Nitrogen fixation genes (nif K,D,H) in the filamentous nonheterocystous cyanobacterium Plectonema boryanum do not rearrange

SHREE KUMAR APTE and JOSEPH THOMAS
Molecular Biology & Agriculture Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085, India

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Abstract. The organisation of the structural genes for nitrogen fixation (nif K,D and H) in a nonheterocystous, filamentous cyanobacterium Plectonema boryanum has been examined in comparison with a heterocystous cyanobacterium, Anabaena torulosa. DNA from repressed (fix-) cultures of A. torulosa showed a discontinuous nif region spread over approximately 18 kb, an arrangement typical of the vegetative cells of heterocystous cyanobacteria. The region contained a contiguous nif/DH separated from nif/K by nearly 11 kb DNA. The intervening 11 kb DNA harboured the gene xisA involved in the rearrangement of nif/K,D,H to form a cluster during differentiation of heterocysts.

DNA from Plectonema boryanum had a small, contiguous nif/KDH cluster spanning a region of approximately 4 kb. DNA homologous to the 11 kb excision with its resident xisA was not present. Nif hybridisation patterns of restriction digests of the DNA isolated from repressed (fix-) or induced (fix+) cultures of P. boryanum were completely identical. These results unequivocally demonstrate that in the nonheterocystous cyanobacterium, unlike in the heterocystous strains, no gene rearrangement, either within the nif/KDH cluster or in its vicinity, accompanies the expression of nitrogenase activity.

Keywords. Filamentous nonheterocystous cyanobacteria; nitrogen fixation genes; P. boryanum; gene rearrangement.

1. Introduction

The ability to reduce atmospheric dinitrogen is restricted to only a few groups of prokaryotes. The reduction of dinitrogen to ammonia is brought about by an oxygen labile enzyme complex consisting of two proteins—nitrogenase and nitrogenase reductase (Eady and Postgate 1974). Nitrogenase is an α2 β2 tetramer encoded by nifK (β subunits) and nifD (α subunits), while the reductase is a α2 dimer encoded by nifH (Dixon 1984). Nitrogenase and nitrogenase reductase from different N2-fixing microbes appear to have been fairly conserved and exhibit considerable homology and cross reactivity both at the level of proteins and their corresponding genes (Orme-Johnson 1985). The organisation of nif structural genes (i.e. nifK,D,H) in various diazotrophs is, however, by no means uniform. For example, the enterobacterium Klebsiella pneumoniae (Merrick et al 1980), the photosynthetic bacterium Rhodopseudomonas capsulata (Avtges et al 1983) and the fast growing rhizobia, Rhizobium meliloti (Corbin et al 1982), R. leguminosarum (Downie et al 1983) or species of Azotobacter (Jones et al 1984) all possess a contiguous nif/KDH cluster. In contrast, in the slow growing rhizobia R. japonicum (Hahn et al 1984), and in the Rhizobium which nodulates the non-legume Parasponia (Weinman et al 1984), nif/KD and nif/H are separated. The functional significance of these arrangements is not yet understood.
Many cyanobacteria, which are the unique photoautotrophic diazotrophs with an oxygenic mode of photosynthesis, show both contiguous and discontinuous type of \textit{nifK,D,H} organisation (Haselkorn 1986; Hallenbeck 1987). Thus, in \textit{Gloeothecae} PCC 6909-1, a unicellular, aerobic, nitrogen-fixing cyanobacterium, \textit{nifK,D,H} occurs in a cluster (Kallas et al 1983) while in the vegetative cells of the filamentous, heterocystous cyanobacterium \textit{Anabaena} 7120 yet another discontinuous arrangement has been reported with \textit{nifK} being separated from \textit{nifDH} by an 11 kb intervening sequence (Rice et al 1982). More interestingly, in heterocysts this intervening sequence (excison) is precisely deleted and \textit{nifK} and \textit{nifDH} get juxtaposed together in a cluster and are transcribed, as in \textit{Klebsiella}, from a single \textit{nifH} promoter (Golden et al 1985). This rearrangement is apparently linked to N\textsubscript{2} fixation since it also occurs in het\textsuperscript{−} mutants of heterocystous cyanobacteria (Damerval et al 1985) and even in \textit{Escherichia coli} wherein a relevant portion of \textit{Anabaena} DNA has been cloned (Lammers et al 1986).

