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I am submitting herewith a thesis written by Kevin F. Duvel entitled "CONSTRUCTION OF A WORKING RESTRICTION MAP OF FRANKIA COSMID CLONE pFAH1 AND MAPPING OF PUTATIVE *nif* GENES." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science, with a major in Botany.

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CONSTRUCTION OF A WORKING RESTRICTION MAP OF FRANKIA COSMID
CLONE pFAH1 AND MAPPING OF PUTATIVE *nif* GENES

A Thesis

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Master of Science

Degree

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ABSTRACT

Cosmid clone pFAH1 is believed to contain Frankia *nif* genes and open reading frames. It was subjected to a variety of single and double restriction enzyme digestions, blotted, and hybridized to various Azotobacter *nif* genes and an unidentified open reading frame orf10. Results from these experiments were used to construct a working restriction map of cosmid clone pFAH1.

The data obtained indicate that orf10, *nifM* and X are adjacent to each other and *nifB* is less than 1kb from *nifX* on an 18kb EcoRI/HindIII fragment. *NifE* is located on an 8.6kb EcoRI/HindIII fragment and is separated from *nifB* by a distance of approximately 10kb.

These data indicate that Frankia *nif* gene arrangement in cosmid clone pFAH1 is unique when compared with other nitrogen-fixing organisms. Further mapping of Frankia *nif* genes will enable researchers to make phylogenetic comparisons between Frankia and other nitrogen-fixing bacteria.

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I. INTRODUCTION

A symbiosis is defined as two or more organisms living together in a way in which all individuals benefit from the relationship. Frankia is a high-mol% G+C gram positive, nitrogen-fixing filamentous bacterium that associates with a variety of perennial dicots (Table 1.1) to create actinorhizal symbioses. These interactions are non-leguminous nitrogen-fixing relationships. In these relationships, Frankia is provided with nutrients and protection, and the host plant receives the excess nitrogen that Frankia does not utilize. To initiate the symbiotic relationship, Frankia must first infect the host plant. Some of the root hairs of the host plant become distorted in a "curling manner" and others form distorted bulging heads with branches in the presence of the bacterium (Berry et al., 1985). This root hair deformation is thought to be a response to some chemical signal released by Frankia to increase infection efficiency. A sticky polysaccharide layer accumulates on the outer surface of the root hair as it deforms (Torrey, 1978). This mucelagenous layer is the site of attachment for the bacterium to the actinorhizal host, and the crevices which form as a result of deformation are believed to be the point of infection. Frankia grows within the root hair toward the hypodermal and cortical cells, which themselves are stimulated to divide.

Table 1.1 - Plants which form root nodules in symbiosis with Frankia.

=====		
Genus	Number of nodulated species in genus	Family

<u>Allocasuarina</u>	58	Casuarinaceae
<u>Alnus</u>	42	Betulaceae
<u>Casuarina</u>	18	Casuarinaceae
<u>Ceanothus</u>	31	Rhamnaceae
<u>Cercocarpus</u>	4	Rosaceae
<u>Ceuthostoma</u>	2*	Casuarinaceae
<u>Chamaebatia</u>	1	Rosaceae
<u>Colletia</u>	3	Rhamnaceae
<u>Comptonia</u>	1	Myricaceae
<u>Coriaria</u>	16	Coriariaceae
<u>Cowania</u>	1	Rosaceae
<u>Datisca</u>	2	Datiscaceae
<u>Discaria</u>	5	Rhamnaceae
<u>Dryas</u>	3	Rosaceae
<u>Elaeagnus</u>	35	Elaeagnaceae
<u>Gymnostoma</u>	18	Casuarinaceae
<u>Hippophae</u>	2	Elaeagnaceae
<u>Kentrothamnus</u>	1	Rhamnaceae
<u>Myrica</u>	28	Myricaceae
<u>Purshia</u>	2	Rosaceae
<u>Retanilla</u>	1	Rhamnaceae
<u>Shepherdia</u>	2	Elaeagnaceae
<u>Talguenea</u>	1	Rhamnaceae
<u>Trevoa</u>	2	Rhamnaceae

=====

(Baker and Mullin, 1992) ref.

* = Lack of adequate collections.

The bacterium proceeds to infect the host by growing through the cortical cell walls of the root. As Frankia penetrates the host cell wall it becomes encapsulated by host derived cell wall-like material and host plasmalemma. The bacterium never comes in direct contact with the host cytoplasm. The bacterium will proliferate in the cortical cells and the resulting infected tissue is called a prenodule. The nodule is initiated below the the prenodule from the pericycle much like a lateral root. As the new lateral root primordium passes through the prenodule cells, its cortical cells become infected by Frankia filaments as it passes through the prenodule. Infected nodule cells are much larger than common cortical cells, stay living and are metabolically active (Berry et al., 1990).

Vesicles are formed by the bacterium inside the nodule cells and are the site for dinitrogen fixation. The vesicles are believed to act as barriers to oxygen diffusion. Parsons et al. (1987) has shown that the laminated lipid monolayers of the vesicle actually increase in number with increasing oxygen concentrations and decrease with decreasing concentrations of oxygen. This feature helps to protect the nitrogenase enzyme which is inhibited by high oxygen concentrations.

The genes which code for the synthesis and functioning of the nitrogenase enzyme in diazotrophic bacteria are termed *nif* genes. *NifH*, *D*, and *K* code for the nitrogenase

enzyme. Nitrogenase is responsible for fixing dinitrogen. The other *nif* genes (Table 1.2) code for the functioning and maintenance of the enzyme. *Nod* genes are required for the nodulation process, and *fix* genes code for products that are necessary for nitrogen fixation in the nodule. Once these genes are correctly mapped in Frankia they can be used for further studying the actinorhizal symbiosis.

Most organisms are unable to reduce dinitrogen into a usable form because they do not possess the ability to produce nitrogenase. As a result, the macroelement nitrogen availability often limits the growth process of many organisms. The enzyme nitrogenase enables Frankia to convert atmospheric dinitrogen into ammonia making Frankia a valuable addition to an ecosystem (Benoit et al., 1990).

Frankia strains have considerable genetic and phenotypic diversity making them intriguing subjects, but their slow growth rate makes them difficult to study. Frankia strains are slow growing in liquid media and even slower in agar based media. The doubling time for Frankia strains is 15 hours or more depending on media, aeration and the strain used. It may take up to a year to obtain a pure culture from a nodule. This is due to the slow growing nature of Frankia in culture and the large number of soil contaminants on the surface of nodules. Problems with surface contamination can be partially overcome by use of proper surface disinfectants to remove fungi and other

Table 1.2 - Genes involved in construction and function of nitrogenase.

<i>Nif</i> gene	Product and Known or Proposed Function
D	MoFe protein α -subunit, forms $\alpha^2\beta^2$ tetramer with β -subunit. FeMo-cofactor is the site of substrate reduction.
K	β -subunit of nitrogenase MoFe protein.
H	Fe protein subunit of nitrogenase. Fe protein is required for FeMo-cofactor biosynthesis.
A	Transcriptional activator of all other <i>nif</i> genes. Positive regulatory element.
L	Required for synthesis of FeMo cofactor. Negative regulatory element.
B	Required for FeMo-cofactor biosynthesis.
M	Required for activation of the Fe protein.
U	Function not known. Appears to be involved primarily in stabilization of the Fe protein.
V	Probably encodes a homocitrate synthase. Homocitrate is an organic component of FeMo-cofactor.
E	Required for FeMo-cofactor biosynthesis. Exhibits a fair level of sequence identity when compared to the MoFe protein α -subunit
N	Required for FeMo-cofactor biosynthesis. Exhibits a fair level of sequence identity when compared to the MoFe protein β -subunit.
Q	Involved in FeMo-cofactor biosynthesis, probably at an early step. Phenotype can be suppressed with elevated levels of Mo, cystine or cysteine.

Table 1.2 (continued):

=====	
<i>Nif</i> gene	Product and Known or Proposed Function

W	Function not known. Required for full activity of the MoFe protein.
Z	Function not known. Required for full activity of the MoFe protein. Might have a function related to FeMo-cofactor formation or insertion.
T	Function not known, not required for diazotrophic growth.
Y	Function not known, not required for diazotrophic growth.
S	Function not known. Appears to be involved in stabilization of Fe protein.
F	Flavodoxin, physiologic reductant of the Fe protein.
J	Couples the oxidation of pyruvate to the reduction of flavodoxin.
X	Specific function not known. Probably a negative regulator of <i>nif</i> gene expression.
=====	
(Dean and Jacobson, 1992)	

bacteria. Obtaining clonal cultures of Frankia is difficult because more than one strain may be present in a nodule. Dilution plating techniques are commonly used to ensure clonal colony isolation, but this process may take weeks or months before results are seen.

Despite the disadvantages, Frankia is still worth studying. Actinorhizal plants are distributed among eight families and seven orders of angiosperms. Most of the host plants are colonizers which add a large amount of nitrogen to deficient soils. The infection and nodulation processes are unique to nitrogen fixing symbioses. This brings up intriguing questions about evolution of the symbiosis which further studies may answer.

Results from studying this system have both environmental and economic benefits. Addition of nitrates to the soil for agricultural purposes is polluting water sources. Nitrates leach from the soil and accumulate in ponds, lakes, and rivers contaminating drinking water. Adding nitrogen to the soil naturally (ie, via actinorhizal symbioses) is a way around this problem. Actinorhizal Alnus rubra trees; for example, have been shown to fix nitrogen up to 300 kg/ha annually (Torrey, 1978). Since alders are a hardy species and add nitrogen to the soil, they are of great value for revegetation projects. Actinorhizal plants are also commonly used for biomass production, fuelwood, timber, browse, pulpwood and fruit (Benoit et al., 1990).

