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Isolation and Characterization of *Frankia* sp. Strain FaC1 Genes Involved in Nitrogen Fixation

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Genomic DNA was isolated from *Frankia* sp. strain FaC1, an *Alnus* root nodule endophyte, and used to construct a genomic library in the cosmid vector pHC79. The genomic library was screened by in situ colony hybridization to identify clones of *Frankia* nitrogenase (*nif*) genes based on DNA sequence homology to structural nitrogenase genes from *Klebsiella pneumoniae*. Several *Frankia nif* clones were isolated, and hybridization with individual structural nitrogenase gene fragments (*nifH*, *nifD*, and *nifK*) from *K. pneumoniae* revealed that they all contain the *nifD* and *nifK* genes, but lack the *nifH* gene. Restriction endonuclease mapping of the *nifD* and *nifK* hybridizing region from one clone revealed that the *nifD* and *nifK* genes in *Frankia* sp. are contiguous, while the *nifH* gene is absent from a large region of DNA on either side of the *nifDK* gene cluster. Additional hybridizations with gene fragments derived from *K. pneumoniae* as probes and containing other genes involved in nitrogen fixation demonstrated that the *Frankia nifE* and *nifN* genes, which play a role in the biosynthesis of the iron-molybdenum cofactor, are located adjacent to the *nifDK* gene cluster.

Frankia is a genus of slow-growing actinomycetes for which identification to species level has not been determined; these organisms are capable of entering into a symbiotic nitrogen-fixing root nodule association with nonleguminous actinorhizal plants (4). These plants are woody pioneer shrubs and trees that include the genera *Alnus*, *Casuarina*, *Comptonia*, and *Elaeagnus*. Actinorhizal plants are important in timber and pulp production and, due to the nitrogen-fixing association they form with *Frankia* spp., they are important species in land reclamation and global nitrogen cycles (42).

Although it was suspected for many years that the nitrogen-fixing root nodules of actinorhizal plants were induced by actinomycetous bacteria, direct evidence for this hypothesis was only recently obtained by Callaham et al. (13), who first cultured *Frankia* spp. from actinorhizal root nodules. Subsequently, there have been many reports on the physiology and ecology of *Frankia* spp. (see reference 29 for a recent review). However, due to a complete lack of known genetic exchange mechanisms in *Frankia* spp. and to the relatively recent culture of the organism, little is known about its genetics. To date, such studies include the detection of plasmids in some *Frankia* strains (30, 39) and the determination of the DNA base composition (68 to 72% guanine plus cytosine) (2) and genome size (7×10^6 to 9×10^6 bases) (1).

Of all biological nitrogen fixation systems, *Klebsiella pneumoniae* is the best characterized, both biochemically and genetically. There are 17 genes in *K. pneumoniae* that have been shown to play a role in nitrogen fixation. Nitrogenase, the enzyme complex that catalyzes biological nitrogen fixation, is composed of two components, both of which are iron- and sulfur-containing proteins (see references, 11, 35 and 36). The nitrogenase Fe protein (component II) is composed of two identical subunits encoded by the *nifH* gene. The Mo-Fe protein (component I) is made up of two dissimilar subunits, $\alpha_2\beta_2$, that are encoded by the *nifD* and *nifK* genes, respectively. The active nitrogenase complex also contains two molecules of an iron-molybdenum (FeMo) cofactor. Although the genes coding for the nitrogenase proteins have been isolated and characterized from many diazotrophic organisms (3, 7, 18, 32–34), work of this nature has not been reported for *Frankia* spp. It has been previously demonstrated that there is significant DNA sequence conservation among the structural nitrogenase genes (*nifHDK*) from *K. pneumoniae* and many other diazotrophs, including *Frankia* spp. (37). While there have been cases in which the sequence conservation among nonstructural nitrogenase genes derived from different organisms is sufficient to allow heterologous hybridization, the level of intergenic homology is, in general, lower than has been the case with the structural nitrogenase genes. In this study, we report the isolation and characterization of gene sequences from *Frankia* spp. that show strong hybridization homology to *K. pneumoniae* structural nitrogenase genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Frankia* sp. strain FaC1 used in this study was isolated in our laboratory from root nodules of an alder tree (*Alnus viridis* subsp. *crispa*), as described before (5). *Escherichia coli* strain HB101 (10) was used as a host organism for plasmid selection and maintenance. *Frankia* sp. strain FaC1 was grown in 500 ml of QmodB medium (24) in 1-liter flasks incubated statically at 28°C. The culture flasks were vigorously swirled one or two times daily to provide aeration and to disrupt hyphal mats and to ensure homogeneous mycelial growth. Nitrogenase activity was induced by culturing FaC1 in a nitrogen-free medium, as previously described (41), and was measured by following the reduction of acetylene (12). *E. coli* HB101 was cultured in liquid LB medium (15) on a gyratory shaker or on LB agar plates at 37°C. When required, ampicillin was included in the medium at a concentration of 50 μg/ml.

