Elements interrupting nitrogen fixation genes in cyanobacteria: presence and absence of a nifD element in clones of Nostoc sp. strain Mac

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Nostoc sp. strain Mac is capable of microaerobic, but not aerobic, nitrogen fixation (Fox−). Nostoc Mac grows as long, relatively straight, filaments that are well dispersed in the culture medium. However, spontaneously-arising revertant strains selected for aerobic nitrogen fixation (Fox+) all grow as coiled filaments that associate in macroscopic clumps or balls of varying dimensions. DNA restriction fragment length polymorphism, using nitrogenase (nif) structural genes as probes, established identity between revertants and the parental culture. Mapping of the fragments and lack of hybridization to specific probes indicated the absence of a DNA sequence interrupting the nifD gene in one Fox+ revertant. Such a nifD element is assumed to be present in essentially all heterocyst-forming cyanobacteria. Only one clone out of 223 Fox− and Fox+ Nostoc Mac clones surveyed lacked the nifD element, indicating that loss of the element is a rare event. The nifD element is present in the same location in the genome of Nostoc Mac as it is in all other heterocyst-forming cyanobacteria analysed. No phenotypic differences could be detected between two Fox+ clones containing or lacking the nifD element, including repression and derepression of nitrogen fixation in response to the presence or absence of combined nitrogen. We suspect that retention of the nifD element in vegetative cells of heterocyst-forming cyanobacteria is a consequence of selective pressure, although such selective conditions in laboratory cultures have not been identified.

Keywords: diazotrophic cyanobacterium, nif gene organization, nifD element, nitrogen fixation, Nostoc sp. Mac

INTRODUCTION

In cyanobacteria, the metabolic process of nitrogen fixation is expressed under anaerobic, microaerobic or aerobic growth conditions by unicellular and filamentous species representative of each of the five major taxonomic Sections (sensu Rippka et al., 1979). In the heterocyst-forming species, nitrogenase (nif) expression appears to be confined to the heterocysts (Elhai & Wolk, 1990), whereas it may be active in any or all cells of the non-heterocyst-forming species. Moreover, organization of the nif structural genes, nifH (dinitrogenase reductase), nifD (α-subunit of dinitrogenase) and nifK (β-subunit of dinitrogenase), in vegetative cells seems to be distinctly different between the heterocyst-forming and the non-heterocyst-forming species (Kallas et al., 1985; Haselkorn, 1992; Tandeau de Marsac & Houmard, 1993). In all non-heterocyst-forming, unicellular and filamentous species examined to date, nifHDK are contiguously organized in the genome and transcribed as an operon (Barnum & Gendel, 1985; Kallas et al., 1985), similar to the situation in most proteobacteria (Dean & Jacobson, 1992). While extensive surveys have not been conducted, in essentially all heterocyst-forming strains examined, the nifD gene is interrupted by a DNA element (Haselkorn, 1992) that must be removed before the nifHDK operon can be transcribed (Golden & Wiest, 1988). Details of the element and its excision have been described only in Anabaena sp. strain PCC 7120 (Golden et al., 1985, 1987, 1988).
1991). In *Anabaena* 7120 the element is 11 kb in size, bounded by an 11 bp direct repeat sequence, and includes the genetic sequence *(xisA)* that encodes the site specific recombinase *(XisA or excisase)* responsible for excision and generation of the transcribed operon (Lammers et al., 1986). Excision occurs during latter stages of heterocyst maturation, but the cellular signal(s) for transcription of *xisA* or activation of XisA is unknown (Haselkorn, 1992).