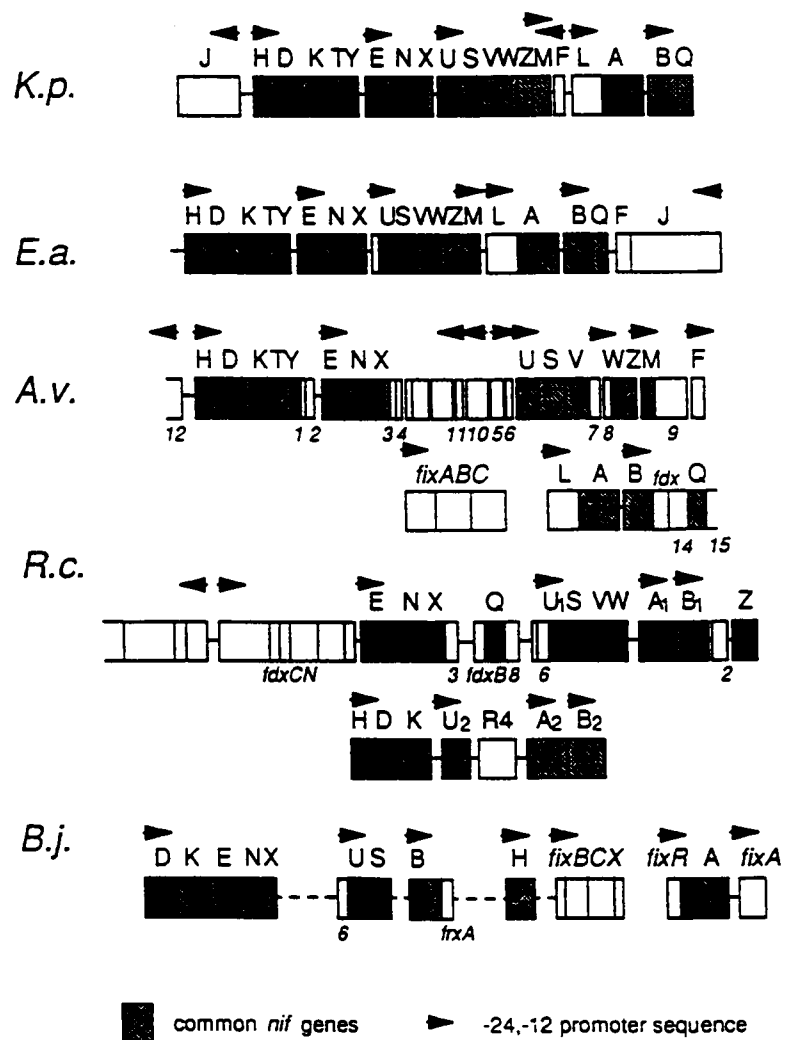
II. LITERATURE REVIEW

Phylogenetic considerations

Azotobacter vinelandii, Enterobacter agglomerans, Rhodobacter capsulatus, Bradyrhizobium japonicum, and Klebsiella pneumoniae *nif* genes have been previously mapped (Figure 2.1) (Merrick et al., 1993). The information obtained from maps such as these including gene order and subsequent sequence analysis has been used to determine the divergence within a genus or between species. This is one way phylogenetic relationships can be determined.

Sequence comparison is considered by some to be the best method for showing similarities or differences between species because the genetic code is the basis for phenotype and remains well conserved throughout time (Benson et al., 1979). Most *nif* genes are especially well conserved in order to retain function. *NifH* has been sequenced and restriction maps have been made for a large number of nitrogen fixing bacteria including Frankia strains HRN18a (Normand et al., 1989), ArI3 (Normand et al., 1988) and FaC1 (Oh et al., 1993). Information from derived amino acid sequence data from strains HRN18a and ArI3 has been used by Normand et al. to predict a divergence time of 100 Myr for these two Frankia strains which have a derived amino acid sequence similarity of 96%. The amino acid sequence was also

Figure 2.1 - Organization of *nif* and related genes in K. pneumoniae, E. agglomerans, Azotobacter, R. capsulatus and B. japonicum. (Taken directly from Merrick et al., 1993).



compared with other nitrogen-fixing prokaryotes to determine which are most closely related in *nifH* sequence. Distance and parsimony analyses grouped Frankia with Anabaena (a filamentous heterocystous bacterium). Phylogenetic trees constructed from rRNA gene sequences place Frankia and Anabaena in entirely different subgroups of eubacteria. Normand et al. (1989) concluded that Frankia received its' *nifH* genes by lateral transfer only 100 Myr ago from a gram-negative soil bacterium. Both Frankia and Anabaena are aerobic soil bacteria, and at least one Frankia strain has been shown to contain *nif* genes on a plasmid (Simonet et al., 1986) suggesting that it might be capable of participating in lateral gene transfer. In a chapter by J. P. Young (1992), this interpretation was challenged. Young stated that their "purely phylogenetic hypothesis" did not have clear support due to standard error of the trees generated, meaning that a different tree could be constructed from the same data set which would not group Frankia and Anabaena together. Young discusses gene duplication and subsequent sequence divergence as a problem with using *nifH* as a phylogenetic tool. Since it is known that more than one copy of nitrogenase genes can be found some a bacteria, it is important to only consider gene homologues when constructing phylogenies. This makes it difficult to construct phylogenetic trees using *nifH* sequence data. The extremely variable G+C content of

bacterial genomes is another reason the conclusions of Normand et al. are not acceptable according to Young. He gives an equally plausible explanation of the data stating that one or more duplications had occurred in a common ancestor of eubacteria and archaeobacteria giving rise to two types of *nifH*. Early in the eubacterial branch, another duplication occurred giving rise to the Clostridium pasteurianum branch, where several more duplications resulted in the family of closely related *nif* genes which include Frankia *nifH*. This explanation of the data does not include lateral gene transfer and shows evidence for Frankia *nifH* integration into the genome at more than 100 Myr ago.

Hybridization and sequencing studies

Genes of a free-living nitrogen-fixing bacterium, Klebsiella, have been studied extensively. Hybridization of bacterial DNA to Klebsiella *nif* genes is frequently used to detect the presence of nitrogen fixing genes in other bacterial species. *NifH*, D and K have been studied more thoroughly than other *nif* genes because they code for nitrogenase. In 1980 Ruvkun and Ausubel reported that Klebsiella *nif* genes H, D and K hybridize with Frankia strain Cp11. More recently, conservation of nitrogenase sequences has been shown by hybridizing Rhizobium meliloti and Klebsiella pneumoniae *nifH*, D, K, A and B to total Frankia (strain Ar13) DNA (Simonet et al., 1988). This does

not definitively prove that these sequences code for nitrogenase in Frankia. All it absolutely says is that some genes in Frankia are similar enough to hybridize to proven *nif* genes in two other nitrogen-fixing bacteria. In all probability, these do represent *nif* genes because of the numerous studies which have compared *nif* genes of various species and found significant compatibility.

J. M. Ligon and J. P. Nakas reported in 1987 the characterization of Frankia species strain FaCl *nifH*, D and K. A genomic library was constructed in the cosmid vector pHc79 and was screened by in situ colony hybridization with structural *nif* genes (*nifH*, D and K) from Klebsiella pneumoniae to identify clones which contained Frankia *nif* genes. Clone pFA28 contained the largest fragment and was used for restriction mapping and hybridization studies. The studies indicated that *nifD* and K were adjacent to each other and the *nifH* gene was located either 33 kb downstream or more than 12kb upstream from the *nifDK* gene cluster. This was unusual because in most diazotrophic bacteria, *nifH*, D and K are contiguous. After the *nifD* and K sequences from this clone were published (Ligon and Nakas, 1988), it became apparent that they did not represent a legitimate Frankia *nifD* and K sequence (Mullin, personal communication) and the results were retracted (Ligon and Nakas, 1990). Clone pFA28 was found to contain a Klebsiella pneumoniae *nif*-containing fragment which was flanked by

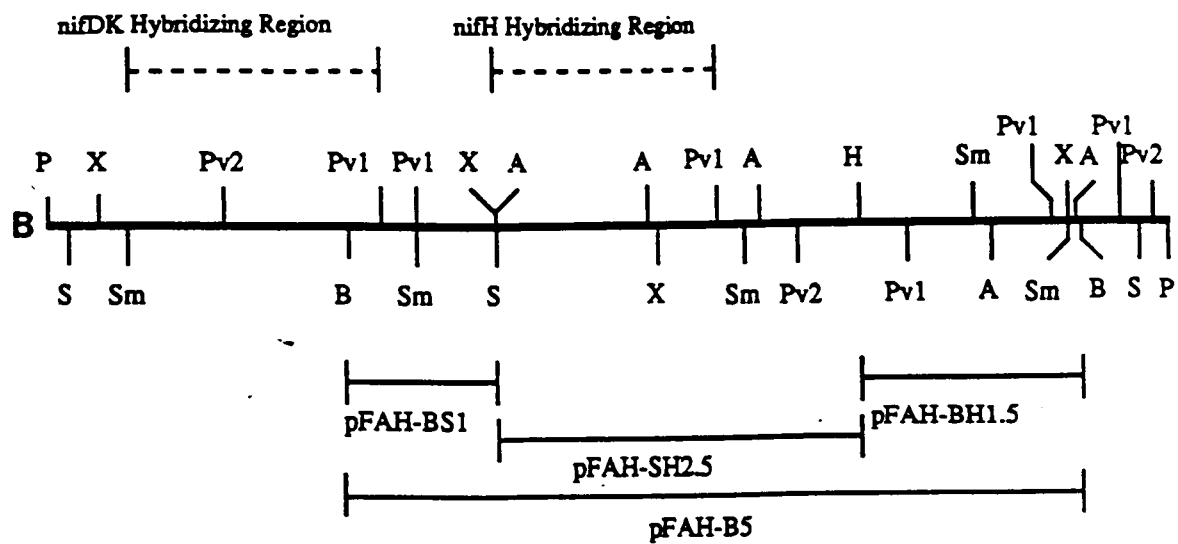
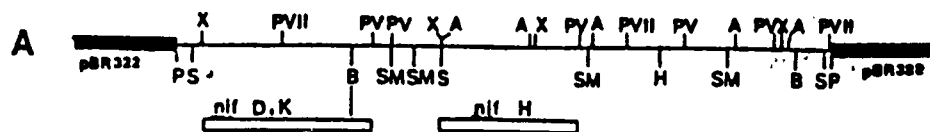
Frankia DNA. In 1990, An et al. and Ligon (personal communication) demonstrated via restriction enzyme digestions and later sequencing (Twigg et al., 1990; Oh et al., 1993) that a 7.2 kb PstI fragment from FaC1 (cosmid clone pFAH1) which was subcloned into pBR322 (pFAH-P7.2) did contain legitimate Frankia *nif* genes (Figure 2.2). This clone has revealed, as in other nitrogen-fixing bacteria, that *nifH*, D and K are adjacent.