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Reagents. Cesium chloride (DNA optical grade) was obtained from Gallard-Schlesinger Chemical Co., Carle Place, N.Y. All enzymes were supplied by Boehringer Mannheim Biochemicals, Indianapolis, Ind., and Bethesda Research Laboratories, Gaithersburg, Md., and were used according to the specifications of the manufacturer. Radiolabeled deoxyribonucleotides were supplied by Amersham Corp., Arlington Heights, Ill., and New England Nuclear Corp., Boston, Mass. All other reagents and chemicals were supplied by Fisher Scientific Co., Rochester, N.Y., and Sigma Chemical Co., St. Louis, Mo.

***Frankia* DNA isolation.** *Frankia* sp. strain FaC1 cells were harvested in late exponential phase (3 weeks old) by centrifugation and suspended in 50 mM Tris–20 mM EDTA–15% sucrose, pH 8.0. The mycelia were broken to create a more homogeneous suspension by forcing the cells several times through an 18-gauge syringe needle. Cells were washed and suspended in the same buffer to an optical density of 100 Klett units (Klett-Summerson colorimeter with a no. 66 red filter; Klett Manufacturing Co., Long Island City, N.Y.). Lysozyme was added to a final concentration of 250 μg/ml, and the suspension was incubated at room temperature for 60 min. RNase A (20-mg/ml stock solution; boiled for 10 min prior to use), proteinase K (20-mg/ml stock solution in 25 mM Tris, 10 mM EDTA, pH 8.0), and sodium Sarkosyl (20% stock solution) were added to final concentrations of 500 μg/ml, 250 μg/ml, and 1%, respectively. The suspension was incubated at 37°C for 1 h until the resulting lysate appeared clear and viscous. Cesium chloride was dissolved in the lysate to 50% (wt/wt), and ethidium bromide was added to 100 μg/ml. Purified DNA was isolated from the lysate by centrifugation in heat-sealable polyallomer centrifuge tubes (2.5 by 9 cm) in a vertical rotor (model VTi 50; Beckman Instruments, Inc., Fullerton, Calif.) at 45,000 rpm for 16 h at 20°C. Ethidium bromide was extracted from the DNA with isoamyl alcohol followed by dialysis for several hours against 100 volumes of 50 mM Tris–10 mM EDTA–50 mM NaCl, pH 8.0, at 4°C. DNA was precipitated by addition of sodium acetate to 0.3 M and 2 volumes of ethanol (95%) followed by incubation at −20°C for several hours. Precipitated DNA was pelleted by centrifugation (8,000 × *g* for 10 min), washed with cold (−20°C) 70% ethanol, and dried under vacuum. The desiccated DNA was then dissolved in an appropriate volume of DNA storage buffer (5 mM Tris, 1 mM EDTA, 5 mM NaCl, pH 8.0) and stored at 4°C. DNA isolated by this procedure routinely yielded high-molecular-weight DNA in the size range of 50 to 100 kilobases (kb) as determined on 0.2 to 0.4% agarose gels.

