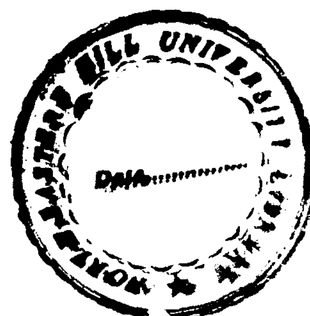
DEVELOPMENT OF MOLECULAR SIGNATURES OF *FRANKIA* STRAINS

ABSTRACT



By

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BOTANY DEPARTMENT
IN PARTIAL FULFILMENT OF THE
REQUIREMENT OF THE DEGREE
OF DOCTOR OF PHILOSOPHY IN BOTANY

OF

NORTH EASTERN HILL UNIVERSITY
SHILLONG, INDIA.

Thesis

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ABSTRACT

The nitrogen fixing actinomycete *Frankia* can live in symbiosis with a large number of woody dicots. These trees, termed actinorhizal trees, are spread over 8 plant families and comprise 22 genera. They are highly valued as timber, fuelwood, windbreaks and as pioneer species in regeneration of wastelands. The symbiotic interaction with *Frankia* leads to the production of root nodules in which nitrogen is converted to ammonia. The genus *Frankia* can be differentiated into host infection groups. Though the quantum of ammonia generated by *Frankia* competes well with that done by the more acclaimed *Rhizobium, Frankia* is not so well known. This is despite the fact that the host range of *Frankia* is much wider and the trees nodulated are important from the point of view of forest regeneration. The obscurity of *Frankia* is attributable to its slow growth rate, lack of knowledge about specific nutritional requirements and the enormous diversity of the genus.

Studies on *Frankia* really took off in 1978, when Callaham and co-workers successfully isolated it from the plant *Comptonia peregrina* (Callaham *et al.*, 1978). The number of *Frankia* strains has grown several fold since then. Today endophytes from *Alnus, Casuarina, Datisca, Myrica, Elaeagnus* and many other host genera are known. The successful isolation of *Frankia* has rekindled hopes of availability of an efficient biofertilizer especially in lands devastated by flooding, fires, landslides and other such natural disasters. Global increase in the actinorhizal population is an added attraction.

Preliminary work on *Frankia* depended heavily on host infection capability. However subsequently, this approach had to be abandoned as many *Frankia* strains failed to

reinfect their original host. This led to the search for new criteria for the classification of *Frankia*. Traditional methods like serotyping, isozyme analysis, protein and fatty acid patterns etc. did help but were later given up as they were cumbersome and difficult to apply in case of *Frankia*. Today, molecular biological approaches have come handy in surmounting these problems. These methods are based on PCR. Since DNA rather than the organism is studied, the method is not limited to pure culture.

PCR relies on the ability of DNA polymerase to amplify any DNA sequence lying between two flanking regions to which primers are available (Mullis *et al.*, 1986). Initially Mullis and associates employed the Klenow fragment of *E.coli* DNA polymerase for amplification. Now cloned versions of a thermally stable and superior enzyme are available. The procedure has undergone a revolutionary transformation. DNA can be amplified from any crude source and if sufficiently pure, the product can be directly sequenced by automated sequencers. Computers can promptly align sequences and decipher phylogenetic relationships. Some well known PCR based methods are:RFLP, DAF, RAPD, rep-PCR, PCR-SSCP, microsatellite analysis and DNA sequence analysis.

Frankia research has greatly benefitted from these recent advances. With the help of PCR and DNA sequencing, charactetrization of many strains has ben possible. Comparative sequence analysis of strains from diverse host specificity groups, has enabled the creation of a phylogenetic tree (Nalin *et al.,* 1995; Normand *et al.*, 1996). All isolates could be differentiated into four major groups. These groups are: (a) A large group comprising *F. alni, Casuarina and Myrica* isolates; (b) the *Elaeagnus* infective group; (c) the unisolated strains of *Dryas, Coriaria* etc.; and (d) the atypical strains (this group includes *Frankia* that lack

nitrogen fixation and or nodulation capacity). The alder group could be further divided into four subgroups. A subsequent emendation of the family *Frankiaceae* also has been done which now encompasses only *Frankia* as the major genus. The differences between *Frankia* and the closely related genus *Geodermatophilus* have been clearly demarcated (Bosco *et al.*, 1992; Normand *et al.*, 1996). At the moment unique probes are available for detection of *Frankia* from disparate host infection groups viz. *Alnus, Casuarina, Elaeagnus, Datisca and Coraria* among others.

But a lot more still needs to be done. *Frankia* speciation is incomplete. Many geographical regions house unknown strains which remain unisolated. Added to this is the fact that there exist some strains that lack nitrogen fixation capacity and/ or nodulation capacity. Many isolates fail to reinfect their original host. Moreover, *Frankia* strains from almost the entire Asiatic zone have not been identified which is a serious lacuna in itself. Furlow (1979) hypothesised that the genus *Alnus* may have originated in the Indo-China region. Therefore, it follows that an immense diversity in case of alder compatible *Frankia* exists in this region..

It is, therefore, pertinent to begin the work of *Frankia* research in India in right earnest. Consequently work relating to collection of alder compatible *Frankia* germplasm was taken up in our laboratory. This led to the isolation of some high nitrogen fixing strains from North East India, particularly Shillong (Ganesh, 1993). Phenotypic and physiological attributes have confirmed the identity of the isolates. In order to release them into soil and assess their competence in inducing nodulation on alder, it is necessary to develop strain specific probes for the amplification of the 16S rRNA and *nif* genes.

One of the approaches in this direction, has been to categorise isolates in accordance with their nitrogen fixation rates and look for distinct molecular signatures that may delineate them. Such approaches can pave the way for the selection of superior strains of *Frankia* which can then be used to nodulate selected host plants.

To look for molecular signatures for *Frankia* strains with distinct nitrogen fixation rates, we chose to undertake a study in a particular geographical site in Shillong. Following were the objectives of the study:

1. Development of molecular signatures for identifying better nitrogen fixers introduced in the field.
2. Assessing feasibility of use of such signatures in estimating the nodulation competence of better nitrogen fixers when present in the soil with other strains.

Briefly the investigation could be divided into the following steps.

1. Collection of nodules from a particular area in Shillong.
2. Assessment of ARA of single nodules and their classification based on nitrogenase activities.
3. DNA extraction from single nodules.
4. Amplification of the *rrn* and *nif* regions in the DNA.
5. Screening for AFLPs.
6. Restriction digestion with appropriate enzymes and screening for RFLPs.

7. To look for any correlation between RFLPs and nitrogenase activity as reflected by ARA.
8. If yes, then inoculation of soil with a mixed culture containing one of the high nitrogenase activity nodule suspension together with others to test the efficacy of the molecular signatures developed as above.
9. If possible use of DNA sequence analysis to design strain specific probes.

With such a purpose in mind, the work was begun. In this direction, nodules were collected from different trees in a particular geographical site in Shillong. Acetylene reduction assays (ARA) were done on single nodules by using the procedure of Stewart *et al.* (1968). Subsequently, DNA was isolated from each single nodule by using a modification of the method of Rouvier *et al.* (1996). The extracted DNA was amplified (Simonet *et al.*, 1991). 16S rRNA and *nif* genes were studied. To test the reliability of PCR amplification, ten individual nodules were prepared and amplification was carried out by the above mentioned procedure. The nodules had developed in dissimilar trees but in field conditions where soil, weather, temperature etc. were closely similar. A total of thirteen different trees were sampled. *Frankia* specific and universal primers were used in various combinations to achieve amplifications of the target sites. Amplified DNA was digested with different restriction enzymes for PCR-RFLP analysis.

The results of the study can be summarised as follows:

1. Trees differed significantly from each other in their mean ARA values. A 'Host' effect was evident. The trees could be classified into two groups : a low and high activity group, based on the nitrogen fixation rates.

2. Individual nodules assayed, could be assorted into 9 PCR-RFLP groups on the basis of differences in the 16S rRNA and *nif* genes.
3. Variability was found in the distal part of 16S rRNA gene. Similarly, variabilities were found in the middle part of the 16S rRNA gene. By the use of a new primer in the *nif* D region, repetitive sequences were found, these sequences originated from the multiple annealing sites that exist for the primers FGPH 750 and FGPD 826' in the *nif* D and H genes respectively.
4. There was no significant difference in the ARA values of different PCR-RFLP groups.
5. Nodules that were highly active and low activity nodules generally fell in the same PCR-RFLP group. Trees differing widely in mean ARA values generally hosted the same strain of *Frankia*. This meant that the host genotype had a dominant influence on the nitrogen fixation rates of nodules.
6. Superior strains (PCR-RFLP groups) detected by this study varied in their nitrogenase activities.Their ARA values varied with the host. Because of this it was not possible to use them for nodulating alder in field conditions. An alternative approach which can be used in the future can be to select for a superior host genotype beforehand, after which different *Frankia* strains can be tested for high nitrogenase activity. Selection of a superior *Frankia* strain for use as a potential biofertilizer would then be possible.
7. In the final analyses, the symbiotic effectiveness of *Frankia*-actinorhizal association seemed to depend on three factors:

   a) Host genotype

   b) *Frankia* genotype

c) Other factors which include environmental factors like soil, edaphic factors and presence of other microbial flora.

Since the host plays a definite role in determining the nitrogen fixation rates of nodules it is not possible to delineate *Frankia* strains simply on the basis of nitrogenase activities of nodules. The host genotype must be kept constant before a search for superior *Frankia* strains is made.

It would be prudent to state that nitrogen fixation inside nodules is an outcome of several factors playing in tandem a complex role. The major factor appears to be the genotype of the participating partners, specifically the genotype of the host and the microsymbiont. Both these factors are influenced by other factors known and unknown, only some of which have been identified. The influence of environmental factors in this connection cannot be ignored. Parameters like time of collection (time, day, month and year), geographical location, soil characteristics, etc. play a major role in regulating the nitrogen fixation rates (Quesada *et al.*,1997 and 1998; Han and New, 1998). Each factor would have to be examined in isolation. As is known, the nitrogen fixation rates of *Frankia* in pure culture may vary drastically from those *in situ* (Sougoufara *et al.*, 1992; Ganesh, 1993; Reddell and Bowen, 1985; Han and New, 1998). The host exerts a control on the entire process of symbiosis. This factor must be taken into consideration while attempting to use *Frankia* as a biofertilizer. Such attempts must however always emphasise both on the host genotype as well as on the genotype of the microsymbiont.

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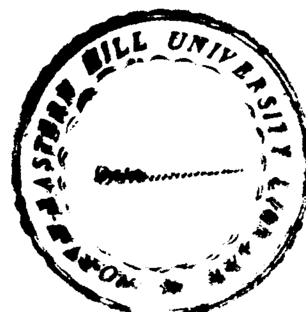

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DECLARATION

I Susamma K. Verghese, hereby declare that the subject matter of this thesis entitled "Development of Molecular Signatures of *Frankia* Strains " is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Botany.

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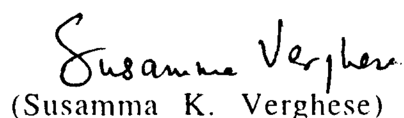
Lastly, I express my gratefulness to my family members for their patience, support and understanding.



(Susamma K. Verghese)

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CHAPTER 1

INTRODUCTION

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The nitrogen fixing actinomycete *Frankia* can live in symbiosis with a large number of woody dicots. These trees, called actinorhizal trees, are spread over 8 plant families and comprise 22 genera. They are highly valued as timber, fuel-wood, windbreaks and as pioneer species in regeneration of wastelands. The symbiotic interaction with *Frankia* leads to the production of root nodules in which nitrogen is converted to ammonia. Although the quantum of ammonia generated by *Frankia* compares well with that done by the more acclaimed *Rhizobium*, *Frankia* is not so well known. This is despite the fact that host range of *Frankia* is much wider and the trees nodulated are more important from the point of view of forest regeneration. The obscurity of *Frankia* is attributable to its slow growth rate, lack of specific nutritional requirements and the enormous diversity of the genus.

Studies on *Frankia* really took off in 1978, when Callaham and coworkers successfully isolated it from the plant *Comptonia* (Callaham *et al.*, 1978). The number of *Frankia* strains has grown several fold since then. Today endophytes from *Alnus, Casuarina, Datisca, Myrica, Elaeagnus* and many other host genera are known.

The relevance of *Frankia* research borders on a twin perspective. The interaction between plant cells and bacterium

during the process of symbiosis is vital for understanding the fundamental basis of cell-cell interactions. The study of the mechanism of nitrogen fixation has its own significance, both in the fields of cellular and molecular biology, as well as for its applied aspects. These studies may finally lead the way to the creation of novel nitrogen fixing plants. The applied aspects of *Frankia* research relate to use of superior *Frankia* strains as bio-fertilizers. These two goals, if realized, have immense potential in improving agriculture, forestry and environment.

Preliminary work on *Frankia* depended heavily on host infection criteria. Subsequently, this approach had to be abandoned, as many *Frankia* strains failed to re-infect their original hosts. This paved the way for the definition of new criteria for the classification of *Frankia*. Traditional methods like serotyping, isozyme analysis, protein and fatty acid patterns, etc., were used, but were later abandoned as they were cumbersome and difficult to apply in case of *Frankia*. Today molecular biological approaches have come handy in surmounting the problems. These approaches include the ones based on the Polymerase Chain Reaction (PCR). Since DNA rather than the organism is studied the method is not limited to pure culture.

PCR relies on the ability of DNA polymerase to amplify any DNA sequence lying between two flanking regions to which primers are available (Mullis *et al.*, 1986). Initially Mullis and associates employed the Klenow fragment of *E. coli* DNA polymerase for amplification. Now cloned versions of thermally stable and

superior enzymes are available. The procedure has undergone a revolutionary transformation. All aspects of this process can be altered to suit the requirements of the individual researcher. DNA can be amplified from any crude source and if sufficiently pure, the product can be directly sequenced by automated sequencers. Computers can promptly align the sequence and decipher phylogenetic relationships, furnishing immediate clues to the taxonomist.

PCR holds enormous potential for research. Using restriction fragment length polymorphism specific DNA fingerprints can be obtained. Sequence polymorphism can be detected by allele specific oligonucleotide (ASO) probes. Length variation among alleles can be detected by variable number of tandem repeats (VNTR) analysis (Boerwinkle *et al.*, 1989). In this, repeated sequences, which are flanked by two primers, are amplified. Random amplified polymorphic DNA (RAPD) analysis exploits the use of random primers to identify DNA polymorphism (Hadrys *et al.*, 1992). In a similar approach, i.e. DNA amplification fingerprinting (DAF), small primers are used to amplify repeated sequences in the DNA. In asymmetric PCR, the amount of one primer is increased 50-100 fold relative to the other. By denaturing gel electrophoresis of PCR products, differences in sequences within populations can be detected, (Muyzer *et al.*, 1993). *Alu* PCR (Nelson and Caskey, 1989), inverse PCR, anchored PCR, RT PCR, (von Beroldingen *et al.*, 1989) etc. are other valuable methods that reveal polymorphisms in the DNA. With the help of labeled probes, increased specificity and sensitivity in PCR can be

achieved. PCR based probes can be used for *in situ* hybridization studies in organisms that are difficult to study in pure culture or where the isolation of sufficient amount of DNA is difficult. Finally cycle sequencing of PCR products can confirm the observed differences. Alternatively the amplified product can be cloned before sequencing, (Ward *et al.*, 1990). All these methods can be applied for inferring phylogenetic relationships between organisms. Besides this, PCR can also be used for altering a particular template sequence. Introduction of restriction sites into amplified products, addition of promoters, specific nucleotide substitutions, insertions and deletions etc. can all be achieved with precision and rapidity. The applications of PCR in the field of recombinant DNA technology are numerous and continue to increase with each day.

Frankia research has greatly benefited from these recent advances in molecular biology. Significant information has accumulated in the past years to enable exploration of phylogeny. Creation of phylogenetic trees by molecular techniques has made it possible to divide all known strains into four groups (Hahn *et al.*, 1988; Nalin *et al.*, 1995; Normand *et al.*, 1996). These groups are: a) A large group comprising *F. alni, Casuarina* and *Myrica* isolates; b) the *Elaeagnus* infective group; c) the unisolated strains of *Dryas, Coriaria* etc.; d) the atypical strains (this group includes *Frankia* that lack nitrogen fixation and/or nodulation capacity). The alder group could be further divided into four groups. A subsequent emendation of the family *Frankiaceae* also has been done which now encompasses only *Frankia* as the major genus.

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The investigation ~~to be~~ carried out could be detailed as follows:

1. Collection of nodules from a particular area in Shillong.
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7. To look for any correlation between RFLPs and nitrogenase activity as reflected by ARA.
8. If yes, then inoculation of soil with a mixed culture containing one of the high nitrogenase activity nodule suspension together with others to test the efficacy of the molecular signature developed as above.
9. If possible use of DNA sequence analysis to design strain specific probes.

CHAPTER 2

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

Symbiotic associations that develop between micro-organisms and higher plants are receiving considerable attention in light of their effect on plant morphogenesis, nutrition and protection against infectious diseases. These associations are distinct cellular recognition processes that arise from specific unions between two taxonomically diverse organism that are often capable of independent existence. The symbiotic associations for augmenting nitrogen supplies are of prime importance. The strong selection pressure on this partnership is the resulting nutritional complementation, the plant can be considered as a carbon rich nitrogen poor autotroph and the symbiont a carbon poor nitrogen rich heterotroph. The ensuing symbiosis makes them nutritionally sufficient, conferring on them definite advantages over others, similarly placed. This process also contributes significantly to the nitrogen pool.

The ability to fix atmospheric nitrogen is restricted to prokaryotes. Eukaryotes do not possess the machinery to reduce nitrogen. This may be due to the incompatibilities of the anaerobic requirements of nitrogen fixation with the highly aerobic environment of eukaryotic cell and also because of the presence of high amount of fixed nitrogen in the environment during evolution. Nitrogen fixing organisms that are unable to support their energy requirements in the free living state form associations with phototrophic eukaryotes, like plants. Thus, one

finds in nature a variety of associations, ranging from colonization of rhizosphere and phyllospere to the endosymbiotic nitrogen fixing state, as in *Rhizobium* and Actinomycetes' associations with legumes and some non-leguminous plants respectively.

2.1 *FRANKIA*-ACTINORHIZAL SYMBIOSIS

The most well acclaimed plant-microbe symbiotic partnership is that between legume-*Rhizobium*. Others like *Nostoc* and *Azotobactor* are also being investigated. A relatively less known association is that between *Frankia and* actinorhizal trees. This too, nowadays, is drawing some attention.

