

DEVELOPMENT OF MOLECULAR SIGNATURES OF
FRANKIA STRAINS

ABSTRACT



By
SUSAMMA K. VERGHESE

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

OF

NORTH EASTERN HILL UNIVERSITY
SHILLONG, INDIA.

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, characterization of many strains has been 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.

REFERENCES

- Bosco, M., M. P. Fernandez, P. Simonet, R. Materassi, and P. Normand.** 1992. Evidence that some *Frankia* strains are able to cross boundaries between *Alnus* and *Elaeagnus* host specificity groups. *Appl. Environ. Microbiol.* **58**: 1569-1576.
- Callaham, D., P. Del Tredici and J.G. Torrey.** 1978. Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. *Science* **199**: 899-902.
- Furlow, J. J.** 1979. The systematics of American species of *Alnus*, (*Betulaceae*). *Rhodora* **81**: 1-151.
- Ganesh, G.** 1993. Study of genetic diversity of *Frankia alni* strains isolated from *Alnus nepalensis* root nodules found in Meghalaya. Ph D thesis. NEHU, Shillong, India.
- Han, S. D., and New, P. B.** 1998. Variation in N₂ fixing ability among natural isolates of *Azospirillum*. *Microb. Ecol.* **36**: 193-201.
- Mullis, K. B., F. A. Faloona, S. Scharf, R. Saiki, G. Horn, and H. A. Erlich.** 1986. Specific enzymatic amplification of DNA *in vitro*: The polymerase-chain reaction. *Cold Spring Harbour Symp. Quant. Biol.* **51**: 263-273.
- Nalin, R., A. -M. Domenach, and P. Normand.** 1995. Molecular structure of the *Frankia* sp. *nif* D-K intergenic spacer and design of *Frankia* genus compatible primer. *Mol. Ecol.* **4**: 483-491.
- Normand, P., S. Orso, B. Cournoyer, P. Jeannin, C. Chapelon, J. Dawson, L. Evtusenko, and A. K. Misra.** 1996. Molecular phylogeny of the genus *Frankia* and related genera and emendation of the family *Frankiaceae*. *Int. J. Syst. Bacteriol.* **46**:1-9.
- Quesada, A., F. Leganes, and E. Fernandez Valiente.** 1997. Environmental factors controlling N₂ fixation in Mediterranean rice fields. *Microb. Ecol.* **34**:39-48.
- Quesada, A., M. Nieva, F. Leganes, A. Ucha, M. Martin, C. Prospero, and E. Fernandez Valiente.** 1998. Acclimation of cyanobacteria communities in rice fields and responses of nitrogenase activities to light regime. *Microb. Ecol.* **35**:147-155.
- Reddell, P., and G. D. Bowen.** 1985. *Frankia* source affects growth, nodulation and nitrogen fixation in *Casuarina* species. *New Phytol.* **100**:115-122.
- Rouvier, C., Y. Prin, P. Reddell, P. Normand., and P. Simonet.** 1996. Genetic diversity among *Frankia* strains nodulating members of the Family *Casuarinaceae* in Australia revealed by PCR and RFLP analysis with crushed root nodules. *Appl. Environ. Microbiol.* **62**: 979-985.

Simonet, P., M. C. Grosjean, A. K. Misra, S. Nazaret, B. Cournoyer, and P. Normand. 1991. *Frankia* genus specific characterisation by polymerase chain reaction. **57**: 3278-86.

Sougoufara, B., L. Maggia, E. Duhoux, and Y. R. Dommergues. 1992. Nodulation and nitrogen fixation in nine *Casuarina* clone-*Frankia* combination. *Acta Oecol.* **13**: 497-503.

Stewart, W.P.D., G. P. Fitzgerald, and R. H. Burris. 1968. Acetylene reduction in nitrogen fixing blue- green algae. *Arch Microbiol* **62**: 336 -348.

NEHU LIBRARY

Acc No. 103804

Acc By... *lv*

Date... 10-9-07

Class by.....

Sub.Heading by.....

Enter by.....

Transcribed by.....