ORGANIZATION OF *nifH* and *nifD* GENES IN *NOSTOC COMMUNE* UTEX 584

by

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(ABSTRACT)

Nostoc commune UTEX 584 is a photosynthetic desiccation-tolerant cyanobacterium capable of fixing nitrogen. Biotinylated *nifH* and *nifD* gene probes from *Azotobacter vinelandii* (a gift from Dr. Dennis Dean) hybridized to *nifH* and *nifD* sequences isolated from *Nostoc commune* UTEX 584. This result supports the view that the nitrogenase structural proteins and their genes are highly conserved in nitrogen-fixing organisms (Rice *et al.*, 1980). Southern transfers of genomic DNA prepared from *N. commune* UTEX 584 were digested with *Hind* III and hybridized with *nifH*-specific and *nifD*-specific probes. Multiple copies of *nifH* (three) and *nifD* (two) were detected. Using colony hybridization, a recombinant *N. commune* UTEX 584 genomic DNA-pBR322 plasmid library was screened with the biotinylated *nifH*-specific probe and positive hybridizing *nifH* clones were isolated. A restriction map of the 3.5 kb *Hind* III insert of the recombinant plasmid (pND001) was produced. From partial sequencing data it was possible to

determine the positions of the N. commune UTEX 584 nifH and nifD genes within the cloned fragment and compare the partial nucleotide sequence and deduced amino acid sequences of the N. commune UTEX 584 nifHD genes with other organisms. Isolation of the nifH and nifD genes from Nostoc commune UTEX 584 now permits a more detailed study of nif gene expression in this desiccation-tolerant photosynthetic microorganism.

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INTRODUCTION

The fixation of nitrogen by both free-living and symbiotic nitrogen-fixing bacteria involves an enzyme complex called nitrogenase. Nitrogenase consists of two proteins, an Fe protein (component II) and a MoFe protein (component I). The Fe protein is composed of two identical subunits with a total molecular weight between 57,000 - 73,000 daltons surrounding an iron-sulfur center consisting of four iron atoms and four acid-labile sulfur atoms. The MoFe protein is considerably more complex. It is a tetramer and has a total molecular weight in the range of 200,000 - 270,000 daltons. The MoFe protein contains four [4Fe-4S] clusters and two iron-molybdenum-sulfur "cofactors" which each contain Fe, Mo and S in the ratio of 8:1:6. The Fe protein and the MoFe protein, including the MoFe cofactor, are irreversibly inhibited by oxygen. The Fe protein supplies reducing equivalents to the MoFe protein which probably binds the substrate and catalyzes its reduction. The transfer of electrons from the Fe protein to the MoFe protein is coupled to Mg-ATP hydrolysis (Bothe, 1982). A more detailed discussion on the structure and reactivity of nitrogenase is given in Mortenson and Thorneley (1979).

Nitrogen fixation requires large amounts of energy with approximately 12 high energy phosphate bonds and 6 low-

potential reducing equivalents in the form of ferredoxin or flavodoxin required for each mole of N_2 reduced (Mortenson and Thorneley, 1979). Since both components are extremely sensitive to oxygen, an additional requirement of the system is an anaerobic environment. Nitrogen-fixing microorganisms have evolved mechanisms to protect nitrogenase from oxygen damage. For example, Rhizobium, a symbiotic nitrogen-fixing bacterium, infects plant roots leading to the formation of root nodules where N_2 fixation occurs (Newcomb, 1981). The leguminous plant produces leghemoglobin, a haemoprotein, which binds O_2 to protect nitrogenase. Azotobacter, a non-photosynthetic aerobic bacterium, is known for its extremely high respiratory activities preventing diffusion of oxygen into the cell (Dalton and Postgate, 1969). Certain cyanobacteria contain specialized cells called heterocysts which provide an anaerobic environment for nitrogen fixation (Fogg, 1949).

Cyanobacteria (blue-green algae) are a diverse group of photosynthetic microorganisms that exist in many environments owing to their ability to tolerate water stress and desiccation and extremes of temperature (Whitton and Potts, 1982). These organisms are characterized as prokaryotes based on their lack of membrane-bound organelles. Their photosynthetic system consists of the pigments chlorophyll a and the phycobiliproteins

(phycoerythrin, phycocyanin and allo-phycocyanin) which absorb light in the range of 560 nm to 680 nm. With a few exceptions, blue-green algae are photoautotrophs, using CO_2 as a carbon source and light as the energy source (Whitton and Potts, 1982). Some cyanobacteria are capable of using organic substrates, in addition to or instead of CO_2, as a carbon source while remaining dependent on light as the energy source; these are classified as facultative photoheterotrophs. Cyanobacteria capable of using organic substrates as both a carbon and energy source in dark metabolism are facultative chemoheterotrophs. Cyanobacteria are capable of switching from a chloroplast-type O_2-evolving photosynthesis (PS I and PS II) to anaerobic photosynthesis (PS I only)-a characteristic of photosynthetic bacteria.

An oxygen-free environment is a requirement for nitrogen fixation in unicellular, filamentous (non-heterocystous), and filamentous (heterocystous) cyanobacteria. The heterocystous forms differentiate heterocysts at regular intervals along the filament under conditions of ammonia or nitrate deprivation (Wolk, 1982). In early differentiation, the vegetative cell undergoes various changes in structure and metabolism. A thick-walled envelope composed of glycolipid and polysaccharide components unique to the heterocyst is formed, which functions to reduce the

diffusion of atmospheric gases through the surface of the cell (Wolk, 1975). Proteases accumulate which degrade both the phycobiliprotein light-collecting apparatus of Photosystem II and enzymes that fix CO_2 (Tel-Or and Stewart, 1976). Consequently, heterocysts rely upon the vegetative cells for their supply of carbohydrate (Wolk, 1968). Heterocysts contain a functional Photosystem I which provides the cell with ATP produced by cyclic photophosphorylation. Reducing power in the form of NADPH is generated from the Pentose Phosphate Cycle. Dinitrogen (N_2) is transported to the site of nitrogen-fixation by diffusion through the heterocyst from the atmosphere and is also transported from vegetative cells through fine channels (microplasmodesmata) that pass through the cross walls between vegetative cells and heterocysts (Fay, 1983). Fixed nitrogen (glutamine) is then transported in the opposite direction.

In nitrogen-fixing organisms, the genes encoding the MoFe protein (*nifK* and *nifD*) and the Fe protein (*nifH*) are highly conserved (Rice *et al.*, 1980). The organization of genes required for N_2 fixation in *Klebsiella pneumoniae* has been studied in detail and the cloned *nifHDK* genes from this organism have served as heterologous probes for study of *nifHDK* genes from other organisms (Cannon *et al.*, 1979). Many organisms show the same physical organization of *nifHDK*

genes as that determined for K. pneumoniae some examples are: Rhodopseudomonas capsulata, a purple nonsulfur bacterium (Avtges et al., 1983), Gloeothece sp. PCC 6909, a unicellular aerobic nitrogen fixing cyanobacterium (Kallas et al., 1983), and Plectonema boryanum, a filamentous, non-heterocystous cyanobacterium (Barnum and Gendel, 1985). A different arrangement of the nifHDK operon has been shown for Anabaena, a filamentous, heterocystous cyanobacterium (Golden et al., 1985), Calothrix sp. PCC 7601, a filamentous heterocystous bacterium (Kallas et al., 1983), and certain slow-growing Rhizobium species (Kaluza et al., 1983). Under conditions of ammonia or nitrate deprivation, certain vegetative cells of Anabaena differentiate into heterocysts and a rearrangement of the nifHDK operon occurs (Golden et al., 1985). It has been shown that 11 kilobases of intervening DNA (called an "excison") interrupts the reading frame between nifD and nifK in the vegetative cell. During heterocyst formation nifD and nifK are brought adjacent to each other by excision of the intervening DNA is excised from the chromosome. In this way a circular molecule is formed and the nifHDK operon is made contiguous. The nif structural genes in Calothrix are arranged similarly to those of Anabaena (Kallas et al., 1983). In Rhizobium species, nifH is separated from nifD by 15 kb, although a

rearrangement of these genes does not occur (Kaluza et al., 1983). In addition to these different arrangements and rearrangements of nif genes, multiple copies of nif - like sequences have been described. Anabaena variabilis PCC 7120 contains two copies of nifH contained on Eco R1 fragments of 10 and 19 kilobases (Golden et al., 1985). A functional nifH gene is contained on the 10 kb Eco R1 fragment while a function for the second nifH-like sequence has not been determined. Rhodopseudomonas capsulata, a photosynthetic bacterium, contains multiple copies of ntr genes (regulatory genes) (Kranz, unpublished results) and non-functional nifH-like sequences on its chromosome (Klipp et al., 1985). Clostridium pasteurianum, a gram-positive anaerobic bacterium, contains six copies of nifH (Chen et al., 1987).

Nostoc commune UTEX 584 is a filamentous, heterocystous cyanobacterium known for its ability to tolerate water stress and the environmental extreme of desiccation (Potts et al., 1983). Colonies of Nostoc commune that are rewetted after periods of drying recover nitrogenase activity (Potts et al., 1984). Studies have shown that nitrogen fixation ceases within 20 min when Nostoc cells are immobilized and dried rapidly at a low matric water potential (Potts and Bowman, 1985). Upon rewetting the desiccated material, a lag period is observed preceding the recovery of nitrogenase activity (Potts and Bowman, 1985). Analysis performed on

rehydrated cells indicates that complex changes in gene expression occur during the lag time (Potts and Bowman, 1984). The Fe protein has been shown to be present in dried (30 min), desiccated and rewetted cells (Potts, 1986). To permit more detailed study of *nif* gene expression during water stress, the *nifHD* genes were isolated from *N. commune*. During the course of the study it became apparent that *N. commune* had multiple copies of *nifH* and *nifD*. The organization of these sequences were then studied further.

MATERIALS AND METHODS

1. Preparation of buffers and reagents

1.1 Buffer-saturated phenol Phenol (Aldrich) was stored at 4°C. The phenol was melted at 60°C (water bath) and 8-Hydroxyquinoline was added to give a final concentration of 0.1% w/v (Maniatis et al., 1982). The melted phenol was poured into a separatory funnel and extracted several times with 0.1 M Tris (pH 8.0) until the pH of the aqueous phase was approximately 7.5.

1.2 Dialysis tubing Tubing was cut to the desired length and boiled for 10 min in a beaker containing 2% w/v sodium bicarbonate and 1 mM EDTA. The tubing was rinsed thoroughly and boiled for 10 min in distilled water. After boiling, the tubing was allowed to cool and then stored at 4°C.

1.3 Formamide (deionized) 10 ml of formamide (Sigma Chemical Co.) was mixed with 1 gram of mixed-bed, ion-exchange resin (Bio-Rad AG 501-X8 (D)) in a covered flask. The mixture was stirred for 1 h at room temperature, filtered through Whatman filter paper and stored at -20°C.

1.4 Salmon sperm DNA Salmon sperm DNA (250 mg) was dissolved in 25 ml of sterile distilled water and mixed gently at 4°C, overnight. The solution was brought to room

temperature and poured into a 50-ml syringe with an 18-gauge needle attached. The DNA was forced through the needle to shear the DNA. After repeating this step four times, the DNA was stored in aliquots at -20°C.

2. Growth of cells

Nostoc commune UTEX 584 was grown in BG-11 (Rippka et al., 1979) medium in axenic batch culture with an air-lift fermentation system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The volume of the vessel was 1.8 liters. Cells were grown at room temperature for two weeks under a continuous photon flux density of 50 umol photons m^{-2} s^{-1}. Anabaena variabilis PCC 7118 (ATCC 27892) was grown under the same conditions as N. commune UTEX 584.