Construction of a *Frankia* gene library. *Frankia* sp. strain FaC1 genomic DNA was partially digested with the restriction endonuclease *Sau*3A. DNA fragments in the size range of 40 to 50 kb were isolated from this partial digest by centrifugation and fractionation of a sucrose density gradient (10 to 40% step gradient with 5% steps) (28). Cosmid vector pHC79 (20) DNA was restricted with *Bam*HI and treated with calf intestinal alkaline phosphatase. Partially digested, size-fractionated *Frankia* DNA and *Bam*HI-restricted, phosphatase-treated pHC79 DNA were ligated with T4 DNA ligase in equimolar concentrations with a total DNA concentration of 1 mg/ml at 15°C for 12 h. Ligated DNA was packaged into lambda phage particles with an in vitro DNA packaging system (Boehringer Mannheim Biochemicals) and *E. coli* HB101 was infected. Ampicillin-resistant, tetracycline-susceptible *E. coli* clones were obtained at a frequency of 5.6×10^6/μg of *Frankia* DNA. Analysis of cosmid DNA isolated from 24 random clones from the gene library demonstrated the average size of the cloned *Frankia* DNA to be 38 kb.

Isolation of plasmids and DNA restriction fragments. Plasmid DNA was isolated by the technique of Bolivar et al. (9) and purified by CsCl-ethidium bromide isopycnic gradient centrifugation. Isolation of small quantities of plasmid DNA from many clones for restriction analysis was performed by the technique of Ish-Horowicz and Burke (21). DNA restriction fragments used for subcloning or for radiolabeled hybridization probes were purified from low-melting-temperature agarose by phenol extraction (28).

DNA-DNA hybridizations. The *Frankia* gene library was screened by the in situ colony hybridization technique of Maas (26) to identify clones carrying sequences homologous to the structural *nif* genes of *K. pneumoniae*. All other hybridizations were performed by transferring DNA in agarose gels to nitrocellulose membranes by the method of Southern (40). Hybridizations were done as described previously (28), using ^{32}P-labeled DNA probes radiolabeled by nick translation (28).

RESULTS

Characterization of *Alnus* root-nodule endophyte. The *Frankia* bacterium cultured from alder root nodules in our laboratory was designated strain FaC1. It has characteristics common to other *Frankia* strains, including a hyphal cell morphology and the ability to produce spores. Strain FaC1 also shares common growth characteristics with other *Frankia* strains, including a lengthy generation time (40 to 45 h) and a preference for organic acids as carbon sources (8). Furthermore, strain FaC1 demonstrated the ability to grow by using dinitrogen gas as a nitrogen source in nitrogen-free medium. Under nitrogen-fixing conditions, specialized vesicle structures unique to *Frankia* spp. during nitrogen fixation (41) and thought to be the site of nitrogenase were observed. Vesicle formation occurred only when FaC1 was grown with dinitrogen as the sole source of nitrogen and coincided with the appearance of acetylene reduction activity. In addition, inoculation of alder seedlings with FaC1 resulted in the formation of root nodules that were absent in uninoculated plants. Finally, FaC1 has been shown to be closely related to other *Frankia* alder root nodule isolates based on two-dimensional polyacrylamide gel electrophoresis of total cell proteins (6).

Isolation and initial characterization of *Frankia nif* clones. Approximately 5,000 *E. coli* clones, representing about 20 genome equivalents, from a gene library constructed with *Frankia* sp. strain FaC1 DNA were screened by in situ colony hybridization, using the *K. pneumoniae nifHDK* gene fragment (Table 1). Several strongly hybridizing clones were identified (data not shown). The clone containing the largest plasmid (pFA28), 55 kb in size, was chosen for further study. Hybridization of *Pst*I-digested, radiolabeled pFA28 DNA to *Pst*I-digested FaC1 total genomic DNA demonstrated that each *Pst*I fragment of pFA28 has a similarly sized counterpart within the FaC1 genome (data not shown). This result indicates that the cloned DNA in pFA28 is representative of the FaC1 genome and is not the product of a ligation of smaller genomic fragments from disparate regions of the genome.

