabaena torulosa* contains sequences similar to the *Anabaena* PCC 7120 *xisA* gene (Apte & Thomas, 1987). Southern analysis of *Nostoc* sp. strain UCD 7801 and a cultured isolate from the symbiotic association with the water fern *Azolla caroliniana* showed hybridization to an *xisA* probe and evidence for rearrangement in samples that contained heterocysts (Meeks *et al.*, 1988). These results indicate that a *nifD* element similar to that found in *Anabaena* PCC 7120 is present in many heterocystous strains and suggest that in these strains the element is excised from the *nifHDK* operon during heterocyst formation.

Rearrangement of the *nifD* element has been directly demonstrated in at least two strains other than *Anabaena* PCC 7120. *Anabaena variabilis* (ATCC 29413) contains an 11 kb element very similar to the *Anabaena* PCC 7120 *nifD* element (Brusca *et al.*, 1989). DNA sequencing showed that the *A. variabilis* element is located at the same position in the 3' region of the *nifD* gene and contains the same directly repeated sequences at the recombination sites as found in *Anabaena* PCC 7120. The *A. variabilis* element contains an *xisA* gene that can functionally replace the *Anabaena* PCC 7120 *xisA* gene and cause site-specific recombination of a PCC 7120 substrate cloned in *E. coli* (Brusca *et al.*, 1989).

Southern analysis of *Nostoc* sp. strain Mac showed the presence of a *nifD* element (Meeks *et al.*, 1994). The common laboratory strain of *Nostoc* Mac is Fox⁻, incapable of aerobic nitrogen fixation. Analysis of spontaneous Fox⁺ revertant clones identified one rare clone, isolate R1, that had lost the element in all cells (Meeks *et al.*, 1994). DNA samples from other revertant clones, which still carry the *nifD* element, showed faint bands on Southern blots that were interpreted as representing excision of the element in heterocysts.

The *Anabaena* PCC 7120 DNA element found within the *fdxN* gene has not been previously described in any other cyanobacterial strain. It has been shown that *A. variabilis* does not contain a 55 kb element in a position similar to that found in *Anabaena* PCC 7120 (Brusca *et al.*, 1989; Herrero & Wolk, 1986). Physical mapping of the chromosome near the *nif* genes showed that the *rbcLS* operon in vegetative cells of *A. variabilis* is located approximately 10 kb away from the *nifS* gene, which is the

same gene arrangement that is found in the *Anabaena* PCC 7120 heterocyst chromosome after excision of the *fdxN* element.

We were interested in determining whether DNA elements similar to the *fdxN* element found in *Anabaena* PCC 7120 were present in other cyanobacterial strains. We report in this paper the presence of an *fdxN*-like element in *Nostoc* Mac and *Anabaena cylindrica* and show that the element rearranges in heterocysts of both strains. A *nifD*-like rearrangement was also detected in *A. cylindrica*. As part of our study, we confirmed the observations of others that *Anabaena* sp. strain M131 is very closely related to *Anabaena* PCC 7120 (Alam *et al.*, 1991; Bancroft & Wolk, 1989; Cai, 1991).

METHODS

Cyanobacterial and bacterial strains and culture conditions.

The cyanobacterial strains used in this study and their sources are listed in Table 1. Fresh water *Anabaena* and *Nostoc* strains were grown in 100 ml liquid cultures of modified Allen & Arnon medium diluted eightfold (AA/8) (Golden *et al.*, 1991), and on plates of modified BG-11 medium solidified with 1.5% (w/v) agar (Brusca *et al.*, 1990). *Anabaena* sp. strain CA was grown in ASP-2 medium (Bottomley *et al.*, 1979). Filter-sterilized sodium thiosulfate (1 mM final concentration) was added to the BG-11 agar medium just before pouring the plates. Large scale cultures of 1–8 l were grown in modified AA/8 medium bubbled with 1% (v/v) CO₂ in air. Heterocyst differentiation was suppressed by the addition of ammonium sulfate (2.5 mM). Cultures were synchronously induced to form heterocysts by transferring vegetative cells from AA/8 medium containing ammonium sulfate to AA/8 medium lacking a source of combined nitrogen. Light levels for the various growth conditions were as previously described (Golden *et al.*, 1991).

Escherichia coli strains carrying plasmid clones were grown in Luria-Bertani (LB) liquid or agar-solidified medium with appropriate antibiotics according to standard procedures. Plasmids were maintained in *E. coli* strains HB101 or DH5 α and cosmids were maintained in strain ED8767 (Golden *et al.*, 1988).