There have been three reports of heterocyst-forming strains in which vegetative cells lack the *nifD* element. Placement in *trans* of a constitutively expressed *xisA* gene in *Anabaena* 7120 resulted in excision of the *nifD* element from all cells of a clonal population, a contiguous *nifHDK* organization and a viable nitrogen-fixing phenotye (Brusca et al., 1990). The moderate thermophile Fischerella sp. ATCC 27929 (PCC 7115) has a contiguous *nifHDK* organization (Saville et al., 1987), but no other related species classified in Section V (Rippka et al., 1979) have been studied. The dominant symbiont (*Anabaena azollae*) in the water fern *Azolla caroliniana* has been shown by restriction mapping to lack a *nifD* element (Meeks et al., 1988) and negative results in Southern hybridizations imply a similar contiguous organization in *A. azollae* from four other *Azolla* species, collected worldwide, that also represent two divergent evolutionary lines of the symbiotic cyanobacterium (Franche & Cohen-Bazire, 1987). In contrast, cyanobacteria cultured from *Azolla caroliniana* contain a *nifD* element (Meeks et al., 1988). Since it is unlikely that cells would acquire DNA in the transition from a symbiotic to a free-living growth stage, these studies defined a negative marker for the dominant symbiotic cyanobacterium in association with *Azolla* spp. and further indicate that this dominant symbiont has not yet been cultured apart from the fern.

We report here a fourth instance of a heterocyst-forming species with a contiguous *nifHDK* organisation in isolation of clonal populations of *Nostoc* sp. strain Mac (PCC 8009). *Nostoc* Mac was originally cultured from symbiotic association within the coralloid roots of the cycad *Macrozamia lucida* L. Johnson (Bowyer & Skerman, 1968). *Nostoc* Mac is among a relatively small group of photoautotrophic cyanobacteria characterized by rapid heterotrophic growth at the expense of exogenous carbohydrate (Bottomley & van Baalen, 1978; cf. also Smith, 1982). At unknown times in its culture history, *Nostoc* Mac accumulated a number of spontaneous mutations, the most obvious being the losses of hormogonia formation and aerobic nitrogen fixation (Fox−; sensu Ernst et al., 1992). Spontaneous Fox− revertants were isolated (Enderlin & Meeks, 1983), but these presumptive revertants have a filament and culture morphology distinct from the parental Fox− strain, implying the possibility of a minor *Nostoc* contaminant in the culture. Therefore, a DNA restriction fragment length polymorphism (RFLP) study was initiated using *nif* structural genes cloned from *Anabaena* 7120 as probes to determine identity between Fox− revertants and the parental culture, as well as to compare with other *Nostoc* strains. The results, detailed here, while establishing such identity, also indicate the absence of a *nifD* element in one revertant, but its presence in the parental culture and all other Fox+ revertants.

**METHODS**

**Culture conditions, mutant selections and cell cloning.** The *Nostoc* sp. strain Mac culture was obtained from Dr L. O. Ingram, University of Florida, via Dr H. T. Bonnett, University of Oregon. It is a direct lineage of the culture obtained by D. S. Hoare (Hoare et al., 1971) from Bowyer & Skerman (1968) and is sister to the culture PCC 8009. The basal medium for growth of all *Nostoc* cultures was that of Allen & Arnon (1955); it was used at full strength when solidified with 1% (w/v) agar (purified by the method of Braun & Wood, 1962) or diluted fourfold in liquid culture. For Fox+ cultures, the basal medium was supplemented with 2.5 mM NH4+ plus 50 mM NO3 (equimolar Na+ and K+ salts) and buffered with 5 mM MOPS, pH 7.5.

To obtain spontaneously-arising Fox+ revertants, NH4NO3-grown *Nostoc* Mac was harvested by centrifugation, washed once in nitrogen-free medium, suspended in that medium and 106 to 5 × 108 cells spread per plate on medium lacking combined nitrogen. The plates were wrapped in parafilm and incubated for 3−4 weeks at room temperature (22−25 °C) under approximately 66 μmol m−2 s−1 of light from 'coolwhite' fluorescent lamps. Revertant colonies were subcloned and maintained under N2 prototrophic growth conditions.

