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Identification of blue-green algal nitrogen fixation genes by using heterologous DNA hybridization probes

(*Klebsiella pneumoniae* DNA probes/recombinant DNA/*Anabaena*)

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ABSTRACT In the filamentous blue-green alga *Anabaena* 7120, aerobic nitrogen fixation is linked to the differentiation of specialized cells called heterocysts. In order to study control of heterocyst development and nitrogen fixation in *Anabaena*, we have used cloned fragments of the *Klebsiella pneumoniae* nitrogen fixation (*nif*) genes as probes in DNA·DNA hybridizations with restriction endonuclease fragments of *Anabaena* DNA. Using this technique, we were able to identify and clone *Anabaena nif* genes, demonstrating the feasibility of using heterologous probes to identify genes for which no traditional genetic selection exists. From the patterns of hybridization observed, we deduced that although DNA sequence homology has been retained between some of the *nif* genes of these divergent organisms, the *nif* gene order has been rearranged.

We have been studying the organization of the nitrogen fixation genes in the photosynthetic blue-green alga *Anabaena* 7120. In the presence of fixed nitrogen, *Anabaena* grows in long filaments uniformly composed of vegetative cells. It differentiates specialized cells, called heterocysts, whenever fixed nitrogen is depleted from its growth medium (1). The heterocysts appear at regular intervals along the filaments; concomitant with their differentiation, the nitrogen fixation genes are induced and nitrogen fixation begins. Nitrogen fixation and differentiation are linked tightly controlled processes; nitrogen fixation and differentiation ordinarily occur only upon depletion of a source of fixed nitrogen, and aerobic nitrogen fixation occurs only in heterocysts (2). The primary role of the heterocyst, in fact, appears to be to provide an anaerobic environment for the oxygen-labile nitrogen fixation reaction. It is believed that a primary product of this reaction, probably glutamine, is responsible for maintaining the heterocyst spacing pattern, by inhibiting further heterocyst development in neighboring vegetative cells when present above a threshold level (3).

In order to understand these coordinate processes in detail it would be useful to isolate mutants defective in each of the genes that must function in heterocyst differentiation and nitrogen fixation and to analyze these mutants by conventional microbial genetic methods. Currier *et al.* (4) and Wilcox *et al.* (5) have isolated mutants of several *Anabaena* strains that display defects in heterocyst development, in the spacing pattern, or in nitrogen fixation. Unfortunately there is not yet a convenient system of genetic analysis in *Anabaena* with which, for example, complementation tests could be performed. We have therefore undertaken the long-term project of cloning and identifying *Anabaena* genes in recombinant DNA molecules propagated in *Escherichia coli*. We have begun our analysis with the *Anabaena* genes for nitrogen fixation.

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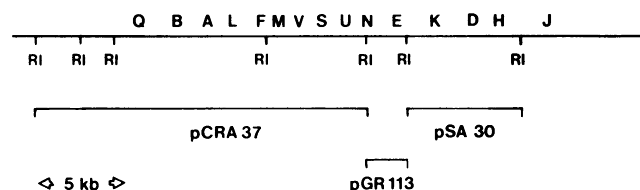

FIG. 1. Map of the *K. pneumoniae nif* region and those regions carried on recombinant plasmids used in this study. RI refers to the *Eco*RI restriction endonuclease sites on the *K. pneumoniae* chromosome. kb, Kilobase pairs. From refs. 11–14.

In order to identify the algal nitrogen fixation (*nif*) genes, we used cloned fragments of the *Klebsiella pneumoniae nif* genes as hybridization probes (6, 7). Because the nitrogenase subunits of the blue-green algae *Anabaena* and *Plectonema* can complement subunits of *Klebsiella* and *Clostridium* nitrogenases *in vitro* (8, 9), we hoped that enough homology might be retained in the DNA of these genes to allow heterologous hybridizations. Our approach was to subject algal DNA to total digestion by restriction endonucleases, separate the resulting fragments by electrophoresis through agarose gels, and then transfer the DNA from the gels to nitrocellulose filters by the technique of Southern (10). The *Anabaena nif* gene fragments were then identified by hybridization with labeled DNA probes carrying segments of the *Klebsiella nif* genes and by subsequent autoradiography. We were greatly aided in this approach by the isolation of cloned *Klebsiella nif* genes on amplifiable plasmids by Cannon *et al.* (6, 7).