2.1 Purification of DNA from N. commune UTEX 584

Cells (40 g wet weight) were harvested by centrifugation and washed once with 50 mM Tris-HCl pH 8.0. The cells were frozen in liquid nitrogen and disrupted by hand grinding with a pestle in a chilled (liquid nitrogen) mortar. The cells were divided into two flasks and to each flask 25 ml of 15% w/v sucrose, 25 mM Tris-HCl pH 8.0, 10 mM Na_2EDTA was added. The cells were exposed to five freeze-thaw cycles in liquid nitrogen. Powdered lysozyme (Sigma Chemical Co.) was added to a final concentration of 15 mg ml^{-1}. The flasks were incubated for 5 h at 37°C with shaking (100 rpm) and

placed at 4°C overnight with continuous shaking at the same speed.

Proteinase K (Boehringer Mannheim) and N-laurylsarcosine (Sigma Chemical Co.) were added to the lysozyme solution at final concentrations of 10 ug ml^{-1} and 5 mg ml^{-1} respectively. The suspension was incubated at 50°C for 4 h. The suspension was diluted with 10 mM Tris-HCl pH 8.0, 5 mM Na_2EDTA and extracted with buffer-saturated phenol. The aqueous supernatant was extracted with 1 volume of 1:1 v/v phenol-chloroform and 1 volume of 24:1 v/v chloroform-isoamyl alcohol. The interphase was re-extracted as above. The nucleic acids were precipitated with an equal volume of isopropanol (-20°C) in the presence of 0.25 M sodium acetate pH 5.2. The nucleic acids were collected on a glass stirring rod, washed in 70% v/v ethanol (-20°C), dissolved in 10 mM Tris-HCl pH 8.0, 0.1 mM Na_2EDTA and stored at 4°C.

2.2 Cesium chloride gradient centrifugation

The crude lysate was mixed directly with cesium chloride and ethidium bromide at a ratio of 4 ml lysate/ 7.42 g CsCl/ 0.2 ml ethidium bromide (10 mg/ml) in a total volume of 10 ml. The sample was centrifuged in a polycarbonate tube at 44,000 rpm (181,000 x g) for 48 h, at 18°C, in a Beckman 50 Ti rotor. The DNA band was visualized by UV light, removed using a Pasteur pipette and freed from ethidium bromide by

repeated extractions with isobutanol. Cesium chloride was removed from the sample with a NAP-25 column (Sephadex G-25 DNA grade, Pharmacia) and dialyzed against 4 L of 10 mM Tris-HCl pH 8.0, 0.1 mM Na_2EDTA, at 4^oC, overnight.

2.3 Purification of DNA from A. variabilis PCC 7118

Due to the thick sheath surrounding the cell wall of N. commune UTEX 584, a rather "harsh" treatment was used to extract DNA (see section 2.1). A more gentle method was used to prepare DNA from A. variabilis. Cells were collected by centrifugation and stored at -20^oC. Cells (4.6 g) were thawed and washed in 0.05 M NaCl, 0.05 M Na_2EDTA, 0.05 M Tris-HCl pH 8.5 (Buffer A). The cells were resuspended in 8 ml of Buffer A and powdered lysozyme (Sigma Chemical Co.) was added to a final concentration of 15 mg ml^{-1}. The flask was incubated for 2 h at 37^oC. After the 2 h incubation period, an equal volume of Buffer A containing 1% w/v sodium dodecyl sulfate (SDS) was added to the cell suspension. The cells were exposed to two freeze-thaw cycles in liquid nitrogen. An equal volume of buffer-saturated phenol was added to the suspension and the lysate was incubated at 4^oC overnight with gentle shaking (50 rpm).

The nucleic acids were precipitated through the addition of an equal volume of isopropanol (-20^oC) in the presence of 0.3 M sodium acetate pH 5.2. The precipitated nucleic acids

were spooled on a glass rod and washed consecutively in 70% v/v, 80% v/v and 90% v/v ethanol (23°C). The nucleic acids were resuspended in 20 ml of 0.1 M NaCl, 0.001 M Na_2EDTA, 0.01 M Tris-HCl pH 8.0 and RNase A (Sigma Chemical Co.) was added to the nucleic acid solution to a final concentration of 400 ug ml^{-1}. The solution was incubated at 37°C for 1 h. Proteinase K (Boehringer Mannheim) was added to a final concentration of 100 ug ml^{-1} and the solution was incubated for an additional 2.5 h at 37°C. The DNA was extracted with buffer-saturated phenol, precipitated onto a glass rod with two volumes of ethanol (-20°C), and washed as above. The DNA was resuspended in 4 ml of 10 mM Tris-HCl pH 8.0, 1 mM Na_2EDTA and further purified on a cesium chloride gradient (see section 2.2).

2.4 Restriction Enzyme Digests

Nostoc commune UTEX 584 DNA was digested with *Hind* III (BRL/Life Technologies Inc., MD) at 2 units ug^{-1} of DNA for 10 and 20 min at 37°C. *Anabaena variabilis* PCC 7118 DNA was digested with *Eco* R1 (BRL/Life Technologies Inc., MD) at 2 units ug^{-1} of DNA for 25 min, at 37°C. The endonucleases were inactivated at 65°C for 10 min. Ten micrograms of DNA were applied to each well of a 10 x 6 x 1 cm-0.7% w/v agarose (Bio Rad) gel and electrophoresis was performed at 7 V cm^{-1} for 3 h in TBE buffer (89 mM Tris base, 89 mM boric

acid, 2 mM EDTA). The gel was stained in ethidium bromide (0.5 ug ml^{-1}) and photographed under UV light through a red filter using Polaroid camera.

3.0 Transfer of Nostoc and Anabaena DNA to nitrocellulose filters

3.1 Southern Transfer Conditions for Southern transfer (Mason and Williams, 1985) were as follows: the gel was immersed in 50 ml of 0.25 M HCl for 10 min at room temperature with gentle agitation. This step was followed by incubation at room temperature in 50 ml of denaturation solution (see Table 3) for 30 min, and in 50 ml of neutralization solution (see Table 3) for an additional 30 min with gentle agitation. Three blotting pads (Schleicher & Schuell) each 6 x 10 cm were saturated with 20x SSC and placed in a tray containing a sufficient volume of 20x SSC to cover the base of the tray. A piece of Whatman filter paper (Type 41) of the same size and saturated with 20x SSC was placed upon the blotting pads. The gel, after neutralization, was placed "well-side" down on the filter paper. A piece of nitrocellulose (Schleicher & Schuell, BA 85, 0.05 um pore size) was cut to the size of the gel and fully wetted with 2x SSC. The wetted filter was placed on top of the gel and smoothed to remove air bubbles between the the nitrocellulose filter and gel. A piece of filter

paper (saturated with 20x SSC) was placed on top of the nitrocellulose followed by seven dry blotting pads. A 200-g weight was placed on top of the sandwich. The DNA was allowed to transfer for 18 h at room temperature. After the 18-h transfer, the gel was restained with ethidium bromide to determine if the DNA had transferred to the filter.

After completion of the transfer, the blotting pads and filter paper were removed. The nitrocellulose filter was marked according to the location of the gel wells and transferred to a dish containing 50 ml of 6x SSC, for 5 min. The filter was allowed to air dry for 15 min. It was then placed between blotting pads and baked at 80°C, in a vacuum oven, for 2 h.

3.2 Dot Blots Optimum conditions for DNA : DNA hybridization were determined using dot blots of genomic DNA hybridized with the biotinylated nifH gene probe from Azotobacter vinelandii (wild type strain UW). A piece of nitrocellulose (Schleicher & Schuell, BA 85, 0.45 um pore size) was cut to the desired size and spotted with DNA samples. If greater than 1 ul of sample was to be spotted on the filter, the filter was air-dried between spotting. The filter was immersed in 0.1 M NaOH/1.5 M NaCl then 0.2 M Tris-HCl pH 7.5, and finally 2x SSC, allowing 30 s for soaking in each solution. The filter was allowed to air dry

for 15 min. It was then placed between blotting pads and baked at 80°C, in a vacuum oven, for 2 h.

3.3 Preparation of biotinylated probes by nick-translation

This procedure was taken from the BluGene Nonradioactive Nucleic Acid Detection System Manual, Bethesda Research Laboratories, Inc., Gaithersburg, MD. The following solutions were pipetted into a 1.5-ml microcentrifuge tube placed on ice: 5 ul of solution A1 (0.2 mM of dATP, dCTP and dGTP), 1 ug of the probe DNA, 2.5 ul of 0.4 mM biotinylated nucleotide (Biotin-11-dUTP) and sufficient H_2O to bring the final volume to 45 ul. The solution was mixed briefly. 5 ul of solution C (DNA Polymerase I and DNA Pol I/DNase I (Nick Translation Grade)) were added in final concentrations of 0.04 units ul^{-1} and 4 pg ul^{-1} respectively. The solution was incubated for 90 min at 15°C. To stop the reaction, 5 ul of stop buffer (0.3 mM Na_2EDTA) and 1.25 ul of 5% w/v SDS were added. The DNA was precipitated by adding an equal volume of isopropanol (-20°C) in the presence of 0.3 M sodium acetate pH 5.2. The sample was placed at -20°C overnight, and the DNA was pelleted by centrifugation at 12,000 x g, for 15 min, at 4°C. The pellet was washed in 70% v/v ethanol, dried under vacuum, and resuspended in 10 ul H_2O.

3.4 Hybridization of biotinylated probes to Southern transfers and dot blots

3.41 Hybridization conditions The baked nitrocellulose filter was placed in a Dazey Products (type 6008) heat-sealable bag containing 5 ml of prehybridization solution (see Table 3) and incubated for 2 h at 42°C (water bath) with gentle agitation. After the 2-h incubation period the prehybridization solution was removed and 3 ml of hybridization solution (see Table 3) were added. Denatured biotin-labeled probe (see section 3.3) was added to the bag to a final concentration of 100 ng ml^{-1}. The bag was resealed and placed in a 42°C water bath for 18 h with agitation. Care was taken to remove bubbles from the bag as they would contribute to background problems. The hybridization solution could be used as many as eight times without any noticeable decrease in signal. In order to reuse,this solution must be boiled for 10 min to denature the probe DNA prior to use.

3.42 Post-hybridization washes The filter was removed from the bag and washed in a siliconized glass tray containing 250 ml of 2x SSC + 0.1% w/v SDS followed by 250 ml of 1x SSC + 0.1% w/v SDS. Both washes were performed at room temperature, for 10 min, with gentle agitation. The filter was transferred to a siliconized beaker containing 250 ml of

0.5x SSC + 0.1% w/v SDS and washed for 90 min, at 50°C, with one buffer change after the first 45 min. The filter was rinsed briefly in 2x SSC.

3.43 Filter Blocking The filter was washed in Buffer 1 for 1 min and incubated in a heat-sealable bag containing Buffer 2, for 30 min, in a 50°C water bath with gentle agitation.

3.44 Application of detection system Streptavidin-Alkaline Phosphatase (SA-AP) conjugate (BRL/Life Technologies Inc., MD) was diluted to 1.0 ug ml^{-1} with Buffer 1. The filter was incubated in 7 ml of diluted SA-AP conjugate (prepared fresh) for 10 min by repeatedly pipetting the solution over the filter in a siliconized petri dish. The filter was washed in 250 ml of Buffer 1 for 10 min at room temperature with agitation. This step was repeated twice followed by a single wash in 250 ml of Buffer 3 for 10 min at room temperature with agitation.