Heterocyst isolation. Heterocysts were isolated from induced filaments by adjusting the original induced culture to 0.25 M sucrose, 50 mM Tris/HCl (pH 8.0), 10 mM EDTA (pH 8.0) and 0.25 mg lysozyme ml⁻¹. The cells were stirred for 30 min at room temperature. They were collected by centrifugation and chilled on ice water. The cell pellet was resuspended in 500 ml 50 mM Tris/HCl (pH 8.0)–10 mM EDTA (pH 8.0)–0.25 M sucrose–1% (w/v) SDS and treated for 2 min with a Sonifier Cell Disruptor 350 (Branson Sonic Power Company) tapered microtip at an output control setting of 4 and 50% duty cycle. The heterocysts were collected by centrifugation at 5000 g for 3 min and the pellet was resuspended in 10 ml cold TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. The heterocysts were washed twice with cold TE buffer by centrifugation at 2000 g for 5 min to remove remaining vegetative cells and debris. A modification of this procedure improves the quality of extracted RNA (Golden *et al.*, 1991).

DNA manipulations. Rapid small-scale DNA preparations from *Anabaena* and *Nostoc* strains were made essentially as previously described (Golden *et al.*, 1991). Larger-scale preparations of vegetative cell and heterocyst DNA were made as previously described (Golden *et al.*, 1985; Golden, S. S. *et al.*, 1987).

Table 1. Occurrence of *nifD* and *fdxN* elements in *Anabaena* and *Nostoc* strains

The presence of the *nifD* and *fdxN* elements and the *xisA* and *xisF* genes was determined by Southern hybridization; probes and hybridization conditions are described in Methods. +, Presence of distinct bands of at least moderate intensities; -, absence of distinct bands, although faint diffuse hybridization was sometimes observed; +/-, faint but distinct bands.

Strain	Source	Heterocysts	<i>nifD</i> element	<i>xisA</i> gene	<i>fdxN</i> element	<i>xisF</i> gene
<i>Anabaena</i> PCC 7120*	R. Haselkorn	+	+	+	+	+
<i>Anabaena</i> M131	C. P. Wolk	-	+	+	+	+
<i>A. cylindrica</i>	C. P. Wolk	+	+	+	+	+
<i>Nostoc</i> Mac	J. C. Meeks	-	+	+	+	+
<i>Nostoc</i> Mac R1	J. C. Meeks	+	-	-	+	+
<i>Nostoc</i> Mac R2	J. C. Meeks	+	+	+	+	+
<i>A. variabilis</i> (ATCC 29413)	C. P. Wolk	+	+	+	-	-
<i>A. torulosa</i>	J. Thomas	+	+	+	-	-
<i>A. azollae</i> isolate A2	J. C. Meeks	+	+	+	-	-
<i>Nostoc</i> UCD 7801	J. C. Meeks	+	+	+	-	+/-
<i>Nostoc</i> ATCC 29133	J. C. Meeks	+	+	+	-	+/-
<i>Anabaena</i> CA	R. Haselkorn	+	-	-	-	-

* Reference strain from which the hybridization probes were obtained.

Fig. 1. Map of the *Anabaena* PCC 7120 vegetative cell chromosome and the cloned DNA fragments used as probes in this study. Vertical arrows indicate the recombination sites for the 11 kb *nifD* (white arrows) and 55 kb *fdxN* (black arrows) rearrangements. The DNA elements are excised from the genome by site-specific recombination during heterocyst differentiation. Genes involved in nitrogen fixation (*nif* and *fdx*) and carbon fixation (*rbc*) are shown (open boxes). The *xisA* and *xisF* recombinase genes (hatched boxes) required for heterocyst-specific DNA rearrangement were used as probes. The fragments An207.6, An256, An154.2 and An155 span the recombination sites and were used as probes to detect excision of the elements. The *nifD* element probe was from a lambda clone, λ HetL47.1-1.4, of the excised *nifD* element linearized at a *Sau*3AI site. The cosmid clones S2A12, T3D4 and T6C6 cover a 106 kb region of the chromosome that contains the *nifD* and *fdxN* elements and flanking regions. Cosmid T3D4 contains sequences entirely within the *fdxN* element.