2.1.1 The Actinorhizal Trees

Actinorhizal plants are woody, dicotyledenous, perennial, angiosperms, spread over eight plant families representing 22 genera (Table 2.1). These plants are nodulated by the filamentous bacterium, *Frankia,* belonging to the family *Frankiaceae*. The term Actinorhiza is given to root nodules that are formed by *Frankia*.

Actinorhizal plants are most valuable in forestry, agriculture, environment and land reclamation. Species of alder are commonly used as pioneer plants in the regeneration of wasted lands. They are used as windbreaks, for pulpwood, timber and fuel wood. *Casuarina* species are used as high quality fuel wood, windbreaks in slope stabilization and as ornamental trees. Other actinorhizal plants have uses in the human diet (*Shepherdia, Elaeagnus, Hippophae)* and as forage for livestock

(*Ceanothus* and *Purshia*). They are also valued for landscaping, provide shade and contribute to the beautification of parks and cities. Actinorhizal plants are able to colonise very different ecosystems ranging from those with organic soils to dry sand dunes. They lend themselves as pioneer species in regenerating lands damaged by flooding, fires, glacial activities, landslides, etc. A well developed root system favors soil binding capacity and the nitrogen fixing capability greatly enhances the quality of impoverished soils.

2.1.2 The *Frankia* Sp.

The name *Frankia* was given by Brunchrost, in 1886 to honor his mentor, A. B. Frank, who coined the word symbiosis (Lechevalier and Lechevalier, 1989). Though the nodules of non legumes were first described in 1829 and nodule formation by an endophyte was demonstrated in 1866, the actinorhizal plants were not considered to be important agriculturally. It was only in 1950s that studies relating to actinorhizal plants began in right earnest. In 1978, the first pure cultures of *Frankia* were available (Callaham *et al.*, 1978). Since then there have been many reports of endophyte isolation from different host species. The genus *Frankia* is now recognized for its formation of nitrogen fixing nodules on suitable host species and its typical morphological and physiological features. The followings are the main features used in the identification of *Frankia* (Lechevalier, 1983; Lechevalier and Lechevalier, 1989):

TABLE 2.1: LIST OF SOME REPRESENTATIVE ACTINORHIZAL PLANTS

Family	Genera
Betulaceae	*Alnus*
Casuarinaceae	*Casuarina, Allocasuarina*
Coriariaceae	*Coriaria*
Datiscaceae	*Datisca*
Elaeagnaceae	*Elaeagnus, Shepherdia*
Myricaceae	*Myrica, Comptonia*
Rhamnaceae	*Discaria, Ceanothus*
Rosaceae	*Dryas, Purshia*

1. Gram positive, actinomycetes.
2. Capable of nitrogen fixation (with a few exceptions).
3. Capable of nodulating actinorhizal plants.
4. Forms sporangia, spores and vesicles.
5. Display host specificity (with some exceptions).
6. Cell Wall Type III.
7. Presence of fucose in cell membrane.
8. G+C % ranges from 66-75.
9 Phospholipid pattern belongs to P I group.
10. Major menaquinone MK 9 (H4).
11. Presence of amino acid A_2pm.
12. Presence of sugar 2-O -methyl mannose

2.1.2.1 Morphology

Frankia display typical actinomycete morphology. The bacteria produce a vegetative mycelium composed of septate filaments On the mycelium may be produced two distinct types of cells:

a) Vesicles : The vesicle is an enlarged spherical cell attached to the substrate mycelium by a stalk cell. Within the cytoplasm of the vesicle, septations may occur that completely or more usually incompletely divide the cell into compartments. The function of these septa is not understood, since fully functional vesicles have been observed without them. Although vesicles traditionally have been thought to serve one function i.e. that of nitrogen fixation,

recent studies appear to indicate that the vesicle may also serve as a propagule, (Torrey, 1985; Zhongze and Torrey, 1985).

b) Spores : The second specialized cell produced by *Frankia* is the spore. Spores are produced in large numbers within the amorphous sporangia. Spores are non-motile and display variable rates of germination. Sporangia may be produced as terminal appendages of the vegetative mycelium or in some strains may occur as intercalary structures within the mycelium. They are 20-60 μm in length and attached to the submerged filaments by thickened sporangiophores, (Diem *et al.*, 1983).

Morphology of *Frankia* within the host cell is similar to that observed *in vitro*. A vegetative mycelium is present in all infected cells. Vesicles are produced in large numbers when nitrogen fixation is high but morphology of the vesicles may be significantly modified by the host. When frankiae occur in plants, they are filamentous and usually surrounded by a host cell membrane and cell wall like material called capsid. Nodules that contain sporangia are referred to as sp^+. Other nodules with few or no sporangia are called as sp^- (the most common type). The sp^+ character is reported to be transmissible since crushed nodules of the sp^+ type when used as innocula for nodulation, produce only sp^+ phenotype. This particular kind of phenotype is difficult to culture (Normand and Lalonde, 1982; Torrey, 1987; Simonet *et al.*, 1994).

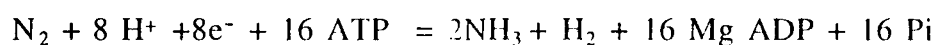
2.1.2.2 Isolation and culture

Attempts at isolating and cultivating *Frankia* in pure culture date back to the late 1800s when interest in symbiotic microorganisms was high. Numerous workers claim to have successfully isolated this filamentous bacterium but none could demonstrate clearly the consistent reinfection of the host plant. In 1978, Callaham *et al.* successfully isolated *Frankia* from the actinorhizal genus *Comptonia*. Since then, large number of successful isolations have arisen and at present many *Frankia* strains are held in collections around the world. Despite this rapid advance, isolation of *Frankia* is still problematic for some actinorhizal genera. This is because of the slow growth, the high variability and requirements of ill defined growth conditions. All cultivation protocols for *Frankia* employ nitrogen deficient media to select against nondiazotrophs. Isolation methods in routine use are 1) Microdissection, (Diem and Dommergues, 1983) 2) Serial filtration, (Benson, 1982). 3) Density gradient centrifugation (Baker and O'Keefe, 1984). Several studies have concentrated on the optimization of growth conditions for the actinomycete (Baker and Torrey, 1979; Baker *et al.*, 1979; Zhongze and Torrey, 1985 ; Ganesh, 1993 and Selim *et al.*, 1996).

2.2 NITROGEN FIXATION IN *FRANKIA*

The task of nitrogen fixation in all diazotrophs is performed by the enzyme, nitrogenase. All known nitrogenases are oxygen sensitive iron - sulphur proteins. The catalytically active moiety

consists of two subunits: Component 1, also termed the dinitrogenase, is a complex of two α proteins (50000 daltons each), two β subunits (60000 daltons each), 24 molecules of iron, 2 molecules of molybdenum and an Iron, molybdenum cofactor often called as FeMoCo. Component 2, known as the Fe protein or the dinitrogenase reductase, is a complex of Iron and two α subunits (32000 daltons each). The molecular weights of the subunits may vary in different organisms (Schwintzer and Tjepkema, 1990). The reduction of nitrogen involves combination of component 1 and 2, with Mg^{++} ATP and a source of reducing equivalents.



$$N_2 + 8\,H^+ + 8e^- + 16\,ATP = 2NH_3 + H_2 + 16\,Mg\,ADP + 16\,Pi$$

Apart form nitrogen, nitrogenase can also reduce acetylene, methyl isocyanide, azide, N_3^-, N_2O, HCN and a host of other substrates.

Mode of Action of nitrogenase

Nitrogenase reduces dinitrogen in a series of steps which involve repeated binding of component 1 and 2 with other proteins. The entire process is thought to be completed in 7 cycles each of which consists of the following steps:

1. The Fe protein is reduced by a Ferredoxin (Reduction of Fe protein).

2. Mg ATP binds to the α subunit of the Fe protein and makes it a more reactive reductant (Activation of Fe protein by ATP).
3. Electron transfer between the two nitrogenase components occurs.
4. Binding of the reducible substrate takes place.
5. Formation of the enzyme complex occurs.
6. Electron transfer to the substrate takes place.
7. Release of the reduced product, ADP and inorganic phosphate occurs.

Concomitant with the reduction of N_2, Hydrogen is also evolved. This process is unidirectional, wasteful of energy and reducing equivalents. Some systems possess an uptake hydrogenase (*hup*) which scavenges the hydrogen and offsets the energy and reductant wasted (Dean and Jacobson, 1992).

Other N_2 fixing complexes that are structurally related to but genetically distinct from the Mo dependent nitrogenases, have recently been recognized. These have catalytic components that are analogous to the Fe protein and the Mo Fe protein. A major difference in these N_2 fixing systems appears to reside within the metal composition of their respective cofactor species. Most possess Vanadium. Interestingly, all of them share some gene products that are required for the maturation of their respective catalytic components.

The primary products of genes encoding the nitrogenase structural components (*nif* H for Fe protein subunit, *nif* D MoFe protein α subunit and *nif* K for MoFe protein β subunit) are not catalytically active. Rather the immature nitrogenase components are processed to active forms through the action of other nitrogen fixation specific (*nif*) genes. All these genes and their functions are enlisted in Table 2.2. There are also *nif* specific regulatory elements that are responsible for activation or inhibition of *nif* gene expression under appropriate physiological conditions. About 20 different *nif* specific genes have been discovered in *K. pneumoniae*, which are divided into 8 transcriptional units. The organization of the *nif* genes in *Frankia* is understood to be similar to that in *K. pneumoniae* (Normand *et al.*, 1988).

2.3 SYSTEMATICS OF *FRANKIA*

Frankiae are typical actinomycetes in terms of the overall physical properties of their genome. The G+C content of *Frankia* DNA (66-75%) is within the range reported for other actinomycetes (An *et al.*, 1985). The genome molecular weights of *Frankia* strains ArI4 and EuI1 as determined by reassociation kinetics analysis are 8.3 X 10^9 and 6.0 X 10^9 respectively (An *et al.*, 1987). Analysis of 16S ribosomal RNA genes of two *Frankia* strains establishes a clear phylogenetic relationship between *Frankia* and the non symbiotic soil actinomycete *Geodermatophilus* (Hahn *et al.*, 1988).

The uniform morphology and growth characteristics of *Frankia* strains in culture gives no hint of the extent of genetic

Table 2.2: *nif* SPECIFIC GENES AND THEIR PRODUCTS
(based on Dean and Jacobson, 1992)

S.No.	Gene	Product and Function
1	*nif* H	Fe protein subunit
2	*nif* D	MoFe protein α subunit
3	*nif* K	MoFe protein β subunit
4	*nif* F	Flavodoxin, physiological reductant of the Fe protein
5	*nif* J	Pyruvate-flavodoxin -oxidoreductase, couples the oxidation of pyruvate to the reduction of flavodoxin
6	*nif* M	Required for activation of the Fe protein
7	*nif* U	Appears to be involved in the stabilization of the Fe protein
8	*nif* S	Function unknown,
9	*nif* V	Probably encodes a homocitrate lyase.
10	*nif* E	Required for FeMo cofactor biosynthesis.
11	*nif* N	-do-
12	*nif* B	-do-
13	*nif* Q	-do-
14	*nif* W	required for full activity of the MoFe protein
15	*nif* Z	-do-
16	*nif* A	positive regulatory element
17	*nif* L	negative regulatory element
18	*nif* X	probably a negative regulatory element
19	*nif* T	Function unknown
20	*nif* Y	Function unknown

diversity that exists within the genus. Hybridization studies revealed a lot of divergence (Fernandez *et al.*, 1989). On an even smaller geographical scale, total protein patterns (Prin *et al.*, 1991, Gardes and Lalonde, 1987), some enzymes (Girgis and Schwencke, 1993), sugars (ST-Laurent *et al.*, 1987), restriction pattern analysis of total DNA (Bloom *et al.*, 1989) and plasmid profiles (Normand *et al.*, 1983) have revealed diversity in *Frankia* isolates from single host plants (Simonet *et al.*, 1989) and in some cases from single nodule lobes (Reddell and Bowen, 1985b). Despite the overall diversity among *Frankia* isolates individual genotypes generally appear to be stable both in culture and in soil environment.

The symbiotic genes of *Frankia* have only been recently subject to study. Structural genes coding for the nitrogenase complex enable to understand the evolutionary origin of the actinomycete. Many other conserved genes like the 16S rRNA, leghemoglobin, *nod* genes are also a subject of intensive study. Several factors have interfered with progress toward understanding the genetics of *Frankia* symbiosis. Symbiotic mutants are not available for study because of the difficulty in generating such mutants and a transformation system suitable for use with *Frankia* has not been identified or created. Until these tools are routinely available or created, progress will continue to be painfully slow.

2.3.1. Molecular Biological Methods In Identification Of Strains.

Modern molecular biological tools, especially DNA sequencing (Sanger *et al.*, 1977) and Polymerase Chain Reaction (Mullis *et al.*, 1986), have revolutionized all areas of biology and medicine. Especially bacterial phylogeny has been greatly transformed. Sequencing has enabled an easier, reliable and precise interpretation of phylogenetic data. Polymerase Chain Reaction (PCR) is a technique that enables the generation of large amounts of genetic material from a slight trace which would otherwise be too small to study. The technique involves extraction of nucleic acids of sufficient purity which are amplified by universal or specific primers. The PCR product is either cloned to generate a sequence library or cycle sequenced to arrive at the requisite sequence information. Automated DNA sequencing methods have facilitated the rapid screening of large gene libraries. Initial screening of the target gene containing clones by restriction fragment length polymorphism can reduce the number of DNA samples that need to be completely sequenced. Complete sequencing of the DNA is facilitated by the presence of conserved sequence domains throughout the molecule allowing primers to be designed that permit the sequencing of the entire gene in a stepwise manner. Once a sequence database has been generated from the clone library, phylogenetic analysis can be carried out and the diversity of the microbial population can be determined

with relation to previously published sequences, (Maidak *et al.*, 1994).

Phylogenetic relationships are routinely analyzed by screening for certain model genes. Termed as molecular clocks, these are ubiquitously present in all organisms and are indispensable for the cell. They have a large size which ensures storage of ample information. Rates of changes in the sequences of such genes are indicative of the rate of evolution. Among the well studied molecular clocks are the 16S rRNA genes (Woese *et al.*, 1983; Woese, 1987), cytochrome C gene, *nif* genes in nitrogen fixers, hemoglobin genes, and *rbc*L genes in plants. 16S rRNA genes are the most popular among these as they are ubiquitously present in almost all life forms. The basic structure of RNA molecules comprises of conserved domains alternating with regions that vary considerably in size and sequence (Gutell *et al.*, 1994). Conserved regions allow identification of universal tracts of sequences in related organisms. Variable domains permit discrimination at different taxonomic levels.

2.3.2 The 16S rRNA Genes As A Taxonomic Tool

The initial sequencing of *rrn* operon of *Frankia* DNA was achieved by using universal prokaryotic primers. The site where the pairing of the mRNA to the small subunit of ribosome takes place is highly conserved. Primers FGPS 849 and 1176' have been designed from this region and have enabled the amplification of the required 325 bp fragment in *Frankia*, (Nazaret *et al.*, 1991).

This fragment was found to be same in length in different *Frankia* strains. Simonet *et al.* (1991) selected a variable region in the rRNA operon and designed primers FGPS 958 and 1093'. These were found to be sufficiently specific for *Frankia* and did not amplify DNA from other bacteria. However they were unable to distinguish between *Geodermatophilus* and *Frankia*. This was later achieved by the work of Bosco *et al.* (1992) who designed primer FGPS 989, located in the helix 31 of domain III of the 16S rRNA gene.

ITS regions of *rrn* operon tend to amass more variability and often reflect strain specific differences. Primers designed in this region are useful for characterization of strains. Simonet *et al.* (1991) designed two primers to amplify the ITS in *Frankia*. They found that the size of the amplicon was conserved in several strains. Mirza *et al.* (1994) studied the rRNA region in many *Coriaria* and *Datisca* isolates and could construct specific primers for identification of such strains. Harry *et al.* (1991) did a partial sequencing of 16S rRNA gene in several strains and discovered variabilities in two distinct segments (140-220 and 980-1060 nt according to the *E. coli* numbering).

The organization of the 16S rRNA operon itself was studied by Normand *et al.* (1992a) who analyzed the gene in *Frankia* strains ORS020606 and Ag/Mut-15 and compared it with the corresponding gene of *Streptomyces*. The length of the 16S rRNA gene was found to be 1513 nt, that of the 23S rRNA 3099 nt and that of the 5S rRNA gene 200 nt. In the entire operon, the 16S rRNA gene was the most conserved especially at the 3' end. Two

potential promoters upstream from the 16S rRNA gene were detected and downstream from the 5S rRNA a *rho* independent terminator was thought to be present. ITS1 that between 16S and 23S genes, was 411 nt and ITS 2, that between 23S and 5S genes, was 68 nt long. In *Casuarina* infective strain ORS020606, only two operons for the 16S gene were found, which explains the slow growth rate of *Frankia*. Sequence homology was found between the gene of the said strain and that of *Alnus* infective strain Ag/mut-15.

Normand *et al.* (1996), analyzed the complete 16S rDNA sequences of a number of *Frankia* strains and divided all the known *Frankia* strains into four groups, namely: 1) A large group comprising *Frankia alni* and related strains (including *Alnus rugosa* Sp+ microsymbionts), 2) unisolated microsymbionts of *Datisca, Coriaria and Dryas* species, 3) *Elaeagnus* infective strains and 4) "atypical" strains (a group that includes an *Alnus* infective non-nitrogen fixing strain).

Honerlage and coworkers studied the 23S region for insertions (Honerlage *et al.*, 1994) and discovered that the 23S region is more variable than the 16S region. A large insertion in the domain III has been found to be specific for the genus *Frankia*. The size of this insertion varies in different species. By sequencing this insertion, the authors were able to divide the genus into seven groups.

A big stumbling block in the study of *Frankia* has been taxonomy. Absence of concrete data and the enormous diversity

of the genus have compounded the ambiguity in the field. Though Lahn *et al.* (1988) attempted a partial nucleotide sequencing of 16S rDNA and emended the family *Frankiaceae*, this was later disputed by some groups. Further efforts in this direction led to the construction of a preliminary phylogenetic tree (Ganesh *et al.*, 1994). Subsequently, 16S rRNA genes from a comprehensive set of *Frankia* strains were completely sequenced (Normand *et al.*, 1996). All known *Frankia* isolates could be delineated into four distinct groups. These groups were found to differ in their host compatibilities as well. Atypical *Frankia* strains could be safely demarcated to a separate category.