Frankia mutants

Accurately mapped *nif* genes can be altered and used to study gene products and functions. *Nif* gene mutations are rarely encountered naturally, so researchers use gene insertions, site directed mutagenesis or a variety of other mutation techniques to produce non-functioning *nif* genes. No *nif* gene mutants have been found to date in Frankia, but an attempt has been made to identify the *nodA* gene in Frankia strain HFPCc13 by cross-species complementation of Rhizobium meliloti (Reddy et al., 1988). A genomic library of Frankia strain HFPCc13 was constructed in the cosmid vector pLAFR3 and was then mated into R. meliloti 5610 *nodA::Tn5 Nod⁻*. The transconjugants were used to inoculate alfalfa plants and a few plants were able to produce nodules, but were unable to fix nitrogen. A plasmid which was capable of complementing R. meliloti 5610 was isolated and named clone pAR1 (24 kb). This clone was probed with a

Figure 2.2 - Restriction maps of pFAH-P7.2. (A) (Oh et al.). (B) Restriction map of *nifH*, D and K from pFAH-P7.2 (Ligon, personal communication).

Restriction sites - A; *Ava*I, B; *Bam*HI, H; *Hind*III, P; *Pst*I, Pv; *Pvu*I, *Pv*II; *Pvu*II; S, *Sal*I, SM; *Sma*I, X; *Xho*I.



3.5 kb BamH1/EcoR1 fragment that contains *nodABC* and part of *nodD* from R. meliloti (pEK12) and showed weak sequence similarity. Clone pAR1 was again introduced into R. meliloti producing nodules, and appeared capable of substituting for the common *nod* genes, but unable to produce a fully functional nodule. It was later discovered that the transposable element fragment (Tn5) which was used to interrupt the R. meliloti *nodA* gene moved out of position allowing the gene to regain activity, not because the Frankia *nodA* was complementing the R. meliloti *nodA* gene.

Plasmid nif genes

Frankia *nif* genes have been shown to be present on a plasmid of 190 kb in at least one Frankia strain. Klebsiella pneumoniae *nifH*, D and K were used to probe plasmid DNA from Frankia strain ULQ0132105009 (Simonet et al. 1986). It was found that a 5.6 kb EcoRI fragment from the plasmid hybridized with the K. pneumoniae probes. The *nif* probes also hybridized with a 10 kb EcoRI fragment from non-plasmid Frankia DNA. This implies that the *nif* genes could be present in more than one copy in the genome at any given time.

III. MATERIALS AND METHODS

pFAH1 clone

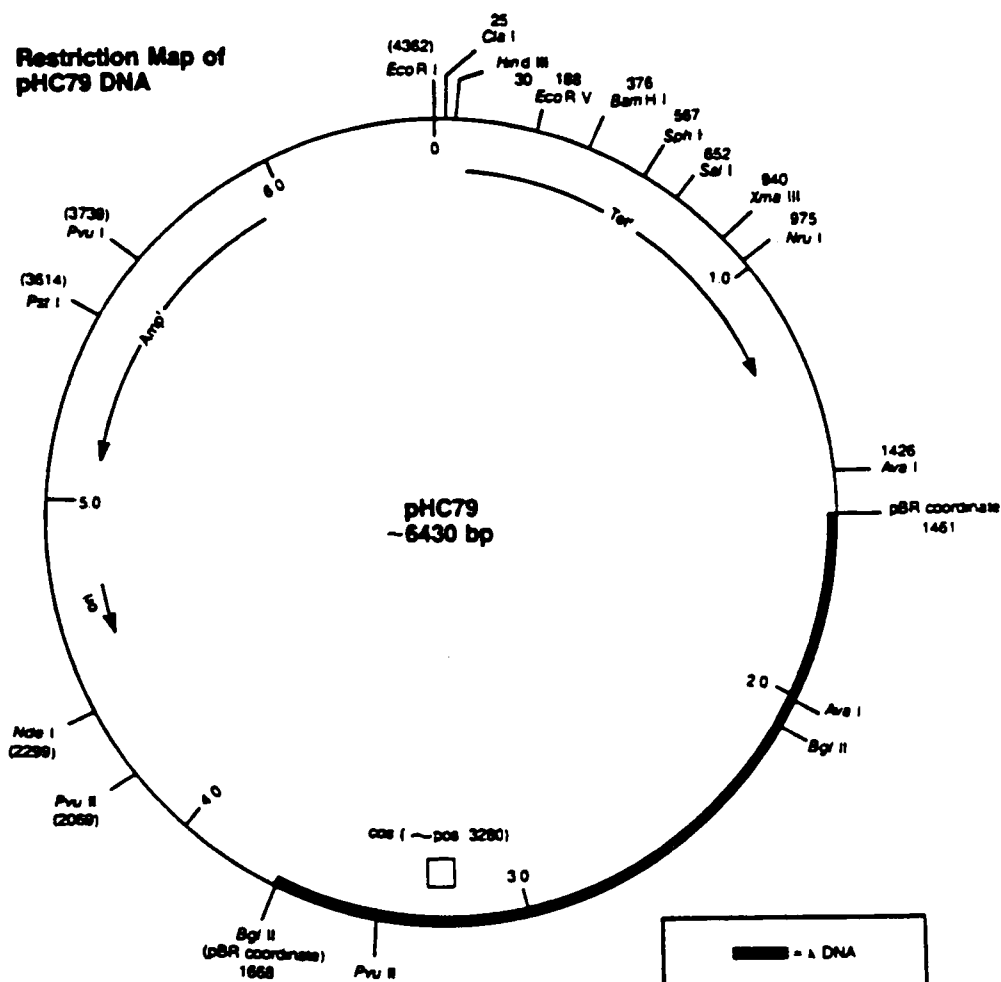
The pFAH1 clone was received from Jim Ligon at Ciba Geigy, Research Triangle, North Carolina. It was isolated from a cosmid library made from Frankia strain FaC1, an Alnus isolate, using Klebsiella nifHDK as a probe (Ligon et al., 1987). The DNA from FaC1 was partially digested with restriction endonuclease Sau3A. 40-50 kb fragments were isolated from this digestion by centrifugation and fractionation of a sucrose density gradient. The pHC79 cosmid vector (Figure 3.1) was digested with BamHI and treated with calf intestinal alkaline phosphatase. Partially digested, size fractionated Frankia DNA and BamHI digested pHC79, treated with calf intestinal alkaline phosphatase, were ligated with T4 DNA ligase. The pFAH1 clone consists of pHC79 cosmid DNA and Frankia nif genes. The pHC79 cosmid clone is approximately 6430 base pairs. Including the Frankia nif genes, it is estimated to be 43-47 kb in length as indicated by restriction digests to date.

Southern blot analysis

Two Southern blots of pFAH1 were prepared by B. C. Mullin (University of Tennessee, Knoxville) and C. S. An (Seoul National University, Seoul, Korea) following

Figure 3.1 - pHC79 cosmid vector. Frankia DNA was inserted at the BamHI restriction site. (Taken directly from Hohn et al., 1980).

Restriction Map of pHC79 DNA



electrophoresis of single and double restriction enzyme digests (Tables 3.1 and 3.2, respectively). Information from ethidium bromide stained gels and from hybridization to the blots was used to construct a working map of the pFAH1 clone. DNA restriction fragment sizes were estimated by measuring the distance in millimeters from the bottom of each well to the middle of each marker band (Φ X-174 DNA digested by HaeIII and lambda DNA digested by HindIII). Since these are markers, the molecular weight of each band is already known. Measuring the distance each marker band has traveled shows how far a fragment of that size will travel on that specific gel. A standard curve was constructed on semi-log paper with distance in millimeters that the band had traveled on the x-axis and molecular weight of the band on the y-axis. Molecular weights of all the bands on that gel were estimated by measuring distances that each of the unknown bands had traveled and interpolation from the standard curve. A simple computer program (DNAsize) was later used to estimate molecular weights from measured band sizes. The molecular weights from both methods were very similar.

Azotobacter probe preparation

Azotobacter nif genes which had been amplified using gene specific PCR primers (Table 3.3) were inserted in a pT7-7 plasmid (Stan Tabor, Harvard Medical School) (Figure

Table 3.1 - Enzymes used for digestion of the pFAH1 clone (blot-1).

Lane	ENZYMES
1	Lambda/HindIII:ΦX-174/HaeIII
2	HindIII
3	EcoRI
4	HindIII/EcoRI
5	BglII
6	BglII/EcoRI
7	BglII/HindIII
8	PstI
9	Lambda/HindIII:ΦX-174/HaeIII
10	PstI/BglII
11	PstI/Xho
12	PstI/HindIII
13	BamHI
14	BamHI/PstI
15	BamHI/BglII
16	BamHI/Xho
17	BamHI/HindIII
18	Lambda/HindIII:ΦX-174/HaeIII

Table 3.2 - Enzymes used for digestion of the pFAH1 clone (blot-2).

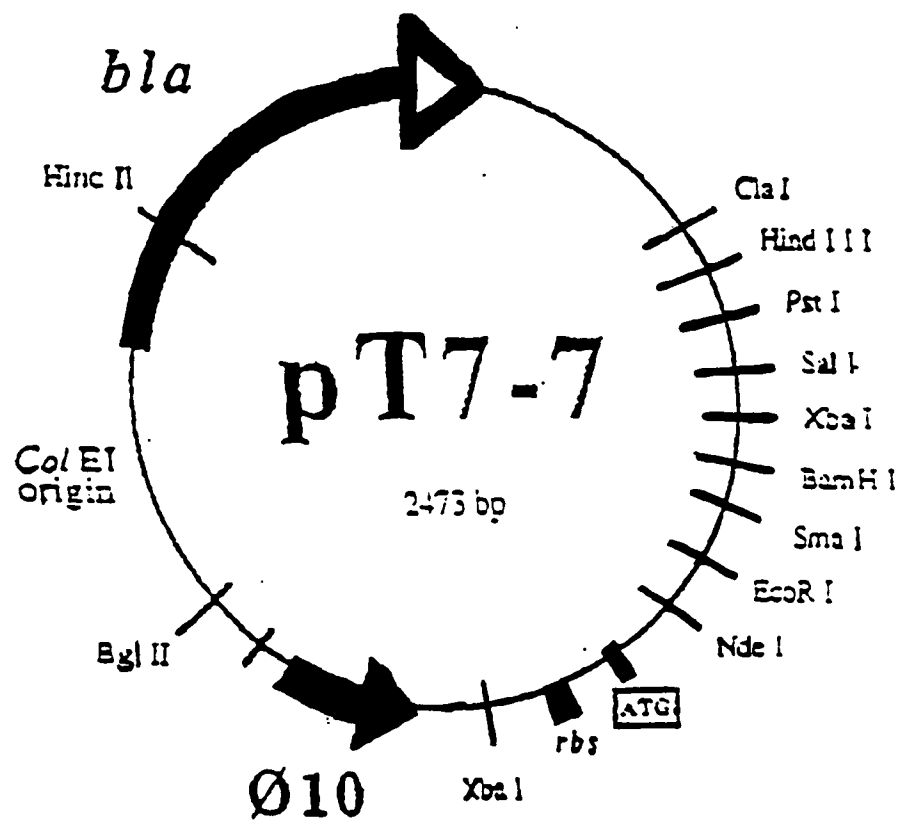
Lane	ENZYMES
1	Lambda/HindIII:ΦX-174/HaeIII
2	HindIII
3	EcoRI
4	HindIII/EcoRI
5	BglII
6	BglII/EcoRI
7	BglII/HindIII
8	PstI
9	PstI/BglII
10	PstI/Xho
11	PstI/HindIII
12	BamHI
13	BamHI/PstI
14	BamHI/BglII
15	BamHI/Xho
16	BamHI/HindIII
17	Lambda/HindIII:ΦX-174/HaeIII

Table 3.3 - Azotobacter vinelandii *nif* genes and open reading frames (orf's). Received from Dennis Dean, Virginia Polytechnic Institute and State University.