Recombinant DNA techniques were performed by standard procedures (Ausubel *et al.*, 1987; Maniatis *et al.*, 1982). Restriction enzymes and other DNA modifying enzymes were obtained from various suppliers and used according to the manufacturers' recommendations. DNA was transferred from agarose gels to GeneScreen Plus (DuPont, NEN Research Products) with 0.4 M NaOH (Reed & Mann, 1985) or 50 mM NaOH/1 M NaCl. DNA probes were radiolabelled with a

random primer kit (Bethesda Research Laboratories, Life Technologies or Boehringer Mannheim). Southern hybridizations were performed at moderate stringency because of the use of heterologous probes. Filters were hybridized in 5 × SSPE (Maniatis *et al.*, 1982)/2% SDS at 55 °C and washed in 5 × SSPE/0.2% SDS at 55 °C. DNA probes were stripped from filters by boiling for 20 min in 1% SDS–10 mM Tris/HCl (pH 8.0)–1 mM EDTA (pH 8.0).

DNA probes. Most of the cloned DNA fragments used as probes in this study are shown in Fig. 1. The probes used to establish the presence or absence of the DNA elements (Table 1) were as follows: the *nifD* element probe was the entire element isolated as an *EcoRI* fragment from the clone λ HetL47.1-1.4 (Fig. 1), which was constructed by cloning the excised *nifD* element linearized at a *Sau3AI* site into the lambda vector L47.1; the *xisA* gene probe was a 2.0 kb *BamHI*–*KpnI* fragment from pAM264 (Brusca *et al.*, 1990); the *fdxN* element probe was the cosmid clone T3D4 (Fig. 1); and the *xisF* gene probe was a 2.5 kb *NcoI*–*XbaI* fragment from pAM896, which is equivalent to pAM1150 (Carrasco *et al.*, 1994). Two additional probes were used in the analysis of *Anabaena* M131: the *Anabaena* PCC 7120 *glnA* probe was a 430 bp *HindIII*–*XbaI* fragment from pCP106 (Ramasubramanian *et al.*, 1994) and the *psbA* probe was from *Synechococcus* sp. strain PCC 7942 (*Anacystis nidulans* R2) (Golden *et al.*, 1986).

Restriction map of *Nostoc* Mac. The restriction map of the *Nostoc* Mac *nif* region (Meeks *et al.*, 1994) was confirmed and extended to include the *fdxN* rearrangement. Southern analysis of single and double restriction enzyme digests with several different DNA probes (data not shown) was used to identify restriction fragment sizes near the *fdxN* element recombination sites in vegetative cell and heterocyst DNA samples.

RESULTS

Detection of *nifD* and *fdxN* elements

We surveyed several common laboratory strains of *Anabaena* and *Nostoc* for the presence of DNA sequences similar to the *Anabaena* PCC 7120 *nif* region, which contains the *nifD* and *fdxN* elements. Initial Southern hybridization experiments used *Anabaena* PCC 7120 cosmid clones S2A12, T3D4 and T6C6 (Golden *et al.*, 1988) as probes (Fig. 1). All tested strains showed hybridization with the S2A12 and T6C6 probes since they contain conserved genes involved in nitrogen and carbon fixation (data not shown). However, the T3D4 probe, which is internal to the *fdxN* element, hybridized with only a subset of strains (Table 1). To further establish the presence or absence of sequences similar to the *Anabaena* PCC 7120 *nifD* and *fdxN* elements, additional Southern experiments were done with probes for the 11 kb *nifD* element and the recombinase genes for the *nifD* and *fdxN* elements, *xisA* (Lammers *et al.*, 1986) and *xisF* (Carrasco *et al.*, 1994), respectively (Table 1).

The *Anabaena* and *Nostoc* strains could be placed into one of three groups: those with both elements, those with only the *nifD* element, and *Anabaena* CA, which lacked both elements. *Anabaena* M131, *A. cylindrica* and *Nostoc* Mac, like *Anabaena* PCC 7120, contain both elements and were further analysed (see below). Three *Anabaena* and two *Nostoc* strains contained the *nifD* element but lacked the *fdxN* element. *A. variabilis* had been shown to contain the *nifD* element and an *xisA* gene (Brusca *et al.*, 1989), and mapping data suggested that it lacked the *fdxN* element (Herrero & Wolk, 1986). Our hybridization experiments confirmed the absence of the *fdxN* element and the *xisF* recombinase gene in the *A. variabilis* genome.