The correspondence between the genetic map of the *Klebsiella nif* region and the *Eco*RI restriction fragments contained in the plasmids used as probes is shown in Fig. 1. The fifteen *Klebsiella nif* cistrons are organized into at least seven transcription units (12–14). Gene *A* appears to code for a *trans*-acting positive control element. Without it, no other *nif* gene is expressed. Genes *K*, *D*, and *H* code for the structural proteins of nitrogenase (*K* and *D*) and nitrogenase reductase (*H*) (15, 16). All of the other genes appear to code for electron transport proteins or proteins involved in the assembly of nitrogenase and its Fe- and Mo-containing prosthetic groups (17). Regulation of the *nif* genes is complex; it may involve a repressor whose corepressor is glutamine, and it may involve glutamine synthetase as a positive gene activator (18–20).

Although the organization of the genes for nitrogen fixation in *Anabaena* is of intrinsic interest, preliminary indications that these genes do not require glutamine synthetase for their ex-

Abbreviations: kb, kilobase pairs; NaDodSO$_4$, sodium dodecyl sulfate; SSPE, 0.15 M NaCl/0.01 M NaH$_2$PO$_4$/0.001 M Na$_2$EDTA, pH 7.0.

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pression make our studies particularly relevant in the context of attempts to engineer microorganisms with high constitutive nitrogen fixing capacity.

MATERIALS AND METHODS

Preparation of Algal DNA. Fifteen-liter cultures of basal salts medium (21) were inoculated with 100 ml of an exponentially growing culture of *Aabaena* 7120 [formerly described as *Nostoc muscorum* (1)] and grown at room temperature with 0.5% CO_2 until stationary phase was reached. Cells were collected at 4°C in a Sorvall continuous-flow centrifuge and washed twice in 0.05 M NaCl/0.05 M Na_2EDTA/0.05 M Tris·HCl, pH 8.5. Cells (15 g) were resuspended in 25 ml of the same buffer, and lysozyme was added to 10 mg/ml. After 2 hr at 37°C, an equal volume of this buffer containing 1% sodium dodecyl sulfate ($NaDodSO_4$) was added, and the lysate was frozen and thawed twice. An equal volume of buffer-saturated phenol was then added, and the lysate was shaken gently at 4°C overnight. DNA from the aqueous phase was then precipitated onto a glass stirring rod with 2 vol of cold ethanol, washed successively in 70%, 80%, and 90% ethanol, and resuspended in 75 ml of 0.1 M NaCl/0.001 M Na_2EDTA/0.01 M Tris·HCl, pH 8.0. Heat-treated (80°C for 10 min) RNase was added to 400 μg/ml; after 1 hr at 37°C, it was followed by proteinase K at 100 μg/ml. Several hours later the DNA was extracted with phenol and then precipitated with ethanol; after resuspension in 0.01 M Tris·HCl, pH 8.0/0.001 M Na_2EDTA it was precipitated with 2-propanol and resuspended in the same buffer (22, 23). DNA from the strains *Plectonema boryanum* 594 and *Anabaena variabilis* Kütz ATCC 29413 was similarly prepared.

Preparation and Labeling of Cloned *Klebsiella nif* DNA. Three *E. coli* strains harboring amplifiable recombinant plasmids containing *Klebsiella nif* genes were used. Strains FMA 185/pCRA37, AB2880/pSA30, and GM4/pGR113 were kindly provided by F. Ausubel and G. Riedel (11). Fig. 1 shows the order of the *Klebsiella nif* genes and the segments present on the three plasmids used.

Plasmid-bearing strains were grown at 37°C in LB or M9 medium (24) containing tetracycline at 10 μg/ml, with 2% casamino acids. At an OD_{600} of 0.6, chloramphenicol was added to a concentration of 100 μg/ml for plasmid amplification. After overnight amplification, the cells were harvested and lysed with lysozyme and $NaDodSO_4$, according to the method of Model and Zinder (25). The cleared lysate was treated with RNase and then extracted with phenol. After CsCl/ethidium bromide equilibrium centrifugation, supercoiled DNA was further purified on a 5–20% sucrose gradient (26).