These findings have raised important questions regarding the organisation of the \textit{nif} region in filamentous, nonheterocystous cyanobacteria, especially the possibility of a rearrangement of the \textit{nif} structural genes. It is interesting to know whether such cyanobacteria (i) have a clustered or a discontinuous arrangement of \textit{nifK,D,H}, (ii) rearrange \textit{nifK,D,H} during N\textsubscript{2} fixation, and (iii) possess excision mechanisms similar to those (\textit{xisA}) responsible for \textit{nifK,D,H} rearrangement during heterocyst differentiation in \textit{Anabaena}. A previous work has shown a contiguous \textit{nifK,D,H} arrangement in repressed (fix\textsuperscript{−}) cultures of two filamentous, nonheterocystous forms (Kallas et al 1985) and in one of these strains the possibility of a \textit{nif} rearrangement has been reported (Barnum and Gendel 1985). But these reports do not provide definitive information on all the three essential points cited above. In the present study these questions are investigated in a filamentous, nonheterocystous cyanobacterium \textit{Plectonema boryanum}-594.

2. Materials and methods

2.1 Organisms and growth conditions

\textit{Anabaena torulosa}, a filamentous, heterocystous, sporulating strain of brackish waters was isolated in this laboratory (Fernandes and Thomas 1982) and used in axenic condition. \textit{Plectonema boryanum}-594, a filamentous, nonheterocystous strain was obtained from Prof R Haselkorn, University of Chicago. Fifteen-litre cultures of both strains were grown in five-fold diluted cyanophagean medium (CM/5) (David and Thomas 1979), with or without 10 mM KNO\textsubscript{3}, under continuous illumination (2-5 mW per square cm) and aeration (2 litres per min) to the stationary phase. \textit{P. boryanum} was induced to fix N\textsubscript{2} microaerobically by growing in combined N-free CM/5 and continually sparging with N\textsubscript{2} for 40 hr as described previously (Apte and Thomas 1984).

2.2 Extraction of chromosomal DNA, restriction digestion and preparation of Southern blots

High molecular weight chromosomal DNA was prepared from approximately 15 g fresh weight of cells harvested from each culture. DNA was extracted by a slight
modification of the procedure described by Mazur et al (1980). Lysozyme
ccentration used and the period of lysis at 37°C was as follows: *A. torulosa*, 5 mg
per ml, 45 min; *P. boryanum*, 10 mg per ml, 2–5–3 hr. Repeated (2–3 times)
spooling of DNA on to glass rods was carried out to obtain a high purity of DNA. In
addition sometimes DNA was further purified on NACS columns (Bethesda
Research Labs, Gaithersburg, Maryland) using the procedure recommended by the
manufacturers. DNA was hydrolysed with EcoRI, Clal, BglII and HindIII (all from
Bethesda Research Labs.) for 5 hr under buffer conditions and temperature
recommended by the supplier. The reactions were terminated by heating for
10 min at 65°C. Five μg DNA was electrophoresed per lane of a 0.7% agarose gel
(20 × 15 × 0.8 cm). Denaturation, neutralisation and unidirectional Southern tran-
fer of DNA to Millipore (HAHY 304, FO HA, 0.4 μm) were performed in 20 × SSC as
described by Maniatis et al (1982).

2.3 Gene probes and hybridisations

All the probes used were recombinant plasmids constructed by the laboratory of
Prof R Haselkorn and contained clones of fragments from the nif region of
*Anabaena* 7120. These gene probes have been described in table 1. A nick
translation kit (N-5000, Amersham International plc., Amersham, England) and α-[32P]dCTP (Isotope Division, BARC, Bombay) were used to radiolabel probes to
1–2 × 10^8 cpmp per μg DNA. Prehybridisation (6 hr) and hybridisation (20 hr) were
performed in 6 × SSC at 65°C as described by Maniatis et al (1982). Blots were
washed in the following sequence: (i) 2 × SSC + 0.2% SDS, three times for 10 min

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<th>Table 1. <em>Anabaena</em> 7120 DNA probes used in this study.</th>
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<td>Recombinant</td>
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<tr>
<td>pAn207-8^b</td>
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|              | the DNA corresponding   | personal communi-
|              | to the 11 kb excision   | cation (see Lam-
| pAn207-3^b   | 2 kb *HindIII* fragment |
|              | located on the 11 kb    | et al 1986, for a |
|              | excision close to nifD   |
| pAn256^b     | A *HindIII* fragment with|
|              | most of nifD (1.4 kb) and|
|              | non-nif/DNA (1.3 kb)    | restriction map of|
| pAn154-3^b   | A *HindIII* segment with |
|              | all of nifH (0.9 kb), 0.15 |
|              | kb of nifD and non-nif |
|              | DNA (0.75 kb)           | the excision)     |

^a All the plasmids were provided by Prof Robert Haselkorn;
^b these *Anabaena* inserts have been cloned into *HindIII* site of pBR322;
^c the *Anabaena* insert was cloned into *SmaI* site of pUC19.
each at 25°C, (ii) 1 × SSC + 0.1% SDS, four times for 30 min each at 65°C, (iii) 0.1 × SSC, two times for 15 min each at 25°C. Dried blots were exposed to X-ray films at −70°C using intensifying screens, for 2–7 days.