Anabaena CA, a marine heterocystous cyanobacterium (Bottomley *et al.*, 1979), did not show hybridization with

Fig. 2. *Nostoc* Mac R2 (Mac) and *A. cylindrica* (A.c.) contain sequences that hybridize with the *fdxN* element. A Southern blot of vegetative cell (V) and heterocyst (H) genomic DNA digested with *EcoRI* was hybridized at moderate stringency as described in Methods with *Anabaena* PCC 7120 cosmid clone T3D4. The positions of DNA size markers are shown on the left in kbp.

probes for either the *nifD* or *fdxN* element, or with probes for the recombinase genes (Table 1).

Vegetative cell and heterocyst DNA was prepared for *A. cylindrica* and *Nostoc* Mac isolate R2 [a Fox^+ revertant containing the *nifD* element (Meeks *et al.*, 1994)] to determine if in these strains the *nifD* and *fdxN* elements are rearranged in heterocysts. Preliminary Southern analysis with S2A12 and T6C6 probes showed evidence for heterocyst-specific DNA rearrangements (data not shown) so these strains were chosen for further analysis.

Nostoc Mac

Southern analysis of *Nostoc* Mac vegetative cell DNA showed hybridization with probes for the *nifD* and *fdxN* elements and their recombinase genes *xisA* and *xisF*, respectively (Table 1). In agreement with a previous report, the *nifD* element has been lost from the Fox^+ revertant isolate R1 (Meeks *et al.*, 1994). Our further analysis of *Nostoc* Mac focused on the Fox^+ revertant isolate R2, which contains a *nifD* element (Meeks *et al.*, 1994). The cosmid T3D4 probe, which contains approximately 35 kb of DNA internal to the *fdxN* element, showed strong hybridization to two fragments in vegetative cell and heterocyst DNA (Fig. 2).

To determine if the *fdxN*-like element underwent rearrangement in *Nostoc* Mac R2 heterocysts, Southern analysis of vegetative cell and heterocyst DNA was performed. The *Anabaena* PCC 7120 fragments An154.2 and An155, which contain the borders of the *fdxN* element, were used as probes against *Nostoc* Mac R2 DNA

Fig. 3. The *Nostoc* Mac *fdxN* element rearranges in heterocysts. Southern blots of vegetative cell (V) and heterocyst (H) DNA from *Nostoc* Mac R2 were hybridized with the *Anabaena* PCC 7120 probes An154.2 (a) and An155 (b). The *Nostoc* DNA was digested with the restriction enzymes *Eco*RI, *Xba*I, *Hind*III, *Hinc*II and *Hpa*I. The An154.2 and An155 probes contain the *nifS* proximal and *nifB* proximal *fdxN* element recombination sites, respectively (see Fig. 1). The positions of DNA size markers are shown on the left in kbp.

digested with several restriction enzymes (Fig. 3). The An154.2 probe showed clear evidence of DNA rearrangement; for example, in the *Eco*RI digest a 6.7 kb band in vegetative cell DNA was replaced by 6.2 kb and 3.1 kb bands in heterocyst DNA. The An155 probe, which is single copy in *Anabaena* PCC 7120 (Carrasco *et al.*, 1994; Golden, J. W. *et al.*, 1987), hybridized to multiple bands in *Nostoc* Mac (Fig. 3). Hybridization to the multi-copy element also occurred under more stringent (65 °C) washing conditions (data not shown). Despite the multiple bands, the An155 probe provided evidence for rearrangement of the *fdxN* element by a reciprocal recombination event; for example, note the 3.1 kb *Eco*RI and 2.2 kb *Hind*III bands identified in heterocyst DNA by both probes.

We have confirmed and extended the map of the *Nostoc* Mac *nif* region (Meeks *et al.*, 1994) to include the borders of the *fdxN* element (Fig. 4). The size of the *fdxN* element in *Nostoc* Mac is unknown. The approximate positions of the *xisF* and *nifS* genes are based on our hybridization results. The distal border of the *fdxN* element in vegetative cells (Fig. 4b) is shown such that rearrangement results in excision of the element, however the orientation of this region is not known. The recombination site of the excised element is shown in Fig. 4(d).

A. cylindrica

Our initial Southern analysis of *A. cylindrica* vegetative cell DNA showed the presence of both the *nifD* and *fdxN*

elements (Table 1). The cosmid T3D4 probe showed strong hybridization to three *Eco*RI fragments in vegetative cell and heterocyst DNA (Fig. 2). Southern blots of *A. cylindrica* vegetative cell and heterocyst DNA were hybridized with DNA fragments containing the borders of the *fdxN* and *nifD* elements to determine if the elements rearranged during development (Fig. 5). The results show clear evidence for rearrangement of both elements; however, our Southern analysis with heterologous probes has not allowed us to construct a restriction map or to establish that the rearrangements result from conservative recombination events.