3.45 Visualization In a polypropylene tube 33 ul of nitroblue tetrazolium (NBT) and 25 ul of 5-bromo-4-chloro-3-indolylphosphate (BCIP) in final concentrations of 0.33 mg ml^{-1} and 0.17 mg ml^{-1} respectively were added to 7.5 ml of Buffer 3. The filter was placed in a heat-sealable bag and incubated at room temperature in the dye solution under low illumination to allow color development. To stop the color

development reaction the filter was washed in 20 mM Tris-HCl pH 7.5, 0.5 mM Na_2EDTA.

4. Preparation of recombinant plasmid library

4.1 Sucrose Gradient Centrifugation N. commune UTEX 584 DNA was digested with Hind III at 2 units ug^{-1} of DNA for 20 min at 37°C. The endonuclease was inactivated by addition of 20 mM EDTA (final concentration). A 37-ml sucrose (10-30% w/v) gradient was prepared in a cellulose nitrate tube using a gradient former. The Hind-III-digested N. commune UTEX 584 DNA was loaded on the sucrose gradient and the gradient was centrifuged at 24,000 rpm (117,300 x g) for 26 h, at 18°C, in a Beckman SW27 rotor. Fractions of 0.5 ml were collected using an Infusion-withdrawal syringe pump connected to a gradient collector. Every third fraction was electrophoresed on a 0.8% w/v agarose gel. Electrophoresis was performed at 7 V cm^{-1} for 3 h in TBE buffer. The gel was stained with ethidium bromide at 0.5 ug ml^{-1} and photographed. Five samples, each containing three consecutive fractions, were placed in separate tubes. Sucrose was removed from each of five samples with a NAP 10 column (Sephadex G-25 DNA grade, Pharmacia). The DNA in each sample was precipitated by adding an equal volume of isopropanol (-20°C). The DNA was pelleted by centrifugation at 16,000 x g for 30 min, at 4°C. Each pellet was washed in

70% v/v ethanol, dried under vacuum and resuspended in 20 ul water. Dot blots were used to determine which of the five samples hybridized with the nifH gene probe (see section 3.2).

4.2 Preparation of competent cells Five ml of LB medium were inoculated with a single colony of E. coli HB101 and grown overnight, with shaking, at 37°C. Fifty ml of LB medium contained in a 125-ml Erlenmeyer flask were inoculated with 0.5 ml of the overnight culture. The cells were grown with vigorous shaking (200 rpm), at 37°C. The absorbance at 600 nm was measured at time of inoculation and at 30 min intervals until the OD_{600} was approximately 0.5. The bacterial culture was transferred asceptically into four sterile 15-ml test tubes with 10 ml of culture in each tube. The cells were centrifuged for 10 min, at 5,000 rpm (4,000 x g), at 4°C, in a Centra 7 table top centrifuge. The supernatant was discarded and the cells were resuspended in 0.5 ml of ice-cold solution 1 (Table 3). The suspension in two tubes were combined and the volume adjusted to 10 ml with solution 1. The cells were then centrifuged as above, the supernatant was discarded and each pellet was resuspended in 1 ml of ice-cold solution 2. The volume in each tube was adjusted to 10 ml with solution 2. The tubes were placed on ice for 30 min and then centrifuged as above.

The supernatant was discarded and the cells were resuspended gently in 2 ml of solution 3. The cells were used immediately or were frozen rapidly in 200 ul aliquots, on dry ice, and stored at -70°C.

4.3 Ligation of insert into plasmid vector

4.31 Preparation of linearized, dephosphorylated vector In a 1.5-ml microfuge tube 1 ul of plasmid pBR322 (1 ug ul^{-1}) was digested with Hind III at 1 unit ug^{-1} of DNA for 1 h, at 37°C, in a total volume of 20 ul. The endonuclease was inactivated at 65°C for 10 min. To the tube was added 3 ul of 10x calf intestinal alkaline phosphatase (CIP) buffer, 1.25 units of CIP (Boehringer Mannheim) and sterile, distilled H_2O to bring the volume to 30 ul. The solution was incubated for 30 min, at 37°C. After the first incubation period, an additional 1.25 units of CIP were added and the solution was incubated for 30 min at 37°C. The DNA was extracted with 1 volume of 1:1 v/v phenol-chloroform and precipitated with two volumes of 95% v/v ethanol (-20°C) in the presence of 0.3 M sodium acetate, pH 5.2, overnight, at -20°C. The sample was centrifuged at 12,000 x g, at 4°C, for 15 min, in a microcentrifuge. The pellet was washed in 70% v/v ethanol, dried under vacuum and resuspended in 50 ul H_2O.

4.32 Cloning genomic fragments into plasmid vectors Genomic Hind III fragments of N. commune UTEX 584 DNA containing nifH sequences (see section 4.1) were cloned into linearized, dephosphorylated pBR322 as follows: in a 1.5-ml microcentrifuge tube, 0.3 ug of the cut genomic DNA fragments were added to 0.1 ug of pBR322, 100 units of T4 DNA ligase (BRL/Life Technologies Inc., MD), 2 ul of 10x ligation reaction buffer and sterile distilled H_2O in a final volume of 20 ul. The mixture was incubated at 4°C overnight.

4.4 Transformation of competent cells Ten ul of the ligation mixture were added to a 1.5-ml microcentrifuge tube containing 200 ul of the cell suspension. The solution was mixed gently by inverting the tube, incubated at 42°C (water bath) for 2 min, and then transferred to ice for 2 min. One ml of LB medium (preheated to 37°C) was added and the tube was incubated for 1 h at 37°C (water bath). The cells were centrifuged at 2,500 rpm (2,000 x g) for 8 min, at 4°C, in a Centra 7 table top centrifuge and the pellet was resuspended in 1 ml of LB medium. An aliquot (200 ul) of the solution was spread on a plate (1.2% w/v agar in LB medium/100 ug ml^{-1} ampicillin).

5. Colony hybridization

5.1 Growth of bacteria A sterile nitrocellulose filter (Schleicher and Schuell, BA 85, 82 mm diam.) was placed on a 1.2% w/v agar plate containing 100 ug ml^{-1} ampicillin (Sigma Chemical Co.). Two hundred ul of transformation mixture were spread evenly over the surface of the filter and the filter was incubated at 32°C, for 15 h, until the colonies were just visible to the naked eye. A replica filter was prepared as follows. The master filter was removed from the agar plate and placed colony side up on a blotting pad. A fresh sterile nitrocellulose filter was placed on top of the master filter followed by a blotting pad. The filters were pressed together using a replicating disc and marked asymetrically with ink. Each filter was placed colony side up on a fresh agar plate containing 100 ug ml^{-1} ampicillin and incubated at 37°C for 4 h, or until the colonies had reached the size of a period. The master filter was stored at 4°C on the agar plate and the replica filter was used for screening library.

5.2 Colony lysis and proteinase K treatment All steps were performed at room temperature unless otherwise noted. The replica filter was placed on a blotting pad saturated with 0.5 M NaOH (freshly prepared) for 8 min, and transferred to a blotting pad saturated with 0.5 M Tris-HCl pH 8.0 for 8

min. The filter was immersed in 30 ml of 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5 for 5 min, and then was transferred to a siliconized petri dish containing 15 ml of 200 ug ml^{-1} proteinase K (Boehringer Mannheim) in 1x SSC and incubated at 37°C, for 1 h. Care was taken not to move the filter while in the proteinase K solution. The filter was removed from the proteinase K solution and immersed in 30 ml of 70% v/v ethanol for 2 min. The wash with 70% v/v ethanol was repeated once. The filter was allowed to air dry for 20 min. One hundred ml of chloroform was then passed through the filter by suction using a Buchner funnel lined with Whatman filter paper (Type 41). The filter was then immersed in 30 ml of 0.3 M NaCl for 5 min and allowed to air dry. It was then placed between blotting pads and baked in a vacuum oven, at 80°C, for 2 h. The filter was prehybridized, hybridized and processed by the Biotin detection system (refer to section 3.4).

6. Confirmation of the identity of recombinant plasmids

6.1 Plasmid extraction Fifty ml of LB broth containing 100 ug ml^{-1} ampicillin were inoculated with 0.1 ml of a 5 ml overnight culture (HB101 containing the recombinant plasmid) in a 125-ml Erlenmeyer flask. The flask was incubated at 37°C, overnight, with vigorous shaking (150 rpm). The cells were pelleted at 8,000 x g for 20 min, at 4°C, and the

supernatant was discarded. The cell pellet was resuspended by mixing in 1.2 ml of an ice-cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0. Powdered lysozyme (Sigma Chemical Co.) was added to a final concentration of 4 mg ml^{-1}. The solution was incubated at room temperature for 5 min, and 2.4 ml of a freshly prepared solution containing 0.2 N NaOH, 1% SDS was added. The solution was mixed carefully by inversion and incubated on ice for 15 min. Ice-cold potassium acetate, pH 4.8 (1.5 ml) was added, the solution was mixed by inversion and incubated on ice for 20 min. The solution was centrifuged at 8,000 x g for 15 min at 4°C. The supernatant was transferred to a new tube and the white precipitate was discarded. RNase A (Sigma Chemical Co.) was added to the supernatant in a final concentration of 20 ug ml^{-1}. The solution was incubated at 37°C for 1 h and then the solution was extracted with an equal volume of 1:1 v/v phenol-chloroform. The aqueous phase was transferred to a fresh tube and the nucleic acids were precipitated with two volumes of 95% v/v ethanol (-20°C), at -20°C, overnight.

The sample was centrifuged at 8,000 x g, for 30 min, at 4°C. The pellet was dissolved in 320 ul H_2O, 80 ul 4 M NaCl, 400 ul 13% w/v PEG_{8000} and the solution was incubated on ice for 60 min. The sample was centrifuged at 12,000 x g, for 30 min, at 4°C. The supernatant was removed and the

pellet was washed in 70% v/v ethanol. The pellet was dried under vacuum and the DNA was dissolved in 50 ul of sterile H_2O.

6.2 Isolation of insert by electroelution Plasmid pND001 was digested with Hind III at 1 unit ug^{-1} of DNA for 3 h at 37°C. The endonuclease was inactivated at 65°C for 10 min. The DNA digest was fractionated on a 0.7% w/v gel in TBE buffer at 7 V cm^{-1} for 3 h. The gel was stained with ethidium bromide at 0.5 ug ml^{-1} and photographed under UV light.

The desired band was cut from the agarose gel using a razor blade and the slice was placed in a dialysis bag with one end of the bag clipped. Five hundred ul of TE buffer were added to the bag and after removing all the air from the bag, the bag was sealed using a clip. The dialysis sac was placed parallel to the plane of electrodes in a mini gel apparatus (Bio Rad). Sufficient TBE buffer was added to cover the bag. The DNA was electroeluted from the gel at 200 V, for 1 h, at room temperature. The DNA was dislodged from the dialysis tubing wall by reversing the polarity for 1 min (200 V). The solution was carefully removed from the bag using a pipette tip and placed in a 1.5-ml microcentrifuge tube. Agarose particles were removed from the sample by passing the solution through siliconized glass

wool packed into a blue pipette tip. The DNA was precipitated by adding an equal volume of isopropanol (-20°C) in the presence of 0.3 M sodium acetate pH 5.2, at -20°C, overnight. The sample was centrifuged at 12,000 x g, at 4°C, for 15 min in a microcentrifuge and the pellet was washed in 70% v/v ethanol, dried under vacuum and resuspended in 20 ul H_2O.