2.3.3 The Nitrogenase Genes As A Taxonomic Tool

nif genes provide additional information to the phylogenetist. This information is more discriminative as the *nif* genes are present in only nitrogen fixers. RFLPs, AFLPs, and DNA sequencing can quickly provide valuable information for strain characterization. In case of *Frankia*, Nick *et al.* (1992) have used this approach for analyzing the *nif* H-D IGS in *Coriaria* infective isolates and developed specific oligos for their characterization. Jamann *et al.* (1993) addressed the problem of strain characterization by using the same technique for typing *Elaeagnus* infective *Frankia* strains. Primers FGPD 807 and FGPK 700' were designed for amplification of the *nif* D-K IGS region. The PCR products were then cleaved by ten 4-base cutting enzymes. The restriction patterns thus obtained allowed the separation of all

the strains into several distinct genomic groups. The estimated data correlated well with the established taxonomic schemes.

A similar exercise conducted by Rouvier *et al.* (1996) enabled a detailed analysis of a number of *Casuarina* infective strains. DNA extracted directly from nodules was amplified using primers specific for the *rrn* and *nif* regions. The amplified products were cut by different restriction enzymes. Five different groups could be recognized on the basis of the restriction patterns. While Fernendez *et al.* (1989) had found a single genomic category for *Casuarina* infective strains, Rouvier *et al.* (1996) demonstrated clear genetic diversity among the same.

The complete sequence of *nif* H was determined from *Frankia* strain HRN18a (Normand and Bousquet, 1989). The open reading frame was found to be 870 bp long and encodes a polypeptide of 270 amino acids. *nif* H was also sequenced from ArI3 (Normand *et al.*, 1988). The amino acid sequence similarity between this sequence and the corresponding sequence from *Frankia* strain HRN18a was found to be 96%. *nif* H was found to be conserved in many nitrogen fixing organisms. *nif* D was sequenced in *Frankia* strain ArI3 (Normand *et al.*, 1992b). The protein coding region was found to be 1458 nt long and encodes a polypeptide of 486 amino acids. A tetranucleotide 8 bp upstream from the ORF provided a ribosome binding site. The length of the IGS between H and D genes was found to be 49 nt. long. The sequence of *nif* D was found to be highly conserved in different nitrogen fixing organisms. The codon usage was found to be highly skewed

towards G or C ending codons. Nalin *et al.* (1995) studied *nif* D-K IGS in *Frankia* strains ArI3 and ACoN24d and found the region to be 265 nt and 199 nt long in the respective strains. They showed that the IGS had no homology with any known DNA sequence. Alignment of sequences from three strains EUNIf, ArI3 and ACoN24d with those other nitrogen fixing bacteria permitted detection of a sequence conserved in *Frankia* but absent in other bacteria. This group also sequenced the *nif* K gene and found it to be 1587 nt long, encoding a 520 amino acid polypeptide. Downstream from *nif* K, an ORF of 38 amino acids, probably coding for *nif* E gene was detected. *nif* K gene sequencing was also done by Hirsch *et al.* (1995) from *Frankia* strain HFPCcI3.

Recently, the organization of *nif* genes was further explored by Harriott *et al.* (1995). They sequenced the entire *nif* region containing *nif* X, W, Z, B and the two adjoining ORFs and reported the absence of *nif* A, known to be present in *Rhizobium* and other nitrogen fixing bacteria.

2.4 HOST - MICROSYMBIONT RELATIONSHIP

The specificity of strains to nodulate certain host plants has been observed since the first *Frankia* strain was isolated. The affinities of pure cultured strains differed significantly from relationships observed using suspensions of crushed root nodules. As a result taxonomy of *Frankia*, which has included specific epithets based on host specificity, has been invalidated (Lechevalier and Lechevalier, 1984). Current belief among *Frankia*

researchers is that host specificity should not be used to define taxa, however there is a strong correlation between host specificities of strains and genetic similarities.

From those *Frankia* strains that have been isolated in pure culture, three or four major host specificity groups can be defined- *Alnus*, *Casuarina* and related strains, *Elaeagnus* group, unisolated strains from *Datisca, Coriaria* and atypical strains (Normand *et al.*, 1996). Additional host specificity groups may be defined in future as strains from *Rhamnaceae*, *Rosaceae*, etc. are isolated and characterized.

Factors that contribute to the commencement of symbiosis may have their origin in the host as well as in the microsymbiont. The physiology of both the partners is a key parameter that must be thoroughly understood The *Frankia*-host alliance is rooted on necessity. The plant keeps a ready supply of carbon and energy under low O_2 tension and the microsymbiont ensures that the plant does not have to look for nitrogen elsewhere.

Signaling molecules secreted by the host and *Frankia* bring about the initiation of symbiosis. Host root exudates regulate *Frankia* genes concerned with nodulation. Specific phenolics may also mediate compatibility between *Frankia* and Actinorhiza. Prin and Rougier (1987) reported that while culture filtrates of *Frankia* had no effect on growth of axenic roots of alder, filtrates from cultures incubated with host root exudates caused extensive root hair deformation. Mucilage secreted by host may also facilitate

bacterial colonization. Binding of *Frankia* to host cell exterior may also trigger host responses. Extra cellular polysaccharides of host origin may provide a substrate for *Frankia* enzymatic activity. Wall degrading enzymes produced by *Frankia* (Seguin and Lalonde, 1989) may help soften host cell walls. The invasion of the microbe itself seems to be delicately controlled, host defense response being under tight regulation. The host cell becomes meristematic and restricts the guest to an extracytoplasmic compartment. Subsequently the bacterium is enclosed in a membrane envelope. This extracytoplasmic space defines a compartment where the microorganism can function as an organelle and the host can organize all the needful for the process of nitrogen fixation. Leghemoglobin and nodulin genes are some of the host genes inductively expressed during the period of nodule formation.

In the endosymbiotic state, the microsymbiont is transformed into a form known as the bacteriod which performs various unique functions in a close co-operation with the host plant and thus behaves practically as an organelle. Though not obligately dependent, the microbe does rely on the plant for several processes. Bacteriod genes exhibit derepression not only of nitrogenase genes but also of those for hydrogenase and specific cytochromes and genes responsible for changes in the outer membrane (Sutton *et al.*, 1981). Metabolism of ammonia is altered as well. Once inside the cell, the bacterial division and differentiation is further influenced by the host. The two partners share several structural components like the peribacteriod

membrane and exchange a number of metabolites too. Thus the microsymbiont can be said to be evolving towards a nitrogen fixing organelle, the ultimate association between the two organisms making them dependent on one another.

2.4.1 Host Specificity

Host specificity is the key which drives the association between *Frankia* and host towards fruition. Some special factors elicited by both the partners have a pivotal role in bringing about the required transitions. Can such unique factors be identified?

Work has already begun in this direction. To arrive at concrete answers, it would be essential to take recourse to molecular biology. Phylogeny of the host in molecular terms needs to be worked out just it has been done in the case of *Frankia*. 16S rRNA sequencing of host chloroplast DNA has already started. An additional phylogenetic tool is the *rbc*L gene. The chloroplast gene encoding the large subunit of the enzyme ribulose 1,5 bisphosphate carboxylase is the enzyme ubiquitously present in all plants and is sufficiently conserved to aid in the elucidation of phylogenetic relationships. Bousquet *et al.* (1992) estimated the phylogeny of the family *Betulaceae* by this technique and found it to be in complete agreement with morphological data and ribosomal DNA ITS sequence data (Savard *et al.*, 1986). *rbc*L gene sequence comparison has been done for many actinorhizal families (Soltis *et al.*, 1995). Preliminary analysis reveals that the diverse actinorhizal hosts harboring *Frankia* are actually closely

related in their *rbcL* sequences. The degree of DNA homology between the different genera varies from 96% to 99% (Maggia and Bousquet, 1994).

Many studies on this aspect are still on and large gaps persist. Preliminary reports by Prat (1989), has revealed that host does have a role in determining the efficiency of symbiosis. More recently Sougoufara *et al.* (1992) experimented with a series of *Frankia* strains and plant host clones and discovered that a particular *Frankia* strain was always a better nitrogen fixer whatever the host clone used. The host effect predominated over all other effects. Sougoufara *et al.* (1992) predicted that improvement of nitrogen fixing ability could be achieved through a two step procedure, starting by screening for the best host clone and subsequently the *Frankia* strain associated with the best clone. Such approaches should significantly facilitate the identification of the best clone X *Frankia* combination.

These and other reports have pointed at the role the host plays in selecting the microsymbiont and later on in determining the progress of the symbiotic relationship inside its cellular milieu. The precise nature of the host-symbiont relationship is intriguing. Is it purely a one-man show? Or is it an equal-equal partnership? Or is there a third dimension in which one partner dominates while the other chooses to play a subdued but significant role?

The answers to these questions are slowly unfolding. Research on nitrogen fixing symbioses has come of age. The cumulative work on *Frankia*-Actinorhiza, legume-*Rhizobium* and other symbiotic systems is ample proof.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

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3.1 COLLECTION OF NODULES

For this study, a natural forest stand in upper Shillong region was selected. This region consisted of mixed forest of *Alnus nepalensis* and *Pinus kesiya* (Fig 3.1a). Other trees that were naturally present were *Myrica* and *Elaeagnus*. Vegetation around the area was marked off. A total of thirteen trees of *Alnus nepalensis* were randomly selected. Nodules were collected during the month of May. Care was taken to see that all factors that could affect the nitrogenase activity of the nodules were constant (e.g. soil, light availability, weather, etc.). The soil was dug to reach the nodulated roots. To avoid any confusion, arising out of the presence of other roots, often leguminous in nature, nodulated roots were traced to the tree of their origin. Ten nodules were taken from each tree. Care was taken to collect only young nodules. Nodule, morphology, color and depth were noted. The nodules thus collected (Fig. 3.1b) were placed in moistened polythene bags and brought to the laboratory on the same day.

3.2 ASSAY FOR NITROGENASE ACTIVITY

Nitrogenase activity was measured by the Acetylene Reduction Assay (ARA) method of Stewart *et al.* (1968). The method relies on the ability of nitrogenase enzyme to reduce acetylene to ethylene. Acetylene and ethylene both can be assayed

Figure 3.1

Fig 3.1 : A Natural Forest Stand Of *Alnus nepalensis* And Nodules Produced By It

a) Trees of *Alnus nepalensis* at the site of study.
b) Nodules produced by *Alnus nepalensis*.

a

b

simply by a gas Chromatograph. For conducting ARA, nodules were collected early in morning and brought to the laboratory in plastic bags. They were washed in running water and detergent to remove adhering soil. This was followed by a brief sterilization with 15 % H_2O_2. The nodules were then washed several times with water. Each nodule was then dried with filter paper and weighed. It was placed in a sterile glass vial fitted with a rubber septum stopper. The vial was closed and sealed with parafilm. 10% volume of the vial air was withdrawn with a syringe and replaced with pure acetylene. Controls without acetylene were also used. The vial was incubated at 37°C for 3 1/2 hours. Using a gas tight syringe, 1 ml of the gas mixture was injected into a Tracor gas Chromatograph, which was fitted with a Porapak T (80-100 mesh) column (6 ft. X 0.125 in.). The injection port, column and detector temperatures were 100. 70 and 120°C respectively. The carrier phase was nitrogen. The flow rates of H_2, air and N_2 carrier gas were 20, 120 and 30 ml/min respectively. Before injecting the sample, the column was standardized with standard ethylene and acetylene. Peak height for 1 nmole of ethylene was calculated. Under the given conditions, ethylene was found to have a retention time of 2 min while acetylene had a retention time of 3.8 minutes.

For each nodule, readings were taken in triplicate. The nitrogenase activity was calculated as follows-

If nitrogenase activity in nmoles of ethylene produced per mg fresh weight of nodule per hour is denoted by NA, then

$$NA = \frac{\text{Peak height of 1 nmole of ethylene x Peak height of sample}}{\text{Incubation period in hours x fresh weight of nodule in mg}}$$

After ARA measurements the nodules were stored in 70% alcohol at -20°C for further use.

Isolation of endophyte:

Nodules, which were found to have high nitrogenase activity, were chosen for the isolation of endophyte. Root nodules were detached into single lobes and sterilized with 30 % H_2O_2. This was followed by 5-6 washes with sterile water. The lobes were peeled in sterile PVP-PBS. Single lobes were put into flasks containing 20 ml of DPM (Baker and O'Keefe, 1984, Appendix 1). The nodule lobes were crushed on the walls of the tubes. The tubes were incubated at 28°C for a period of 2 months without agitation. They were examined periodically and those showing contamination were discarded. The tubes which showed suspected frankiae were saved and examined for the presence of the actinomycete.

Reference strains and *Frankia* isolates:

Reference strains of *Frankia* and Laboratory isolates were used in the present study. All were cultured at 28°C in DPM (Baker and O'Keefe, 1984, Appendix 1). The list of reference strains is given in Table 3.1.

TABLE 3.1: BACTERIAL STRAINS USED IN THIS STUDY AND THEIR SOURCES

	Bacteria	Strain	Source
1.	*Frankia* sp.	ACN1AG	Lyon, France
		ARgP51AG	Lyon, France
		AnpST11	Shillong, India
2.	*Rhizobium* sp.	Rmd1021	IARI, New Delhi
3.	*E. coli*	HB101	IARI, New Delhi

Rhizobium meliloti strain Rmd1021 was grown in TYS and *E. coli* strain HB101 was grown in LB medium, (Appendix 2).

3.2 EXTRACTION OF NUCLEIC ACID

3.2.1 Isolation Of DNA From Nodules

The procedure of Rouvier *et al.* (1996) was used. Care was taken to avoid any exogenous DNA during DNA extraction. All solutions were autoclaved and sterile gloves were used for handling DNA.

Previous methods used for *Frankia* DNA extraction from root nodules relied on the use of a mixture of Lysozyme and achromopeptidase for effecting lysis, Simonet *et al.* (1988b). Bosco *et al.* (1996) preferred the use of thermal shocks in combination with a detergent in their procedure. Giasson and Lalonde (1987) achieved the same using hot alkali in combination with SDS. Benson *et al.* (1996) standardized the procedure for obtaining DNA from a single hypha. Besides these methods, PCR can also be done without DNA isolation. This can be accomplished by analyzing an aliquot of a crushed nodule suspension (Simonet *et al.*, 1988a) or by doing an *in situ* PCR of protoplasts (Hodson *et al.*, 1995). All these procedures were tried in the present study.

Nodule DNA extraction is plagued by the presence of phenolics which not only impede extraction but also amplification. Some workers have used poly-vinylpyrrolidone to precipitate phenolics (Rouvier *et al.*, 1996). The use of ascorbic acid also decreases the oxidation of phenolics (Benson *et al.*, 1996). Following is the method used for DNA isolation in the present study:

1. Nodules were washed several times with detergent and water. They were then sterilised with 30 % H_2O_2 and rinsed several times with water.
2. A single nodule was selected and outer layers were removed in sterile PVP-PBS or TEA (Appendix 1). In case of use of TEA,, nodules were washed several times in this buffer.
3. Each lobe was crushed in 300 µl of extraction buffer (100 mM Tris, 20mM EDTA, 1.4M NaCl, 2% [wt/vol.] polyvinyl pyrrollidone) in a 1.5 µl eppendorf tube.
4. The homogenate was incubated at 65°C for 30 min and then treated with 10 µl of 20% Sodium dodecyl sulphate.
5. The tube was kept at 4 °C for an additional 10 min.
6. It was centrifuged at 1200 g for 5 min.
7. The supernatant was extracted with equal volume of chloroform : isoamyl alcohol 24:1.
8. The aqueous phase was transferred to a fresh tube and the DNA precipitated with two volumes of ice cold absolute ethanol.
9. The tube was kept at -20°C for an hour and the DNA pelleted by centrifugation at 13000 g for 30 min.
10. Pellet was washed with 70% alcohol and vacuum dried.
11. DNA was dissolved in 10 µl of ultra pure water and stored at -20 °C till further use.
12. The preparation was run on a 0.8% agarose gel to check for purity.

3.2.2 Isolation Of DNA From Bacterial Cultures

Extraction of DNA from bacterial cultures can be achieved in the same manner as done for nodules except for the use of PVP (Bosco *et al.*, 1996). In the present study, a modification of the procedure of Simonet *et al.* (1984) was used. The method involved following steps:

1. Bacterial suspension was pelleted by centrifugation at 1200 g for 10 min.
2. The pellet was resusupended in 1ml of TE8 buffer (Appendix 2). The suspension was rapidly forced through a sterile, 1 ml syringe to break the colonies.
3. Lysozyme was added at a final concentration of 0.5mg/ml and the tube incubated at 37°C for 30 minutes.
4. 150 µl of 20% SDS was added and the whole mixture divided into two tubes. To each tube, 625 µl of buffer saturated phenol was added. The tube was inverted gently thrice.
5. This was followed by centrifugation at 1200 g for 5 minutes.
6. The aqueous phase was transferred to a new tube. Equal volume of chloroform was added and the tube was inverted gently four or five times.
7. The tube was centrifuged at 1200 g for 5 minutes at 4°C.
8. The aqueous phase precipitated by the addition of two volumes of ice cold absolute ethanol.
9. The DNA was pelleted by centrifugation at 4°C for 30 minutes at 13000 g. The DNA pellet thus obtained was washed with 70% alcohol and vacuum dried.

10. DNA was dissolved in 10μl of ultra pure water.

3.3 DNA AMPLIFICATION :

Double stranded DNA amplification was performed by using the procedure of Simonet *et al.* (1991). Primers used corresponded to the *nif* H-D, *nif* D-K IGS regions, the 16S rRNA gene and 16S-23S ITS region. These primers were chosen after careful comparison of the conserved and variable regions in the *Frankia* genome from a number of strains and also from other bacteria.

PCR amplification was performed in a total volume of 25 μl in a thin wall PCR tube, using a Thermal Cycler (GeneAmp PCR2400, Perkin Elmer, USA). Extreme care was exercised to avoid any source of exogenous DNA during experiments. PCR mix was prepared in a UV hood. Sterile gloves were used during all handling of DNA. The standard PCR solution contained 1X PCR buffer (10X buffer containing 100mM Tris-HCl, pH 8.3, 500mM KCl, 15mM $MgCl_2$ and 0.01% gelatin), 125mM deoxynucleotide triphosphates (dNTPs), 0.5 mM each primer, 1μl of template DNA and 1U *Taq* DNA Polymerase (Bangalore Genei, India).

<u>Stock Primer Solutions</u>:

Primers in the lyophilized state were acquired from Bangalore Genei, India. Stock primer solutions of 25mM and working solution of 5mM were made in ultra pure water and stored at -20°C for further use.

Deoxyribonucleotide solutions:

Individual dNTPs in powder form were procured from Boehringer Mannheim, Germany. A 5mM stock solution was made in water and the pH adjusted to 8.0. From the stock, working solution containing all the four dNTPs at a final concentration of 1.25mM was prepared and stored at -20°C.