3. Results

Figure 1 shows the hybridisation of various Anabaena 7120 probes to Southern blots of HindIII digests of A. torulosa DNA. A. torulosa was used here as a representative of heterocystous cyanobacteria and as a positive control for the various Anabaena probes employed. All the probes hybridised well to A. torulosa DNA but showed a HindIII restriction pattern quite distinct from that of Anabaena 7120 (Golden et al 1985). EcoRI and BglII did not hydrolyse A. torulosa DNA properly. HindIII sites in the nif region of A. torulosa were less frequent than in Anabaena 7120 thus yielding larger nif positive fragments (2.8–3.2 kb) than those observed in Anabaena 7120. In spite of this, no single fragment hybridised to all three probes of the nif structural genes (nifK, D as well as nifH; figure 1) thus indicating that they were not clustered. Two fragments hybridised to nifH probe. We have evidence now that the larger fragment (3.2 kb) is involved in the rearrangement in heterocysts (data not included) and is therefore associated with nifD while the 1.2 kb fragment probably indicates a second copy. Two probes internal to the 11 kb intervening sequence (excision) between nifK and nifD of Anabaena 7120 were used to locate homologous sequences in A. torulosa. To our knowledge the data in figure 1 is the first evidence of occurrence of genes homologous to fragments 207.3 and 207.65 outside Anabaena 7120. Interestingly, a summation of fragment sizes hybridising to these two probes yield 11.2 kb suggesting that the dimensions of such excisons may be identical in many heterocystous cyanobacteria. The overall results suggest a discontinuous nifK,D,H arrangement similar to other heterocystous cyanobacteria (Hallenbeck 1987).

Figure 2 shows the hybridisation of Anabaena 7120 nifK (2b), nifD (2c) and nifH (2d) probes to Southern blots of ClaI, EcoRI and HindIII single and double digests of DNA isolated from repressed (fix−) cultures of P. boryanum (2a). All these probes hybridised to a single ClaI fragment of 12.1 kb. In HindIII digests both nifD and nifH were found located on the same 3.3 kb fragment indicating a close linkage between these two genes. No evidence for an association between nifK and nifD genes was found in these digests.

P. boryanum DNA did not hybridise to the probes pAn207.3 and pAn207.65 (data not shown) even at a much lower stringency of washing (5 × SSC + 0.5% SDS) although positive bands were observed with A. torulosa DNA digests. Clearly, therefore, DNA homologous to the sequence intervening between nifK and D of Anabaena 7120 and more specifically to xisA was absent in P. boryanum.

Figure 3 provides a comparison of the hybridisation of Anabaena 7120 nifK,D,H probes to DNA isolated from repressed (fix−) or induced (fix+) cultures of P. boryanum. All the three probes hybridised to either a single 12.1 kb ClaI fragment (3a) or a single 3.4 kb BglII fragment (3b). This pattern of hybridisation was completely identical both in fix− as well as fix+ cultures and no evidence of a gene rearrangement within (3b) or nearby (3a) to the nifKDH cluster was detected. BglII data clearly showed that all the three genes (nifK,D,H) were contiguous.
Figure 1. Hybridisation of *Anabaena* 7120 gene probes to DNA obtained from the vegetative cells of the heterocystous cyanobacterium *Anabaena torulosa*. The lanes from left to right are: a stained agarose gel showing electrophoretic fractionation of *A. torulosa* vegetative cell DNA digested with *Hind*III (lane 1); autoradiograms showing hybridisation of replicate blots of lane 1 to pAn207-8 (*nif*K, lane 2), pAn207-3 (fragment internal to the 11 kb excision located close to *nif*D, lane 3), pAn256 (*nif*D, lane 4) and pAn154-3 (*nif*H, lane 5). Lane 6 shows hybridisation of pAn207-55 (xisA) to a separate blot. DNA fragment sizes (in kb) shown to the right of lane 5 correspond to lanes 2-5 and those shown to the right of lane 6 correspond to lane 6 only.