6.3 Restriction mapping All digestions were performed at 37°C in a total volume of 10 ul unless otherwise noted. Refer to Table 2 for the conditions of the restriction enzyme digestions. The DNA digests were fractionated on a 0.7% w/v agarose gel in TBE buffer at 8 V cm^{-1} for 3 h. The gels were stained with ethidium bromide at 0.5 ug ml^{-1} and photographed.

7. Subcloning in plasmid pGEM-4

7.1 Preparation of competent cells Five ml of M9 Medium (Maniatis et al., 1982) were inoculated with a thick suspension of E. coli JM109 and grown overnight, with shaking (100 rpm), at 37°C. A sample of the overnight culture (0.1 ml) was transferred to five ml of LB medium which was then grown overnight, with shaking, at 37°C. Fifty ml of LB medium were inoculated with 0.5 ml of the overnight culture. The cells were grown with shaking (200

rpm), at 37°C, until the OD_{600} was in the range of 0.13-0.15. The remaining procedures for preparing competent cells of JM109 were the same as given for HB101 (section 4.2).

7.2 Preparation of linearized, dephosphorylated vector In a 1.5-ml microfuge tube, plasmid pGEM-4 was digested with Eco Rl at 1 unit ug^{-1} of DNA for 1 h, at 37°C. In a second 1.5-ml microfuge tube, pGEM-4 was digested with Eco Rl and Hind III each at 0.5 unit ug^{-1} of DNA for 1 h, at 37°C. The vectors were dephosphorylated (section 4.31).

7.3 Extraction of DNA from Low-melting agarose Fragments of DNA containing portions of nifD and nifH from N. commune UTEX 584 were isolated from low-melting agarose as follows (Maniatis et al., 1982): In a 1.5-ml microfuge tube plasmid pND001 was digested with Eco RI at 0.5 unit ug^{-1} of DNA for 3 h, at 37°C. In a second 1.5-ml microfuge tube, pND001 was digested with Eco Rl and Hind III each at 0.25 units ug^{-1} for 3 h, at 37°C. The DNA digests were fractionated on a 0.7% w/v Sea Plaque low melting agarose gel (FMC BioProducts) in TBE buffer at 7 V cm^{-1}, for 2 h, at 4°C. After staining the gel with ethidium bromide at 0.5 ug ml^{-1} the bands were located under UV light. Areas containing the desired DNA were excised from the gel and transferred to pre-weighed 1.5-ml microfuge tubes.

After melting each slice at 70°C, for 5 min, 2 volumes of pre-warmed (70°C) TE buffer were added to each tube followed by an equal volume of buffer-saturated phenol (pre-warmed at 37°C). The solutions were mixed briefly and the samples were centrifuged in a microfuge at room temperature for 5 min. The aqueous phases were transferred to new tubes and the phenol phases were extracted with TE buffer (pre-warmed). One tenth of a volume of 3 M Na-Acetate (pH 5.2) was added to each tube, the samples were then extracted with 1 vol of 1:1 v/v phenol-chloroform and centrifuged in a microfuge for 2 min, at room temperature. The aqueous phases were then extracted with an equal volume of 24:1 v/v chloroform-isoamyl alcohol. Isopropanol (-20°C) was added to precipitate the DNA, which was then mixed and centrifuged in a microfuge, for 5 min, at 4°C. The pellets were washed in 70% v/v ethanol (-20°C), dried under vacuum and suspended in TE buffer.

DNA fragments containing portions of nifD and nifH were ligated into linearized, dephosphorylated pGEM-4 under the same conditions as those described in section 4.32. The ligated DNA was transformed into competent cells of JM109 (section 4.4).

7.4 Screening for recombinant plasmids(Smith, 1987)
Approximately 20 bacterial colonies, selected on the basis

of their small size, were streaked onto agar plates (1.2 % w/v agar in LB medium/100 ug ml^{-1} ampicillin). The plates were incubated overnight at 37°C. Using a sterile toothpick, an inoculum of each colony was placed in a 1.5-ml microfuge tube containing 10 ul of protoplasting buffer (Table 3). A 0.7% w/v agarose gel containing TBE buffer and 0.05% w/v SDS was prepared. Each gel slot was pre-loaded with 4 ul of lysis buffer (Table 3) followed by a sample in protoplasting buffer. Electrophoresis was performed at 50 V for the first 15 min, and then increased to 120 V for 2.5 h, at room temperature. The gel was stained with ethidium bromide at 0.5 ug ml^{-1} and photographed under UV light.

8. Sequencing of DNA

The dideoxy-chain termination method (Sanger et al, 1977) was used to sequence DNA fragments which were inserted in pGEM-4 recombinant plasmids (Promega Biotec Technical Manual, 1986). Deoxy-adenosine 5'-(alpha-thio) triphosphate (1300 Ci $mmol^{-1}$) was obtained from NEN Research Products (Boston, MA) and DNA polymerase I (Klenow fragment) was used in the sequencing reaction. The gel was pre-electrophoresed for 30 min at 85 watts before loading the samples. Electrophoresis was performed at 65 watts for 4°h, with a duplicate set of reactions loaded after the first 2 h. After completing electrophoresis, one plate was removed from

the gel and the gel (adhered to the second plate) was immersed in fixing solution (5% v/v acetic acid and 5% v/v methanol). A piece of blotting pad was placed on top of the gel removing the gel from the glass plate. The mounted gel was dried with a gel dryer for 30 min, placed in a casette containing Kodak SB 5 film, and developed for 3 days. DNA sequence data were analyzed using the IBI DNA/Protein Sequence Analysis System (IBI, New Haven, Ct.).

RESULTS

The biotinylated A. vinelandii nifH DNA probe hybridized with Anabaena variabilis PCC 7120 genomic Eco R1-digested DNA to give two distinct bands on a Southern transfer (Figs. 2 a,b). These bands correspond to DNA fragments with sizes of 18 kb and 11 kb. The same nifH probe also hybridized with Nostoc commune UTEX 584 Hind III-digested genomic DNA to give bands of 9 kb, 3.5 kb and 1.1 kb (Figs. 2 a,b). There was no change in these patterns when N. commune genomic DNA was digested with Hind III for longer periods prior to the Southern transfer procedure. Under the same hybridization conditions, a similar pattern was detected on Southern blots of N. commune DNA digested with Eco R1 and Eco R1-Hind III (Fig. 3). With Eco R1-digested N. commune DNA the nifH probe hybridized to three fragments of 9 kb, 5.5 kb and 1.5 kb. Three fragments of 3.2 kb, 2.4 kb and 1.2 kb were observed when Eco R1-Hind III-digested N. commune DNA was transferred and probed with nifH. Hybridization of the biotinylated A. vinelandii nifD probe to a Southern blot of Nostoc commune UTEX 584 DNA digested with either Hind III, Eco R1, or Hind III-Eco R1 gave the following results: with Hind III-digested DNA, nifD showed a strong signal of 3.5 kb and a weaker signal of 1.9 kb (Fig. 4a,b). Similarly, Eco R1-digested DNA probed

with nifD showed a strong signal of c 4 kb and a weaker signal of 2.3 kb. One band of 1.1 kb was detected on a Southern blot containing Eco R1-Hind III-digested N. commune DNA probed with nifD.

A recombinant library of E. coli containing N. commune genomic DNA inserted into the Hind III site of plasmid pBR322 was screened for N. commune nifH clones by colony hybridization (Fig. 5). The biotinylated A. vinelandii nifH gene was used as the hybribization probe. DNA prepared from hybridizing four nifH-clones were with Hind III, transferred to Southern blots and probed with nifH (Figs. 6 a,b). Three of these clones, isolated from the recombinant pBR322 plasmid library were named pND001, pND002 and pND003, and analyzed further. Results obtained after analyzing the restriction pattern of pND002 indicated the presence of a mixture of two plasmids; a recombinant and a non-recombinant. Further analysis of this preparation was discontinued. Preliminary results from pND003 indicated that the insert was arranged in reverse orientation when compared to pND001. This plasmid was not studied further.

From information provided by single and double restriction enzyme digestions and partial sequencing analysis, a restriction map was produced for the cloned Hind III fragment contained on pND001 (Fig. 7). The cloned Hind III fragment was 3.5 kb, and contained single Eco R1

and Acc I sites. Approximately 840 bp of the nifH gene is contained within a 1.0 kb Eco Rl-Pvu I fragment. There is at least one additional Pvu I site contained within the adjacent 1.0 kb Pvu I fragment, but the exact location was not determined. Preliminary data indicate the presence of two, possibly three internal Hinc II sites contained within a 980 bp Hinc II fragment. The insert is cut extensively with Hpa II; four Hpa II sites have been localized from the data and are shown on the restriction map. The DNA was not susceptible to digestion with any of the following enzymes: Ava I, Bam HI, Bgl II, Pst I, Sac I, Sma I or Xba I. The position of the N. commune nifH sequence within the insert was determined through hybridization with the nifH probe with single and double restriction enzyme digestions (Figs. 8 a,b; 9 a,b). The nifH gene probe hybridized to a Southern blot of pND001 digested with Eco Rl, Acc 1 and Hinc II (Figs. 8 a,b). The nifH probe hybridized with a 6.7 kb Eco Rl fragment, a 3.5 kb Acc I fragment and approximately three Hinc II fragments (Fig. 8 b). Hybridization of the nifH probe to a Southern blot containing Eco Rl, Hpa II, Eco Rl-Hpa II and Eco Rl-Pvu I digested DNA provided the results shown in Figures 9 a and b. An Eco Rl-Pvu I fragment of c 1 kb and a Hpa II (Eco Rl-Hpa II) fragment of c 400 bp showed strong signals when hybridized with the nifH probe, while a

c 900 bp Hpa II (Eco Rl-Hpa II) fragment showed a weaker signal with the probe (Fig. 9 b).

Strong nifD identity was detected exclusively within a 1.1 kb Eco Rl fragment, and nifH homology was located within a 2.4 kb Eco Rl-Hind III fragment (Fig. 7). These two fragments were isolated, cloned in pGEM-4 (pNDD1 and pNDH1 respectively), and partially sequenced (Figs. 10, 11). Strong homology was observed when comparing amino acid sequences of polypeptides encoded by nifH and nifD of N. commune UTEX 584 with those of A. variabilis nifH (Mevarech et al., 1980) and nifD (Lammers et al., 1983), A. vinelandii nifH and nifD (Brigle et al.,1985), K. pneumoniae nifH (Scott et al., 1981) and R. meliloti nifH (Torok and Kondorosi, 1981) (Figs. 12,13). From this information the positions of nifH and nifD genes in N. commune UTEX 584 were determined. The entire nifH gene and c 860 bp of the nifD gene are located on the original 3.5 kb Hind III fragment subcloned in pBR322.

Figure 1. Heterocysts (H) - sites of nitrogen fixation in Nostoc commune. Heterocysts provide an anaerobic environment for the fixation of atmospheric nitrogen. They are differentiated from vegetative cells (V) and are formed at regular intervals along the filament. The figure shows heterocysts which have been stained with triphenyl tetrazolium chloride (TTC) - deposition of formazan indicates reducing activity.