Clones of Fox+ *Nostoc* Mac for colony hybridizations were obtained by first fragmenting filaments to an average chain length of 1−2 cells by sonic cavitation using a microprobe and a model W 225R sonicator (Heat Systems-Ultra Sonics). The fragmented cultures were washed by centrifugation and various dilutions plated on basal medium supplemented with NH4NO3 and 1% purified agar. The plates were incubated as above. Individual colonies (205) were picked to 2 ml of liquid culture and incubated in the light at room temperature until they reached an average chlorophyll *a* (Chl *a*) density of approximately 8 μg ml−1.

**Nitrogen fixation.** Nitrogenase activity was estimated by whole-cell reduction of acetylene to ethylene and the rates normalized to Chl *a* content as described previously (Meeks et al., 1983). To examine repression of nitrogenase activity, 5 mM MOPS-buffered (pH 7.5), 3 mM NH4+ or 5 mM NO3 was added to exponential cultures and the rates of acetylene reduction monitored at various times for up to 192 h on 20 ml subsamples. In derepression experiments, cultures were transferred from NH4NO3 medium to medium lacking combined nitrogen and acetylene reduction monitored as before.

**In vitro DNA manipulations.** All solutions and basic *in vitro* DNA manipulations were according to Maniatis et al. (1982). Cells grown with N2 (all Fox− clones) or NH4NO3 (all Fox+ clones) were lysed and the DNA purified by the procedure of Golden et al. (1985), as modified by Meeks et al. (1988). The restriction endonucleases *EcoRI*, *HindII* and *HindIII* were used as recommended by the manufacturers (Bethesda Research Laboratories; Boehringer Mannheim; New England Biolabs). Electrophoretic separation of the fragments in 0.7% (w/v) agarose, transfer to nylon membranes (Gene Screen Plus; DuPont NEN Products) and hybridization under stringent conditions were as described by Meeks et al. (1988).

For colony hybridizations, 1-5 ml of cell suspension from 20 ml cultures was harvested by centrifugation for 1 min at maximum speed in a microcentrifuge. The cell pellet was washed once with
nif gene organization in Nostoc sp. strain Mac

Fig. 1. Autoradiogram of Anabaena 7120 nifH (pAn154.3) 32P-labelled probe hybridized to endonuclease-digested DNA extracted from Nostoc strain R1 (R1), strain R2 (R2), strain Mac parental culture (Fox-), and strain ATCC 29133 (29133). The DNA was digested singly with the endonucleases EcoRl (1), Hincll (2), Hindlll (3) or in combinations with EcoRl and Hincll (1/2), EcoRl and Hindlll (1/3) and Hincll and Hindlll (2/3). The numbers on the side refer to size in kb of fragments migrating the distance shown as determined by digestion of lambda phage DNA with BstEII.

RESULTS AND DISCUSSION

Isolation and morphological characteristics of Fox+ revertants

Under our liquid culture conditions, Nostoc Mac does not attain high population densities; thus, high-volume cultures must be concentrated and plated to detect spontaneously-arising mutants. Reversion to Fox+ occurred at a frequency of about $2 \times 10^{-8}$. Approximately 40 Fox+ revertants of Nostoc Mac were accumulated in two sets of platings separated by a 6 year period. Of the 20 revertants obtained in the initial set of platings, two were retained and were designated Nostoc Mac strain R1 and Nostoc Mac strain R2. The parental strain grows as relatively long filaments of similar-shaped cells and the filaments are well dispersed in the culture medium. All Fox+ revertants display variable vegetative cell sizes and the coiled filament morphology typical of many Nostoc strains (Rippka et al., 1979), although none appeared to have regained hormogonia formation. The coiled filaments also associate in macroscopic clumps or balls of varying dimensions and densities whether grown with N₂ or NH₄⁺ as nitrogen source.