Gene		orf	
H	pDB567	1	pDB575
D	pDB336	2	pDB569
K	pDB537	3	pDB574
E*	pDB558	6	pDB570
N	pDB560	7	pDB571
X*	pDB553	8	pDB572
U	pDB525	10*	pDB556
S*	pDB551	11	pDB582
V	pDB555	12	pDB573
W	pDB550	13	pDB576
Z	pDB547		
M*	pDB559		
B*	pDB548		
Q	pDB554		

* - Indicates *nif* genes and orf used for hybridizations.

Figure 3.2 - pT7-7 plasmid. Harbors Azotobacter *nif*
genes between restriction sites BamHI and NdeI
(Stan Tabor, Harvard Medical School).



3.2) between BamHI and NdeI sites in the laboratory of Dennis Dean. DNA samples were stored in TE buffer at -20°C at the University of Tennessee until use. Competent cells were made and transformations were performed with *nif* clones E, N, X, U, S, V, W, N, M, Q and B; and orf clones 1, 3, 8, 10, 11 and 12 (see Appendix for detailed procedures). The transformants were plated on LB-AMP media and put in an incubator overnight at 37°C. The principle behind this selection method is that only the cells which contain the pT7-7 plasmid will grow because they carry the ampicillin resistance gene. Six of the largest colonies were chosen from each transformation and plated separately on LB-AMP and incubated as before. A loopfull of cells was used to inoculate LB-AMP liquid media (2 ml LB and 5 µl ampicillin from 20 mg/ml stock) which was incubated overnight at 37°C in a shaker. The plasmids were extracted from the cells via an alkaline lysis miniprep protocol (see Appendix), and run on a minigel (see Appendix) to confirm the approximate plasmid size and to check for continuity between actual and expected band insert sizes. Lanes one and eight commonly contained the marker lambda/HindIII digest, and lanes two-seven were loaded with a small portion of the DNA from the plasmid extraction. When the fragments were not the predicted size (vector DNA: 2473 bp and *nif* gene), the plasmid prep was discarded. Azotobacter *nif* clones of correct size were preserved in glycerol culture (see

Appendix) for long term storage.

To remove the Azotobacter *nif* probes from the PT7-7 plasmid, a double digestion with BamHI/NdeI was performed (see Appendix). The digested DNA was analysed on a minigel to see if the *nif* gene was completely separated from the vector. Two bands were apparent when digestions were complete (vector and *nif* gene) and three bands were observed when the digestions were not complete (vector, *nif* gene, and vector + *nif* gene). Usually the later was observed, but experiments did not need to be repeated because in each case an adequate amount of *nif* gene DNA was able to be recovered.

Isolated *nif* inserts were removed from the minigel by either agarose digestion or electroelution (see Appendix). A small portion (1 μ l insert DNA in 8 μ l TE and 1 μ l 10x sample loading buffer, BIOS, New Haven, Connecticut) of the insert was run on a minigel with lambda/HindIII digested marker DNA (8 ng/ μ l, 10 μ l loaded), stained with ethidium bromide and observed under ultraviolet light for quantitative analysis. The *nif* gene band was compared to the lambda/HindIII band which it most closely resembled in intensity. Calculations were performed to determine the amount of DNA in ng/ μ l for the remainder of the insert DNA. 15ng of insert DNA was radiolabelled (see Appendix) and used to probe blot-1.

Azotobacter nif gene hybridization

Azotobacter nif genes (E_s , E_L , B, S, X and M) and orf10 were used to probe blot-1 (Table 3.3). This was done to aid in the mapping process. Following *nif* probe preparation, as described before, the probes were radiolabelled with ^{32}P dCTP (see Appendix). Each probe was then added (on separate occasions) to a heat sealable plastic bag containing the blot which had been prehybridized for at least 24 hours (see Appendix for protocol details). Blots were hybridized in a shaking water bath at 65°C for 24 hours. Following hybridization the radioactive solution was removed from the bag and the blot was washed (see Appendix). The blot was resealed in a Real Value Snap'n Seal storage bag (one gallon size) with a small amount of 1xSSC, 0.1% SDS to keep the blot from desiccating and was exposed to Kodak X-Omat RP diagnostic film with two Cronex Xtra life intensifying screens (DuPont) and placed in the -80°C freezer. The exposure time varied depending on the age of the ^{32}P and possibly the extent to which the Azotobacter probes bound to the Frankia DNA. The autoradiographs were developed and used as additional information for mapping.

Mapping

Once the fragment lengths were estimated from the bands on the gel, the information was used to create a map. Determining a map is often a very time consuming process

involving a lot of trial and error. The way to create a map is to use all the information available and logically fit all the fragments together. Single and double digests with restriction enzymes, complete and incomplete digestions, and hybridizing with probes aided in the mapping process.

IV. RESULTS/DISCUSSION

Ethidium bromide stained gel analysis

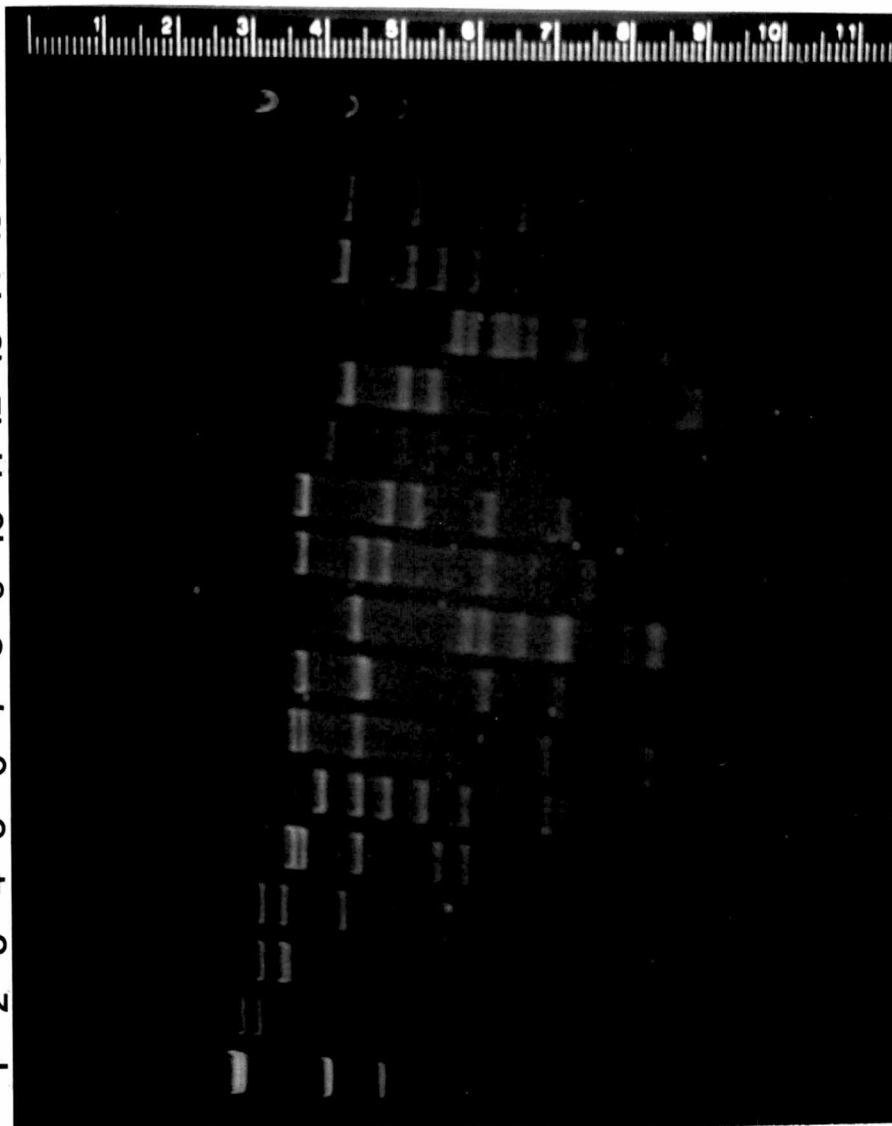
The pFAH1 cosmid clone was subjected to single and double restriction enzyme digestions by B. C. Mullin (Table 3.1) and C. S. An (Table 3.2). Both gels were stained with ethidium bromide and photographed (Figure 4.1 - C. S. An. Bands from B. C. Mullin's gel photograph were too light to reproduce (data not shown). Band migration was measured from the photographs, and fragment sizes were estimated for both gels (Table 4.1). The combined data from the gel photographs was used to help recognize partial digestions and identify bands which were not able to be seen in one of the gels. Due to the complexity of the data additional information was needed; therefore, hybridizations with Azotobacter nif probes had to be performed.

pFAH1 clone hybridization with Azotobacter probes

Results of the pFAH1 clone hybridization with Azotobacter nif genes are shown in Figure 4.2B-G. Azotobacter nifE was digested with SalI to produce three fragments of 531 bp (E_S : Figure 4.2D), 711 bp (E_L : Figure 4.2C) and 252 bp (too small to identify on minigel). This was done because it has been shown that Azotobacter nifE shows sequence similarity with Azotobacter nifD in a region