For the *fdxN* element, the An154.2 probe showed the appearance of 3.6 kb and 2.3 kb *Eco*RI fragments in the heterocyst chromosome (Fig. 5). Although the An155 probe seemed to show the appearance of the same two *Eco*RI fragments in heterocyst DNA, which would be indicative of a conservative recombination event, the data are difficult to interpret because this probe hybridized to multiple bands in *A. cylindrica* similar to the results found with *Nostoc* Mac (compare Figs 3 and 5). The data for *Hind*III- and *Xba*I-digested DNA also show clear changes between vegetative cells and heterocysts indicating DNA rearrangement.

The probes for the *nifD* element, An207.6 and An256, showed that this element rearranges in *A. cylindrica* heterocysts (Fig. 5). The An207.6 probe showed striking changes in all three digested DNA samples. The An256 probe showed clear evidence of rearrangement only in

Fig. 4. Map of the *Nostoc* Mac regions involved in the *nifD* and *fdxN* rearrangements. The restriction enzymes *EcoRI* (R), *HpaI* (H1), *HincII* (H2) and *HindIII* (H3) were used to construct a restriction map of the chromosomal regions involved in the *nifD* and *fdxN* rearrangements in vegetative cell (a, b) and heterocyst (c, d) DNA. The map shows the approximate positions of the *nifK*, *xisA*, *nifD*, *nifH*, *nifS* and *xisF* genes as determined by Southern analysis and comparison to *Anabaena* PCC 7120. Vertical arrows indicate the approximate positions of the recombination sites for the *nifD* (white arrows) and *fdxN* (black arrows) rearrangements. (a) Vegetative cell chromosomal region containing the *nifD* element and the *nifS* proximal recombination site of the *fdxN* element. (b) Region containing the *nifS* distal recombination site. (c) Heterocyst chromosome after rearrangement of the *nifD* and *fdxN* elements. (d) Rearranged *fdxN* element junction presumed to be on the excised *fdxN* element. The excised *nifD* element is not shown.

Fig. 5. *A. cylindrica* *fdxN* and *nifD* elements rearrange in heterocysts. Southern blots of *A. cylindrica* vegetative cell (V) and heterocyst (H) DNA either uncut or digested with the restriction enzymes *EcoRI*, *HindIII* or *XbaI* were probed with the *Anabaena* PCC 7120 fragments An154.2 (a) and An155 (b), which contain the *fdxN* element recombination sites, and An207.6 (c) and An256 (d), which contain the *nifD* element recombination sites (see Fig. 1). The positions of DNA size markers are shown on the left in kbp.

*Eco*RI-digested DNA by the appearance of a 4.0 kb band in heterocyst DNA.

Anabaena M131

Anabaena M131 is a filamentous cyanobacterial strain that does not form heterocysts and cannot fix nitrogen aerobically. Our initial Southern analysis of *Anabaena* M131 (obtained from R. Haselkorn's laboratory) showed no differences between it and *Anabaena* PCC 7120 (data not shown). To confirm these results, we obtained an independent culture from C. P. Wolk's laboratory and performed RFLP analysis. Southern blots of *Anabaena* M131 and *Anabaena* PCC 7120 DNA samples were digested with the four restriction enzymes (*Eco*RI, *Xba*I, *Hind*III and *Cl*aI) and probed with three cosmid clones (S2A12, T3D4 and T6C6) that cover the *nif* and *rbc* region (Fig. 1), and with probes for the *glnA* and *psbA* genes. Over 100 restriction fragments were detected with these probes. The analysis showed no RFLPs between *Anabaena* PCC 7120 and *Anabaena* M131, and showed that *Anabaena* M131 contains the *nifD* and *fdxN* elements.

DISCUSSION

In *Anabaena* PCC 7120, 11 kb and 55 kb DNA elements are found within the ORFs of the *nifD* and *fdxN* genes, respectively. The elements excise from the chromosome during the late stages of heterocyst differentiation allowing the correct expression of the *nifHDK* and *nifB-fdxN-nifSU* operons. We have shown that two additional cyanobacterial strains, *Nostoc* Mac and *A. cylindrica*, contain both of these DNA elements and that the elements are rearranged in heterocysts. A third strain, *Anabaena* M131, also contained both DNA elements but RFLP analysis showed it to be very closely related to *Anabaena* PCC 7120. Our study identified three classes of strains: those mentioned above that contain both elements, those that contain only the *nifD* element, and *Anabaena* CA which lacks both elements.