Restriction patterns of the nifH gene and relatedness of Nostoc Mac clones

To verify that the distinct morphological differences between presumptive revertants and the parental culture were not a consequence of low-level contamination of a different, but poorly competitive, Nostoc strain, RFLPs were analysed. The restriction patterns using a nifH probe are complex in heterocyst-forming cyanobacteria due to the existence of extra copies of the nifH gene; thus this
probe is highly discriminatory in strain comparisons (Meeks et al., 1988). The \( nifH \) restriction pattern was identical in \( \textit{Nostoc} \) Mac and revertant strains R1 and R2 (Fig. 1). The restriction patterns were also identical in the three cultures when fragments of \( nifK, glnA \) (glutamine synthetase) and \( rbcS \) (small subunit of ribulose bisphosphate carboxylase) genes from \( \textit{Anabaena} \) 7120 were used as probes (data not shown). However, the \( nifH \) pattern differed from that of \( \textit{Nostoc} \) sp. ATCC 29133 (PCC 73102) (Fig. 1), which was also isolated as a symbiont from \( \textit{Macroymagia} \) sp. (Rippka \textit{et al.}, 1979), and from those of four other \( \textit{Nostoc} \) sp. strains (data not shown). These results establish clear identity between the Fox- and Fox+ cultures of \( \textit{Nostoc} \) Mac and lack of identity to closely related strains.

\( \textit{Nostoc} \) Mac is capable of nitrogenase expression under microaerobic incubation conditions (data not shown). Thus, the Fox- mutation appears to result in formation of an altered heterocyst wall and decreased oxygen protection of nitrogenase (Murry & Wolk, 1989). We do not know the specific nature of the mutation or of the reversion(s) that corrects the oxygen-sensitive phenotype. Attempts to complement the mutation by conjugation of cosmid-cloned genomic DNA from revertant strain R2 have been unsuccessful (N. Hommes & J. C. Meeks, unpublished results). The observation that spontaneous reversion to a Fox+ phenotype also results in a pleio-

\[ \text{Fig. 2. Autoradiogram of \textit{Anabaena} 7120 nifD (pAn256) 32P-labelled probe hybridized to endonuclease-digested DNA from \textit{Nostoc} Mac Fox+ revertant strains R1 and R2. The endonuclease digests and size markers are as in Fig. 1. The membrane in Fig. 1 was stripped of the nifH probe and reprobed with nifD.} \]

morphic phenotype may seem unusual. However, we and our co-workers (Chapman, 1984; Wallis, 1993) have noted that selection of a nitrogen-fixation phenotype often yields colonies with altered morphologies, especially when it involves mutants that form defective heterocyst walls with oxygen-sensitive nitrogenase activity. It would appear that some reactions of the synthetic pathways resulting in functional heterocyst envelopes are also involved in determining the morphology of the vegetative filament. We speculate that this is the situation in \( \textit{Nostoc} \) Mac and its revertants.

\[ \text{Restriction patterns of the nifD and xisA genes and map of the nifHDK region in revertant strains R1 and R2} \]

Hybridization with a \( nifD \) probe resulted in a different pattern of \( \text{EcoRI}, \text{HincII} \) and \( \text{HindIII} \) restriction fragments between the two Fox+ revertant strains R1 and R2 (Fig. 2). A faint 0.35 kb band was present in \( \text{EcoRI} \) digests of strain R1, but not of strain R2. In single \( \text{HincII} \) digests, strain R1 had a single band at 2.3 kb, while strain R2 had a major band at 4.8 kb and a minor band at 2.3 kb. Digests with \( \text{HindIII} \) give identical 3.7 kb bands, but additional 0.9 or 2.2 kb bands were present in strains R1 or R2, respectively. Identical bands of 1.7, 2.9 (plus 0.4) and 1.3 kb were present in \( \text{EcoRI} + \text{HincII}, \text{EcoRI} + \text{HindIII} \) and \( \text{HincII} + \text{HindIII} \) double digests, respectively, of the
two strains. However, the EcoRI + HincII and EcoRI + HindIII digests gave unique bands of 2.9 and 1.85 kb, respectively, in strain R2, while strain R1 yielded a 0.35 kb band characteristic of EcoRI digestion alone. The 0.9- and 2.2 kb bands in HincII + HindIII digests of strains R1 and R2, respectively, are those characteristic of digestion with HindIII alone. The banding patterns in the parental Fox+ Nostoc Mac cultures were identical to those of revertant strain R2 (data not shown). As will be detailed below, fragments in common in the double digests of strains R1 and R2 map to the nifH end of the nifD probe, while those that differ map toward the nifK end.