Amplifications were performed under standard conditions described by Simonet *et al.* (1991). The list of primers used is given in Table 3.2.

A total of 35 PCR cycles were run, during which the entire PCR solution was denatured at 93°C for 3 min followed by 35 cycles each of which consisted of a denaturation for 30 seconds at 93°C, annealing for 30 seconds and elongation at 72°C for 30 seconds. This was followed by a final elongation at 72°C for 4 min. PCR products were detected by using 2% (wt/vol) agarose gel electrophoresis in TBE buffer (Appendix 2) at 10 V/cm. The gels were stained in aqueous solution containing 0.5 mg /ml ethidium bromide, destained in distilled water and visualized with a Transilluminator. A *Hind*III- *Eco*RI double digest of λ DNA was used as a molecular weight marker.

Annealing Temperatures:

For each primer pair, specific annealing temperatures were used which were calculated as follows:

TABLE 3.2: LIST OF PRIMERS USED IN THE PRESENT STUDY

16 S *rrn* Operon

A . Total 16S rRNA gene

1. FGPS 6	5' TGGAAAGCTTGATCCCTGGCT 3'	(Normand *et al.*, 1996)
2. FGPS 1490'	5' AAGGAGGGGATCCAGCCGCA 3'	(Normand *et al.*, 1996)

B. *Frankia* specific distal 16S rRNA gene region

1. FGPS 989	5' GGGGTCCGTAAGGGTC 3'	(Bosco *et al.*, 1992)
2. FGPS 1490'	5' AAGGAGGGGATCCAGCCGCA 3'	(Normand *et al.*, 1996)

C. Proximal 16S rRNA gene region

1. FGPS 485	5' CAGCAGCCGCGGTAA3'	(Normand *et al.*, 1996)
2. FGPS 910'	5' AGCCTTGCGGCCGTACTCCC3'	(Normand *et al.*, 1996)

D. 16S -23S ITS region

1. FGPS 989	5' GGGGTCCGTAAGGGTC 3'	(Bosco *et al.*, 1992)
2. FGPL 2054'	5' CCGGGTTTCCCCATTCGG 3'	(Simonet *et al.*, 1991)

1. FGPS 989	5' GGGGTCCGTAAGGGTC 3'	(Bosco *et al.*, 1992)
2. FGPS.2536'	5' GAACAGCCCAACCCTTGGAAC 3'	(Normand *et al.*,1992a)

nif Operon

E. *nif* D-K IGS (*Alnus* specific)

1. FGPD 807	5' CAGTGCTACCGGTCGATGAA 3'	(Jamann *et al.*, 1993)
2. FGPK 333'	5' CCGGGCGAAGTGGCT 3'	(Nalin *et al.*, 1995)

F. *nif* H-D IGS

1. FGPH -750	5' GAAGACGATCCCGACCCCGA 3'	(Simonet *et al.*, 1991)
2. FGPD - 826'	5' TTCATCGACCGGTAGCAGTG 3'	(This study)

Annealing temperature = 4 (G+C) + 2(A+T) -5

When annealing temperatures of the two primers were different, the lower temperature was used as a general rule. However, more often than not, annealing temperatures were raised in order to increase specificity.

Typically the following temperatures were used for each primer pair:

Frankia specific 'distal 16S rRNA region using primers FGPS 989 and FGPS 1490'	-	59°C
Proximal 16S rRNA region using primers FGPS 485 and FGPS910'	-	65°C
Total 16S rRNA gene using primers FGPS 6 and FGPS 1490'	-	55°C
All *nif* region amplifications	-	55°C

Biphasic Amplification strategies

In order to increase specificity and yield of amplification, following modifications were made in the basic PCR protocol:

1. Nested PCR: Initially a standard PCR was performed, the PCR products were then investigated as targets in a second cycling phase with a new set of more specific primers annealing within the initially amplified region, (Haqqi *et al.*, 1988).

2. Booster PCR: A first set of PCR cycles was performed with a low concentration of primers followed by a second cycling phase with increased primer concentration, (Rauno *et al.*, 1989).

3. Alterations in the $MgCl_2$ concentration: $MgCl_2$ requirements varied from one primer pair to the other. Changes which ranged from a final concentration of 1.5 mM to 4 mM helped in increasing stringency.

4. Hot start PCR: PCR mix was prepared with the omission of one component. The mix was heated to denaturation for 3 minutes. Normal PCR was then resumed with the addition of the missing component.

Design of new Primer in the *nif* D region

A new primer in the *nif* D region was synthesized. This was to basically enable the amplification of the *nif* H-D IGS, which had not been done so far. This primer is complementary to the primer FGPS 807' (Jamann *et al.*, 1993). The sequence of the primer is given below:

FGPD - 826'   5' TTCATCGACCGGTAGCAGTG 3'

3.4 PURIFICATION OF PCR PRODUCTS:

PCR products that were not pure, were purified before restriction analysis. Typically a DEAE paper method (Dretzen *et al.*, 1981) for purification was chosen. The procedure is simple, efficient and yields DNA that is extremely pure. DNA molecules that are longer than 15 kb or are single stranded are not purified by this process. The method involves the following steps.

1. The DNA of interest was run on an agarose or acrylamide gel of appropriate percentage.

2. The band of interest was located with a hand held long wavelength UV lamp.
3. Using a sharp scalpel or blade, an incision was made in the gel directly in front of the leading edge of the band of interest and about 2mm wider than the band on either side.
4. Wearing gloves, a piece of DEAE paper of the same width as the incision and slightly deeper than the gel was cut. Using blunt ended forceps the walls of the incision were pulled apart and the membrane was inserted. The forceps were removed and the incision was closed making sure not to trap air bubbles.
5. Electrophoresis was resumed until the band migrated well into the membrane. The progress was followed with a hand held UV lamp.
6. When all the DNA migrated, the electric current was turned off. Using blunt ended forceps the membrane was removed. It was rinsed in the low salt buffer (Appendix 3) to dislodge any agarose sticking to the paper.
7. The membrane was transferred to a microfuge tube. Enough high salt buffer (Appendix 3) was added to cover the membrane completely. The tube was capped and incubated at 65°C for 5 minutes.
8. The fluid was transferred to a fresh tube. A second aliquot of the high salt buffer was added to the membrane and incubation repeated. The two aliquots were pooled. The membrane was checked to ensure no DNA was left on it. The membrane was then discarded.

9. The eluate was then extracted with phenol: chloroform.

10. The aqueous phase was transferred to a fresh microfuge tube. The DNA was precipitated with two volumes of ice cold ethanol and centrifuged at 13000 g at 4°C for 30 minutes.

11. The final pellet was washed with 70% ethanol and dried under vacuum. The DNA was redissolved in 5-10 μl of TE buffer.

12. The purity of the preparation was checked by loading an appropriate amount on an agarose gel.

3.5 RESTRICTION DIGESTION OF PCR PRODUCTS

Restriction endonucleases were chosen after scanning the DNA sequences with MacVector® software. Typically enzymes likely to give variability after comparison of a number of *Frankia* sequences were selected. Five microlitre of the PCR products were digested with 5U of the respective restriction enzyme in a total volume of 20 μl for 1 hour at the optimal temperature suggested by the manufacturer. The following restriction enzymes were used: *Bso*F1, *Bst*U1, *Rsa*1, *Nru* 1, (New England Biolabs, USA), *Eco* R1 and *Xho* 1 (Bangalore Genei, India). The sites and the reaction conditions for these enzymes are given in Table 3.3. Restriction fragments were separated by electrophoresis on a 4% [wt/vol.] horizontal agarose gel. Gels were run at 5V/cm and visualized with a transilluminator at 312nm. An *Eco*R1, *Hind*III, double digest of λ DNA was used as a molecular weight marker.

TABLE 3.3: RESTRICTION ENZYMES USED IN THIS STUDY

Enzyme	Target Sequence	Reaction Conditions*
Rsa 1	GT↑AC	NE Buffer 1, DNA, 37°C
*Bst*U 1	CG↑CG	NE Buffer 2, DNA, 60°C
*Bso*F 1	GC↑NGC	NE Buffer 2, DNA, 65°C
*Eco*RI	G↑AATC	Buffer B, DNA, 37°C
Xho 1	C↑TCGAG	Buffer E, DNA, 37°C
Nru 1	TCG↑CGA	*Nru* 1 Buffer, DNA, 37°C

* Composition of buffers is given in Appendix 2

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 4

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4.1 COLLECTION OF NODULES

Shillong is rich in the population of the actinorhizal genera. These trees appear interspersed with those of Khasi pine (*Pinus kesiya*) in all parts of Shillong. Especially in upper Shillong, from where the nodules were collected, forests comprised almost exclusively of trees of *Alnus nepalensis* (Fig 3.1, A). The soil of this area is rich and the moisture content is high. This probably accounted for the occurrence of large number of nodules in all trees. Nodules were of the "Alnus" type as described by Zhongze and Torrey (1985). They occurred in clusters of lobes with each lobe varying in diameter from 1 mm to 4 mm (Fig. 3.1, B). The colour was usually pale yellow to brown. They were located at depths ranging from 2-9 cm. The fresh weight of the nodules varied considerably, with some with a mass of 2 mg and some of 17 mg. On storage, they rapidly turned dark brown owing to the oxidation of phenolics.

4.2 ASSAY FOR NITROGENASE ACTIVITY

Acetylene reduction assays (ARA) were conducted on single nodules using the procedure of Stewart *et al.* (1968). These tests were done on young and visibly healthy nodules. Old nodules tended to have more dead and woody tissues. Thirteen trees were sampled in the study. Ten nodules were taken from each tree.

Trees were numbered 1 to 13. Nodules from each tree were numbered 1 to 10. Thus each nodule was assigned an individual number, for example nodule no. 10 from tree 1 was numbered 1-10 and nodule no. 5 from tree no. 3 was numbered 3-5.

The nitrogenase activities for the nodules are presented in Fig. 4.1. The nitrogenase activities for the trees 6, 10, 11, 12 and 13 were not detectable so they have not been shown here.

Nitrogenase activities for single nodules were low. These values were comparable to similar work done earlier (Normand and Lalonde, 1982; Ganesh, 1993). Many nodules did not display a detectable nitrogenase activity. Variability existed, which ranged from minor to significant differences. Pairwise comparison of ARA values from different trees was made statistically by student's "t" test ($P > 0.5$). From the data thus obtained two main conclusions could be drawn:

A) Variability between trees:

Significant variability existed in the ARA values between trees. On the basis of these differences it was possible to divide the trees into two groups - the high nitrogenase activity group (0.1-2 nmols/mg fresh weight h) and the low activity group (0-0.1 nmols/mg fresh weight hour). This is seen from ARA values of different trees (Fig 4.1). Fig. 4.2 displays the mean ARA values of trees. Each tree had its own range of nitrogen fixation rates, giving rise to the speculation that there may be a "tree" effect here.

Figure 4.1

Fig. 4.1: Nitrogenase Activities Of Nodules From Different Trees.

Ten individual nodules were taken from each tree. Nitrogenase activities were measured by the method of Stewart *et al.*, 1968. Values in cases of some trees are not presented as they were not detectable.

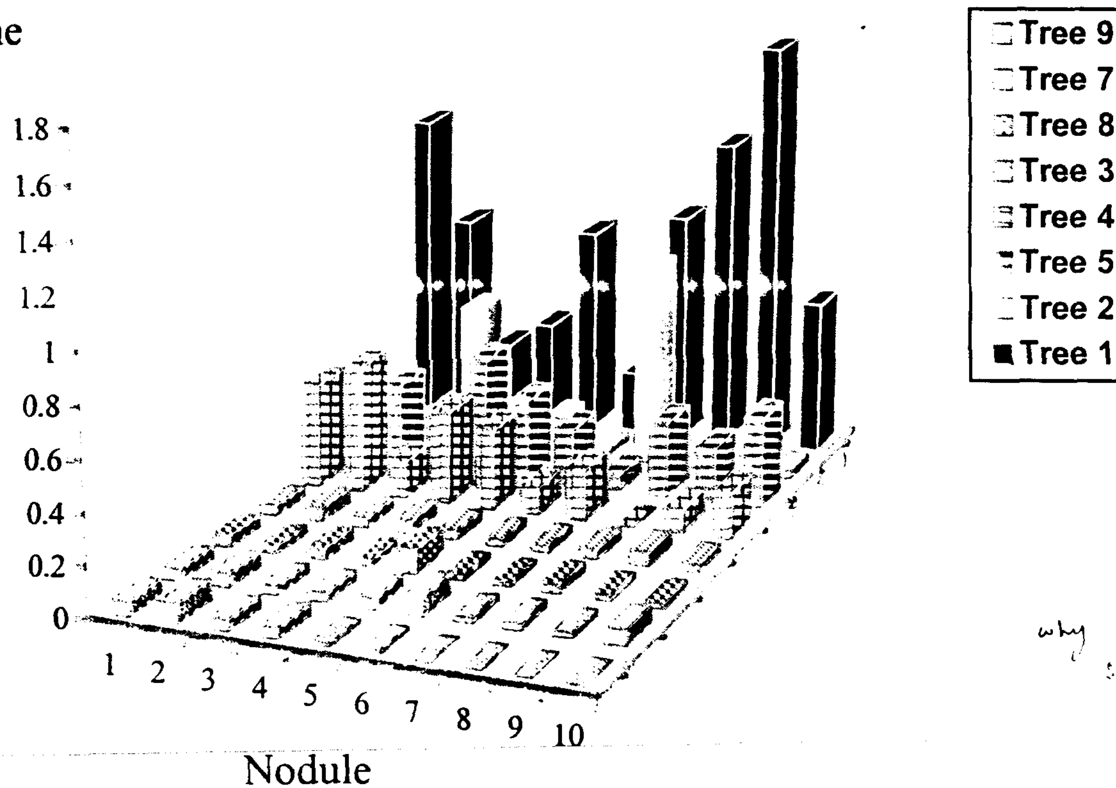


Fig. 4.1 Nitrogenase activities of nodules from different trees

Figure 4.2

Fig 4.2 : Mean Acetylene reduction in different trees.

Ten individual nodules were taken from each tree. Nitrogenase activities were measured by the method of Stewart *et al.,* 1968. Mean values were calculated for each tree. Thirteen different trees from a particular geographical site in Shillong were sampled.

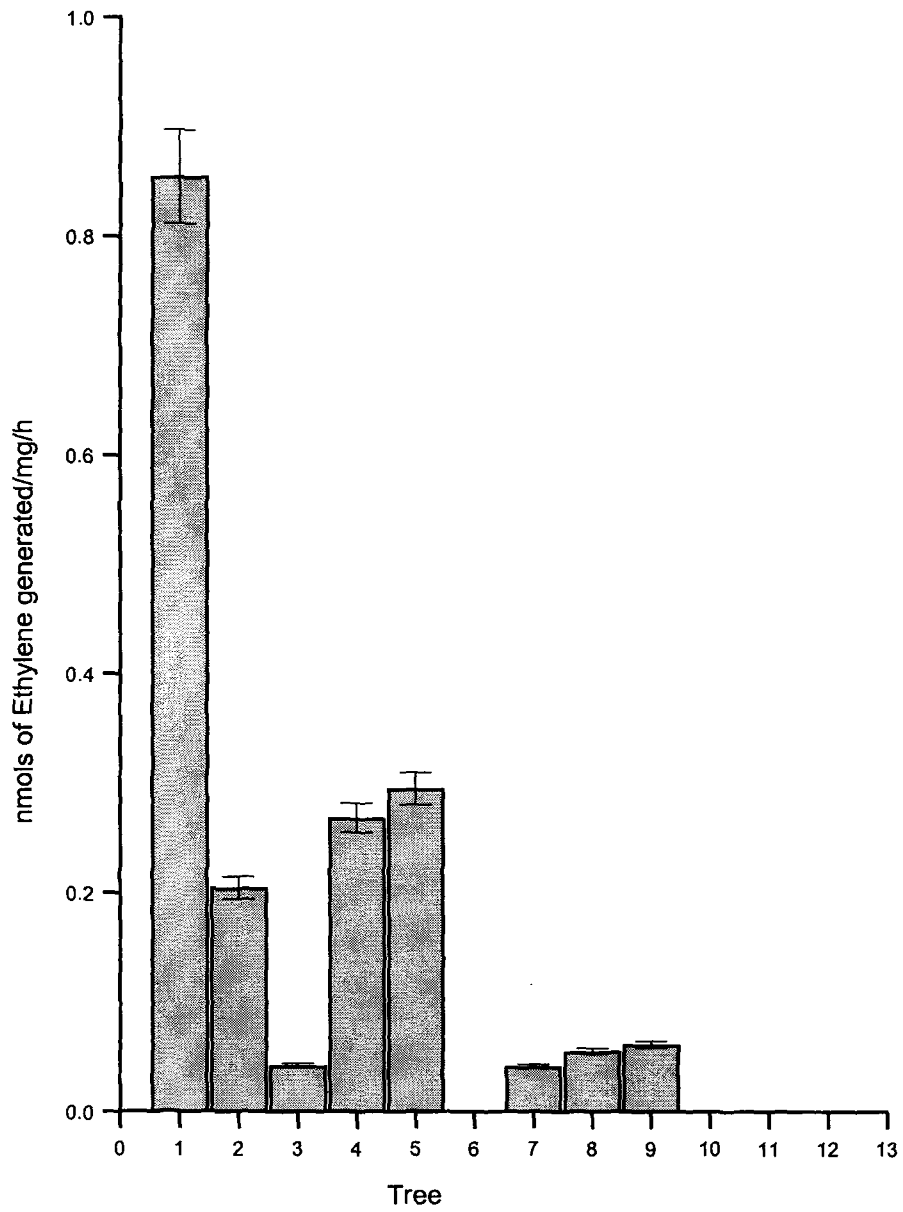


Fig. 4.2 MEAN ACETYLENE REDUCTION IN DIFFERENT TREES.

B) Variability within trees:

Variability also existed within single trees. Significant variability was found in case of trees 1, and 2. This may be due to the presence of different *Frankia* strains as other conditions including the genotype of the host were constant.

4.3 EXTRACTION OF NUCLEIC ACID

Two impediments to isolating DNA are the presence of plant phenolics and presence of contaminating bacteria in the nodule. To minimize these problems the nodule was sterilized with H_2O_2 and rinsed several times with water. The periderm was peeled away and each naked lobe was washed in several changes of sterile PVP-PBS. DNA was isolated by a modification of the procedure of Rouvier *et al.* (1996). This method was found to be better than other methods tried. Addition of ascorbic acid in the pre-wash step significantly improved the yield and quality of DNA. The expected 20 kb band was obtained for most of the cases, (Fig. 4.3, A). Where a visible DNA was absent positive amplifications were obtained from one of the dilutions tested.