A partial restriction map of pFA28 was determined (Fig. 1) by using known restriction sites within the vector as reference points. Ligation of pFA28 DNA restricted with these enzymes followed by transformation of *E. coli* HB101 re-

TABLE 1. Characteristics of the *nif* gene fragments used as hybridization probes[a]

Gene fragment	Characteristic(s)	Reference or source
nifHDK	6-kb *Eco*RI fragment from pSA30	14
nifH	0.8-*kb Bgl*I/*Eco*RI fragment from pSA30	14
	1.1-kb *Sma*I/*Eco*RI fragment derived from *A. vinelandii*, pMJH1	Gift of M. Jacobson, Virginia Polytechnic Institute
nifD	1.2-kb *Pvu*I/*Bam*HI fragment from pSA30	14
nifK	1.4-kb *Sal*I fragment from pSA30.1	3
nifA	0.7-kb *Pvu*I fragment from pJC072	Gift of J. Collins, University of Wisconsin
nifXN	2.1-kb *Sal*I fragment from pJC212	Gift of J. Collins, University of Wisconsin
nifLFM	2.7-kb *Sal*I fragment from pJC271	Gift of J. Collins, University of Wisconsin
nifNE	2.7-kb *Sal*I fragment from pJC277	Gift of J. Collins, University of Wisconsin
nifBAL	3.0-kb *Sal*I fragment from pJC303	Gift of J. Collins, University of Wisconsin
nifJ	3.7-kb *Sal*I fragment from pJC373	Gift of J. Collins, University of Wisconsin
nifMVSU	3.9-kb *Sal*I fragment from pJC391	Gift of J. Collins, University of Wisconsin
nifQB	3.9-kb *Pvu*I fragment from pJC399	Gift of J. Collins, University of Wisconsin

[a] Unless otherwise noted, all DNA fragments are derived from *K. pneumoniae*.

sulted in clones carrying only the border regions of pFA28 with pHC79 as the vector. Hybridization data (not shown) indicated that the *Frankia nifD* and *nifK* genes reside within a 7.2-kb *Bgl*II-*Eco*RI fragment of pFA28 (Fig. 1). This fragment was subcloned by the ligation of *Bgl*II-*Eco*RI-digested pFA28 DNA, transformation, and subsequent selection of ampicillin-resistant HB101 transformants. This was possible because pHC79 contains a 2.7-kb *Bgl*II-*Eco*RI fragment that includes the origin of replication and the ampicillin resistance gene, thus allowing this fragment to serve as a vector. A plasmid containing the 7.2-kb *Bgl*II-*Eco*RI *nif* gene fragment was subsequently identified and designated pFADK3. A detailed restriction map of the 7.2-kb insert fragment of pFADK3 is shown in Fig. 1.

Hybridizations were performed with *Pst*I-restricted pFA28 and pFADK3 DNA, using purified DNA fragments from *K. pneumoniae* representing the individual structural *nif* genes *nifH*, *nifD*, and *nifK* (Table 1) as hybridization probes. Both the *K. pneumoniae nifD* and *nifK* gene fragments demonstrated strong hybridization to similarly sized fragments from pFA28 and pFADK3 (Fig. 2). The *nifD* probe hybridized to a 2.7-kb *Pst*I fragment in pFA28 and pFADK3, while the *nifK* probe hybridized to 1.1- and 0.4-kb *Pst*I fragments from both plasmids. These data, coupled with data from similar hybridization experiments with pFA28 and pFADK3 DNA restricted with other restriction endonucleases (data not shown), and the restriction map of pFADK3 (Fig. 1) indicate that the *Frankia* sequences that show strong homology to *K. pneumoniae nifD* and *nifK* genes are contiguous.