Figure 4.1 - Photograph of ethidium bromide stained gel of pFAH1 following a series of single and double restriction enzyme digestions.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



231

94

66

44

23

20

Table 4.1 - pFAH1 cosmid clone digestions. Restriction enzymes used to digest pFAH1, estimated fragment sizes in kilobases and migration distances in millimeters of each observed band from blot-1. (P) indicates probable partial digestions and (*) represents bands that were observed from gel-2 but not from blot-1. (x2) following an estimated band size indicates that it was of a stronger intensity and was counted as two bands instead of one.

```
=====
Enzymes/          Migration
Fragment Number    Distance      Predicted Length
-----
```

LANE 3 - EcoRI

1	41.0	38.64-P
2	45.0	28.05-P
3	52.0	18.56
4	57.5	14.43
5	*	14.16
		TOTAL-47.15

LANE 4 - HindIII/EcoRI

1	49.0	21.79-P
2	53.5	17.27
3	58.0	14.16
4	*	8.70
5	*	4.66
		TOTAL-44.79

LANE 5 - BglII

1	46.0	26.08-P
2	48.0	23.06-P
3	52.0	18.59-P
4	54.5	16.51-P
5	57.5	14.49-P
6	60.5	12.86
7	63.5	11.52
8	70.0	9.27-P
9	74.0	8.22-P
10	76.5	7.65
11	83.5	6.33-P
12	98.0	4.45
13	103.0	3.97
14	143.0	1.66 (x2)
		TOTAL-43.77

Table 4.1 (continued):

Enzymes/ Fragment Number	Migration Distance	Predicted Length
-----------------------------	-----------------------	------------------

LANE 6 - BglIII/ EcoRI

1	60.0	13.18-P
2	63.0	11.79-P
3	67.5	10.11
4	77.0	7.65
5	84.5	6.21
6	94.0	4.91
7	98.0	4.47
8	104.0	3.90
9	121.5	2.65
10	123.0	2.56
11	126.0	2.40
12	139.5	1.78
13	141.0	1.72
14	143.0	1.64
		TOTAL-50.00

LANE 7 - BglIII/HindIII

1	50.5	19.65-P
2	56.0	15.29-P
3	60.0	13.04
4	62.5	11.90
5	68.5	9.74-P
6	76.5	7.68
7	97.5	5.52
8	104.5	3.86-P
9	120.5	2.71
11	134.0	2.00
13	140.0	1.75
14	141.5	1.69
		TOTAL-46.29

LANE 8 - PstI *

1	*	12.44
2	*	7.64 (x2)
3	*	3.81
4	*	2.65
5	*	1.32
6	*	1.04
		TOTAL-36.54

Table 4.1 (continued):

=====		
Enzymes/ Fragment Number	Migration Distance	Predicted Length

<u>LANE 10 - PstI/BglII</u>		
1	69.0	9.40
2	77.5	7.43
3	103.0	3.95
4	106.5	3.65
5	113.0	3.15 (x2)
6	121.5	2.61
7	134.0	1.97
8	138.5	1.77
9	144.0	1.55
10	151.0	1.30
11	157.0	1.11
12	167.5	0.82
13	176.5	0.59
		TOTAL-42.45

LANE 11 - PstI/Xho

1	43.5	28.80-P
2	52.0	17.73-P
3	63.0	11.37
4	81.0	6.60
5	101.0	4.05
6	119.5	2.68
7	122.0	2.53
8	138.5	1.74
9	141.5	1.62
10	150.0	1.32
11	156.0	1.13
12	164.5	0.89
13	174.5	0.64
		TOTAL-34.57

Table 4.1 (continued):

=====		
Enzymes/ Fragment Number	Migration Distance	Predicted Length

LANE 12 - PstI/HindIII

1	43.5	28.80-P
2	63.0	11.17
3	84.5	5.88
4	91.0	4.99
5	104.5	3.64
6	118.5	2.66
7	132.0	1.97
8	138.0	1.72
9	143.0	1.53
10	149.0	1.32
11	155.5	1.12
12	166.0	0.83
13	175.0	0.62

TOTAL-37.42

LANE 13 - BamHI

1	56.0	14.27-P
2	70.0	8.63-P
3	78.0	6.80
4	87.0	5.39
5	93.5	4.59
6	99.5	3.99
7	107.0	3.36
8	118.5	2.60
9	123.0	2.36
10	127.5	2.13
11	133.5	1.87
12	139.0	1.64
13	143.0	1.50
14	152.0	1.20
15	166.5	0.80

TOTAL-38.23

Table 4.1 (continued):

Enzymes/ Fragment Number	Migration Distance	Predicted Length
-----------------------------	-----------------------	------------------

Lane 14 - BamHI/PstI

1	74.5	7.38
2	86.0	5.02
3	92.5	4.62
4	133.0	1.86
5	138.5	1.64
6	142.5	1.50 (x2)
7	151.0	1.22
8	154.0	1.13
9	165.0	0.84
10	174.0	0.63
		TOTAL-27.34

LANE 15 - BamHI/BglII

1	94.0	4.33
2	97.0	4.04
3	102.0	3.60
4	109.0	3.08
5	116.0	2.64
6	119.5	2.44
7	125.5	2.14
8	131.0	1.90
9	134.0	1.78
10	140.0	1.55
11	143.0	1.45
12	149.0	1.25
13	151.0	1.19
14	161.0	0.92
		TOTAL-32.31

Table 4.1 (continued):

Enzymes/ Fragment Number	Migration Distance	Predicted Length
-----------------------------	-----------------------	------------------

Lane 16 - BamHI/Xho

1	41.5	30.77-P
2	56.0	13.29-P
3	67.0	8.31
4	83.0	5.54
5	89.0	4.76
6	96.0	4.03
7	104.0	3.35
8	116.5	2.54
9	121.0	2.31
10	125.0	2.11
11	132.0	1.81
12	137.0	1.62
13	148.0	1.26
14	150.0	1.20
15	152.0	1.14
16	153.0	1.11

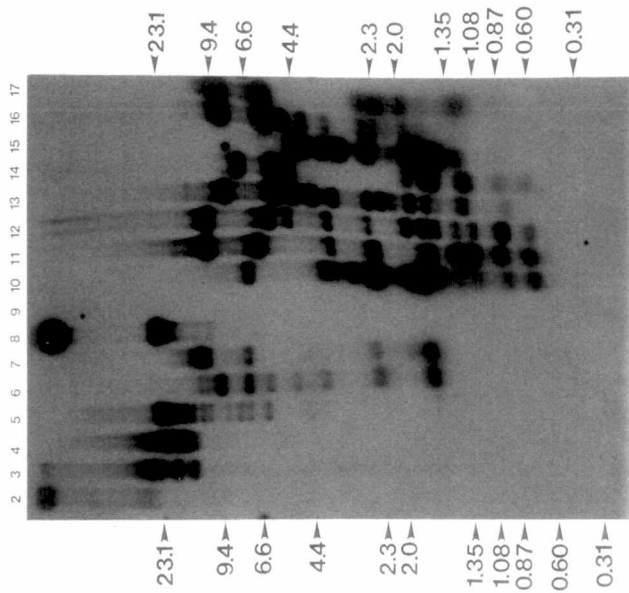
TOTAL-41.09

LANE 17 - BamHI/HindIII

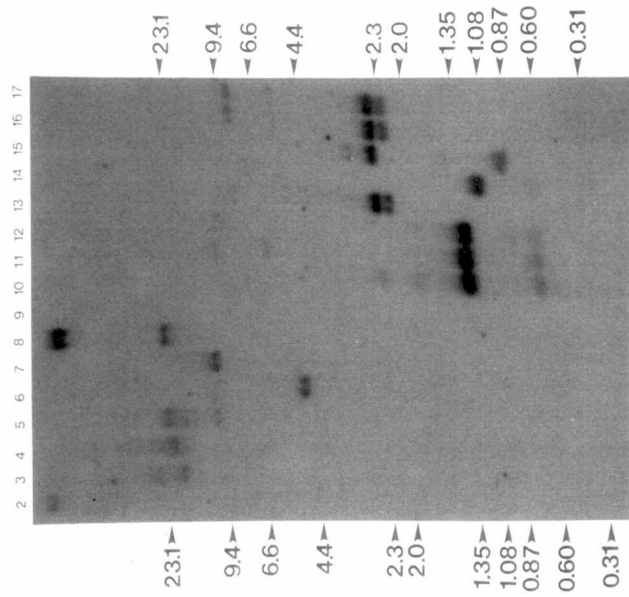
1	66.0	9.04 (x2)
2	81.5	5.74 (x2)
3	115.0	2.62
4	120.0	2.35
5	123.5	2.18
6	130.0	1.89
7	137.0	1.61
8	148.0	1.25
9	151.0	1.16
10	162.5	0.86

TOTAL-43.48

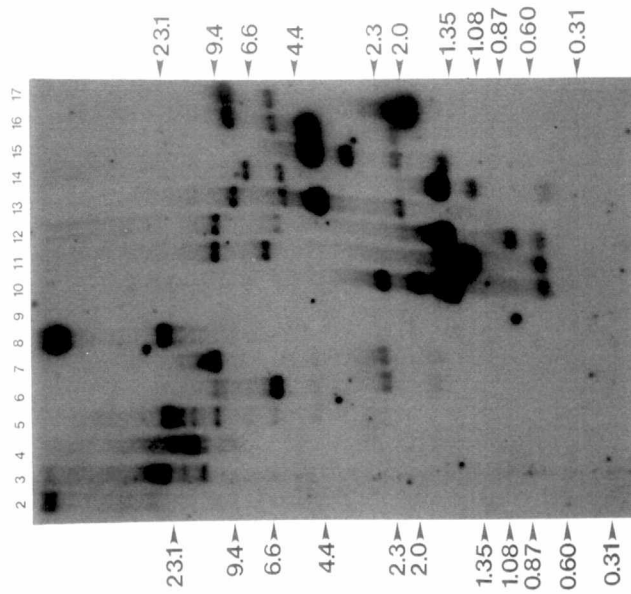
Figure 4.2 - Photographs taken of autoradiographs which had been hybridized to radiolabelled probes. The following were used as radiolabelled probes: (A) pFAH1 cosmid clone; (B) *nifB*; (C) *nifE_L*; (D) *nifE_S*; (E) *nifX*; (F) orf 10; (G) *nifM*; (H) lambda/HindIII digested DNA.