4.4 DNA AMPLIFICATION

Isolated DNA was directly used for PCR without further purification. A 1:100 to 1:1000 dilution usually proved adequate enough to rid the preparation of impurities. The quantity of the DNA in most of the cases was enough for amplification. To test the

reliability of PCR amplification. ten individual nodules from 13 different trees were prepared and amplification was carried out as described above. The nodules had developed in dissimilar trees but in field conditions where soil, weather, temperature etc. were closely similar. Initially *Frankia* specific primers were employed to demonstrate the presence of *Frankia* DNA in nodules. Subsequently, employment of various combinations of universal and *Frankia* specific primers, enabled amplification of target sites.

4.4.1 Total 16S rRNA Gene

Total 16S rRNA gene was amplified with the help of the two universal 16S primers FGPS 6 and FGPS 1490' (Normand *et al.*, 1996). The molecular weight of the resultant band corresponded to 1500 bp (Fig 4.3, B). The length of the 16S rRNA gene in *Frankia* is stated to be 1513 bp (Normand *et al.*, 1992a).

4.4.2 Total 16S rRNA And 16S-23S ITS

Amplification of the region encompassing the total 16S rRNA gene and the adjoining 16S-23S ITS was achieved using primers FGPS 6 (Normand *et al.*, 1996) and FGPL 2054' (Simonet *et al.*, 1991). A 2.1 kb fragment resulted (Fig. 4.3, C, D). Other bands were also seen. Moreover, all nodule samples did not yield positive signal. Hence this region was not analyzed further.

4.4.3 *nif* D-K IGS

Nalin *et al.* (1995) have characterized primer FGPK 333' for amplification of *Frankia* DNA from alder compatible strains. This primer can be used in combination with primer FGPD 807 (Jamann *et al.*, 1993), to achieve amplification of the *nif* D-K IGS. In the present study, a band with an approximate molecular weight of 740 bp was obtained (Fig 4.3, E). The previous reports on this IGS region revealed the sequence diversity in this region. The length of the IGS varies considerably from strain to strain (Nalin *et al.*, 1995).

4.4.4 Distal 16S rRNA Gene

On the basis of conserved sequences found in the *Frankia* 16S rRNA gene, Bosco *et al.* (1992) were able to characterize a 16-nucleotide sequence in the distal region for *Frankia* strain identification. Alder and *Elaeagnus* host infection groups could be easily differentiated on the basis of variability in this sequence. Accordingly primer FGPS 989ac and FGPS 989e were designed on the basis of this sequence. Primer FGPS 989ac was used with primer FGPS 1490' in the present study to amplify DNA from alder nodules. The expected 521 base pair band was obtained (Fig. 4.4, A). This enabled confirmation of the presence of *Frankia* in the nodules. Amplification with primer FGPS 989 e did not yield any result. This demonstrates the absence from these nodules of *Frankia* strains belonging to the *Elaeagnus* group.

Amplifications yielded the same 521 base pair band in all nodules. Only in one particular case an additional 1200 bp band was seen. The same did not amplify when it was amplified at higher annealing temperature after purification from the gel (Fig. 4.4, B). However, the smaller fragment was seen, clearly indicating that the smaller fragment was included in the larger fragment. Obviously, the larger fragment was a product of alternate annealing sites for the primer pair such that these sites were flanking the target annealing sites. Based on the published sequence information, it was seen that some amount of mismatches between primer FGPS 989 and the template were possible. These mismatches occur at different positions in different strains. (Give reference)

4.4.5 23S rRNA Gene

Attempt was made to study the 23S rRNA region by using the primer FGPS 2536' (Normand *et al.*, 1992b) in conjunction with FGPS 989 (Bosco *et al.*, 1992) and also FGPS 6. However, the expected 4 Kb fragment could not be obtained. This could have been due to fragmentation of DNA.

4.4.6 Proximal End Of 16S rRNA Gene

Proximal segment of the 16S rRNA gene was amplified using primers FGPS 6 and FGPS 505' (Normand *et al.*, 1996). A band with an approximate molecular weight of 540-bp was obtained (Fig. 4.5, A). This is similar to the expected size according to previously published information (Normand *et al.*, 1992a).

4.4.7 Partial 16S rRNA Gene

Partial 16S rRNA gene was amplified using primers FGPS 6 and FGPS 910' (Normand *et al.*, 1996). Multiple bands were seen in a few cases while others displayed single bands (Fig. 4.5, D & E). This region was, therefore, not analyzed further by restriction digestion.

4.4.8 Middle 16S rRNA Gene

The middle part of the 16S rRNA gene, lying between primers FGPS 485 and FGPS 910', was amplified. This region is a relatively invariable region. Though a single band was expected, multiple bands were seen (Fig. 4.6, A, B, C). The enzyme *Bst*U1 has two sites in *Frankia* DNA. The corresponding sites are absent in chloroplast DNA. Similarly the enzyme *Eco*R1 has a single site in the middle part of *Frankia* 16S rDNA, which is absent in plant DNA. Before further amplification attempts, we did a preliminary search for these sites in our amplicons. When amplification products were treated with the two enzymes cleavage was seen (lane 3 and 4, Fig 4.6, D), though a part of the amplicons remained undigested. It can be presumed that the amplification products had originated both from *Frankia* as well as plant DNA. Subsequently rest of the DNA samples were subjected to amplification under stringent conditions by the same primers. The aim being to decrease the chances of plant DNA amplification. Multiple bands were obtained for all samples tested which persisted even when the annealing temperature was raised to 61°C (Fig. 4.6, A, B, C and D). This

implied that the sequence was G+C rich. Comparative sequence analysis revealed that primer 910' could bind to 5-8 sites per molecule of template. These mismatch sites varied from strain to strain. (Table 4.1).

As annealing temperature was raised, the specificity of the reaction increased (Fig. 4.6, E). However, single bands were obtained only at an annealing temperature of 65°C (Fig. 4.6, F). The amplified bands were run on a 4% agarose gel which revealed amplified fragment length polymorphism (Fig. 4.7, A). Mainly two kinds of bands were observed : 415 bp and 330 bp.

4.5 PURIFICATION OF PCR PRODUCTS

Extraneous bands present in the amplification products were removed by DEAE cellulose paper method as detailed in section 3.4. A pure DNA band was obtained (Fig. 4.4, E). The yield of the method was 60-65%. The purified band when amplified gave the expected products.

4.6 RESTRICTION DIGESTION OF PCR PRODUCTS

PCR-RFLP technique has shown great potential for the detection of variation at the infra specific level as well as for genotyping of clonal material and for epidemiological studies. In case of *Frankia*, the intensive characterization of the genomes at the DNA level is yet to be done. Hence, the RFLP technique has emerged as an indispensable tool for preliminary identification and classification of isolates. The method relies on the judicious

TABLE 4.1 : ALTERNATE ANNEALING SITES FOR PRIMER FGPS 910' FOUND IN THE 16S rRNA GENE OF A FEW *FRANKIA* STRAINS.

S.No.	Strain	Site	Sequence*	No. Of Mismatches
1	ORS020606	275	GGGcGacCGGCCaCActGgg	8
	$ARgP5_{IAG}$	275	GGGcGacCGGCCaCActGgg	8
	AVN17	275	GGGcGacCGGCCaCActGgg	8
2	ORS020606	382	GGGAtgACGGCCttcgGGtT	7
	$ARgP5_{IAG}$	382	GGGAtgACGGCCttcgGGtT	7
	AVN17	381	GGGAtgACGGCCttcgGGtT	7
3	ORS020606	409	aGGttTACaaCCcgAAGGCc	8
	$ARgP5_{IAG}$	409	aGGttTACaaCCcgAAGGCc	8
	AVN17	408	aGGttTACaaCCcgAAGGCc	8
4	ORS020606	842	GGGAGTACGGCCGCAAGGCT	0
	$ARgP5_{IAG}$	842	GGGAGTACGGaCGCAAGGCT	1
	AVN17	841	GGGAGTACGGCCGCAAGGCT	0
5	ORS020606	1320	GtGAaTACGttCcCggGcCT	8
	$ARgP5_{IAG}$	1320	GtGAaTACGttCcCggGcCT	8
	AVN17	1325	GtGAaTACGttCcCggGcCT	8

*Residues in small case letters indicate mismatches with the corresponding residues in the primer. Sequences of the above strains are available in Internet data bank.

use of a combination of restriction enzymes leading to the detection of sometimes even single base changes in the DNA.

4.6.1 Map Locations Of Restriction Sites In The rRNA Region:

Map locations were inferred from the known sequence of *Casuarina* nodulating *Frankia* strain ORS020606 (GeneBank accession number M 58598). For this sequence and for each enzyme, the IBI MacVector® software (IBI, NewHaven, Conn.) gave, the locations of the restriction sites and the sizes of the fragments expected. Fragment sizes were calculated by Log Molecular weight method.

4.6.2 *Bso*F1 Digestion Of Distal 16S rRNA Gene

Restriction digestion was carried out using the enzyme *Bso*F1, on all the amplicons of distal 16S rRNA region obtained by PCR as mentioned in section 4.3.7. The amplicons that were not pure were subsequently purified. Digestion was performed as detailed in section 3.5. Digestion by *Bso*F1 resulted in 3 bands, the molecular weights of which were 290, 180 and 120 bp respectively. In some cases only two bands were seen. In one particular tree all the nodule samples gave a pattern containing six bands. In other cases a different pattern containing 3 bands was in evidence while in some others definite conclusions could not be made. This indicated that there was considerable variability in the nodule samples. Based on this study the nodules were divided into five groups:

Group II: nodules 2-1 to 2-10, 9-1 to 9-10.

Group III: nodules 3-1 to 3-10.

Group IV: nodules 5-1 to 5-10, 6-1 to 6-7.

Group V: nodules 8-1 to 8-10.

4.6.3 *Rsa* 1 Digestion Of Distal 16S rRNA Gene

The amplicons for distal part of 16S rRNA gene were digested with *Rsa* 1. Two kinds of patterns were obtained (Fig. 4.4, C, and D). The molecular weights of the bands were: 240, 155 and 120 bp in Group I and 230, 155 and 130 bp in group II. The nodules were divided into two groups according to the results obtained.

Group I: nodules 2-1 to 2-10, 9-1-9-10

Group II: nodules 1-1 to 110, 3-1 to 310, 4-2 to 4-10, 5-1 to 510, 6-1 to 6-7, 7-1 to 7-5, 8-1 to 8-10, 10-1 to 10-5, 11-1 to 11-5, 12-1 to 12-5, 13-1 to 13-5.

Based on the above two results the total groups remained five :

Group I: nodules 1-1 to 1-10, 4-2 to 4-10, 7-1 to 7-5, 10-1 to 10-5, 11-1 to 11-5, 12-1 to 12-5, 13-1 to 13-5.

Group II: nodules 2-1 to 2-10, 9-1 to 9-10

Group III: nodules 3-1 to 3-10.

Group IV: nodules 51-51. 61-67

Group V: nodules 8-1 to 8-10

Restriction analysis of this region thus has revealed differences. This segment has been shown to be highly variable (Hahn *et al.*, 1989, Nazaret *et al.*, 1991 and Bosco *et al.*, 1992) in *Frankia*. It

contains sufficient number of informative sites to allow grouping of all strains according to their sequence homologies. In previous studies, this permitted demarcation of *Frankia* strains into 8 different genomic groups (Nazaret *et al.*, 1991 and Bosco *et al.*, 1992). Alignment and comparative sequence analysis has shown that *Frankia* strains belonging to same genomic species shared similar sequences while strains belonging to different genomic species exhibited distinct sequences. This region is therefore a region of choice for designing of synthetic oligonucleotide probes. Such probes can increase specificity and reliability required for detection and identification tests conducted on *Frankia* strains inside nodule or in soil.

4.6.4 *Bst*U 1 Digestion Of Middle Part Of 16S rRNA Gene

Amplicons obtained after PCR of the middle segment of 16S rRNA gene were purified before restriction analysis. Digestion was performed using the enzyme *Bst* U1. Digestion patterns that were obtained are presented in Fig 4.7. B, C, D and E. The 415 bp band was cleaved to 350, 44 and 16-bp respectively. The 330 bp band was cleaved to 190 and 140 bp. In some cases there was no digestion.

The results obtained enabled differentiation of all nodules into three groups.

Group I: nodule 4-5

Group II: nodules 1-1 to 1-10, 2-1 to 2-10, 3-1 to 3-10, 4-2 to 4-10, 5-1 to 5-10, 6-1 to 6-7, 7-1 to 7-5, 8-1 to

8-10, 9-1 to 9-10, 10-1 to 10-5, 11-1 to 11-5, 12-1 to 12-5, 13-1 to 13-5.

Group III: nodules 3-6, 3-7

Based on the above three results, the total groups obtained became seven:

Group I: nodules 1-1 to 1-10, 4-2 to 4-10, 7-1 to 7-5, 10-1 to 10-5, 11-1 to 11-5, 12-1 to 12-5, 13-1 to 13-5.

Group II: nodules 2-1to 2-10, 9-1 to 9-10

Group III: nodules 3-1 to 3-5, 3-8, 3-9, 3-10.

Group IV: nodules 5-1 to 5-10, 6-1 to 6-7

Group V: nodules 8-1 to 8-10.

Group VI: nodules 3-6, 3-7

Group VII: nodule 4-5.

Thus restriction analysis of this region revealed some variability. Though RFLP analysis of the middle segment of 16S rRNA gene is not reported in literature, sequencing results of the total gene have shown that this particular region is relatively conserved. However, differences do exist. This can be seen if we compare the restriction patterns of three different DNAs for example ARgP5^{1AG}, AVN17 (Reference strain, source : University of Lyon, France) and Lawjenriew 13 (our lab., unpublished). While restriction digestion of first two DNAs with the enzyme *Bst*U1 are not likely to yield visible differences, the length of the digested bands being different only by 2 nt, digestion of Lawjenriew 13 will result in a fragment which differs from others by 28 nt. Probably this difference is

reflected in differences in genomic groups of the said strains too. Thus, significant differences can be detected by restriction analysis of the middle segment of 16S rRNA gene. These should enable the designing of specific oligonucleotides for *Frankia* strain characterization.

4.6.5. *Alu* 1 Digestion Of Proximal 16S rRNA Gene

Alu 1 digestion was performed on amplicons obtained by amplification of proximal region of 16S rRNA gene. A single digestion pattern was obtained with the appearance of two bands having band lengths of 280 and 250 bp respectively (Fig 4.5, B: Lane 4 and Fig 4.5, C). Thus no RFLPs were obtained in this region (Fig. 4.5, C). To discount the possibility that the amplified fragments might have arisen from host DNA, we cut the amplicons with *Rsa* 1, which lacks restriction sites in this region in case of *Frankia*. The results showed that amplicons obtained did belong to *Frankia* DNA, (lane 3, Fig 4.5, B). However some amount of plant DNA might have been present as restriction digestion with *Alu* 1, did leave some partially digested DNA (Fig. 4.5, C).

The absence of differences in this segment contrasts with earlier reports which have shown this region to be highly variable (Harry *et al.*, 1991, Hahn *et al.*, 1989). The reason for this anomaly may be because of the fact that the reported region of variability lies between 140-220 nt, while in our case the *Alu* 1 site happens to be at 250 nt. The region lying between 220 nt to 400

nt is understood to be constant (Normand *et al.*, 1992a; Nazaret *et al.*, 1991).

4.6.6 *nif* H-D IGS REGION

nif H-D IGS was probed with a new primer. This primer, FGPS 826', being complementary to the primer FGPS 807' reported by Jamann *et al.* (1993), enables amplification of the *nif* H-D IGS. For *nif* H gene, primer FGPH 750 was used (Simonet *et al.*, 1991). Instead of the expected single band, a pattern of multiple bands were seen. The pattern differed slightly in different strains and nodules. The largest band in the pattern varied from 1.5-kb in some cases to 900-bp (Fig.4.8, A, B and C). Fragments with molecular weights of 400-bp, 250-bp and 89-bp were common to all, while others differed from nodule to nodule. By scanning multiple aligned sequences it was discerned that *nif* H primer FGPH 750 could anneal to many sites in *nif* D gene. Similarly mismatch sites for *nif* D primer FGPD 826' were present in the *nif* H gene. Table 4.2 presents the locations and sequences of these sites in some *Frankia* strains. When amplified, *E. coli* and *Rhizobium* DNA did not give any positive signal. It can be assumed that this pattern pertained only to *Frankia*. Amplifications with primers FGPH 750 and FGPK 333', produced a similar pattern with some differences revealing the considerable homogeneity between the three *nif* genes.

Figure 4.3

Fig. 4.3: **DNA ISOLATION AND AMPLIFICATION.**
(Molecular weights in base pairs are included.)

A) Total DNA extraction from nodules of alder.
Lane 1: Molecular weight standard.
Lane 2-8: DNA isolated from different nodules.

B) Total 16S rDNA region amplified using primers FGPS 6 and FGPS 1490'.
Lane 1: Molecular weight standard.
Lane 2-7: Amplified DNA from different nodules.
Lane 8: Negative control.

C) Amplification of 16S rRNA and 16S-23S ITS region using primers FGPS 6 and FGPL 2054'.
Lane 1: Molecular weight standard.
Lane 2-5: Amplifications, negative result.
Lane 6: Amplification, positive result.
Lane 7: Negative control.

D) Amplification of 16S rRNA and 16S-23S ITS region using primers FGPS 6 and FGPL 2054'.
Lane 1: Molecular weight standard.
Lane 2-6: Amplified DNA from different nodules.
Lane 7: Negative control.

E) Amplified *nif* D-K IGS region using primers FGPD 807 and FGPK 333'.
Lane 1: Molecular weight standard.
Lane 2-7: Amplified DNA from different nodules.
Lane 8:Negative control.