Hybridizations with *nifH*. Attempts to demonstrate hybridization of *nifH* gene fragments derived from *K. pneumoniae* and *Azotobacter vinelandii* (Table 1) to pFA28 and other uncharacterized *nifD* and *nifK* hybridizing cosmid clones

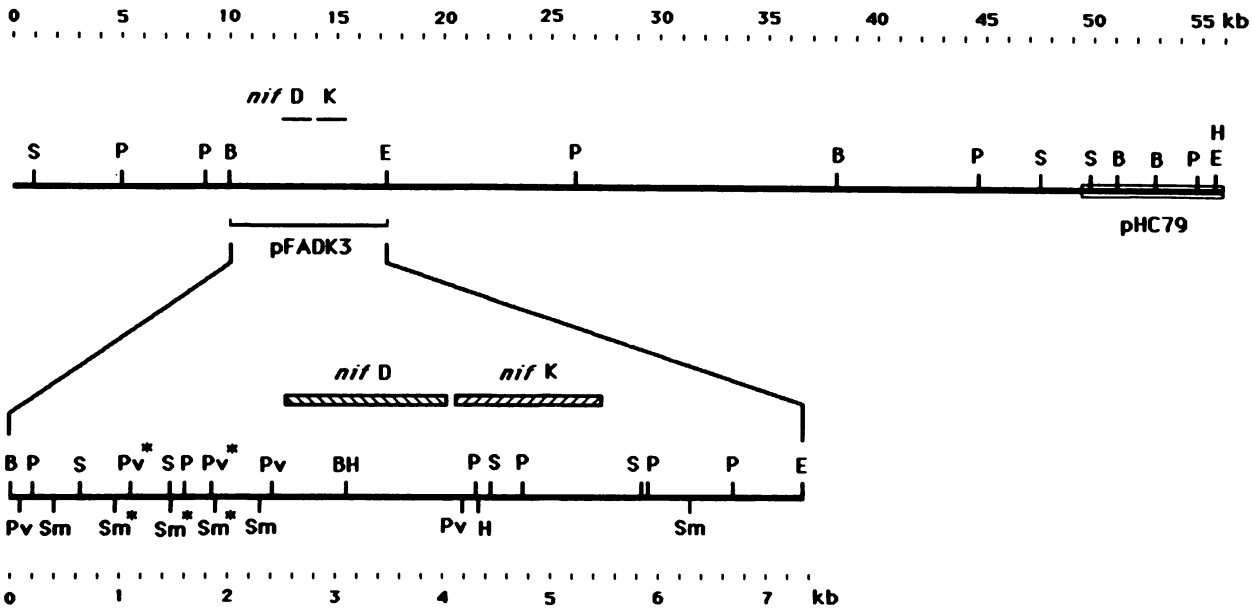

FIG. 1. Partial restriction map of pFA28 (top) and complete restriction map of pFADK3 (bottom). The pHC79 vector is shown as an enclosed box at the right edge of the pFA28 map. The scale in kilobases for pFA28 and pFADK3 is shown above and below their respective maps. The restriction sites indicated are as follows: B, *Bgl*II; BH, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*I; S, *Sal*I; Sm, *Sma*I. The precise placement of restriction sites marked with an asterisk is unknown.