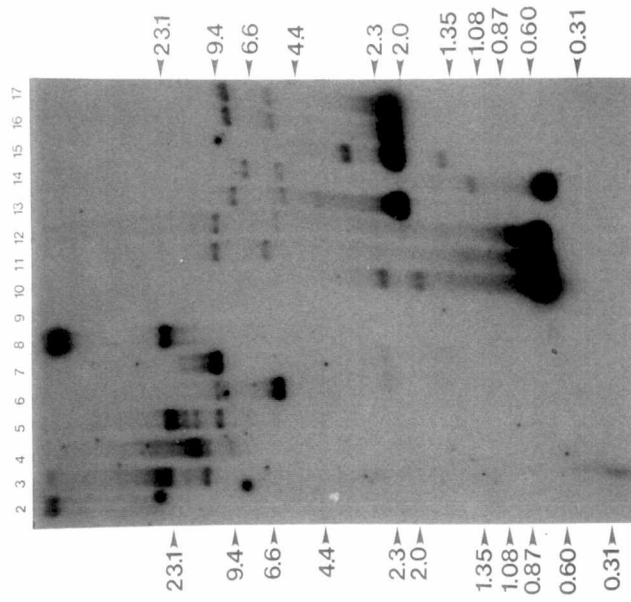
A



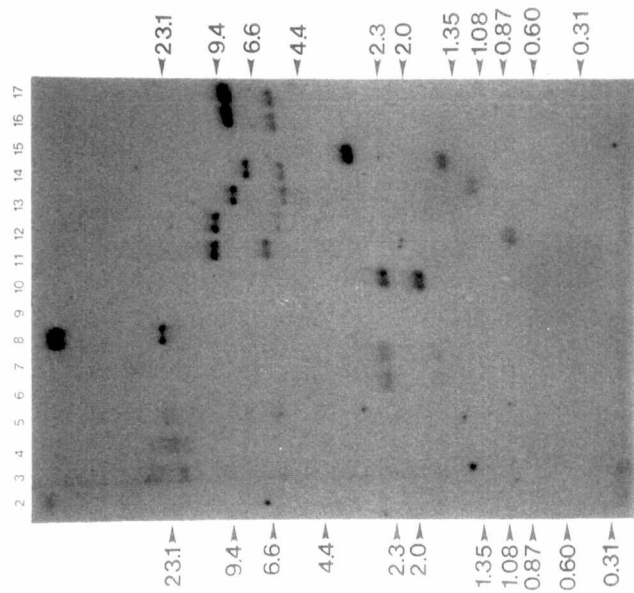
B



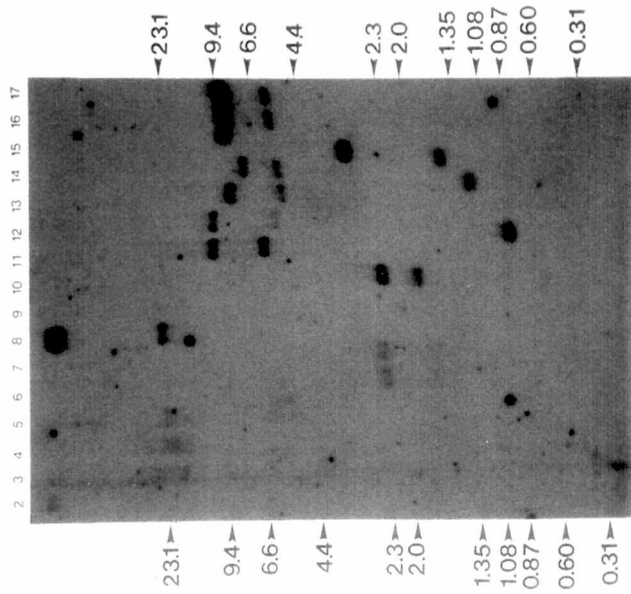
C



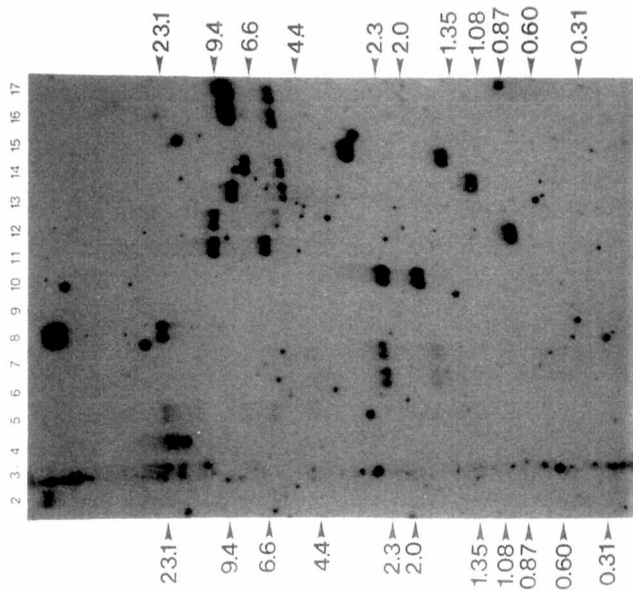
D



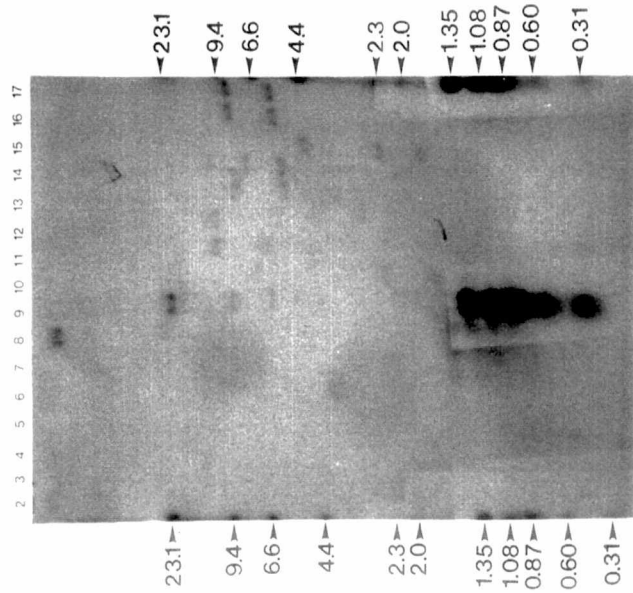
E



F

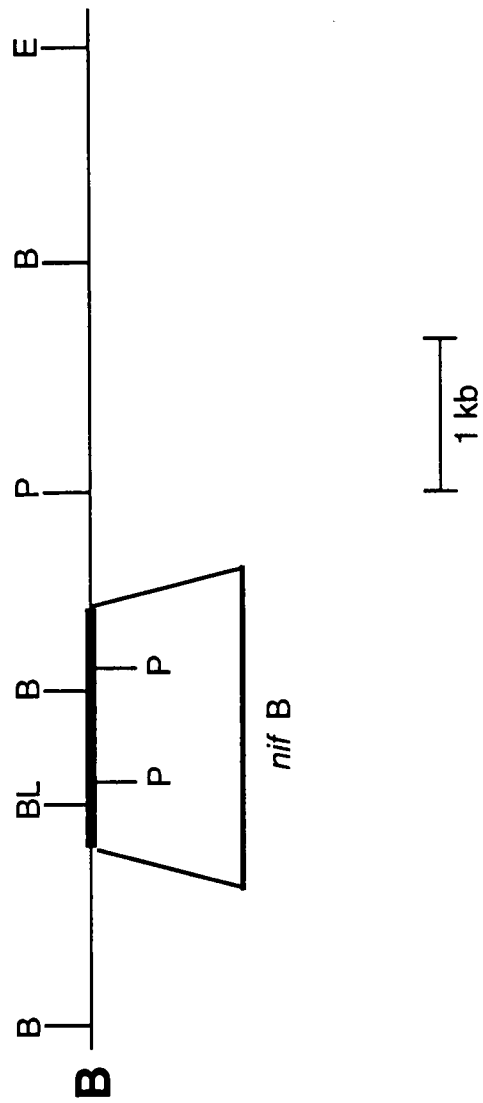
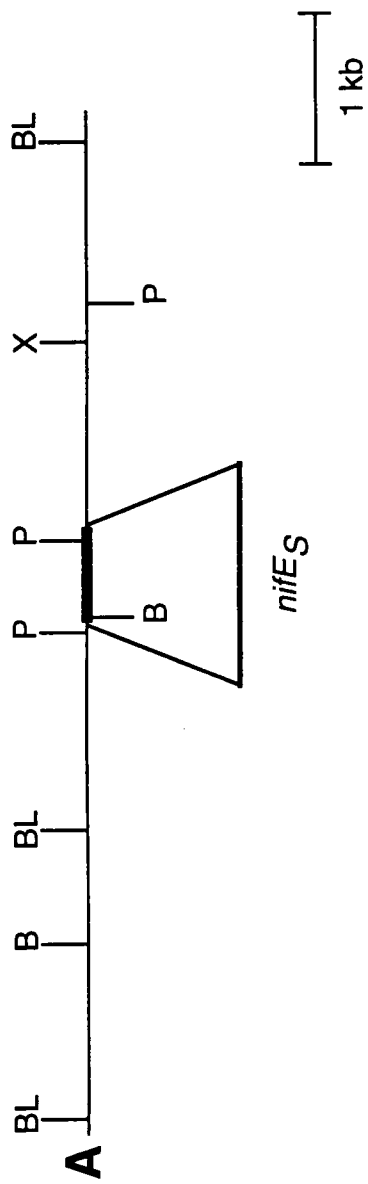