A difference in spacing between nifD and nifK was implied by probing the same blots with a fragment of xisA: this probe hybridized to genomic DNA from strain R2, but not to that from strain R1, under high-stringency (Fig. 3) and low-stringency (data not shown) conditions. Moreover, a similar lack of hybridization to strain R1 DNA, but hybridization to strain R2, was observed when a fragment of pAn207.3 was used as a probe (data not shown). In Anabaena 7120, 207.3 maps at the opposite end from xisA within the nifD insertion element (Lammers et al., 1986). These data indicate that genomic DNA of strain R1, but not strain R2, lacks two of the genes located within the nifD insertion element of Anabaena 7120.

Restriction maps of the nifHDK region in the two strains, based on data from Figs 1, 2 and 3, plus blots of nifK (data not shown), are depicted in Fig. 4. These maps emphasize the presence of a nifD element absent in strain R1, but that one is present in strain R2 and the parental Fox- culture. Similar restriction maps of the nifHDK region, showing the presence of a nifD element containing xisA and 207.3, have been constructed with DNA from Nostoc sp. ATCC 29133 and Nostoc sp. ATCC 27896 (PCC 6310) (J. C. Meeks, unpublished results). Thus, based on restriction maps of Nostoc strains Mac R2, ATCC 29133 and ATCC 27896, the nifD element is present in the same genomic location as it is found in Anabaena 7120 (Golden et al., 1985), A. variabilis (ATCC 29413) (Brusca et al., 1989), and Nostoc sp. strains N1 (UCD 120) and UCD 7801 (Meeks et al., 1988). Based on incomplete maps, a nifD element in the same location was also inferred in Nostoc sp. strains PCC 7121 and PCC 7906, as well as Calothrix sp. strain PCC 7601 (Kallas et al., 1985). These observations support the conclusion that the nifD element is widely distributed in heterocyst-forming species, at least those of Section IV (Rippka et al., 1979). Absence of the element in Fischeraella sp. ATCC 27292 (Saville et al., 1987) renders it unclear whether the element is present in heterocyst-forming strains classified in Section V since no other strains have been examined. Although it is not known when insertion of the nifD element occurred in species of Section IV, it appears to have been conserved in its genomic location while strains or species have evolved, as evidenced by mutations reflected in different restriction patterns in and around the nif genes.

The restriction map in Fig. 4 allows identity of the minor or weakly hybridizing bands to the Anabaena 7120-derived An256 probe of strain R2 in Fig. 2. For examples, the weakly hybridizing 2.3 kb band in HincII digests is probably a consequence of a small fraction of the DNA having rearranged in heterocysts. Conversely, the faint bands of 2.9 kb in EcoRI + HincII and 1.85 kb in EcoRI + HindIII double digests result from weak hybridization to the region of the nifD element of An256 between the nifD insertion point and 207.3, which also shows low homology to other Nostoc strains (Meeks et al., 1988). This low homology implies evolutionary divergence of the sequences within the nifD element.

**nifD restriction patterns and hybridization of xisA to additional Fox+ and Fox- clones**

To assess the frequency at which loss of the nifD element may occur in the Nostoc Mac population, additional Fox+ revertants were isolated and cloned some 6 years after isolation of strains R1 and R2. The genomic DNA of these revertants was digested with HindIII, which yields distinct banding patterns in strain R1 compared to strain R2 when probed with nifD (Fig. 2). The results with 16
such randomly selected revertants, plus strains R1 and R2, are shown in Fig. 5. In all cases the restriction pattern of these revertants was characteristic of strain R2, implying the presence of a nifD element.