For *in vitro* labeling, the DNA was incubated with DNA polymerase I (Boehringer Mannheim) in the presence of [α-^{32}P]dCTP (300 Ci/mmol, Amersham; 1 Ci = 3.7×10^{10} becquerels) as described by Maniatis *et al.* (27). Labeled DNA was separated from unincorporated nucleotides on a Sephadex G-50 column equilibrated with 0.01 M Tris·HCl, pH 8.0/0.001 M Na_2EDTA/0.1% Sarkosyl NL-97.

Restriction Digests, Electrophoresis, and Hybridizations. Algal DNA was digested with *Eco*RI (New England BioLabs) at 5 units/μg of DNA, *Hin*dIII (New England BioLabs) at 2 units/μg of DNA, or *Xba* I (Bethesda Research Laboratories) at 10 units/μg of DNA. After approximately 3 hr of digestion at 37°C in the buffer recommended by the supplier, the nucleases were inactivated at 65°C for 10 min. Three micrograms of DNA was applied per sample slot of a 20 × 15 × 0.7 cm 0.7% agarose gel and electrophoresed at 35 V for 16 hr in 0.04 M Tris/0.036 M NaH_2PO_4/0.001 M Na_2EDTA. The gels were then stained in ethidium bromide at 0.5 μg/ml and photographed under long-wavelength UV light. The gels were denatured in 0.2 M NaOH/0.6 M NaCl for 1.5 hr and neutralized in 1 M Tris·HCl, pH 7.5/0.6 M NaCl for 1.5 hr, and the DNA was then transferred to Millipore HAWP filters with 20× SSPE, according to the technique of Southern (10); 1× SSPE is 0.15 M NaCl/0.01 M NaH_2PO_4/0.001 M Na_2EDTA, pH 7.0. After overnight transfer, the filter was briefly rinsed in 2× SSPE, baked at 80°C for 4 hr in a vacuum oven, and then prehybridized at 65°C for 12 hr in 200 ml of 6× SSPE containing 0.5% $NaDodSO_4$, Denhardt's solution (28), and denaturated calf thymus DNA at 15 μg/ml. The filter was then transferred to 20 ml of fresh buffer containing approximately 2×10^7 cpm of ^{32}P-labeled DNA, and the bound DNA was hybridized at 65°C with shaking for 24 hr in a boilable plastic pouch (Seal-a-Meal, Dazey Products). The filter was then rinsed overnight at 65°C in at least three changes of 6× SSPE/0.5% $NaDodSO_4$ (200 ml each) and was finally rinsed three times for 1 hr each in 200 ml of 2× SSPE at room temperature. The dried filter was placed in a cassette with Kodak XR-5 film and a Du Pont Cronex Lightning Plus intensifying screen at −70°C for at least 1 week, and the film was then developed.

Electron Microscopy. Heteroduplex molecules were annealed and spread according to Davis *et al.* (29), except that formamide concentrations were 25% (vol/vol) in the spreading solution and 5% in the hypophase.

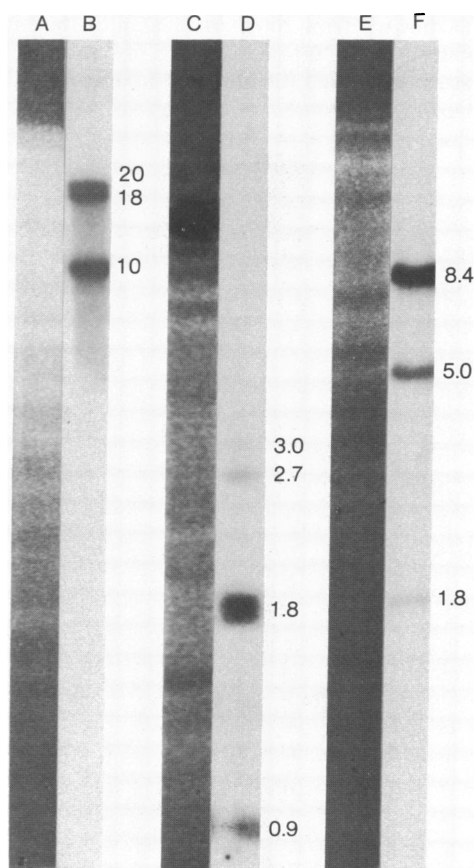

FIG. 2. Hybridization of ^{32}P-labeled pSA30 DNA to restriction fragments of *Anabaena* 7120 DNA. Lanes A, C, and E show the ethidium bromide staining patterns after agarose gel electrophoresis of *Anabaena* 7120 DNA digested with *Eco*RI, *Hin*dIII, and *Xba* I restriction endonucleases, respectively. Lanes B, D, and F show corresponding autoradiograms after transfer of the DNA restriction fragments to nitrocellulose filters and hybridization with ^{32}P-labeled pSA30 DNA. Sizes of bands are given in kb.