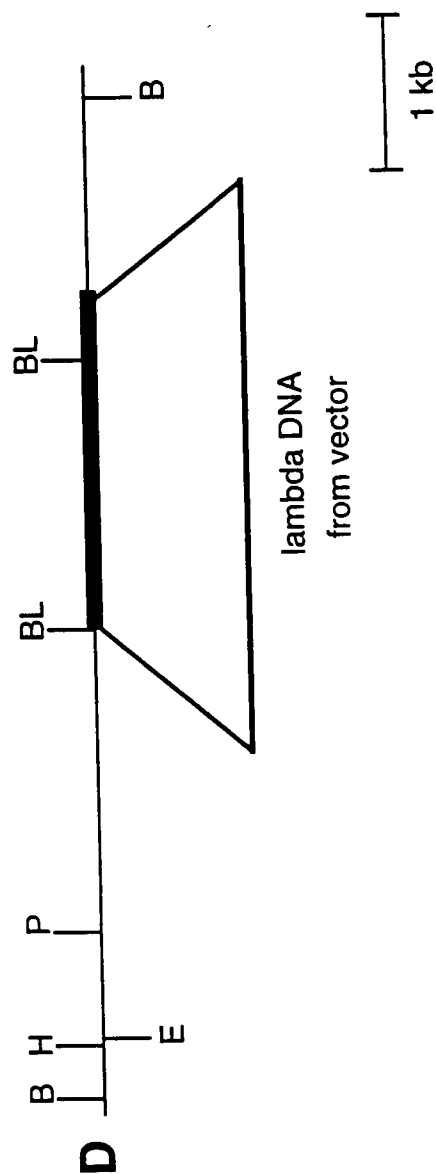
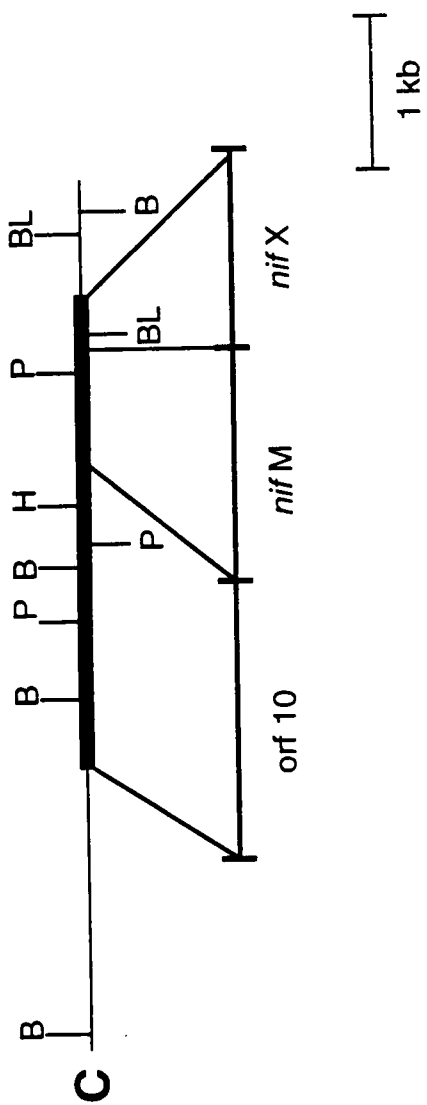
G



H

Figure 4.3 - Restriction maps. (A) *nifE*_s, (B) *nifB*, (C) *nifX*, M and *orf10*, (D) pHC79 lambda DNA restriction map.
Restriction sites - B, BamHI; P, PstI; BL, BglII; H, HindIII; E, EcoRI; X, Xho.





lambda DNA
from vector

bands which were homologous to vector lambda DNA (Figure 4.2H). This information was used to construct a restriction map around the cos site within the pHC79 vector (Figure 4.3D).

All the band distances were measured from blot-1 and fragment sizes were estimated using DNAsize (computer program) (Table 4.1). Fragment sizes were used in combination with the results obtained from the autoradiographs to create the various maps (Figure 4.3A-D and Figure 4.4). Strongly hybridizing bands were considered to extend the entire length of the probes, and weakly hybridizing bands were mapped as partially hybridizing to the probes. *NifS* was used as a probe, but the signals were very weak and not able to be measured accurately. This may be due to a lack of *nifS* in the pFAH1 cosmid clone or very low sequence similarity. Another explanation is that the quantity of probe that was used to hybridize was smaller than estimated (less than 15 ng) and consequently strongly hybridizing bands were not observed.

Comparing the location of the *nif* genes from the pFAH1 restriction map with other nitrogen-fixing bacterial maps indicates that Frankia *nif* gene arrangement is unique in part. No other maps have *nifM*, X, B and *orf10* adjacent to each other (Figure 4.4). To date, *nifM* has only been observed in A. vinelandii, A. chroococcum and K. pneumoniae (Dean et al., 1992). Heterologous hybridization of *nifM*

Figure 4.4 - HindIII/EcoRI restriction map of cosmid clone pFAH1. Bold/italic numbers represent approximate locations of *nifM*, X, B, E, orf10 and pHC79 vector DNA (V).

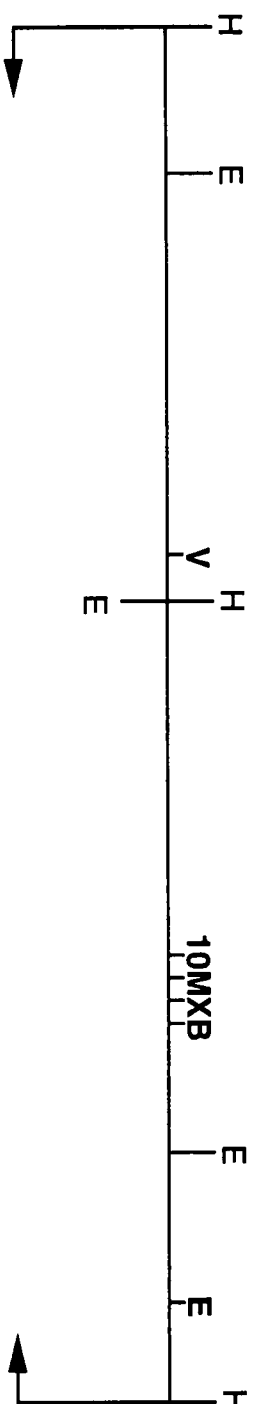


Figure 4.4 - HindIII/EcoRI restriction map of cosmid clone pFAH1.
 Bold letters and numbers represent approximate locations of
nif M, X, B and E, *orf10* and pHC79 vector DNA (V).

with other organisms has not been fruitful; therefore, this observation was both encouraging and unexpected. The function of *orf10* has not yet been determined, but the primary sequence has been shown to be similar with a family of hydrophilic inner-membrane binding proteins that demonstrate ATP-binding functions and are involved in active transport (Dean et al., 1992).

Mapping *nifX* next to *nifB* may have resulted due to sequence identity between a conserved region of *nifX* and *nifB* product primary sequence (Dean et al., 1992). This is doubtful; however, because this observation is based on DNA product comparison and not DNA:DNA hybridization, and the probe hybridization information that was used in mapping also had *nifM* and *orf10* hybridizing to the same fragments as *nifX* and B.

NifE and B are approximately 9.2 kb apart, and both hybridize to a 11.52 kb *Bgl*III fragment. This is consistent with other maps because these genes are usually not contiguous, and are often separated by 10 kb or more.

The data obtained from this study are encouraging. It gives further support for Frankia being a unique nitrogen-fixing organism at the molecular level, and once more genes from pFAH1 are mapped, phylogenetic relationships may be made between Frankia and other nitrogen-fixing bacteria.

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BIBLIOGRAPHY

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APPENDIX

Section 1: Preparation of media, solutions and buffers

References: Maniatis et al., 1982.

I. Media

A) LB Medium

10.0 g Bacto-tryptone
5.0 g Bacto-yeast extract
10.0 g NaCl
Adjust pH to 7.5 with NaOH, and bring up to 1 L.

B) 2xYT

16.0 g Bacto-tryptone
10.0 g Bacto-yeast extract
5.0 g NaCl
Adjust pH to 7.0, and bring up to 1 L.

C) LB-AMP Medium

Follow protocol for LB media then add filter sterilized ampicillin from an aqueous stock. Bring to a final concentration of 35-50 ug/ml. Be sure the media is 50°C before adding the ampicillin.

D) Antibiotics

Ampicillin: Prepare a stock solution of 20 mg/ml filter sterilize and store in aliquots at -20°C. Add to sterile cooled media (50°C) to a final concentration of 35-50 ug/ml.

E) S.O.C.

Add the following to 97 ml of distilled water:
2 g bactotryptone
0.5 g yeast extract
1 ml of 1 M NaCl
0.25 ml of 1 M KCl
Stir to dissolve, autoclave, and cool to room temperature. Add 2 M Mg⁺⁺ stock to make medium 20 mM in Mg⁺⁺. Add 2 M glucose stock to make a final medium concentration of 20 mM. Filter through a 0.2-μm filter and adjust pH to 7.0.

II. Solutions

A) Miniprep solution I

50 mM Glucose
10 mM EDTA (pH 8.0)
25 mM Tris-HCl (pH 8.0)

B) Miniprep solution II

0.2 N NaOH
1% SDS

C) Miniprep solution III

3 M Potassium
5 M Acetate

D) Prehybridization solution

60 ml 20 x SSC
10 ml 100 x Denhardt's solution
10 ml 10% SDS
4 ml EDTA
Make up to 200 ml with sterile distilled water.

E) 10 x SSC (concentrated stock):

Add the following to 800 ml water:
87.65 grams sodium chloride
44.10 grams sodium citrate
Adjust pH to 7.0
Bring total volume to 1 liter, autoclave.

F) Denhardt's solution (50x)

5 g Polyvinylpyrrolidone
5 g BSA (Pentax Fraction V)
Make up to 500 ml with sterile distilled water.
Filter through a disposable filter.
Dispense into 25 ml aliquots and store at -20°C.

G) Chloroform

24:1 - chloroform:isoamyl alcohol

H) Phenol

Equilibrate with TNE buffer.

III. Buffers

A) TE buffer

10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

B) TAE buffer

Working Solution: 0.04 M Tris-acetate
0.002 M EDTA

Concentrated Stock:
(50x per liter) 242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8.0)

C) TNE buffer

10 mM Tris-HCl
100 mM NaCl
1 mM EDTA

D) beta agarase buffer

10 mM BisTris (pH 6.5)
1 mM EDTA

Section 2: Preparation of competent cells

Streak DH5 α (Gibco BRL, Gaithersburg, MD) cells from a glycerol culture on an LB plate, and incubate overnight at 37°C to obtain single colonies. Transfer colony to 5 ml of 2xYT media and incubate in shaker (200 RPM) at 37°C overnight. Add 50 μ l of overnight culture to 5 ml of 2xYT to create a 1/100 volume and shake in the incubator at 37°C for 90 minutes. Transfer 1 ml of this culture to a cold Eppendorf tube and keep on ice from this point on. Let the cells cool and then spin for 45 seconds at 4°C. Pour off supernatant and be sure to remove all media before placing the tubes back on ice. Add 1 ml of cold CaCl₂ (0.1 M) to pellet, and resuspend by pipetting up and down gently. Put on ice for at least 30 min., spin for 30 seconds and decant supernatant. Add 200 μ l of 0.1 M CaCl₂ and resuspend gently with pipettor.