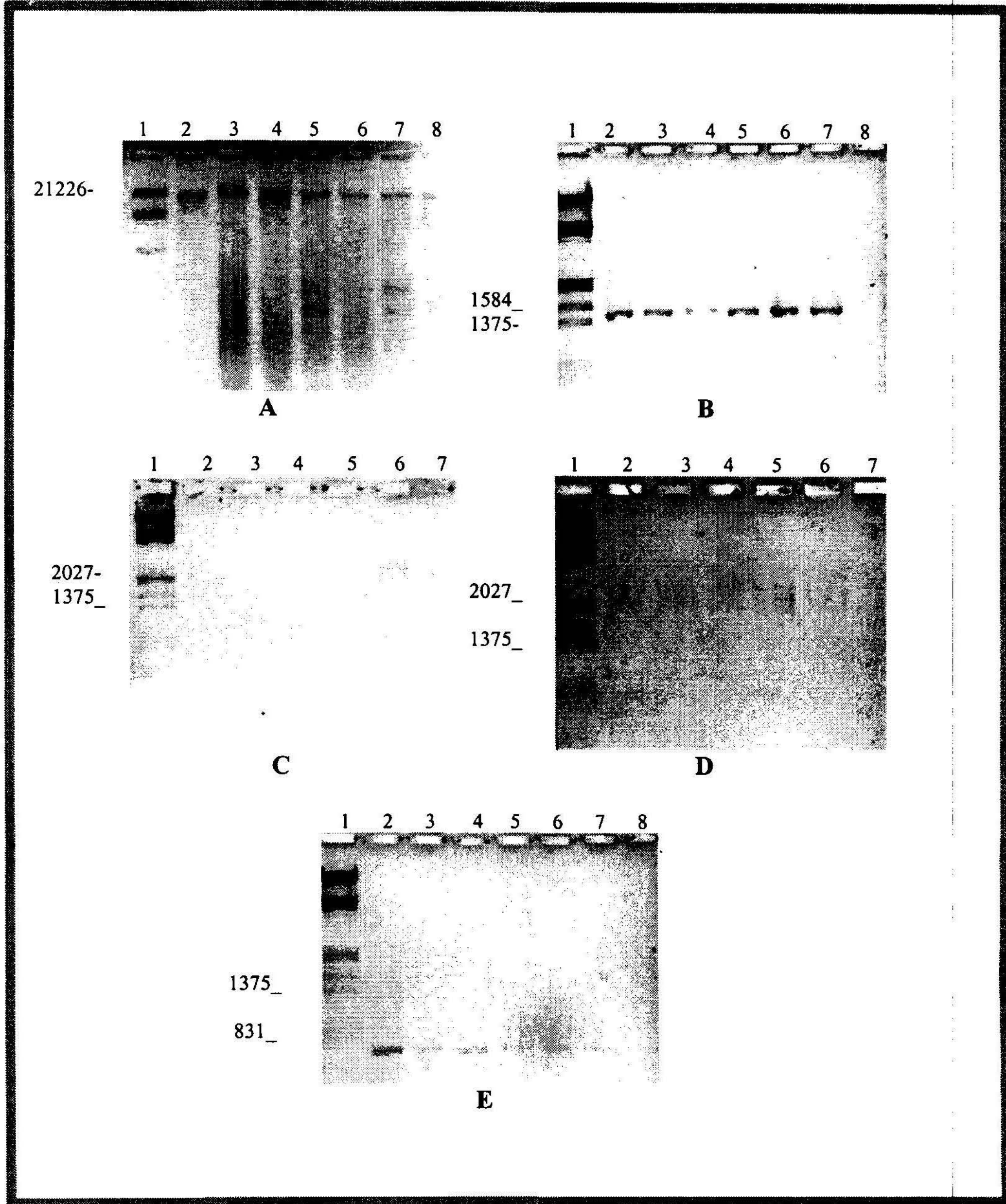

1 2 3 4 5 6 7 8
21226-
A
1 2 3 4 5 6 7 8
1584_
1375-
B
1 2 3 4 5 6 7
2027-
1375_
C
1 2 3 4 5 6 7
2027_
1375_
D
1 2 3 4 5 6 7 8
1375_
831_
E

Figure 4.4

FIG 4.4 **AMPLIFICATION AND RESTRICTION ANALYSIS OF THE DISTAL 16S rRNA GENE.**

(Molecular weights in base pairs are included.)

A) Amplification of distal segment of 16S rRNA gene using primers FGPS 989 and FGPS 1490'.
Lane 1: *Eco* R1 and *Hind* III digest of Lambda DNA.
Lane 2 :Positive control using DNA from *Frankia* strain ACN<u>1AG</u>.
Lane 3-7 : Amplified DNA from different nodules.
Lane 8: Negative control.

B) Reamplification of the 1200 bp band from Lane 4 in A) under stringent conditions.
Lane 1: Molecular weight standard.
Lane 2: Amplified DNA.
Lane 3: Negative control.

C) *Rsa* 1 digestion of amplicons obtained in A).
Lane 1: Digested sample.
Lane 2: Molecular weight standard:
Lane 3: Undigested 521 bp band.

D) *Rsa* 1 digestion of amplicons obtained in A).
Lane 1: Molecular weight standard;
Lane 2: Undigested 521 bp band.
Lane 3: Digested sample.

E) Purification of DNA by DEAE cellulose paper method.
Lane 1: Molecular weight standard.
Lane 2: Purified DNA.

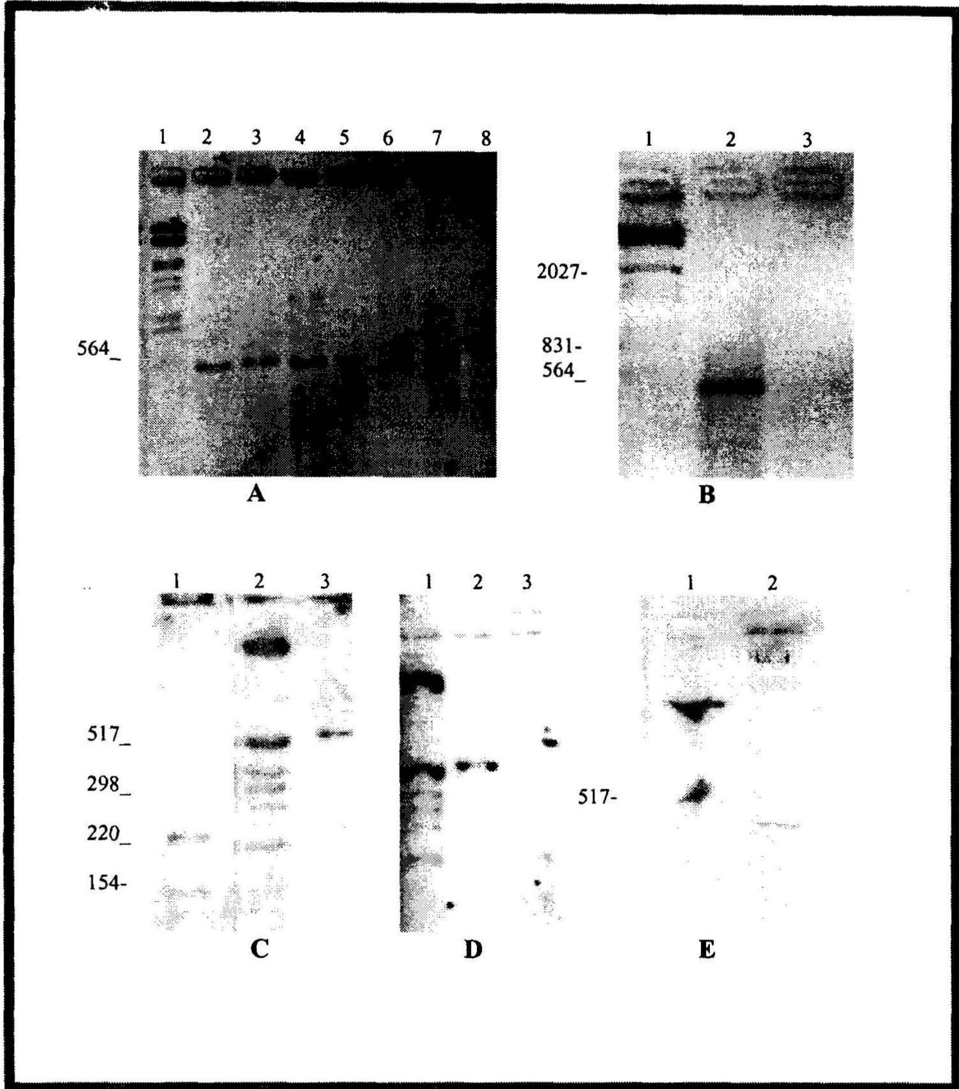

1
2
3
4
5
6
7
8
564_
A
1
2
3
2027-
831-
564_
B
1
2
3
517_
298_
220_
154-
C
1
2
3
D
1
2
517-
E

Fig. 4.5 **AMPLIFICATION AND RESTRICTION DIGESTION OF PROXIMAL 16S rRNA GENE.**

(Molecular weights in base pairs are included.)

A) DNA amplified using primers FGPS 6 and FGPS 505'.
Lane 1: Molecular weight standard
Lane 2-4: Amplifications, negative result.
Lane 5-6: Amplification, positive result.

B) Restriction digestion of Amplicons obtained in A)
Lane 1: Molecular weight standard
Lane 2: Undigested 540 bp amplicon
Lane 3: Amplicon digested with *Rsa* 1.
Lane 4: Amplicon digested with *Alu* 1.

C) Restriction digestion of amplicons obtained in A)
Lane 1: Undigested 540 bp amplicon.
Lanes 2-8: Different amplicons digested with *Alu* 1

D) Amplification of DNA isolated from nodules using primers FGPS 6 and FGPS 910'.
Lane 1: Molecular weight standard
Lane 2-7: Amplified DNA from different nodules.

E) Amplification of DNA isolated from nodules using primers FGPS 6 and FGPS 910'.
Lane 1: Molecular weight standard
Lane 2-4: Amplified DNA from different nodules.

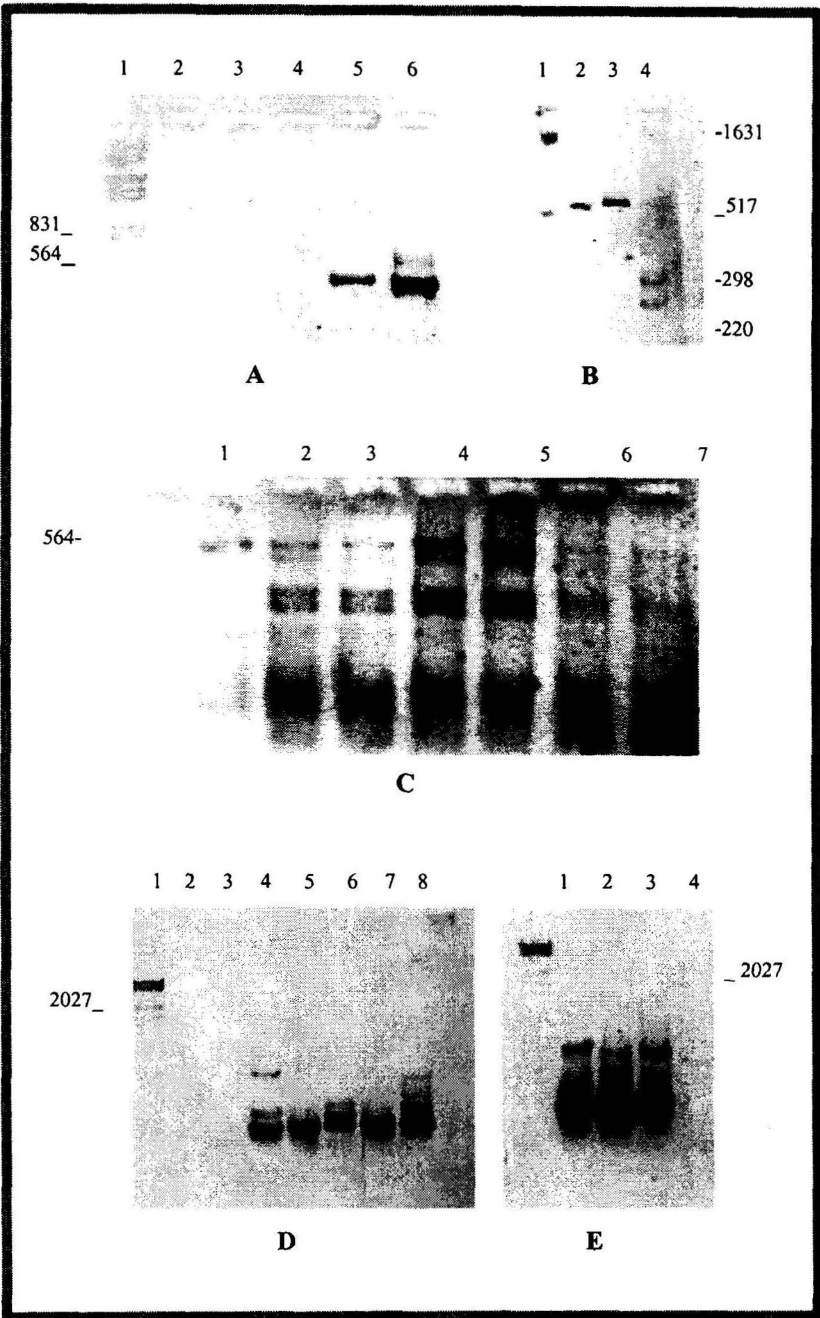

1
2
3
4
5
6
831_
564_
A
1
2
3
4
-1631
_517
-298
-220
B
1
2
3
4
5
6
7
564-
C
1
2
3
4
5
6
7
8
2027_
D
1
2
3
4
_ 2027
E

Fig. 4.6 AMPLIFICATION OF MIDDLE SEGMENT OF 16S rRNA GENE.

(molecular weights in base pairs are included.)

A) Amplification of middle segment of 16S rRNA gene using primers FGPS 485 and FGPS 910'.
Lane 1: Molecular weight standard.
Lane 2-7: Amplified DNA from different nodule samples.

B, C: Middle part of 16S rRNA gene amplified using primers FGPS 485 and FGPS 910'.

Lane 1: Molecular weight standard.
Lane 2-7: DNA amplified from different nodules.
Lane 8: Negative control.

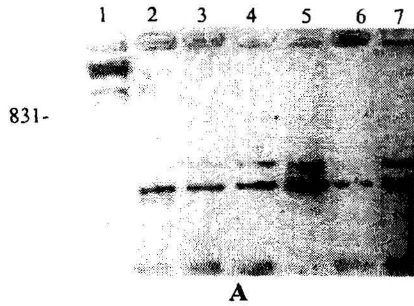
D:Amplification of middle part of 16S rRNA gene using primers FGPS 485 and FGPS 910'.
Lane 1: Molecular weight standard.
Lane 2, 5-8: Amplified DNA from different nodule samples.
Lane 3: *Eco* R 1 digest of DNA from lane 2.
Lane 4: *Bst* U 1 digest of DNA from lane 2.

E: Amplification of DNA using primers FGPS 485 and FGPS 910', at different annealing temperatures .

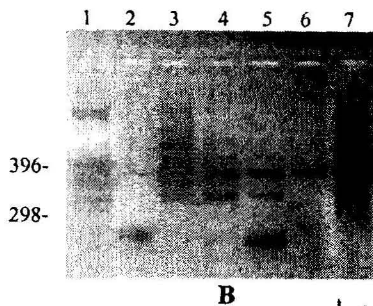
Lane 1: Molecular weight standard.
Lane 2: DNA amplified at an annealing temperature of 57 °C.
Lane 3: DNA amplified at an annealing temperature of 59 °C.
Lane 4: DNA amplified at an annealing temperature of 61 °C.

F: Middle part of 16S rRNA gene, amplified at an annealing temperature of 65 °C.

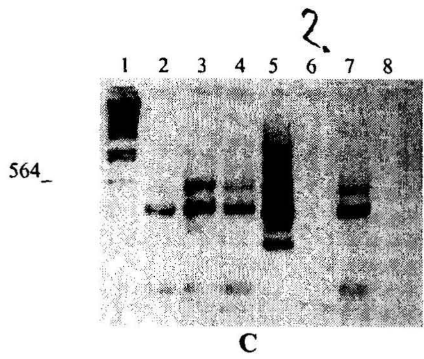
Lane 1-4: DNA amplified from different nodules.
Lane 5: Molecular weight standard.



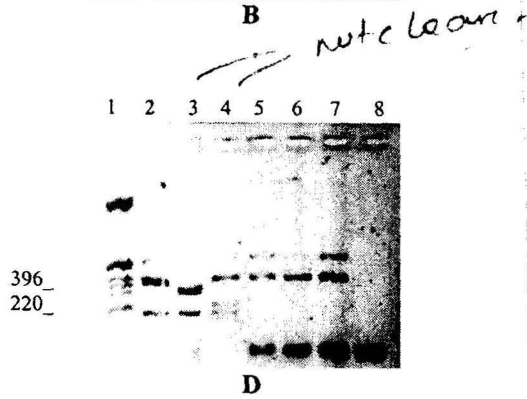
A



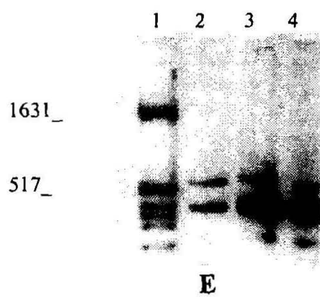
B



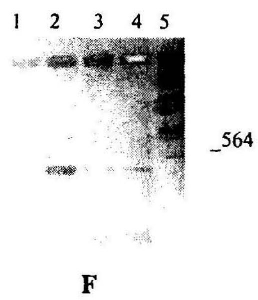
C



D



E



F

FIG: 4.7 AMPLIFICATION AND RESTRICTION ANALYSIS OF MIDDLE PART OF 16S rRNA GENE.
(Molecular weights in base pairs are included.)

A: AFLPs obtained during amplification of middle segment of the 16S rDNA, using primers FGPS 485 and FGPS 910'.
Lane 1-8 : Amplified DNA from different nodules.

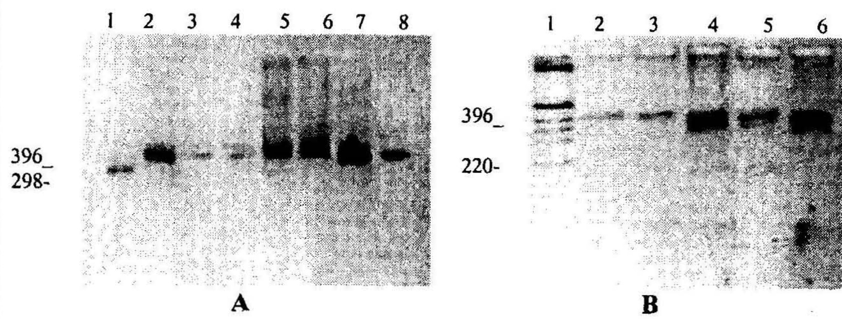
B: *Bst*U1 digestion of amplicons obtained as in A).
Lane 1: Molecular weight standard .
Lane 2-8 : Amplified DNA from different nodules treated with *Bst*U1.