RESULTS

As discussed in the Introduction, our approach to identifying those algal DNA fragments that contain nitrogen fixation (*nif*) genes was to use plasmids carrying defined segments of the *K. pneumoniae nif* operons as heterologous probes. We digested *Anabaena* 7120 DNA with restriction enzymes, subjected the DNA fragments to agarose gel electrophoresis, transferred the arrayed fragments to nitrocellulose paper, and then hybridized the fragments with labeled DNA probes containing *Klebsiella nif* genes. Fig. 2 shows the electrophoretic patterns of *Anabaena* 7120 DNA fragments after digestions with *Eco*RI, *Hin*dIII, or *Xba* I endonuclease along with the corresponding hybridization patterns for a pSA30 DNA probe. Plasmid pSA30 contains a 6-kb fragment of *Klebsiella nif* DNA, extending from genes *E* through *H* (Fig. 1). As can be seen in Fig. 2, *Anabaena* 7120 *Eco*RI fragments 10, 18, and 20 kb long hybridized with pSA 30. We sometimes also saw a low level of hybridization with a 6-kb *Eco*RI fragment; we do not know whether this band is a distinct *Eco*RI fragment or a cleavage product of one of the larger *Eco*RI fragments. Similarly, *Anabaena* 7120 *Hin*dIII fragments 3.0, 2.7, 1.8, and 0.9 kb long hybridized to pSA30, as did *Anabaena* 7120 *Xba* I fragments 8.4, 5, and 1.8 kb long.

The specificity of this hybridization for *nif* genes was demonstrated by recloning the *nif* gene segment of plasmid pSA30 into a phage λ gt7 vector (unpublished). This DNA was designated λ gt7-Kp1; when labeled and used as a hybridization probe, the same algal DNA bands hybridized as with the pSA30 probe (not shown). Thus, the hybridization pattern is specifically produced by the *nif* gene portion of pSA30.

Further evidence that the observed hybridization is due to homologies between the *nif* genes of the two organisms was obtained by using a probe carrying another segment of the *Klebsiella nif* operon, plasmid pCRA37. As shown in Fig. 1, pCRA37 contains the left-most portion of the *Klebsiella nif* operon, extending from gene *Q* through gene *U*. As shown in Fig. 3, pCRA37 appears to hybridize to the same 10-kb *Eco*RI fragment of *Anabaena* 7120 DNA as does pSA30, supporting the idea that the observed hybridization is due to *nif* gene homologies between *Klebsiella* and *Anabaena*. No hybridization could be detected when *Anabaena* 7120 DNA fragments were probed with labeled pGR113 DNA, which contains parts of *Klebsiella nif* genes *N* and *E* (Fig. 1).

Because three *Eco*RI fragments of *Anabaena* 7120 DNA, each 10–20 kb long, hybridized with the 6-kb *Klebsiella nif* fragment on pSA30, we suspected that although a high degree of homology exists between the *nif* genes of these two organisms, the order of their *nif* genes had probably been rearranged relative to each other. That is, if the *Klebsiella nif* gene order *E, K, D, H* had been retained in *Anabaena*, no more than two *Anabaena Eco*RI fragments should have hybridized with the 6-kb *nif* segment of the pSA30 probe. Because three fragments, all greater than 6 kb in length, hybridized with this probe, we believed that gene rearrangements had probably occurred.

Such rearrangements were confirmed by heteroduplexing studies. The 10-kb fragment of *Anabaena* 7120 DNA was isolated on a λ gt7 cloning vector from a pool of recombinant λ phages. Cloning details will be presented in a separate paper. The DNA from this phage, designated λ gt7-An154, was then hybridized with the DNA from λ gt7-Kp1, the λ phage carrying the pSA30-derived *nif* genes. Fig. 4 shows one such

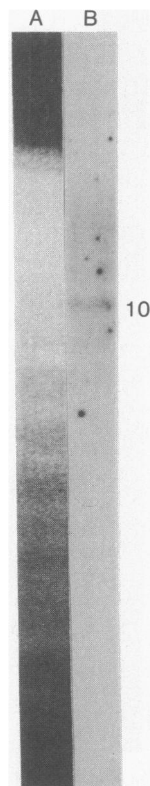

FIG. 3. Hybridization of ^{32}P-labeled pCRA37 DNA to *Eco*RI restriction fragments of *Anabaena* 7120 DNA. Lane A shows the staining pattern after agarose gel electrophoresis. Lane B shows the corresponding autoradiogram after transfer of DNA to a nitrocellulose filter and hybridization with ^{32}P-labeled pCRA37 DNA. Sizes of bands are given in kb.