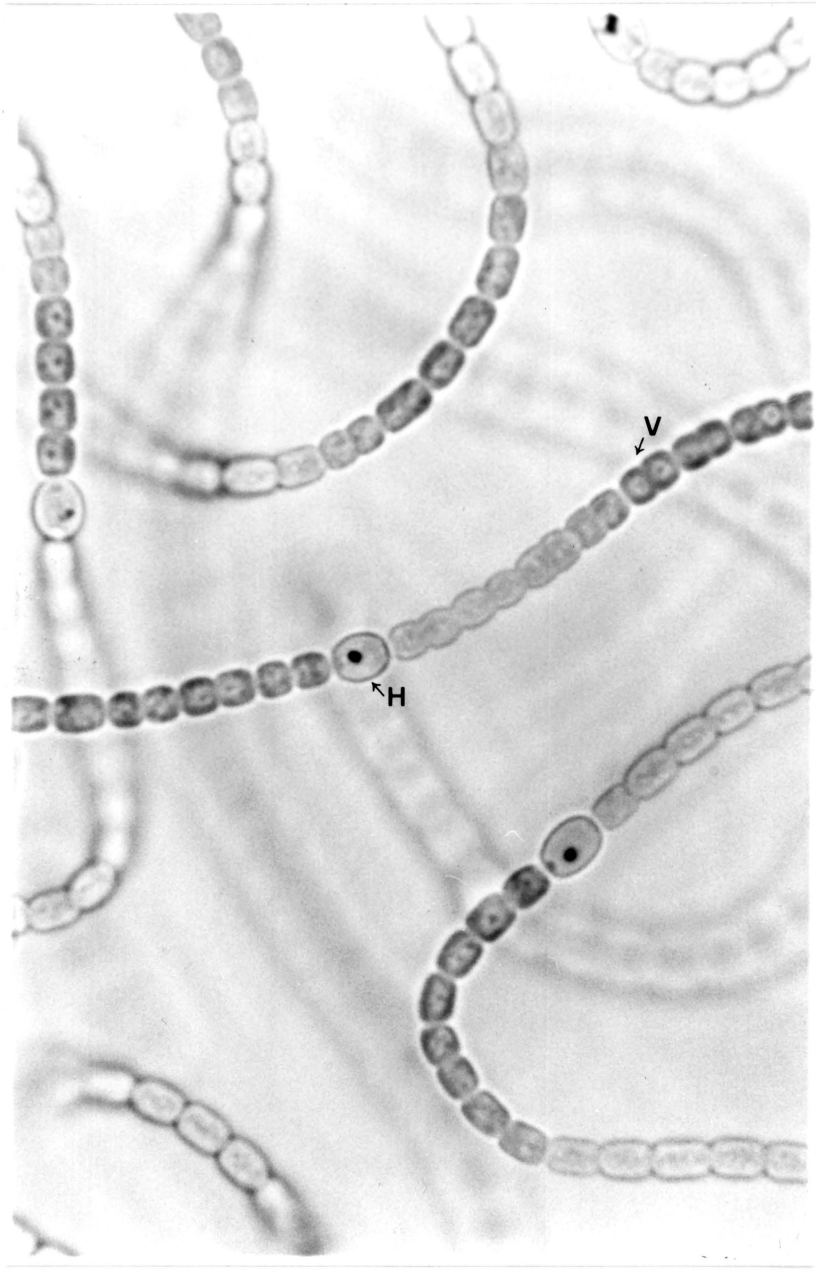

V
H

Figure 2. Restriction enzyme digests and Southern blot hybridization of DNA from Nostoc commune UTEX 584 and Anabaena variabilis PCC 7118 to the Azotobacter vinelandii nifH probe. a) Lane 1 - lambda DNA digested with Hind III; Lane 2 - positive marker (pMJH5) containing the Azotobacter vinelandii nifH DNA fragment; Lane 3 - Anabaena variabilis DNA digested with Eco Rl; Lanes 4 and 5 - Nostoc commune DNA digested with Hind III for 10 min (Lane 4) and 20 min (Lane 5). b) Hybridization of the nick-translated 0.6 kb A. vinelandii Eco Rl/Hind III nifH DNA fragment to a Southern blot of the gel shown in Fig. 2a.

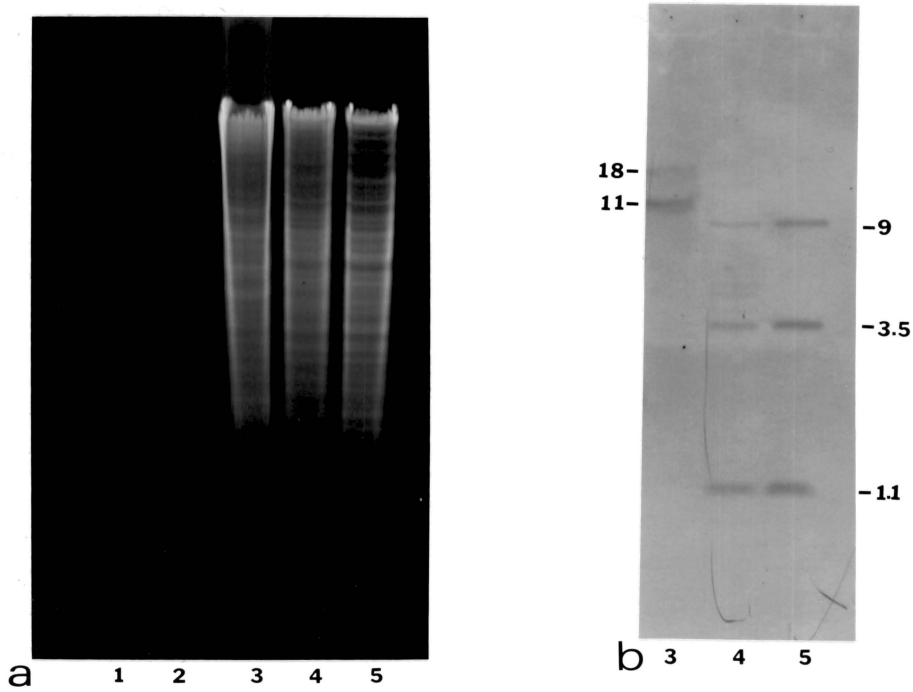
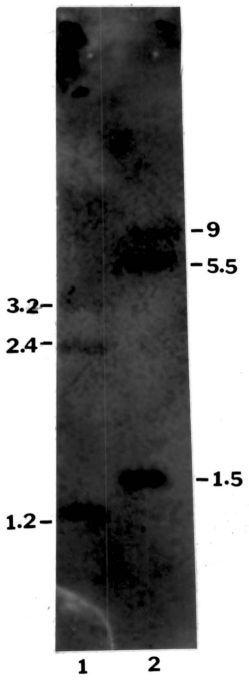
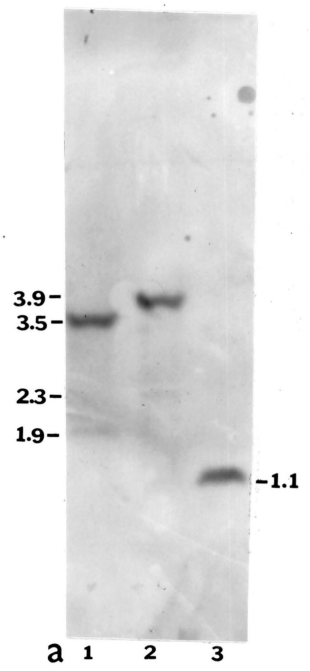

a
1
2
3
4
5
18-
11-
-9
-3.5
-1.1
b
3
4
5

Figure 3. Southern blot hybridization of the A. vinelandii nifH probe to DNA from N. commune UTEX 584 digested with Eco Rl - Lane 1; Eco Rl-Hind III - Lane 2.

Figure 4. Southern blot hybridization of A. vinelandii nifD probe to N. commune UTEX 584 DNA. a) N. commune DNA digested with Hind III - Lane 1; Eco Rl - Lane 2; Eco Rl-Hind III - Lane 3. b) As in Fig. 4a, the weaker signals of 2.3 kb and 1.9 kb in Fig. 4a are more apparent in this Southern blot. Lane 1 - pND001 digested with Eco Rl; Lane 2 - N. commune DNA digested with Hind III; Lane 3 - N. commune DNA digested with Eco Rl.


-9
-5.5
3.2-
2.4-
-1.5
1.2-
1
2


3.9-
3.5-
2.3-
1.9-
-1.1
a
1
2
3

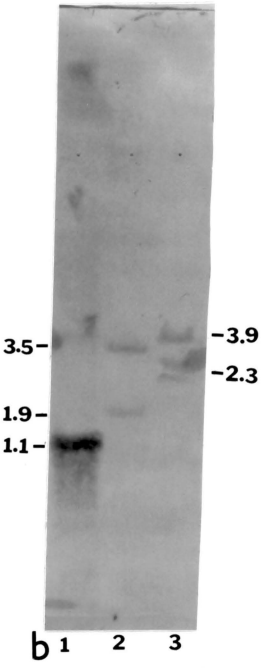

3.5-
-3.9
-2.3
1.9-
1.1-
b
1
2
3

Figure 5. Colony hybridization. A screen of the recombinant library of *E. coli* containing *N. commune* genomic DNA inserted into the *Hind* III site of plasmid pBR322. Biotinylated *A. vinelandii nifH* gene was the hybridization probe. Arrows indicate positive clones.

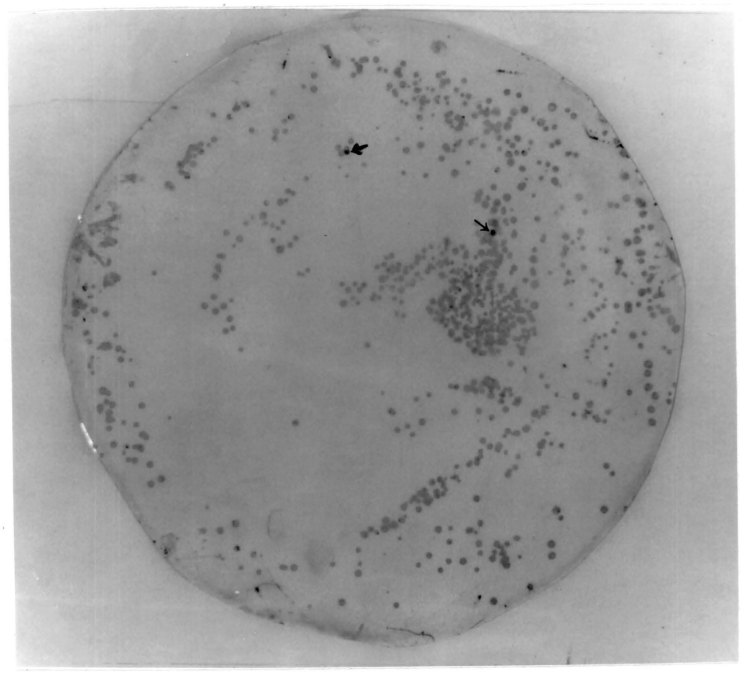
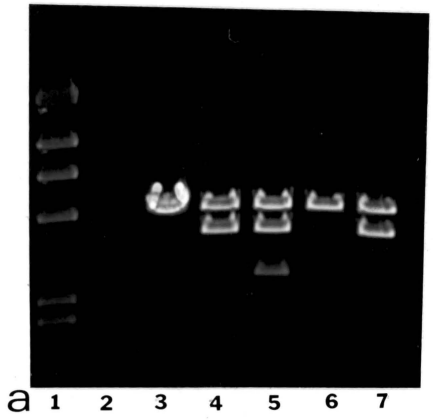


Figure 6. Hybridization of the biotinylated A. vinelandii nifH gene probe to a Southern blot of plasmid DNA from four positive nifH clones. a) Lane 1 - lambda DNA digested with Hind III; Lane 2 - positive control marker pMJH5; Lane 3 - plasmid pBR322 digested with Hind III; Lanes 4-7 - four recombinant plasmids digested with Hind III. b) Hybridization of the A. vinelandii nifH probe to a Southern blot of the gel shown in Fig. 6a.


a
1
2
3
4
5
6
7

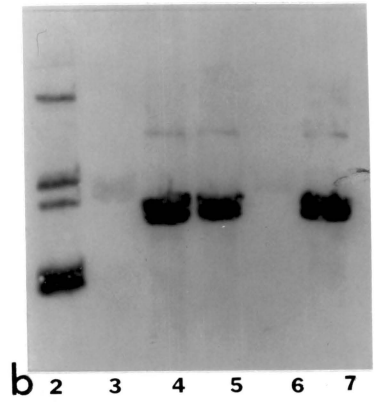

b
2
3
4
5
6
7

Figure 7. Physical map of the nifH and nifD gene cluster of Nostoc commune UTEX 584 present on the 3.5 kb Hind III - Hind III insert in pND001.