Section 3: Transformation protocol

Add 1 μ l of plasmid DNA to 100 μ l of competent cells in an Eppendorf tube and put on ice for 35

minutes. Heat shock the cells for 90 seconds at 42°C and place back on ice for 2 minutes. Add 1 ml of room temperature SOC and incubate for 1 hour at 37°C so the transformants can express AMP resistance. Plate 50 ul on LB-AMP media and incubate at 37°C overnight. Choose six colonies to streak separately on an LB-AMP plate which is divided into six sections.

Section 4: DNA Precipitation

Use 5 M or 1 M NaCl stock to change the concentration of the DNA solution to 0.1 M NaCl. Mix well, then add two volumes of ice cold 100% EtOH. Mix again and then freeze to -80°C for at least two hours (the smaller the fragment size the longer it should be frozen). Spin at 4°C for at least 30 minutes and discard the supernatant. Rinse the pellet with ice cold 70% EtOH and remove any excess EtOH by aspiration. Dry the pellet and resuspend in 20 µl TE.

Section 5: Random primer labeling (Gibco BRL, Gaithersburg, MD, Random Primers DNA Labeling System)

Denature 25 ng of DNA dissolved in 5-20 ul of TE by heating for 5 min. in a boiling water bath, then cool immediately on ice. Perform the following additions on ice:

- 2 ul dATP solution
- 2 ul dGTP solution
- 2 ul dTTP solution
- 15 ul Random Primers Buffer Mixture
- 5 ul alpha-32P dCTP, 3000 Ci/mmol, 10 uCi/ul (ICN, Irvine, CA)
- Distilled water to a total vol. of 49ul.

Mix briefly. Add 1 ul Klenow fragment, mix gently but thoroughly. Incubate at 25°C for 1 hour, then add 5 ul stop buffer and put on ice. Dilute 2 ul of the mixture with 498 ul of distilled water or TE buffer. Spot 5 ul of this dilution on a glass fiber filter disk (Whatman GF/C or equivalent). Wash the filter disk 3 times with 1% sodium pyrophosphate, 0.7% hydrochloric acid and 5% acetic acid for 15 min. Rinse 10 min. with 95% ethanol at room temperature. Dry the filter under a heat lamp while in a slight breeze, and determine the precipitable radioactivity by liquid scintillation counting.

Section 6: DNA blot hybridization (Amersham, Arlington Heights, IL)

Make up pre-hybridization solution as shown in Section 1 part II-D and add it to the membrane. Denature 0.5 ml of a 2 mg/ml solution of sheared (salmon sperm) DNA by heating in a boiling water bath for 5 min. Chill the salmon sperm DNA and add it to the pre-hybridization solution. Shake for at least 1 hour at 65°C in a heat-sealed plastic bag (use 5 ml of sol./ 100 sq. cm of membrane). Denature heterologous DNA as before but add the radiolabeled probe. Shake for at least 12 hours in water bath at 65°C.

Section 7: Washing radiolabeled blot

Remove blot from water bath and carefully cut open the bag. Pour liquid contents into radioactive waste jug and wash blot by quickly pouring 200 ml of 2xSSC into bag while carefully moving the solution over the blot. Once again discard the solution into the jug and wash with 200 ml of 2xSSC. This waste may be discarded in the sink while water is running. Carefully remove the blot from the bag and place into a dish. Quickly add enough 2xSSC, 0.1% SDS to cover the blot so it will not dry and shake for 30 min. at 65°C. Remove the dish from the water bath and pour liquid contents into the sink. Add 1xSSC, 0.1%SDS to a depth of 2-3 cm and shake for 15 min., repeat. Place the blot in a sealable bag with a small amount of 1xSSC + 0.1%SDS and expose to Kodak X-Omat RP diagnostic film using one or two Cronex Xtra life intensifying screens (DuPont) for the required amount of time.

Section 8: Preservation of glycerol culture (Maniatis, 1982)

Add 5 ul of 20 mg/ml ampicillin to 2 ml LB in an autoclaved vial, and inoculate with a loop of the transformed cells. Incubate overnight at 37°C in the shaker. Add 0.15 ml of autoclaved glycerol to each of the six vials, and then add 0.85 ml of the overnight culture to each vial and vortex. Quick freeze in either dry ice or liquid nitrogen and store in -80°freezer indefinitely.

Section 9: Extraction of plasmid DNA (Maniatis, 1982)

The alkaline lysis miniprep protocol described was taken from Maniatis. Inoculate 2 ml of LB-AMP

media with a loop of transformed cells and incubate overnight at 37°C while shaking at 200 RPMs. Spin down the culture at 4°C for 30 seconds and remove the supernatant completely. A suction hose may be used for this procedure to remove small drops because problems may arise during restriction digests if all the supernatant is not removed. Resuspend the pellet in 100 ul ice cold solution I and vortex. Add 200 ul of freshly made solution II and mix gently (do not vortex!). Add 200 ul of ice cold solution III and mix by inverting and shaking. Store on ice for 3-5 minutes then spin for 5 minutes at 4°C. Quickly transfer the supernatant to a fresh tube. Add 500 ul of a 1:1 mixture of phenol:chloroform to the tube and vortex. Spin for 2 min. at 4°C and transfer the top layer to a new tube. Precipitate the DNA by adding two volumes of room temperature ethanol, vortex, and let sit at room temperature for two minutes. Spin for 5 minutes at 4°C and remove the supernatant completely. Rinse pellet in 70% ethanol for approximately 10 minutes at 4°C and dry under a vacuum. Redissolve the pellet in 40 ul TE and add 10 ul of 100 ug/ml RNase A (Sigma, St. Louis, Mo).

Section 10: Minigel protocol (Maniatis, 1982)

Filter 1xTAE buffer, and put 30 ml of it in a 125 ml Erlenmeyer flask. Add 0.24 grams of electrophoresis grade agarose (Gibco BRL, Gaithersburg, MD) to the 30 ml of TAE to create an 0.8% solution. Cool down to 50°C and pour into a 6.3 cm x 10.2 cm taped tray. Place comb in gel so it is 0.5 mm from the bottom and let sit for 30 minutes. Put a thin layer of buffer on the top of the gel and pull the comb out. The buffer layer helps the comb slip out more easily. Put gel in container and pour in enough buffer to have a thin layer coating the gel. Add 80 ng marker DNA (from 8 ng/ μ l stock) to either outside lane or both outside lanes after heating to 65°C. The middle six lanes should contain the plasmid DNA (2 ul), TE (8 ul), and 10xdye (1 ul) mixtures. After carefully pipetting the marker and plasmid DNA into the wells, hook up the electrodes and turn on voltage so the DNA will run toward the positive pole because DNA is negatively charged. Turn off the voltage after the gel is finished running, and pour the gel and the buffer into a tupperware container. Add 12.5 ul of EtBr (10 mg/ml stock) to an edge away from the gel, wrap container in

foil and shake for 30 minutes. Pour the EtBr into a waste container and rinse three times with tap water. View gel in darkroom under ultraviolet light.

Section 11: Recovery of plasmid inserts

I. Agarose digestion

Cut desired band from low melting agarose gel with a razor blade and put it in an Eppendorf tube. Weigh the contents of the tube to determine the amount of beta agarase that must be added ($5\mu\text{l} = 1\text{Unit}$). [1Unit is defined as the amount of beta agarase (New England Biolabs, Beverly, MA) required to digest $200\mu\text{l}$ of agarose in one hour at 40°C]. Before adding the enzyme, equilibrate gel slice by adding beta agarase buffer (see section 1) and let sit for 15 min., then repeat. Next, melt the agarose in a 65°C water bath for 10 min. Quickly transfer the tube to a 40°C bath and add the required amount of enzyme. Let sit for one hour then remove and adjust the salt concentration to 0.5 M NaCl while on ice. After the 15 min. ice treatment, spin at 4°C for 15 min. Remove the supernatant and perform an ethanol precipitation to collect the DNA. Resuspend the pellet in $20\mu\text{l}$ TE.

II. Electroelution

Prepare a $6.3\text{ cm} \times 10.2\text{ cm}$ 0.8% agarose gel and load $10\mu\text{l}$ of a $25\text{ ng}/\mu\text{l}$ DNA stock in each well. Run gel as usual and stain with EtBr to observe under ultraviolet light. While observing, cut a well with a sharp utensil in the gel next to the band which is to be removed. Place gel back onto the tray and put into electrophoresis setup not allowing the buffer to cover the top of the gel. To the newly cut well add $0.5 \times \text{TAE}$ buffer and allow the current to run for 1 min. at 100 volts. Recover buffer from well and put into Eppendorf tube. Replace buffer in well and repeat twice. Observe under UV light to confirm electroelution of the band. Perform an ethanol precipitation on the pooled fractions and resuspend in $10\mu\text{l}$ TE.

Section 12: Restriction enzyme digestion to remove Azotobacter nif genes

Begin with approximately 200-325ng plasmid DNA. Add TE to bring up to a total volume of $20\mu\text{l}$ in

an Eppendorf tube. Add 2.5 μ l of 10x sample loading buffer (BIOS, New Haven, CT) which is supplied by New England Biolabs (NEB, Beverly, MA) and 1.5 μ l autoclaved distilled water. Add 1 μ l NdeI, and incubate 1.5 hours in water bath at 37°C. Remove from water bath and add 2.5 μ l 1 M NaCl to bring to a final concentration of 0.1 M NaCl. Perform a DNA precipitation as described before. Resuspend in 20 μ l TE and add 2.5 μ l of 10x buffer which is supplied for BamHI (NEB, Beverly, MA). Add 1.5 μ l autoclaved distilled water and 1 μ l BamHI. Incubate for 1.5 hours in a water bath at 37°C. This solution may be placed in the -20°C freezer or run on a minigel immediately.

VITA

Kevin Frank Duvel was born in Elgin, Illinois on June 20, 1970. He attended St. Johns elementary school in Algonquin, Illinois while growing up in West Dundee. He graduated from Jacobs High School in June 1988 and continued his education at Eastern Illinois University. After three years at Eastern Illinois University he chose to pursue studies at the University of West Florida in Pensacola, Florida. After one semester, he returned to Eastern Illinois University to complete a Bachelor of Science degree in Botany with a minor in Chemistry. This degree was awarded in May 1992. The author then accepted a graduate teaching assistantship in the Botany Department at the University of Tennessee, Knoxville in the fall of 1992 and began work towards a M.S. degree.