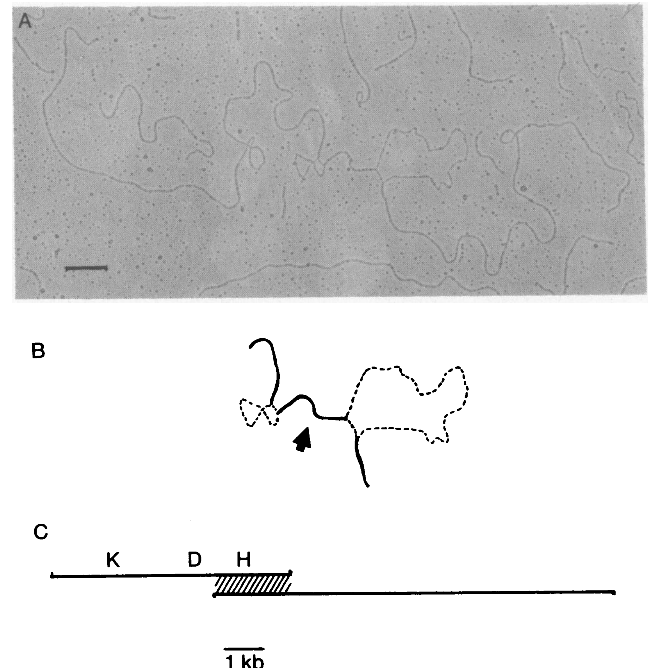

FIG. 4. (*A*) Heteroduplex molecule formed from DNAs of λgt7-An154, a phage λ vector containing a 10-kb *Eco*RI restriction fragment of *Anabaena* 7120, and λgt7-Kp1, the same phage vector but containing the 6-kb *Eco*RI *nif* fragment recloned from pSA30. Bar indicates 1 μm. (*B*) Drawing showing the region of homology present (see arrow) in the *Eco*RI inserts of the two phages. Broken lines indicate regions of single-stranded DNA. (*C*) The overlapping region of homology in the two *Eco*RI DNA fragments. The upper line represents the 6-kb *K. pneumoniae Eco*RI DNA fragment. The letters *K, D,* and *H* mark the approximate positions of the *K. pneumoniae nif* genes. The lower line represents the 10-kb *Eco*RI fragment of *Anabaena* 7120 DNA.

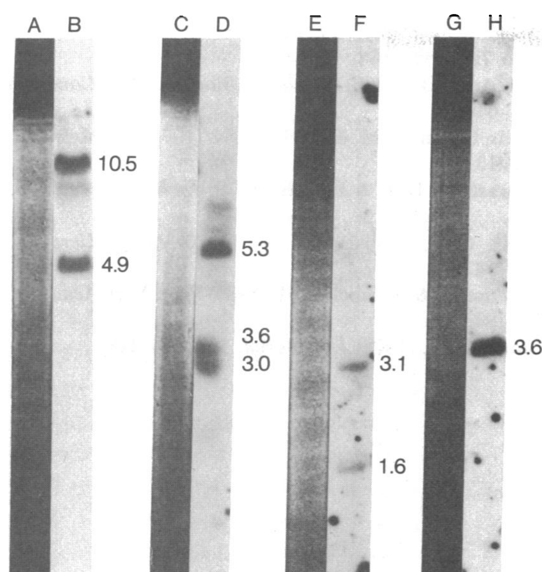

FIG. 5. Hybridization of ^{32}P-labeled pSA30 DNA to restriction fragments of *A. variabilis* 29413 DNA and *P. boryanum* DNA. Lanes A, C, E, and G show staining patterns of restriction fragments after agarose gel electrophoresis. Lanes A and C are *A. variabilis* DNA digested with *Eco*RI and *Hin*dIII, respectively. Lanes E and G are *P. boryanum* DNA digested with *Hae* II and *Bgl* II, respectively. Lanes B, D, F, and H show corresponding autoradiograms after transfer of the DNA to nitrocellulose filters and hybridization with ^{32}P-labeled pSA30 DNA. Sizes of bands are given in kb.