Anabaena CA is distinct from the other strains examined in this study. It is a marine strain and shows differences in the regulation of heterocyst development and nitrogen fixation compared to fresh water strains (Bottomley *et al.*, 1979). Recent analysis of its *rbcL-rbcS* and *rca* genes showed significant differences from *Anabaena* PCC 7120 and *A. variabilis* (Li & Tabita, 1994). It would not be too surprising if *Anabaena* CA does not contain either the *nifD* or *fdxN* elements in its genome. However, we cannot exclude the possibility that the elements are present but have diverged to such an extent that they cannot be detected by our methods.

Evidence supporting the presence of a *nifD* element had been previously shown for some strains. *A. torulosa* has an 11 kb gap between the *nifHD* and *nifK* genes, and hybridizes with an *xisA* probe (Apte & Thomas, 1987). *Nostoc* UCD 7801 and cultured isolates from *A. caroliniana* were shown to have a gap between *nifHD* and *nifK*, to hybridize with *xisA*, and also to show evidence of

rearrangement to form a contiguous *nifHDK* operon (Meeks *et al.*, 1988). *Nostoc* ATCC 29133 was also reported to contain a *nifD* element (Meeks *et al.*, 1994). In agreement with these earlier results, our hybridization experiments showed the presence of the *nifD* element and the *xisA* gene in each of these strains. Additionally, our results with the *fdxN* element and *xisF* probes indicate that this element is absent from these strains.

All tested strains other than *Anabaena* CA contain the *nifD* element. Of these, three *Anabaena* and two *Nostoc* strains were found to be missing the *fdxN* element. We know of no other characteristic that would group these five strains together. For example, *Nostoc* UCD 7801, *Nostoc* ATCC 29133 and *Anabaena azollae* isolate A2 are symbiotically competent (Meeks *et al.*, 1988, 1994), but *A. variabilis* and *A. torulosa* are not known to be capable of symbiosis. Although it is not understood why these strains lack the *fdxN* element, they clearly demonstrate that the *fdxN* element is dispensable. The *nifD* element is widely distributed in Section IV (Rippka *et al.*, 1979) non-branching, heterocyst-forming species (Meeks *et al.*, 1994). Our data suggests that the *fdxN* element is not as widely distributed among Section IV strains.

Anabaena M131, *A. cylindrica* and *Nostoc* Mac contain both elements. The *fdxN* element is therefore not unique to *Anabaena* PCC 7120. The *fdxN* elements in *Nostoc* Mac and *A. cylindrica* showed only limited similarity to the *Anabaena* PCC 7120 *fdxN* element when the cosmid T3D4 was used as a probe (Fig. 2) indicating significant divergence between the elements. The size of the *fdxN* elements in *Nostoc* Mac and *A. cylindrica* was not determined. Although the *fdxN* elements in *Nostoc* Mac and *A. cylindrica* have diverged significantly from the *Anabaena* PCC 7120 element, they show heterocyst-specific rearrangement. This shows that the different elements have retained this aspect of their regulation.

It is thought that selective pressure, of unknown nature, accounts for the retention of the *nifD* element in the vegetative cell chromosome (Meeks *et al.*, 1994). The *fdxN* element has been retained in the strains discussed above throughout years of laboratory culturing, which also suggests that some type of selective pressure retains the element. However, the selection must be weak since the *fdxN* element is absent from many strains and may only benefit the element itself. Perhaps the *fdxN* element is similar to a lysogenic phage and has acquired developmental regulation as part of its life-cycle to keep it from harming its host under nitrogen-deficient conditions.

Anabaena M131 is very similar to *Anabaena* PCC 7120; our results showed no RFLPs between the two strains. Other investigators have previously noted a high degree of similarity between these strains by genome mapping experiments (Bancroft & Wolk, 1989) and analysis of multicopy insertion sequences (Alam *et al.*, 1991; Cai, 1991). *Anabaena* M131 does not form heterocysts or fix nitrogen aerobically suggesting that the strain has accumulated one or more spontaneous mutations during

laboratory culturing. *Anabaena* PCC 7120 is known to contain active insertion sequences that could provide one mechanism for the proposed mutations (Cai, 1991).

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