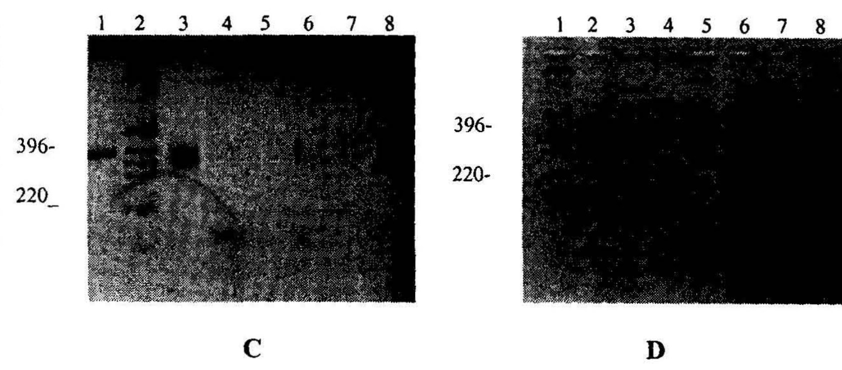
C: *Bst*U1 digestion of amplicons obtained as in A).
Lane 1: DNA isolated from *Frankia* strain ACN1AG, untreated control.
Lane 2: Molecular weight standard.
Lane 3: DNA isolated from *Frankia* strain ACN1AG, treated with *Bst* U1.
Lane 4-8: Amplicons treated with *Bst*U1.

D: *Bst*U1 digestion of amplicons obtained as in A)
Lane 1: Molecular weight standard .
Lane 2-8 : Different amplicons treated with *Bst*U1.

E: *Bst*U1 digestion of amplicons obtained as in A)
Lane 1: Molecular weight standard.
Lane 2-7: Different amplicons treated with *Bst*U1.

Figure 4.7


1 2 3 4 5 6 7 8
396_
298-
A
1 2 3 4 5 6
396_
220-
B


1 2 3 4 5 6 7 8
396-
220_
C
1 2 3 4 5 6 7 8
396-
220-
D

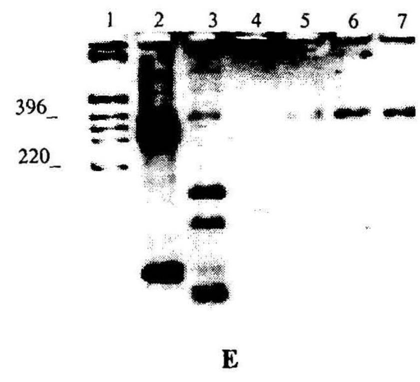

1 2 3 4 5 6 7
396_
220_
E

Fig. 4.8 **AMPLIFICATION AND RESTRICTION ANALYSIS OF *nif* H-D IGS REGION.**

(Molecular weights in base pairs are included.)

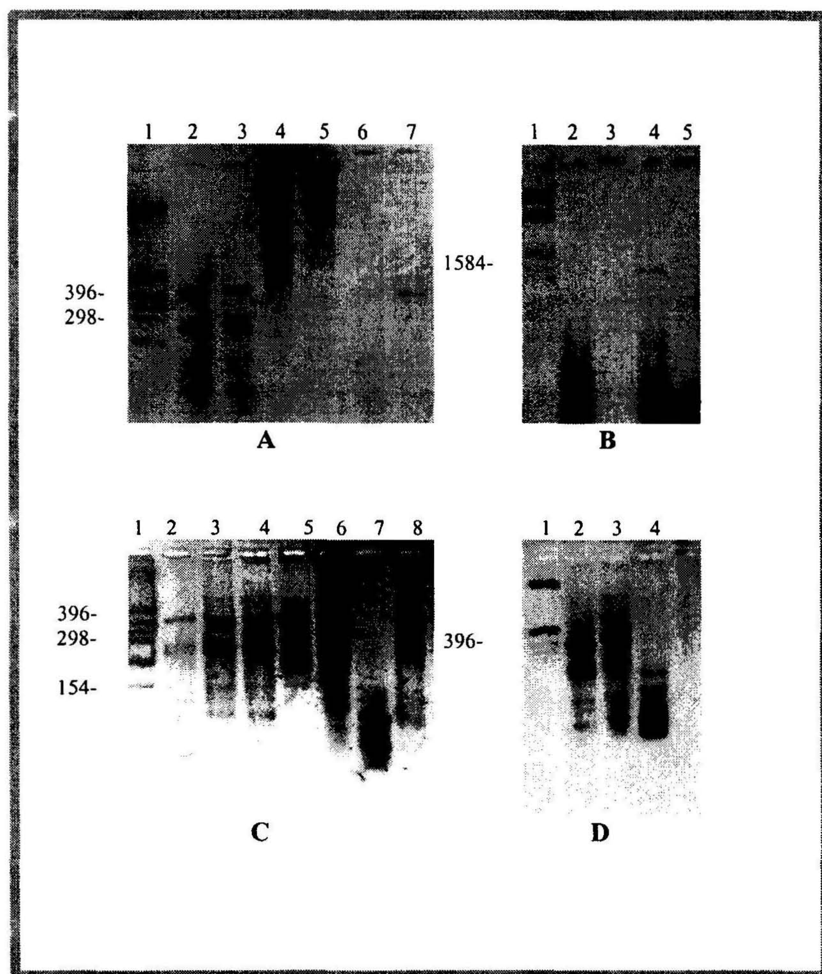
A) *nif* H-D IGS region amplified using primers FGPH 750 and FGPD 826'.
Lane 1:Molecular weight standard.
Lane 2-7:DNA amplified from different nodules.

B) Amplification of *nif* H-D IGS region using primers FGPH 750 and FGPD 826'.
Lane 1: Molecular weight standard.
Lane 2, 3 Amplifications, negative result.
Lane 4: Amplification, positive result.
Lane 5: Negative control.

C) *nif* H-D IGS region amplified using primers FGPH 750 and FGPD 826'.
Lane 1: Molecular weight standard.
Lane 2-8 :DNA amplified from different nodules.

D) Restriction digestion of *nif* H-D IGS amplification products.
Lane 1: Molecular weight standard.
Lane 2: *nif* H-D IGS Amplicon, untreated control.
Lane 3: Amplicon 1, digested with *Xho* 1.
Lane 4: Amplicon 2, digested with *Xho* 1.

Figure 4.8



Photographs taken on GelDoc 1000, Biorad, USA
and printed on HP LaserJet 6MP.

TABLE 4.2 : ALTERNATE ANNEALING SITES FOR PRIMER FGPD 826' FOUND IN THE *nif* H GENE OF SOME *FRANKIA* STRAINS.

S.No.	Strain	Site	Sequence*	No. of Mismatches
1	ArI3	170	tcCaGCTcgCGGcCGAgaAg	9
	HRN18a	170	tcCaGCTcgCGGcCGAgaAg	9
	Frankia El	164	CggTGaTcCaGcTCGcTGcc	9
2	ArI3	239	CACTGgccCTCGaCGAgcAc	9
	HRN18a	239	CACTGgccCTCGaCGAgcAc	9
	Frankia El	239	CACTGgccCTCGaCGAgcAc	9
3	ArI3	242	CcCcaCTggCccTCGAcGAg	9
	HRN18a	242	CcCcaCTggCccTCGAcGAg	9
	Frankia El	242	CcCcaCTggCccTCGAcGAg	9
4	ArI3	278	CcCgGCTcCaGGcCaccGgA	9
	HRN18a	278	CcCgGCTcCaGGcCaccGgA	9
	Frankia El	278	CcCgGCTcCaGGcCaccGgA	9
5	ArI3	332	gcCTcCTcCaGGTaGgTGAt	8
	HRN18a	332	gcCTcCTcCaGGTaGgTGAt	8
	Frankia El	332	gcCTcCTcCaGGTaGgTGAt	8
6	ArI3	773	CAaTGgacgaGcTCGAgGAg	9
	HRN18a.	773	CAaTGgacgaGcTCGAgGAg	9
	Frankia El	776	CAaTGgacgaGcTCGAgGAg	9

*Residues in small case letters indicate mismatches with the corresponding residues in the primer. The sequences of the above strains are available in Internet data bank.

Presence of such repeats in *Frankia* DNA is interesting and is useful for studying DNA polymorphisms in closely related strains. Just as the analysis of repetitive sequences has been helpful in detection of polymorphisms in *Frankia* isolates (Murry *et al.*, 1995), the repeats in *nif* genes are also informative of strain specific differences. Amplification of such regions can generate genomic fingerprints for strains. Sequencing of different samples can reveal more variability.

Another way of scanning for differences would be use of specific restriction enzymes that may differentiate between bands that look similar on the agarose gel. We selected the *nif* H-D region patterns that were identical on the gel and subjected each to digestion with the enzyme *Xho* 1. This enzyme has a single site in the distal end of *nif* H and in the proximal part of *nif* D. Therefore, it was expected that *Xho* 1 would cut one or more of the bands. The results indicated that most of the DNAs lacked the restriction sites except some which showed a distinct cut in all bands (Fig 4.8, D). This could be attributed to strain specific differences. There may be two strains, one possessing the *Xho* 1 sites and the other lacking it. Digestion with the restriction enzyme *Nru* 1 did not yield any cleavage (data not shown). Since the published sequences of *nif* H and *nif* D possess *Xho* 1 and *Nru* 1 sites, it seems that the *nif* H or *nif* D sequences in the present set of nodule samples were different.

Results obtained herein permitted the separation of all the nodules into three groups:

Group I: nodules 1-1 to 1-10, 2-1 to 2-10, 9-1 to 9-10, 4-2 to 4-10, 7-1 to 7-5, 10-1 to 10-5, 11-1 to 11-5, 12-1 to 12-5, 13-1-13-5.

Group II: nodules 4-9, 4-10.

Group III: nodules 9-6, 9-8, 9-9.

Ligon and Nakas (1987) reported that the *nif* H gene was not always contiguous with *nif* D and *nif* K in some species of nitrogen fixers. The differences in the *nif* H region are apparently much more wide than is presently known. Cournoyer *et al.* (1993) reported that the *nif* H-D IGS varies in length from 43 bp in HRN18a to 68 bp in ARgP5LAG. Most strains have their own *nif* H-D IGS. It is important to remember that *Frankia* strains from many geographical regions have not been analyzed so far. The free living status of *Frankia* also has received little attention. While it has been established that the host plant has had an impact on the evolution of *Frankia* (Cournoyer *et al.*, 1993), it is also thought that the adaptations under free living conditions may have been influential in its evolution. Examination of *Frankia* from all geographical sites, soils and sediment samples may allow one to make more definite conclusions about *nif* genes in the genus.

In the final analysis the total number of PCR-RFLP groups obtained on the basis of the above criteria was found to be 9 (Table 4.3). Table 4.4 presents the nitrogenase activities of different groups tree wise.

Table 4.3

Table 4.3: Summary Of Results Obtained In The Present Study.

Individual nodules were separated into nine different PCR-RFLP groups as detailed in the text. Different RFLP patterns are numbered as I, II, III etc. BF, R, BU, X, A and N refer to the different patterns obtained with *Bso*F 1, *Rsa* 1, *Bst*U 1, *Xho* 1, AFLPs in the 16S rRNA genes and different patterns in the *nif* H-D IGS regions. For instance BF I refers to RFLP pattern I obtained with the enzyme *Bso* F1. - indicates inconclusive results.

TABLE 4.3: SUMMARY OF RESULTS OBTAINED IN THE PRESENT STUDY

Parameter → Groups ↓	*Bso*F1	*Rsa*1	*Bst*U1	*Xho*1	AFLPs in middle 16S	Patterns in *nif* region
Group 1	BFI	RI	BUI	XI	AI	NI
Group 2	BFI	RI	BUII	XI	AI	NI
Group 3	BFII	RII	BUI	XI	AI	NI
Group 4	BFII	RII	BUI	XII	AI	NI
Group 5	BFIII	RI	BUI	XI	AI	NI
Group 6	BFIII	RI	BUIII	XI	AII	NI
Group 7	BFIII	RI	BUI	XI	AI	NII
Group 8	BFIV	RI	BUI	XI	AI	NI
Group 9	-	RI	BUI	XI	AI	NI

TABLE 4.4: NITROGENASE ACTIVITIES OF NODULES FROM DIFFERENT TREES AND THEIR RESPECTIVE PCR-RFLP GROUPS.

TREE 3

S.No	Ethylene produced (nmols/mg fresh weight of nodule/hour	PCR-RFLP Group
1	0.0407	1
2	0.0555	1
3	0.0474	1
4	0.0555	1
5	0.0407	1
6	0.02841	2
7	0.02664	2
8	0.03996	1
9	0.0562	1
10	0.0296	1
Mean	0.04206	

TREE 2

S.No	Ethylene produced (nmols/mg fresh weight of nodule/hour	PCR-RFLP Group
1	0.01916	3
2	0.05673	3
3	0.5823	3
4	0.05106	3
5	0.1435	3
6	0.02442	3
7	0.8302	3
8	0.06845	3
9	0.0674	3
10	0.0296	3
Mean	0.20453	

TABLE 4.4 (Continued)

TREE 9

S.No	Ethylene produced (nmols/mg fresh weight of nodule/hour	PCR-RFLP Group
1	0.05522	3
2	0.07812	3
3	0.0451	3
4	0.0432	3
5	0.1334	3
6	0.01312	4
7	0	3
8	0	4
9	0	4
10	0	3
Mean	0.03249	

TREE 1

S.No	Ethylene produced (nmols/mg fresh weight of nodule/hour	PCR-RFLP Group
1	1.2809	5
2	0.8539	5
3	0.319	5
4	0.4269	5
5	0.8539	5
6	0.2553	5
7	0.95460	5
8	1.2809	5
9	1.702	5
10	0.6364	5
Mean	0.8542	

TABLE 4.4 (Continued)

TREE 4

S.No	Ethylene produced (nmols/mg fresh weight of nodule/hour	PCR-RFLP Group
1	0.4269	ND
2	0.5320	5
3	0.14948	5
4	0.3766	5
5	0.3293	6
6	0.1080	5
7	0.2109	5
8	0	5
9	0.0999	7
10	0.1879	7
Mean	0.2422	

TREE 5

S.No	Ethylene produced (nmols/mg fresh weight of nodule/hour	PCR-RFLP Group
1	0.3455	8
2	0.3367	8
3	0.2427	8
4	0.485	8
5	0.3359	8
6	0.2368	ND
7	0.0666	8
8	0.3108	8
9	0.2249	ND
10	0.37	8
Mean	0.2952	

TABLE 4.4 (Continued)

TREE 8

S.No	Ethylene produced (nmols/mg fresh weight of nodule/hour	PCR-RFLP Group
1	0.0483	9
2	0.0297	9
3	0.0449	9
4	0.0278	9
5	0.0985	9
6	0.022	9
7	0.0197	ND
8	0.0371	ND
9	0.0232	ND
10	0.203	ND
Mean	0.05537	

4.7 CORRELATION BETWEEN ARA VALUES AND PCR-RFLP GROUPS

The ARA values of each group are presented in Table 4.4. Significance tests were done for each pair of groups. The relationships, if any, between the observed nitrogenase activities and the PCR-RFLP patterns were assessed.

4.7.1. Nitrogenase Activities Of Nodules From Individual Trees:

As far as nodules from a single tree are concerned, significant differences were obtained in case of nodules originating from Tree 1 and Tree 2. Fig. 4.9 presents the means of ARA values of nodules from different trees and their PCR-RFLP patterns. In case of the rest of the trees, the differences obtained were insignificant. In case of Tree 1 and 2 the nodules that showed differences in ARA values did not show any variability in PCR-RFLP patterns. This can be because of absence of other strains in the nodules studied. It is also possible that other strains were present but not detected by our technique. PCR-RFLP approach has its own limitations in identification of organisms. Besides this, it is possible that the primers used in the present case were unable to detect novel strains.

4.7.2. Variability Found In Case Of Nodules With Similar Nitrogenase Activities

In case of nodules 4-5 and 4-10, the nitrogenase activities of

Figure 4.9

Fig 4.9 : Average ARA Values And PCR-RFLP Groups Obtained For Different Trees.

Ten individual nodules were taken from each tree. Nitrogenase activities were measured by the method of Stewart *et al.*, 1968. Mean values were calculated for each tree. Thirteen different trees from a particular geographical site in Shillong were sampled. DNA extractions, amplifications and restriction digestions were performed as detailed in the text.

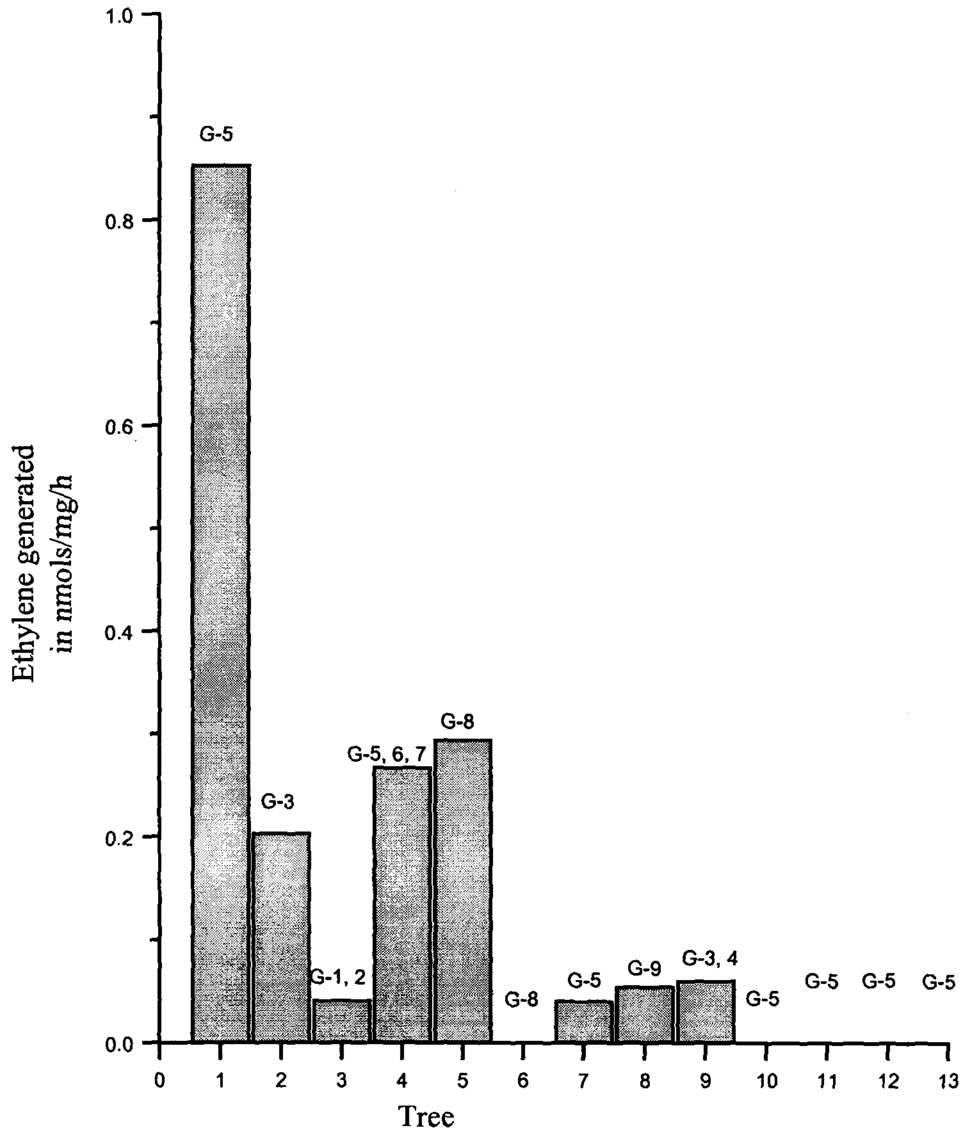


Fig. 4.9 AVERAGE ARA VALUES AND PCR-RFLP GROUPS OBTAINED FOR DIFFERENT TREES.

whom were comparable and which were from the same tree, the PCR-RFLP patterns were found to be different. Similarly nodules 3-6 and 3-7, while belonging to Tree 3, displayed same range of nitrogenase activities as done by other nodules of the same tree, *but corresponded to different PCR-RFLP groups. It can be* concluded that these differences originated from the differences in strains.