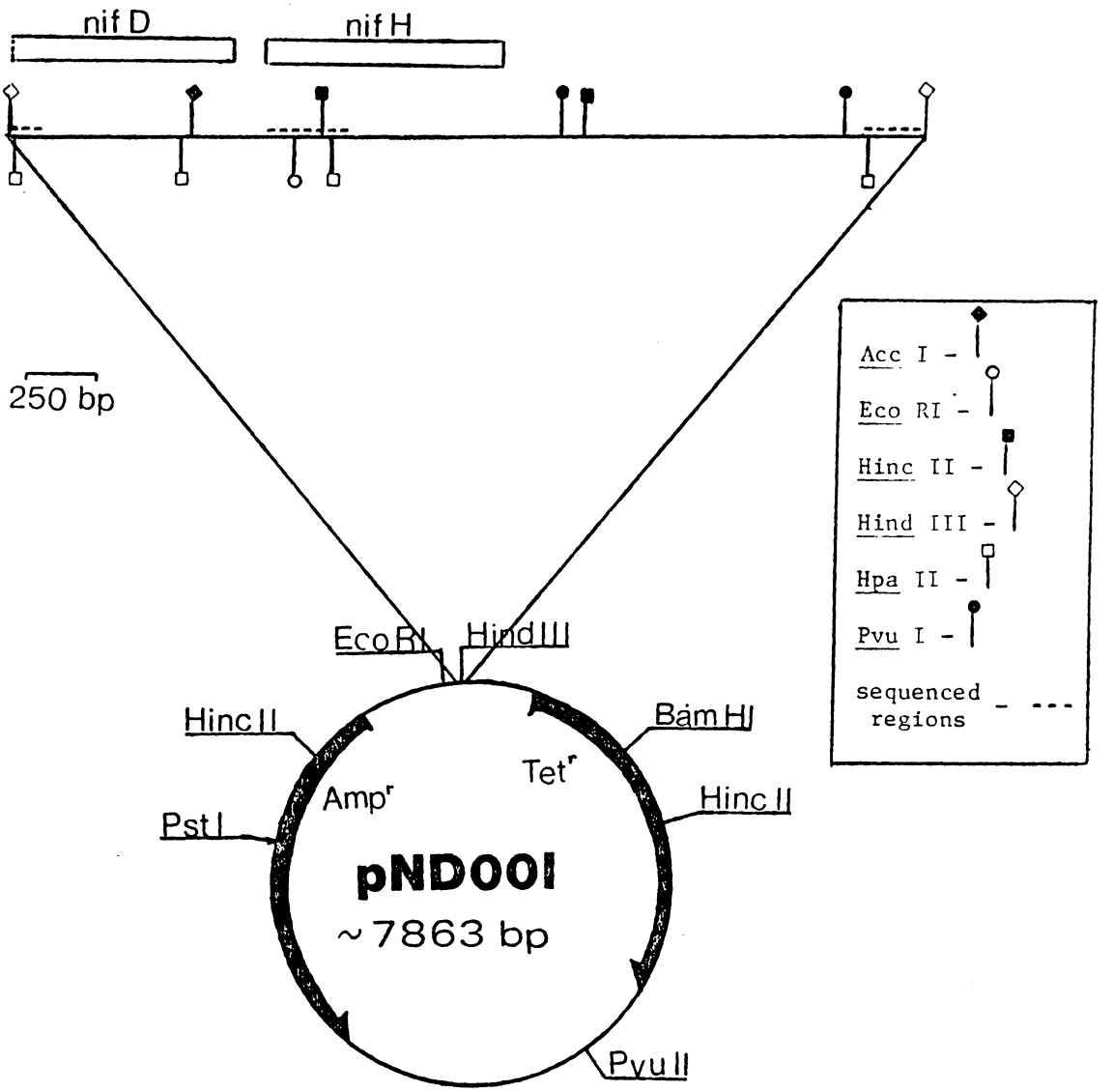

nif D
nif H
250 bp
Acc I -
Eco RI -
Hinc II -
Hind III -
Hpa II -
Pvu I -
sequenced regions
Eco RI
Hind III
Hinc II
Bam HI
Amp^r
Tet^r
Hinc II
Pst I
pND001
~7863 bp
Pvu II

Figure 8. Restriction enzyme digestions and Southern blot hybridization of the A. vinelandii nifH probe to two recombinant plasmids. a) Lane 1 - lambda DNA digested with Hind III; Lane 2 - pND001 digested with Eco R1; Lane 3 - pND003 digested with Eco R1; Lane 4 - pND001 digested with Acc I; Lane 5 - pND003 digested with Acc I; Lane 6 - pND001 digested with Hinc II; Lane 7 - pND003 digested with Hinc II; Lane 8 - pND001 digested with Hind III. b) Hybridization of the nifH probe to the Southern blot of the gel shown in Fig. 8a.

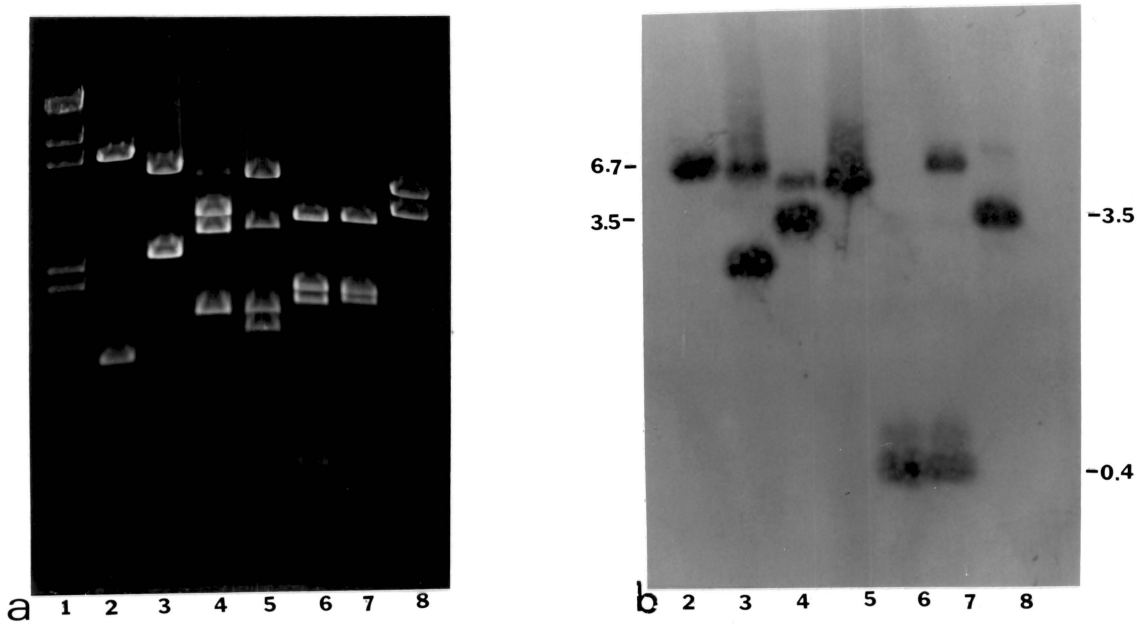

a
1 2 3 4 5 6 7 8
6.7-
3.5-
-3.5
-0.4
b
2 3 4 5 6 7 8

Figure 9. Restriction enzyme digestions and Southern blot hybridization of the A. vinelandii nifH probe to the 3.5 kb insert (isolated from pND001) of N. commune UTEX 584 DNA. a) The 3.5 kb insert digested with Eco Rl (Lane 1), Hpa II (Lane 2), Eco Rl-Hpa II (Lane 3), Eco Rl-Pvu I (Lane 4). b) Hybridization of the nifH probe to a Southern blot of the gel shown in Fig. 9a.

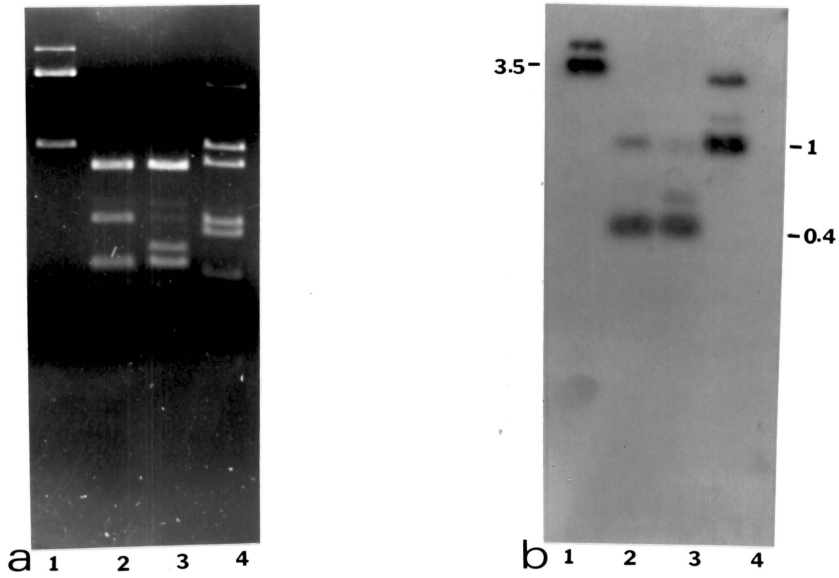

a
1
2
3
4
3.5
1
0.4
b
1
2
3
4

Figure 10. Comparison of the partial nucleotide sequences of the COOH terminus of the N. commune and A. variabilis nifH genes. The numbering indicates the nucleotides beginning with the 5' ATG start codon of the A. variabilis nifH gene. The DNA strands shown are the coding strands (identical to mRNA).

```
                    640         650         660         670
                     *           *           *           *
An   AATGATTCACTTCGTACCTCGTGACAACATCGTTCAACACGCAGAATTGCGCC

Nc   AAatgaTCACTaCGTACCcCGTGACAACATtGTTCAACACGCAGAATTGCGCC

     680         690         700         710         720             730
      *           *           *           *           *              *
An   GTATGACCGTTAACGAGTACGCACCAGACAGCAACCAAGGT---CAAGAGTACC

Nc   GgATGACtGTTAACGAGTACGCACCAGAaAGCAACCAAGcTaacgAAtAtcgga

               740         750         760         770         780
                *           *           *           *           *
An   GCGCATTAGCTAAGAAGATCAACAACGACAAGCTCACCATTCCTACACCAATG

Nc   ttttggctcaaAAaAttATCgACAACaAaAAtCTggCtATTCCTACACCcATc

          790         800         810         820         830
           *           *           *           *           *
An   GAAATGGATGAACTAGAAGCTCTGAAGATCGAATACGGTCTATTAGACGACGAC

Nc   GAAATGGAaGAACTAGAAGaatTGttGATtGaaTtCGGTaTccTcGAaagCGAC

      840         850         860         870         880         890
       *           *           *           *           *           *
An   ACCAAGCACTCTGAAATCATCGGTAAGCCCGCAGAAGCTACCAATAGGTCATGC

Nc   gaaAAtactgCcatgctggttggtaagac----------------------TGC

           900         910         920         930         940
            *           *           *           *           *
An   CGTAATTAGGAGACACGGAGACAGGAGATGAGGAGCAATTCCTCTTCCCACT

Nc   tactgaagctccgtAgtctaAgtcGcGcTaAGaAatAAcgCtTtagCaCAg
```