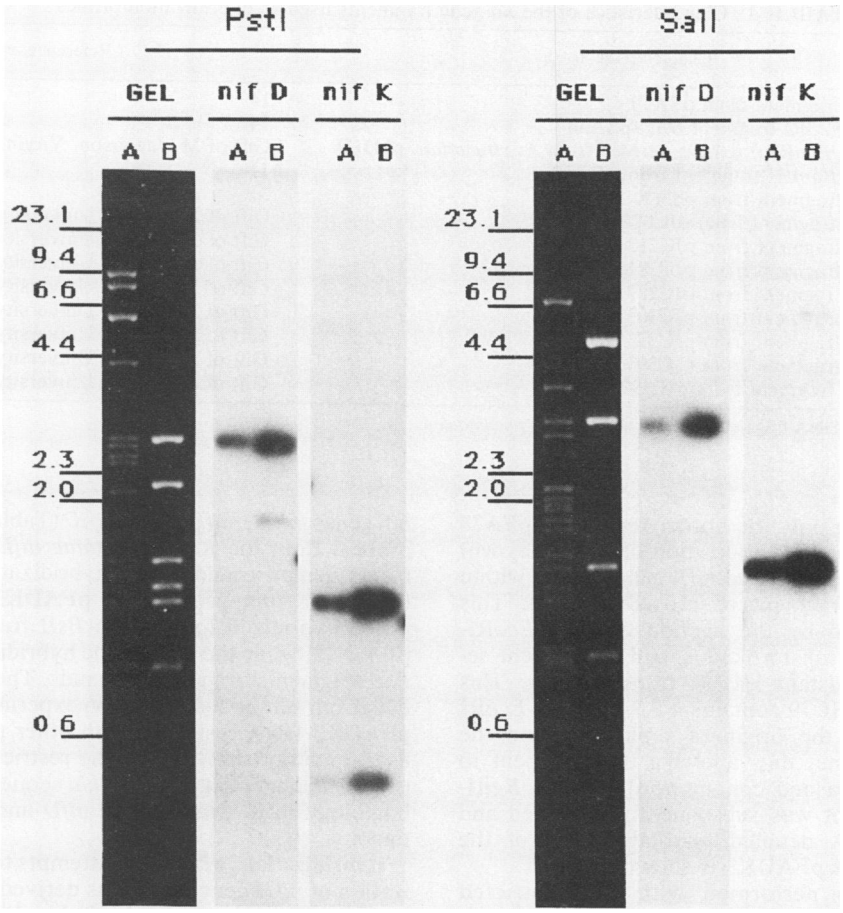

FIG. 2. Hybridizations of *K. pneumoniae nifD* and *nifK* gene fragments to pFA28 and pFADK3. Hybridizations with the *nifD* and *nifK* gene probes are shown to *Pst*I- and *Sal*I-digested pFA28 DNA (A lanes) and pFADK3 DNA (B lanes). The restricted DNA is shown on the left after electrophoresis through agarose, with the migration of DNA size standards indicated on the left (in kilobases).

were negative. However, the *nifH* gene fragments did hybridize to a 7-kb *Pst*I fragment from FaC1 genomic DNA (Fig. 3).

Hybridization with nonstructural *nif* gene fragments. Nonstructural *nif* gene fragments representing all other genes known to be involved in nitrogen fixation in *K. pneumoniae* (Table 1) were hybridized to *Pst*I-digested pFA28 and genomic FaC1 DNA. Only the *nifNE* gene fragment demonstrated significant hybridization. Examination of the hybridization results shown in Fig. 4 reveals that the *K. pneumoniae nifNE* gene fragment hybridized to *Pst*I fragments of pFA28 of approximately 6.2 and 0.8 kb whereas with pFADK3 it hybridized to 1.3-, 1.1-, and 0.8-kb *Pst*I fragments. In the latter experiment, hybridization was strongest to the 1.3- and 0.8-kb fragments. Since the *nifNE* gene fragment also contains a small portion of the C-terminal end of the *nifK* gene, hybridization was detected to the 1.1-kb *Pst*I fragment that contains similar *Frankia nifK* sequences. In the restriction map of pFADK3 (Fig. 1) the 0.8-kb fragment is adjacent to the 1.1-kb *nifK*-containing *Pst*I restriction fragment. The *nifNE*-hybridizing 1.3-kb *Pst*I fragment of pFADK3 contains the *Eco*RI site that served as one of the cloning sites used in the subcloning of the 7.2-kb *Bgl*II-*Eco*RI *nifDK* fragment. Although the restriction map of pFA28 is incomplete, the *Pst*I fragment containing this *Eco*RI site is known to be 6.2 kb in size (Fig. 1) and to be the same fragment that demonstrates homology with the *nifNE* gene probe. Thus, the 6.2- and 0.8-kb *Pst*I fragments of pFA28 represent the same *nifNE* homologous region in pFADK3 covered by the 1.3- and 0.8-kb fragments. This region is located immediately downstream from the *nifK* gene of *Frankia* spp. relative to *nifD*.