4.7.3. Variability Between Trees With Respect To The ARA Values And The PCR-RFLP Grouping

Table 4.4 shows that different trees may possess nodules that have widely varying nitrogen fixation rates. So much so that each tree may exhibit a specific range of ARA values. Trees could be divided into two groups on the basis of their ARA values. Within each group were present trees that were apparently genotypically different from each other.

When each nodule was examined for the *Frankia* strain by PCR-RFLP study, it was discerned that nodules with high as well as low activity fell in the same PCR-RFLP group. As far as nodules from a single tree are concerned, the nodules that showed differences in ARA values did not show any variability in PCR-RFLP patterns except in two cases. By applying student's "t" test, it was seen that there was no significant difference in the mean ARA *values from two different PCR-RFLP groups. At the same time, trees* differing in ARA values generally hosted the same strain of *Frankia*. This has a single implication: the same strain of *Frankia*,

if nodulating different trees, will display different nitrogen fixation rates. Simply put this means that the genotype of the host determines the nitrogen fixation rates of the nodules.

The variations in ARA values within the same tree and the same PCR-RFLP group are obviously the result of other factors. The role of various environment factors cannot be ignored. These factors, obviously in isolation as well as in combination, exert a far greater influence on the symbiotic partnership than is presently assumed.

In the present study we were unable to identify any superior nitrogen fixing nodules. Nodules that were high in activity in one host were low in activity in another. Thus assignment of molecular signatures to superior strains was not possible. An alternative approach would be to keep the host genotype constant and search for superior *Frankia* strains. Needless to say, that prior selection of a superior host genotype would be necessary before such a study can be undertaken. The present investigation has pointed at one superior host genotype (Tree 1) in the site of study.

4.8 DISCUSSION

From the results obtained in the present investigation, it is clear that plant-microbe symbiotic interactions are influenced at various levels by many factors. These elements either in isolation or in togatherness exert their influences on either or both of the symbiotic partners. Nitrogen fixation is overwhelmingly a fraternal

endeavour. The photosynthetic activity of the plants is linked togather with the nitrogen fixation activity of bacteria to reduce dinitrogen. There are pressures on both the partners to continue in the symbiotic state though on the surface it may appear that the microbe is more committed towards symbiosis. There is a division of labour between the two organisms, a careful orchestration of which permits optimum benefits to both. In participating in mutual association, they derive impetus from the surrounding environment which not only is the bearer of nutrients but also takes away some of the fixed nitrogen. Our knowledge of the plant genes involved in symbiosis is fragmentary as is our information about frankial genes and their expression. we can at best glance at the morphological and physiological features of some of the steps involved and try to extrapolate it to the composite endosymbiotic system.

In order to zero on to constituents that have a bearing on the symbiotic efficiency, it would be pertinent to examine the entire process of symbiosis vis-a-vis the physiology of participating partners. The symbiotic relationship between *Frankia* and Actinorhiza seems to be an outcome of mainly three components : host genotype, *Frankia* genotype and other elements.

Host effects:

Previous work on other nitrogen fixing systems throws more light on these aspects. Many reports on *Frankia* have shown that the nitrogenase activities of *Frankia* are different in pure culture

and in *in situ* conditions. Obviously, this effect is brought about by the host. The host genotype controls nodule morphology. Kondorosi *et al.* (1977) showed that infection of *Rhizobium* on pea root cells was affected by changes in the cell wall chemistry so much so that some strains produced effective nodules while others produced ineffective nodules on the same host plant. In case of *Frankia*, different strains of the bacterium produced different responses in the same host clone of *Casuarina equisetifolia* (Reddell and Bowen, 1985a). Similar results were obtained in some species of *Alnus* by Dawson and Sun (1981). Ganesh (1993) found a single *Frankia* strain to be highly active in symbiotic condition, as compared to others. This strain had low activity in culture but when different host genotypes were nodulated, the nitrogenase activity *in situ* was high. Sougoufara *et al.* (1992) selected a combination of high and low nitrogen fixing *Frankia* strains and tested them on three *Casuarina* host clones. They discovered that a certain host clone always produced the maximum nitrogen fixing nodules irrespective of the strain used. Similarly, there was a host clone which always produced the lowest activity nodules.

The physiology of the host is the machinery that is availed of by the endosymbiont during the reduction of nitrogen. The demand for fixed nitrogen comes in a major way from the host. Obviously the microbe must, therefore, work to the dictates of the host. The plant is known to exert its influence on the entire process of symbiosis in a series of steps (Regensburger *et al.*, 1986, Murry *et al.*, 1985):

a) Selection of the microsymbiont for infection and nodulation.

b) Control of the growth of microsymbiont inside the host cell.

c) Control of O_2 concentration inside the nodule by regulating the levels of leghemoglobins.

d) Controls of the levels of Glutamine synthase, Glutamate synthase and Glutamate dehydrogenase.

e) Control of the export of ammonia outside the cell.

f) Control of the levels of ATP inside the vesicle.

g) Regulation of the synthesis of carbon compounds for the utilization of the microsymbiont.

Role of *Frankia*:

During infection, *Frankia* cells are known to elicit protein factors that mediate the selection of the host for infection. Though many strains of *Frankia* may infect, only a few are known to form nodules, out of which fewer produce effective nodules. During the entire process of symbiosis a continuos interplay between frankial determinants and the host-related phytohormones ensues. The host and the microbe also mutually enhance their individual characters (Sprent *et al.*, 1987). This regulatory process is modulated by temperature, nitrate concentration in the soil, moisture and other environmental factors.

The central fact, however, is that *Frankia* is the contributor of the nitrogen fixation apparatus. This makes it a direct player in

the process of nitrogen reduction. Obviously strains that have an efficient nitrogenase system would produce more fixed nitrogen as compared to others when all other factors are constant. The role of the microsymbiont is thus vital in nitrogen fixation. Screening of a large number of isolates under diverse environmental conditions and use of different host genotypes, can help in identification of superior nitrogen fixing *Frankia* strains. By following such a strategy Han and New (1998) were able to obtain a single high nitrogen fixing strain of *Azospirillum*, among a collection of 285 others. Similar results were produced in case of *Rhizobium* by Batzli *et al.* (1992).

Combined effects:

The functional relationship between *Frankia* and plant is far from simple. The microbe and plant may show complete compatibility as far as establishment of infection is concerned, but the resulting association may not provide optimal benefit to either partner. The physiological activity of the two partners must have a say in determining functional compatibility. This compatibility will be a prerequisite for the phenotypic expression of the frankial association (frankial effectiveness) and will depend not only on the genetic make up of *Frankia* and the plant involved but also on factors external to the association. External factors have the potential to bring about an imbalance in the symbiotic relationship. The role of pH, soil, soluble phosphorus, Calcium levels, available nitrogen, conductivity of water, time of collection, climate, light availability, canopy cover, etc., are

significant in this regard. The role of these factors in different host-microsymbiont systems has been examined by some workers (Quesada and Valiente, 1996; Quesada *et al.*, 1997, 1998; Olson *et al.*, 1998; Han and New, 1998). They found significant variations in ARA values with changes in the above mentioned parameters. Some of these elements may combine with each other or the organism to produce secondary effects. Other factors that may similarly influence the outcome of symbiosis are age of the tree, age of the nodule and presence of other microbial flora in the vicinity of the nodule.

To conclude, the symbiotic efficiency of any partnership between the actinorhizal host and the *Frankia* strain is defined by the functional capacity of the association and environmental factors. The functional capacity is itself a function of the host genotype, the *Frankia* genotype, and the environmental factors. This has been graphically presented in Fig. 4.10. Any attempt to use *Frankia* as a biofertilizer must take into account these factors. A comprehensive study of all associated determinants in the *Frankia*-Actinorhizal symbiotic system would not only go to increase our fundamental knowledge about plant-microbe symbiosis but also facilitate selection of efficient strains (both host and *Frankia*). This may pave the way for provision of efficient biofertilizers for soil improvement.

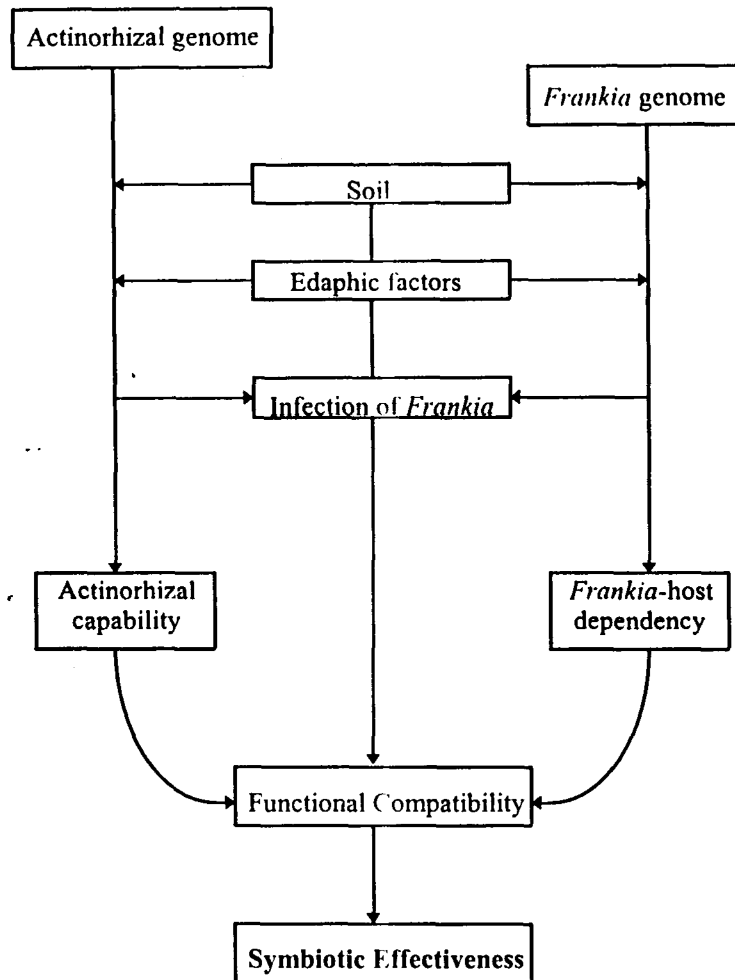


Fig. 4.10 Factors controlling the effectiveness of *Frankia*-Actinorhizal symbiosis.

CHAPTER 5

CONCLUSIONS

CHAPTER 5

CONCLUSIONS

The purpose of the present study was to look for molecular signatures for nodules with differing nitrogenase activity. In this direction, nodules were collected from different trees in a particular geographical site in Shillong. ARA and PCR-RFLP analyses of individual nodules were done.

The results of the study can be summarized as follows:

1. Trees differed significantly from each other in their mean ARA values. A 'Host' effect was evident. Based on the nitrogen fixation rates, the trees could be classified into two groups : the low and the high activity groups.

2. Individual nodules assayed, could be assorted into 9 PCR-RFLP groups on the basis of differences in the 16S rRNA and *nif* genes.

3. Variability was found in the distal part of 16S rRNA gene. Variability was also found in the middle part of the 16S rRNA gene. By the use of a new primer in the *nif* D region, repetitive sequences were found, these sequences originated from the multiple annealing sites that exist for the primers FGPH 750 and FGPD 826' in the *nif* D and *nif* H genes respectively.

4. There was no significant difference in the ARA values of different PCR-RFLP groups.

5. Nodules that were highly active and low activity nodules generally fell in the same PCR-RFLP group. Trees differing widely in mean ARA values generally hosted the same strain of *Frankia*. This meant that host genotype had a dominant influence on the nitrogen fixation rates of nodules.

6. In the final analyses, the symbiotic effectiveness of a certain *Frankia*-actinorhizal association seemed to depend on three factors:

   a) Host genotype
   b) *Frankia* genotype
   c) Other factors like soil, edaphic factors and presence of other bacteria.

Since the host plays a definite role in determining the nitrogen fixation rates of nodules, it is not possible to delineate *Frankia* strains simply on the basis of nitrogenase activities of nodules. The host genotype must be kept constant before a search for superior *Frankia* strains is made.

It would be prudent to conclude that nitrogen fixation in the nodules is an outcome of several factors playing a complex role in tandem. The major factor appears to be the host genotype which is fine tuned by the microsymbiont genotype. Both these factors are acted upon by numerous other known and unknown factors only some of which have been identified. Each factor would have to be examined in isolation. As is known, the nitrogen fixation rates of *Frankia* in pure culture may vary drastically from

those *in situ*, this factor must be taken into consideration while attempting to use *Frankia* as a biofertilizer. Such attempts must however always emphasize on both, the host genotype and the genotype of the microsymbiont.

APPENDICES

APPENDIX 1

COMPOSITION OF MEDIA

1. DEFINED PROPIONATE MEDIUM (Baker and O'Keefe, 1984)

COMPONENT	AMOUNT/LITRE
KH_2PO_4	1.0g
$MgSO_4.7H_2O$	0.1g
$CaCl_2.2H_2O$	0.01g
Sodium Propionate	1.2g
Hoagland's Microelement Stock	1ml
Fe Na EDTA Stock	1.8ml

pH-6.8

2. HOAGLAND MICROELEMENT STOCK (Hoagland and Arnon, 1950)

COMPONENT	AMOUNT/LITRE
H_3BO_3	2.86g
$MnCl_2.4H_2O$	1.81g
$ZnSO_4.7H_2O$	0.22g
$CuSO_4.5H_2O$	0.08g
$NaMoO_4$	0.025g
$CoCl_2.6H_2O$	0.025g

3. FERRIC SODIUM EDTA STOCK

$FeNa_2EDTA$	0.010g/l

APPENDIX 2

COMPOSITION OF BUFFERS USED FOR ELECTROPHORESIS

1. TBE BUFFER (Sambrook *et al.*, 1989)

COMPONENT	AMOUNT/LITRE
Tris base	54.0g
Boric Acid	27.5g
EDTA solution, 0.5M, pH 7.8	20ml

pH 7.8

2. TE8 BUFFER

Tris base	50mM
EDTA	20mM

pH 8.0

3. EXTRACTION BUFFER FOR ISOLATION OF DNA (Rouvier *et al.*, 1996)

Tris base	100mM
NaCl	1.4M
EDTA	20mM
CTAB[1]	2% [w/v]
PVP[2]	1% [w/v]

pH 8.0

1 - Cetyl Trimethyl Ammonium Bromide
2 - Polyvinylpyrrolidone

4. PVP-PBS

NaCl	0.8g

NaH_2PO_4	1.16g
KH_2PO_4	0.2g
PVP	30.0g

5 TEA (pH 7.6 at 25°C)

	AMOUNT/LITRE
Tris HCl	10mM
Na_4EDTA	1mM
Ascorbic acid	20mM

6. LURIA BERTANI BROTH (Sambrook *et al.*, 1989)

	AMOUNT/LITRE
Tryptone	10g
NaCl	10g
Yeast Extract	5g

This was added to 900 ml of water. The pH was adjusted to 7.0 using 4M NaOH. Volume was made up to 1000 ml. The medium was autoclaved and filtered through a 0.2µm filter.

7 TYS Medium (Sambrook *et al.*, 1989)

Tryptone	8g
Yeast Extract	5

This was added to 900 ml of water. The pH was adjusted to o 7.0 using 4M NaOH. Volume was made up to 1000 ml. The medium was autoclaved and filtered through a 0.2µm filter.

A stock solution of 10mg/ml streptomycin in water was prepared and sterilized by filtration through a 0.2µm filter. It was added to the above medium at a final concentration of 10µg/ml.

APPENDIX 3

BUFFERS FOR PCR AND RESTRICTION ANALYSIS

1. 10 X PCR BUFFER (pH 8.3 at 25°C)

Tris-HCl	100mM
KCl	500mM
$MgCl_2$	15mM
Gelatin	0.01% [w/v]

2. 1 X NE BUFFER 1 (pH 7.0 at 25°C)

Bis Tris Propane-HCl	10mM
$MgCl_2$	10mM
DTT	1 mM

3. 1 X NE BUFFER 2 (pH 7.9 at 25°C)

NaCl	50mM
Tris-HCl	10mM
$MgCl_2$	10mM
DTT	1 mM

4. 1 X NE BUFFER 4 (pH 7.9 at 25°C)

Potassium acetate	50mM
Tris-acetate	20mM
Magnesium acetate	10mM
DTT	1 mM

5 1 X BUFFER B (pH 8.0 at 25°C)

Sodium Chloride	100mM
Tris-HCl	10mM
Magnesium Chloride	10mM
β-Mercaptoethanol	5 mM

6 1 X BUFFER E (pH 7.9 at 25°C)

Potassium acetate	66mM
Tris-acetate	33mM
Magnesium acetate	10mM

DTT	0.5 mM

7 1 X BUFFER FOR *Nru* 1 (pH 7.7 at 25°C)

Tris-HCl	50mM
KCl	100mM
$MgCl_2$	10mM

8 LOW SALT WASH BUFFER FOR PURIFICATION OF DNA

Tris-HCl	50mM
NaCl	0.15M
EDTA	10mM

pH 8.0

8 HIGH SALT BUFFER FOR PURIFICATION OF DNA

Tris-HCl	50mM
NaCl	1M
EDTA	10mM

pH 8.0

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