Figure 11. Comparison of the partial nucleotide sequences of the N. commune and A. variabilis nifD genes. Numbering indicates the nucleotides beginning with the 5' ATG start codon of A. variabilis nifD. The DNA strands shown are the coding strands (identical to mRNA).

```
      880         890         900         910         920         930
       *           *           *           *           *           *
An    ACGTGTAGTAGCTCAGTGGTCTGGTGATGGTACACTCAACGAGTTGATCCAA

Nc    gCGcGTAGTcGCTCAGTGGTCaGGTGATGGcACcaTCAACGAaaTGtTgatg


              940         950         960         970         980
               *           *           *           *           *
An    GGCCCTGCTGCTAAGTTAGTCCTCATCCACTGCTACCGTTCTATGAACTACAT

Nc    acCCCcaaTGtgAAGaTgaaCCTCATCCACTGtTACCGgTCgATGAACTACAT


           990        1000
            *           *
An    CTGCCGTAGTTTGGAAG

Nc    CaGCCGTcGcaTGGAAG
```

Figure 12. Comparison of the deduced amino acid sequence of the COOH terminus of the N. commune nifH gene product with the nifH gene products of A. variabilis, A. vinelandii, K. pneumoniae, and R. meliloti. Perfect homologies of N. commune and A. variabilis amino acid sequences are boxed. A sequence of c 80 residues of the COOH terminus portion of nifH is shown for each organism. Numbering indicates the distance of the amino acid residue from the N terminus. For N. commune, the data were obtained by sequencing upstream and downstream from the single Eco R1 site indicated in Fig. 7.

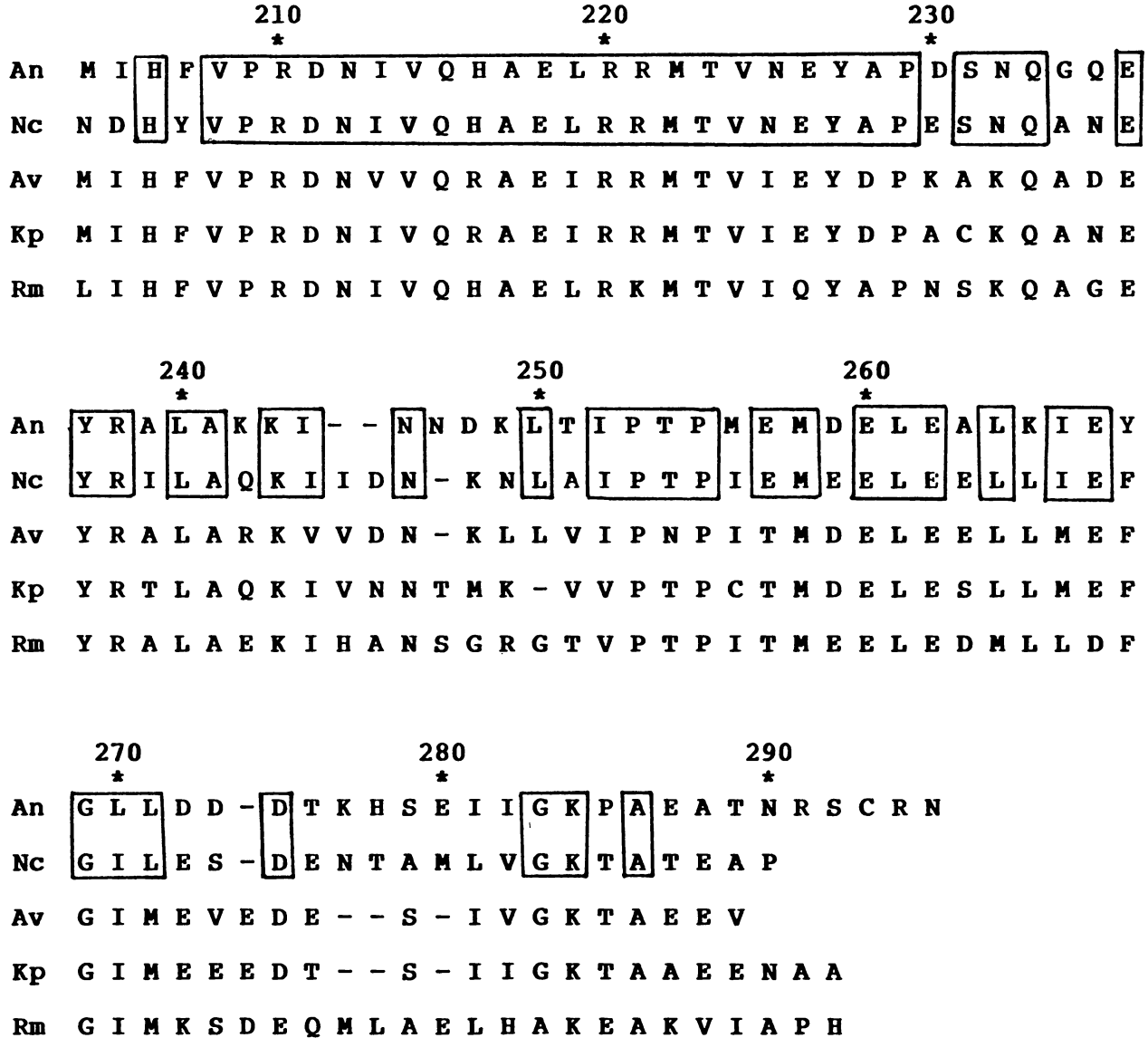

210
220
230
An M I H F V P R D N I V Q H A E L R R M T V N E Y A P D S N Q G Q E
Nc N D H Y V P R D N I V Q H A E L R R M T V N E Y A P E S N Q A N E
Av M I H F V P R D N V V Q R A E I R R M T V I E Y D P K A K Q A D E
Kp M I H F V P R D N I V Q R A E I R R M T V I E Y D P A C K Q A N E
Rm L I H F V P R D N I V Q H A E L R K M T V I Q Y A P N S K Q A G E
240
250
260
An Y R A L A K K I - - N N D K L T I P T P M E M D E L E A L K I E Y
Nc Y R I L A Q K I I D N - K N L A I P T P I E M E E L E E L L I E F
Av Y R A L A R K V V D N - K L L V I P N P I T M D E L E E L L M E F
Kp Y R T L A Q K I V N N T M K - V V P T P C T M D E L E S L L M E F
Rm Y R A L A E K I H A N S G R G T V P T P I T M E E L E D M L L D F
270
280
290
An G L L D D - D T K H S E I I G K P A E A T N R S C R N
Nc G I L E S - D E N T A M L V G K T A T E A P
Av G I M E V E D E - - S - I V G K T A E E V
Kp G I M E E E D T - - S - I I G K T A A E E N A A
Rm G I M K S D E Q M L A E L H A K E A K V I A P H

Figure 13. Comparison of partial amino acid sequence data for nifD of N. commune with those of A. variabilis and A. vinelandii. Perfect homologies of the N. commune and A. variabilis nifD gene products are boxed. The numbering refers to amino acid residues (see Fig. 12).

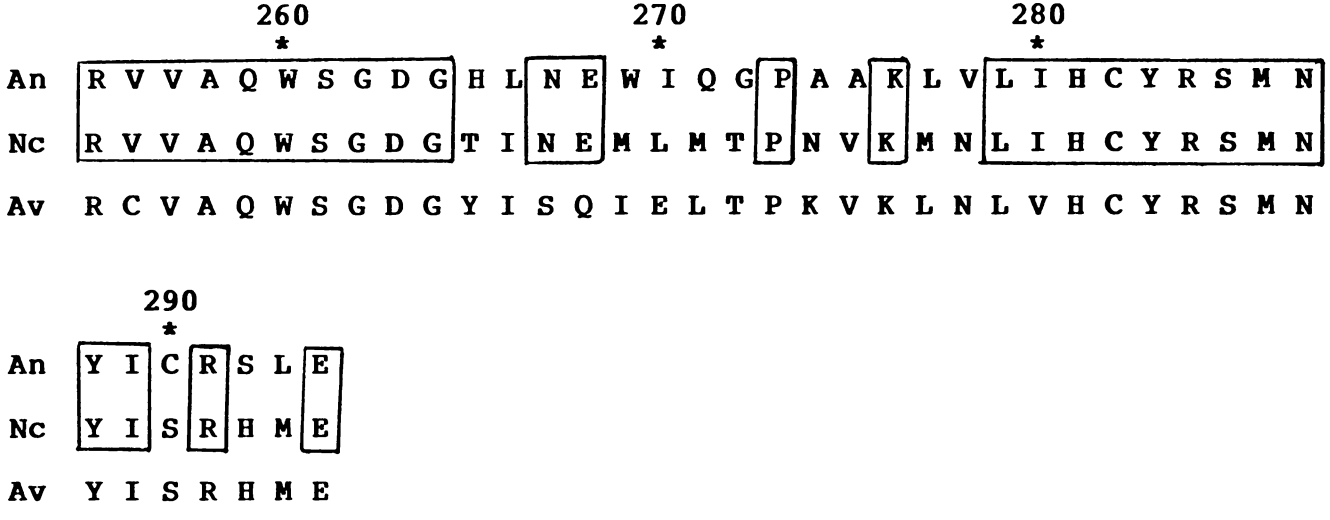

260
270
280
An R V V A Q W S G D G H L N E W I Q G P A A K L V L I H C Y R S M N
Nc R V V A Q W S G D G T I N E M L M T P N V K M N L I H C Y R S M N
Av R C V A Q W S G D G Y I S Q I E L T P K V K L N L V H C Y R S M N
290
An Y I C R S L E
Nc Y I S R H M E
Av Y I S R H M E

TABLE 1

Strains	Characteristics	Source
Nostoc commune UTEX 584	(Het^+ Nif^+)	M. Potts
Anabaena variabilis PCC 7118 (ATCC 27892)	(Het^- Nif^-)	M. Potts
E. coli HB101	F^-, hsdS20 (r^-_B,m^-_B), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl-5, mtl-1, sup E44, lambda	Maniatis *et al.*, 1982
E. coli JM109	$recA^-$ (recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lambda-, (lac-pro AB), {F', traD36, proAB, $lacI^Q$, lacZ M15})	Maniatis *et al.*, 1982
Plasmids		
pBR322	4363 bp, amp^r, tet^r	Promega Biotech
pGEM-4	2870 bp, amp^r, T7 and SP6 promoters flanking a pUC18-derived multiple cloning region	Promega Biotech
pMJH5	604 bp *Bgl* II-*Eco* R1 of *nifH* of *A. vinelandii* in pUC-8	Dr. D. Dean, VPI & SU
pDB6	822 bp *Kpn* I-*Kpn* I of *nifD* of *A. vinelandii* in pUC-8	Dr. D. Dean, VPI & SU
pND001	3500 bp *Hind* III insert containing *nifH*, intervening sequence, and *c* 860 bp of *nifD* of *N. commune* in pBR322	This study
pND002, pND003	additional recombinant *nifH* clones in pBR322	This study

Table 1 conti.