DISCUSSION

We have constructed a gene library of DNA from *Frankia* sp. strain FaC1, an alder root nodule isolate. Clones containing the genes that appear to encode the α and β subunits of the MoFe protein (*nifD* and *nifK*) of nitrogenase were identified by heterologous hybridization with *K. pneumoniae*-derived *nif* gene fragments. Restriction endonuclease mapping (Fig. 1) and hybridization studies (Fig. 2) indicate that the *nifD* and *nifK* genes are located next to each other. Although hybridization could not be detected with *K. pneumoniae* or *A. vinelandii nifH* gene fragments to pFA28 (the *nifDK* cosmid clone) or to other uncharacterized *nifDK* hybridizing clones, hybridization to a 7-kb *Pst*I fragment from FaC1 genomic DNA was detected (Fig. 3). This result suggests that the lack of hybridization of the *nifH* probes to pFA28 DNA is not due to low DNA sequence homology between the *Frankia nifH* gene and the *nifH* gene fragments used as hybridization probes. Therefore, it can be concluded

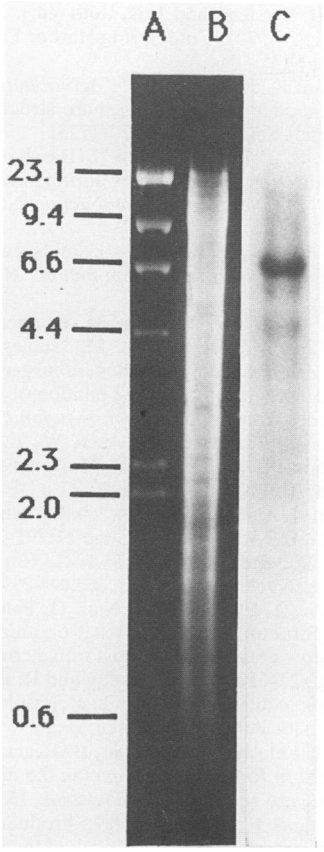

FIG. 3. Hybridization of a *nifH* gene fragment to *Frankia* sp. strain FaC1 genomic DNA. Lane B depicts *Pst*I-digested genomic DNA from *Frankia* sp. strain FaC1 after electrophoresis through agarose. *Hin*dIII-digested lambda DNA is shown in lane A, with the size of each fragment indicated on the left (in kilobases). Hybridization of the *nifH* gene fragment derived from *A. vinelandii* to the *Frankia* DNA in lane B is shown in lane C.

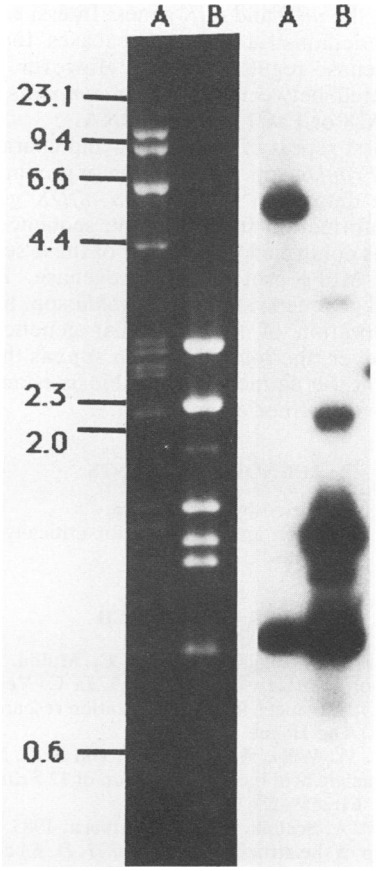

FIG. 4. Hybridization of a *nifNE* gene fragment to pFA28 and pFADK3 DNA. *Pst*I-digested pFA28 (A lanes) and pFADK3 (B lanes) DNA is shown in the left panel after electrophoresis through agarose. The panel on the right depicts hybridization of the *K. pneumoniae nifNE* gene fragment to the DNA in the left panel. The migration of DNA molecular mass size standards is shown on the left (in kilobases).