4. Discussion

The hybridisation patterns described in figures 2 and 3 have allowed the construction of a restriction map for the *nif* region of *P. boryanum*-594 (figure 4a). The map is complete for a region of approximately 8 kb spanning the EcoRI site to the left of *nif*K to the EcoRI site to the right of *nif*H. The two *Cla*I sites which lie outside this region cannot be mapped and their proposed location is tentative (indicated by dotted line). Based on the BglII data, the *nif*KDH cluster should span 3.4 kb which cannot possibly accommodate all the information necessary to make active nitrogenase proteins. In *K. pneumoniae*, the *nif*KDH cluster spans 4 kb (Roberts and Brill 1981) and it is likely that the physical limits of *P. boryanum* *nif*KDH cluster are similar to or greater than those described by figure 4a. A similar restriction map of the *nif* region of *A. torulosa* could not be determined due to incomplete hydrolysis and the absence of overlapping restriction fragments. Yet a tentative *Hind*III map has been prepared by lining up *Hind*III fragments in the order in which they are found in *Anabaena* 7120 (Golden et al 1985). This is presented in figure 4b for comparison. A total span of 18 kb appears to accommodate the *nif*K,D,H and intervening sequence (with *xis*A) of *A. torulosa*. This is in agreement with the dimensions of corresponding DNA in *Anabaena* 7120 (Haselkorn...
Figure 2. Localization of nifK,D and H genes in the repressed (fix⁻) cultures of the nonheterocystous cyanobacterium *Plectonema boryanum*-594. (a) Stained agarose gel showing electrophoretic fractionation of *P. boryanum* DNA digested with *Clal* (lane 1), *Clal* + *EcoRI* (lane 2), *EcoRI* (lane 3), *EcoRI* + *HindIII* (lane 4), *HindIII* (lane 5) and *HindIII* + *Clal* (lane 6). Lane 7 contains size markers; a mixture of bacteriophage lambda DNA digested with *XhoI* and *BstEII*. To the right are autoradiograms showing hybridisation of replicate blots of the gel shown in (a) to *Anabaena* 7120 probes pAn207-8 (b), pAn256 (c) and pAn154-3 (d). DNA fragment sizes in kb are shown to the right of each autoradiogram.
Figure 3. Hybridisation of Anabaena nifK,D and H probes to DNA isolated from repressed (fix⁻) or induced (fix⁺) cultures of P. boryanum-594. DNA from fix⁻ (lanes 1,3,5 and 7) and fix⁺ (lanes 2,4,6 and 8) cultures was digested with either Clal (a) or BglII (b). Lanes 1 and 2 show electrophoretic fractionation of the restriction digests on agarose gels. Lanes 3 to 8 show autoradiograms of hybridisation of replicate blots of lanes 1 and 2 to pAn207.8 (lanes 3,4), pAn256 (lanes 5,6) and pAn154.3 (lanes 7,8). The numbers indicate DNA fragment sizes in kb.
Figure 4. Restriction maps of nif region of Plectonema boryanum (a) and Anabaena torulosa (b). The P. boryanum map has been constructed from the data shown in figures 2 and 3 and is complete for an 8 kb region (indicated by solid lines). The location of Clai sites is tentative and is indicated by dotted lines. The BglII sites are shown to be within nif cluster since 3-4 kb DNA cannot accommodate the information pertaining to all three nif genes. The physical sizes (shown as cross-hatched rectangles) of nifK and D are based on present data and that of nifH is based on known molecular weight of nitrogenase reductase. A tentative map of A. torulosa (b) obtained by lining up all HindIII fragments (figure 1) hybridising to various gene probes in the same order in which they are located on Anabaena 7120 chromosome. The fragments within each gene can be oriented either way. The map is given for comparison with Plectonema (a) and shows the important differences in nif organisation: i.e., a total span of = 18 kb with an intervening sequence of = 11 kb in A. torulosa as against a small, contiguous cluster of = 4 kb in P. boryanum.

et al 1986) except that the latter has many more HindIII sites (13) than has A. torulosa (7) in this region.

It is quite clear now that the heterocystous and nonheterocystous cyanobacteria differ strikingly in their organisation of nif structural genes. In the heterocystous forms, the vegetative cell DNA shows a characteristic discontinuous arrangement (figure 4b) of nifK,D and H. An intervening sequence between nifK and D approximately 11 kb in size, with a resident xisA may also be a general feature of all heterocystous forms including their het- mutants. In contrast, the filamentous, nonheterocystous cyanobacteria show a contiguous arrangement of nifK,D,H within a small cluster (figure 4a) similar to unicellular cyanobacteria (Kallas et al 1983) or Klebsiella (Merrick et al 1980). The total size of fragments hybridising to all the three nif structural genes in P. boryanum ranges from 3-4 kb (BglII) to 4-2 kb (HindIII) which cannot accommodate any more than the structural genes. Clearly, therefore, a rearrangement within the nif cluster is improbable and is proved to be so by the BglII data (figure 3b). The possibility of a reorganisation close to the nifKDH cluster, if not within, was suggested by Barnum and Gendel (1985) who showed that the fix+ cultures of
P. boryanum showed a 4.5 kb Clal fragment containing all three nif genes in addition to the 12 kb Clal fragment found in fix^− cultures. The results shown in figure 3 discount this observation. It is possible that the 4.5 kb fragment was a result of some non-specific hybridisation at a relatively low stringency employed by the above workers. The present study unequivocally demonstrates that filamentous, nonheterocystous cyanobacteria exhibit no reorganisation of genes, either within the nif/KDH cluster or in its close vicinity during the expression of nitrogenase activity.

Acknowledgements

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