pNDD1	1100 bp *Eco* RI insert containing *c* 60 bp of *nifH* and *c* 860 bp of *nifD* of *N. commune* in pGEM-4	This study
pNDH1	2400 bp *Eco* RI-*Hind* III containing *c* 840 bp of *nifH* of *N. commune* in pGEM-4	This study

Table 2

Conditions for restriction enzyme digestions

enzyme	plasmid DNA	units ug^{-1} of DNA	10x buffer	digestion time (h)
Hind III	01,02,03	16	React 2	3
Pst I	01,02,03	16	React 2	3
Kpn I	01,02,03	16	React 4	3
Hinc II	01,02,03	16	React 4	3
Sal I	01,02,03	16	React 3	4
Bam HI	01,02,03	16	React 3	4
Sph I	01,02,03	16	React 6	4
Eco RI	01,02,03	16	React 3	4
Acc I	01,03	12	Low	4
Xba I	01,03	16	React 2	4
Ava I	01,03	16	React 2	4
Sma I	01,03	20	React 4	4
Sac I	01,03	20	Low	4
Bgl II	01,03	16	React 3	4
Pvu I	01,insert	13	High	3
Hpa II	insert	16	React 8	3
Ava II	insert	16	React 2	3
Acc I-Eco RI	01	26	Low* +	3
Acc I-Hind III	01	26	Low* +	3
Hpa II-Eco RI	insert	33	React 8* +	3
Eco RI-Pvu I	01,insert	30	React 3 +	3

Table 2 conti.

enzyme	plasmid DNA	units ug^{-1} of DNA	10x buffer	digestion time (h)
Eco RI-Hinc II	insert	33	React 4 +	3

note: 01, 02, 03 refer to plasmids pND001, pND002, pND003

insert = 3500 bp Hind III insert containing nifH, intervening sequence and c 860 bp of nifD

* = with modifications
+ = total volume of 15 ul

TABLE 3

Solutions

10x React 2 Buffer: 0.5 M Tris-HCl pH 8.0, 0.1 M $MgCl_2$, 0.5 M NaCl (Hind III, Pst I, Xba I, Ava I, Ava II)

10x React 3 Buffer: 0.5 M Tris-HCl pH 8.0, 0.1 M $MgCl_2$, 1.0 M NaCl (Sal I, Bam HI, Eco RI, Bgl II)

10x React 4 Buffer: 20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 50 mM KCl (Kpn I, Hinc II, Sma I)

10x React 6 Buffer: 20 mM Tris-HCl pH 7.4, 6 mM $MgCl_2$, 50 mM KCl, 50 mM NaCl (SpH I)

10x React 8 Buffer: 20 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$ (Hpa II)

10x Low Salt Buffer: 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT (Acc I, Sac I)

10x High Salt Buffer: 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT (Pvu I)

Denaturation solution: 1.5 M NaCl, 0.5 M NaOH

Neutralization solution: 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5

1x SSC: 0.15 M sodium chloride, 0.015 M sodium citrate pH 7.0

Prehybridization solution:
30% w/v deionized formamide
5x SSC
0.1% w/v SDS
1 mM Na_2EDTA pH 8.0
10 mM Tris-HCl pH 7.5
1x Denhardt's solution
0.5 mg ml^{-1} denatured Salmon sperm DNA (Sigma Chemical Co.)

Hybridization solution:
30% w/v deionized formamide
5x SSC
0.1% w/v SDS
1 mM Na_2EDTA pH 8.0
10 mM Tris-HCl pH 7.5

Table 3 conti.

1x Denhardt's solution
5% PEG_{8000}
0.2 mg ml^{-1} denatured Salmon sperm DNA (Sigma Chemical Co.)

Denhardt's solution (50X): 1% w/v Ficoll
1% w/v Polyvinylpyrrolidone
1% w/v Bovine serum albumin (BSA)

Buffer 1: 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl

Buffer 2: 3% w/v BSA in Buffer 1

Buffer 3: 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$

Solution A1: 0.2 mM non-labeled dNTPs in 500 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$, 100 mM 2-mercapto-ethanol, 100 ug ml^{-1} BSA (BRL/Life Technologies Inc., Md)

Solution C: 0.4 units ul^{-1} DNA Pol I, 40 pg ul^{-1} DNase I, 50 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 50% w/v glycerol and 100 ug ml^{-1} nuclease-free BSA (BRL/Life Technologies Inc., Md)

Stop Buffer: 300 mM Na_2EDTA (pH 8.0) (BRL/Life Technologies Inc., Md)

TBE buffer: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA

LB medium:

Bacto-Tryptone	10 g l^{-1}
Bacto- Yeast extract	5 g l^{-1}
NaCl	10 g l^{-1}

Solution 1: 10 mM morpholinopropanesulfonic acid (MOPS) pH 7.0, 10 mM rubidium chloride

Solution 2: 10 mM MOPS pH 6.5, 50 mM calcium chloride, 10 mM rubidium chloride

Solution 3: solution B with the addition of 10 % v/v glycerol

Table 3 conti.

10x CIP buffer: 0.5 M Tris-HCl pH 9.0
10 mM $MgCl_2$
1 mM $ZnCl_2$
10 mM spermidine

10x ligation buffer: 0.3 M Tris-HCl pH 7.6
0.1 M $MgCl_2$
0.1 M DTT
5 mM ATP pH 7.0

Potassium acetate, pH 4.8: 60 ml of 5 M potassium acetate
11.5 ml glacial acetic acid
28.5 ml H_2O

M9 Medium: Per liter:

Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g

Adjust pH to 7.4, autoclave, cool, and then add:

1 M $MgSO_4$	2 ml
20% glucose	10 ml
1 M $CaCl_2$	0.1 ml

Protoplasting buffer: 30 mM Tris-HCl pH 8.0
5 mM Na_2EDTA
50 mM NaCl
20% w/v sucrose
50 ug ml^{-1} RNase A (Sigma)
50 ug ml^{-1} lysozyme (Sigma)

Lysis buffer: 89 mM Tris
89 mM boric acid
2.5 mM Na_2EDTA
2% w/v SDS
5% w/v sucrose
0.04% w/v bromophenol blue

DISCUSSION

The nifH gene which encodes the Fe protein of nitrogenase (component II; dinitrogen reductase), and the nifD and nifK genes which encode the alpha and beta subunits of the MoFe protein are highly conserved among diverse nitrogen - fixing organisms (Rice et al., 1980). This study also confirms this finding by comparing the partial amino acid sequences of nifH and nifD gene products from N. commune UTEX 584 (deduced from DNA sequence analysis) with those of A. variabilis, A. vinelandii, K. pneumoniae and R. meliloti. Partial amino acid sequences of the nifH and nifD genes of N. commune show particularly strong conservation when compared to those of A. variabilis.

Although the genes for nitrogen fixation are conserved among different organisms, their arrangements and copy numbers in the genomes vary. A comparison between non-heterocystous and heterocystous cyanobacteria indicated that non-heterocystous cyanobacteria have a contiguous nifKDH gene cluster, whereas heterocystous cyanobacteria show a different organization of their nif structural genes (Kallas et al., 1983). DNA from vegetative cells of A. variabilis PCC 7120 contains linked nifDH genes with nifK separated from nifD by approximately 11 kb. Under conditions of nitrogen deprivation, some vegetative cells differentiate

into heterocysts, the site of nitrogen fixation, and their nif DNA is rearranged to form a complete nifKDH operon (Golden et al , 1985). Two Nostoc strains PCC 7121 (Het^- mutant) and PCC 7906 (Het^+ wild type) exhibited the same nif gene organization as A. variabilis PCC 7120 (Kallas et al, 1983). Therefore, it seems likely that other Nostoc strains undergo a similar type of nif gene rearrangement. While the functional significance of nif gene rearrangements is not well understood, it is hypothesized that they serve some function in gene regulation (Kallas et al., 1983).

A study of the organization of nif genes in A. variabilis led to the discovery of multiple copies (two) of nifH genes in the genome (Golden et al., 1985). Three copies of nifH, one functional nifH gene and two nifH-related structures, were detected in the anaerobic gram-positive bacterium, Clostridium pasteurianum (Chen et al., 1986). In the present study, three copies of the nifH gene and at least two copies of the nifD gene were detected in N. commune UTEX 584. All three copies of the nifH gene, located on 9 kb, 3.5 kb and 1.1 kb Hind III fragments, respectively showed strong hybridization signals of equivalent intensity with the A. vinelandii nifH probe. Only one copy of nifD, located on a 3.5 kb Hind III fragment, hybridized strongly with the nifD probe. The 3.5 kb Hind III fragment that showed hybridization to nifH and

nifD probes contains the entire nifH gene and a partial nifD gene. These linked genes presumably form the functional nifDH portion of the nif operon. As for the function of the other nifH sequences, they could be non-functional pseudogenes or second and third copies of a functional nifH gene (Rice et al.,1982). It is possible that an alternate nitrogen fixation system exists in N. commune, as was found in Azotobacter vinelandii, which functions under Mo deprivation growth conditions (Bishop, 1986).

The data suggests that the 3.5 kb Hind III fragment contains the functional nifD gene adjacent to one of the copies of nifH. Three possibilities could account for the presence of the additional weak hybridization signals detected on Southern blots of N. commune DNA digested with Hind III and Eco Rl and probed with nifD. The first possibility is that the nifD probe, which extends 800 bp within the reading frame of nifD, hybridizes to a second Hind III fragment of 1.9 kb, which contains the 3' end of N. commune nifD. This possibility could not account for detection of hybridization with a 2.3 kb Eco Rl fragment in addition to the c 4 kb fragment which contained all of nifD. The weak hybridization signals may also come from a different gene whose sequence is homologous with nifD. The third possibility is that the weak signals correspond to a

nifD-like sequence that has less sequence identity with the nifD probe than the nifD sequence which is adjacent to nifH. The data support the second and third possibilities, although further studies need to be performed to confirm this unequivocally. The reasoning which rules out the first possibility and supports either the second or third possibilities is summarized as follows: the 4.0 kb Eco R1 fragment which hybridizes strongly with the nifD probe must contain the entire nifD gene based on partial sequence analysis of the 3.5 kb insert of pND001. DNA sequences and sequence identity data indicate that the start codon of nifD begins within the 1.1 kb Hind III-Eco R1 fragment and extends 860 bp to the Hind III site; 550 bp of the 820 bp nifD probe of A. vinelandii hybridizes to this fragment. The 4.0 kb Eco R1 fragment extends downstream from the Hind III site approximately 2.9 kb and, therefore, must contain the remaining c 600 bp of nifD. Of this 600 bp of nifD, only 270 bp would hybridize to the Azotobacter probe if one assumes sufficient homology.

If there is a second nifD sequence, is it located adjacent to one of the copies of nifH? If the Eco R1 site that cleaves nifH c 60 bp from the 5' end in pND001 is conserved, then it is not possible for nifD to lie adjacent to nifH contained on the 9 kb Hind III fragment, but it is possible for nifD to lie adjacent to nifH contained on the

1.1 kb Hind III fragment. If the latter were true, then N. commune would contain two nifDH operons. It would then be interesting to determine under what conditions these operons function. Bishop (1986) described a second nitrogen fixation system in Azotobacter vinelandii which operates under conditions of molybdenum deficiency (Bishop, 1986). If an alternate nitrogen fixation system also existed in Nostoc commune, would it require a second nifDH operon? Before addressing these questions, a more detailed study of multiple nifH and nifD sequences in Nostoc commune is required.

The isolation of the nifH and nifD genes from desiccation-tolerant Nostoc commune now permits the use of these genes as specific homologous probes in the study of nif expression during various stages of water stress.

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