heteroduplex molecule. λgt7-An154 is homologous to λgt7-Kp1 along a 2-kb section of the DNA extending from the middle of *Klebsiella* gene *D* through gene *H*. Similar heteroduplexing studies between λgt7-An154 and *Eco*RI fragments of pCRA37 cloned into λ gt7 phages have shown that λgt7-An154 also contains genes homologous to the central portion of the *Eco*RI fragment containing *Klebsiella nif* genes *M, V, S,* and *U*. λ gt7-An154 does not possess any other regions homologous to pCRA37 or pGR113. These studies confirm both the homology between *Klebsiella* and *Anabaena nif* genes and the relative rearrangement of the gene order.

We also isolated DNA from two other blue-green algal strains, in order to learn whether the *nif* gene homologies also occurred between *Klebsiella* and other nitrogen-fixing blue-green algae. *Plectonema boryanum* 594 is a filamentous alga that lacks heterocysts but can reduce nitrogen under anaerobic conditions (30). *Anabaena variabilis* Kütz ATCC 29413 is a heterocystous alga capable of heterotropic growth (31). As can be seen in Fig. 5, the DNA from both of these strains also hybridized to the pSA30 *Klebsiella nif* gene probe; the interspecies homology among *nif* genes appears to be tightly conserved.

DISCUSSION

This work has demonstrated that heterologous DNA can be used as a probe for genes on which evolutionary constraints have been exercised. *Klebsiella* and the blue-green algae occupy divergent evolutionary branches; *Klebsiella* is a unicellular enterobacterium that fixes nitrogen only under anaerobic conditions, whereas *Anabaena* is a multicellular filamentous blue-green alga that fixes nitrogen aerobically. Yet their *nif* genes have been sufficiently constrained from diverging so as to allow detection of *Anabaena nif* genes with *Klebsiella nif* gene probes. The use of heterologous probes may prove useful in other systems lacking an easily isolatable messenger RNA or other direct selection procedures. The accompanying paper by Ruvkun and Ausubel also demonstrates that homology between *nif* genes has been retained in a wide variety of prokaryotic organisms (32).

Although homology has been retained between some of the *nif* genes, their genetic order has not been conserved. This was evident from the pattern of hybridizations between *Eco*RI-digested *Anabaena* 7120 DNA and the *Klebsiella* pSA30 probe. Three *Eco*RI fragments, 10, 18, and 20 kb long, hybridized with the 6-kb *nif* segment of the pSA30 probe. The third hybridizing fragment indicated that gene rearrangement had probably occurred. The possibility remained that multiple copies of the *nif* operons, bounded by different nucleotide environments, existed in *Anabaena*. This was ruled out, however, by hybridizing λgt7-An154, the cloned 10-kb *Eco*RI fragment of *Anabaena* 7120, to a total *Anabaena* 7120 *Eco*RI digest; only the 10-kb fragment hybridized with the probe. Similarly, gene rearrangement was confirmed by heteroduplexing studies between λgt7-An154 and vectors carrying defined segments of the *Klebsiella nif* operons, heteroduplexes were formed between contiguous segments of *Anabaena* DNA and non-contiguous segments of *Klebsiella* DNA.

We have long considered the possibility that the *nif* genes in *Anabaena* are carried on a naturally occurring plasmid. Despite the fact that a large number of plasmid size classes occur in the alga (ref. 33; unpublished results) we were unable to demonstrate any hybridization between plasmid bands on nitrocellulose blots of gels, and *nif* gene probes of either *Klebsiella* or *Anabaena*. Thus, our available evidence indicates that the *nif* genes of *Anabaena* are chromosomally located.

We are greatly indebted to Fred Ausubel and coworkers for sending us strains containing cloned *K. pneumoniae nif* genes. This work was supported by Grant GM 21823 from the U.S. Public Health Service and Grant 5901-0410 from the U.S. Department of Agriculture. B.M. was supported during part of this work by U.S. Public Health Service Training Grant GM 7190. D.R. was supported by U.S. Public Health Service Training Grants GM 780 and GM 07183.

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