To increase the sample size and uncouple two potentially different alterations, excision of the nifD element and reversion of the Fox- phenotype, in the genomic DNA of Nostoc Mac, 205 clones were isolated on NH₄NO₃-supplemented medium. These clones, plus strain R1 as the negative control, were subjected to colony lysis and probed with xisA, yielding a positive result in all cases except strain R1 (data not shown). These results support the idea that loss of the nifD element in vegetative cells is a rare event.

**Nitrogen repression phenotype of revertant strains R1 and R2**

Heterocyst differentiation and subsequent nitrogenase expression are regulated by the presence or absence of combined nitrogen in the medium (Wolk, 1982). To determine if the nifD element is directly involved in nitrogen control, repression and derepression of nitrogenase activity was examined in strains R1 and R2 (Fig. 6).

The kinetics of repression by NH₄⁺ or NO₃⁻ of nitrogen-fixing cultures were identical in the two strains, as were the kinetics of derepression when cultures were transferred from NH₄NO₃ to combined-nitrogen-free medium. These results indicate that genetic information within the nifD element does not influence other genes in response to combined nitrogen availability. It is noteworthy that NO₃⁻ was as effective as NH₄⁺ in repression of nitrogenase expression in the Nostoc Mac clones; in many diazotrophic cyanobacteria, NO₃⁻ is a less effective repressor (van Baalen, 1987).

We have superficially surveyed other phenotypic characteristics in comparative experiments with strains R1 and R2. These include heterotrophic growth with glucose or sucrose as carbon sources, complementary chromatic adaptation and viability after desiccation, with no discernible phenotypic differences. Phage susceptibility has not been examined.

**Significance of the nifD element**

The results of our limited screening of Fox⁻ and Fox⁺ Nostoc Mac clones indicate that loss of the nifD element is a rare event; nevertheless, the isolation of strain R1
indicates that it does occur on a random basis. Since the excision is a deletion event, and there is no experimental evidence for reintegration of the element, cells that have excised the element should accumulate in the population, unless there is some selective pressure for its retention. If the frequency of random excision is similar to that of spontaneous mutation (about 10^{-6}) and 1–10 ml of about 10^7 cells ml^{-1} are transferred at each subculturing, it is possible that cells lacking the nifD element are rarely carried in the subcultures. Thus, the 223 clones examined would be an insufficient population size from which to draw meaningful conclusions. However, the lack of accumulation of such cells seems unlikely given the length of time Nostoc Mac has been maintained in culture collections and subcultured (6 years of subculturing by us prior to establishing -80 °C stocks).

Although selective pressure seems the most likely rationale for retention of the nifD element, such selective conditions are obscure. We have seen no phenotypic differences between separate cultures of strains R1 and R2 under our benign culture conditions, but we have not examined growth in dual culture. Haselkorn (1992) has suggested that sequences in the nifD element may provide a growth advantage similar to the situation with DNA repair by the bleomycin resistance gene when in Escherichia coli. The presence of a gene homologous to cytochrome P-450 in the element could confer an advantage (Lammers et al., 1990). A competitive advantage would explain the rare presence of cells lacking the nifD element that could have accumulated in the Nostoc Mac cultures. It is worth noting that the two natural populations of heterocyst-forming cyanobacteria lacking the nifD element occur in moderately extreme habitats. The moderate thermophilic Fischerella sp. ATCC 27929 may face less competitive pressure in thermophilic waters than heterocyst-forming strains in mesophilic or soil habitats. Additional cultures from thermophilic environments should be examined. Anabaena azollae may be an obligate symbiont in Azolla spp. with no other growth environment (Meeks et al., 1988). In this case, the fern may limit competition by providing barriers against colonization and growth by other diazotrophic cyanobacteria. This idea is supported by the observation that minor symbionts, cultured from Azolla caroliniana, are present in such low population densities that they cannot be detected by molecular probes in total symbiont preparations from the association (Meeks et al., 1988).

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