that the *nifH* gene of *Frankia* sp. strain FaC1 is not located on the pFA28 clone. All clones identified by in situ screening of the *Frankia* gene library with the *K. pneumoniae nifHDK* gene fragment contained the *nifDK* gene cluster, while none contained the *nifH* gene. Given the size of the cloned FaC1 DNA in pFA28 (49 kb) and the location of the *nifDK* gene cluster within it, we conclude that the *Frankia nifH* gene must be located more than 12 kb upstream or 33 kb downstream from the *nifDK* gene cluster.

A similar split organization of the structural nitrogenase genes has been described in other symbiotic nitrogen-fixing bacteria. In *Bradyrhizobium* species, there is a distance of 17 kb separating the *nifH* gene and the *nifDK* gene cluster in *Bradyrhizobium japonicum* strain 110 (23, 31) and a distance of 11 kb in the cowpea *Rhizobium* sp. strain IRc78 (22). The nitrogenase structural genes have been shown to be unlinked in *Parasponia Rhizobium* (38). It has also been reported that there is a separation of 11 kb between the *nifK* gene and the *nifHD* gene cluster in the cyanobacterium *Anabaena* sp. strain 7120 (33) that is deleted during the differentiation of vegetative cells to heterocysts, the nitrogen-fixing cells (17), to create a unified *nifHDK* structural gene cluster. Many analogies, both structural and functional, can be made between *Frankia* and *Anabaena* spp. concerning the differentiation of specialized cells that are the location of nitrogen fixation activity. Whether there exists a similar genetic rearrangement of the *nif* structural genes in *Frankia* spp. during the differentiation of nitrogen-fixing vesicles is unknown. Since the gene library used in this study was made from a heterotrophically grown *Frankia* culture that lacked vesicles, the detection of a *nif* gene rearrangement similar to the one found in *Anabaena* spp. would require comparison with the *nif* gene organization of DNA isolated from vesicles.

Of the nonstructural *K. pneumoniae nif* gene fragments used to probe the pFA28 clone, only the *nifNE* fragment showed strong homology (Fig. 4). Hybridization and restriction endonuclease mapping data indicate that the *nifNE* hybridizing fragments are contiguous with one another and are located immediately downstream of the *nifDK* gene cluster. A similar location of the *nifE* and *nifN* genes has been shown in *B. japonicum* (19), *A. vinelandii* (16, 25), and *K. pneumoniae* (27). Based upon the work of Dean and Brigle (16), the *nifE* and *nifN* genes might be expected to have a high degree of interspecies homology. They demonstrated that the *nifE* and *nifN* genes of *A. vinelandii*, which code for proteins involved in the biosynthesis of the FeMo cofactor of nitrogenase, share regions of DNA homology with the *nifD* and *nifK* genes, respectively. The FeMo cofactor is closely associated with the MoFe proteins that

are encoded by the *nifD* and *nifK* genes. Interspecies homology has been demonstrated in some cases for *nifA*, the positive nitrogenase regulatory gene. However, no homology was detected between the *K. pneumoniae nifA* gene probe and pFA28 or FaC1 genomic DNA.

This is the first report of the cloning and characterization of *nif* genes in *Frankia* spp. Comparison of preliminary DNA base sequence data from the *Frankia nifDK* gene cluster with similar information from *nif* gene sequences of other diazotrophs has confirmed the identity of these sequences as coding for the MoFe proteins of nitrogenase. The lack of well-developed genetic systems in *Frankia* spp. has discouraged the exploration of the molecular genetics of these bacteria. However, the role of *Frankia* spp. as the nitrogen-fixing partner in the actinorhizal symbiosis is an important one that deserves further examination.

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