USE OF MOLECULAR CHARACTERISTICS IN *FRANKIA* FOR STRAIN IDENTITY

ABSTRACT

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THESIS SUBMITTED
IN FULFILMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BOTANY

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1999

ABSTRACT

Nitrogen is one of the most important elements for living organisms. Vast amount of nitrogen is available in the atmosphere, but the plants are unable to fix it for their use. Somehow this ability has been confined to micobes only. Actinomycete *Frankia*, is one of the microorganisms that fix atmospheric nitrogen. *Frankia* can enter into symbiotic association with some plants, called as actinorhizal plants. This association leads to the formation of root nodules.

Actinorhizal plants belong to eight families and 25 genera (Lechevalier, 1994). These actinorhizal plants have potential applications in reforestation and soil improvement. *Alnus, Hippophae* and *Elaeagnus* have been used for land stabilization, timber production and improvement of soil fertility.

Frankia are filamentous, branched, Gram positive bacteria. On the basis of the actinomycetous nature of *Frankia*, Becking (1970) placed them in the family Frankiaceae in the order Actinomycetales. In symbiotic association *Frankia* infect the cortical region of nodule tissue.

The isolation of *Frankia* proved difficult until the first reproducible isolation from *Comptonia peregrina* (Callaham *et al.*, 1978). In pure culture they have branched and septate hyphae ranging from 0.5-2.0 μM in diameter. The formation of sporangia, which are spherical in shape (3-4.05 μM diameter), is a characteristic feature of the genus *Frankia* (Lechevalier and Lechevalier, 1990). *Frankia* also produce swollen structures

called as vesicles. Vesicles are the site of nitrogenase activity, the enzyme responsible for nitrogen fixation (Meesters *et al.*, 1985). Vesicles are analogous to heterocysts of cyanobacteria because of their ability to restrict the diffusion of oxygen to the site of nitrogenase activity.

Frankia strains have been isolated from a large number of species within *Alnus* (Lechevalier, 1986). Extensive investigations on all aspects of *Frankia*-actinorhizal plant symbiosis have been taken up in the last fifteen years. Much of the work done on *Frankia* has been carried out using isolates generated by crushing nodules in suitable culture medium. In the present study also the nodules of *Alnus nepalensis* were collected from Arunachal Pradesh, India. The microorganism was isolated using the same method and its identity was confirmed. Our contention was that the very nature of this technique was likely to generate genetically mixed cultures. Unfortunately, very little work had been done with the view to purify the cultures genetically. Although Tzen *et al.* (1991), Prin *et al.* (1991) and Lumini and Bosco, (1996) carried out investigations on generation of single spore cultures, their attempts were confined to *Elaeagnus* and *Hippophae*. No reports were available on genetic purification of the *Alnus* infective *Frankia* cultures.

The Random Amplified Fragment Length Polymorphism (RFLP) technique has been used as a powerful tool to identify and distinguish closely related isolates (Bloom *et al.*, 1989). Restriction digestion of a given DNA fragment can give rise to a pattern of fragments that is informative of the location of target sites in the sequence. Mutation may lead to the change in the target sequence. DNA from different members of a genus were analyzed by such methods (Dobritsa *et al.*, 1985). Jamann *et al.* (1993) used such an approach for typing *Elaeagnus* infective *Frankia* strain.

The present investigations were taken up with the following objectives:

1. To obtain genetically pure cultures by generating single spore lines of some alder compatible *Frankia* strains.
2. To investigate the presence of variability in the original mother strain by using the molecular patterns and nitrogenase activities of the single spore lines derived from it.
3. Based on the variability observed, try to develop strain specific molecular markers.

For the present study the mother cultures were sporulated using 2μg/mL ampicilline. The sporulated cultures were filtered through 0.9μM glass filters and the spores were trapped on the glass filter. The filter was then shaken in fresh DPM medium and spore density counted on a haemocytometer. Appropriate dilution was done to achieve a spore density of 2 spores per drop of 5μL volume. This spore suspension was then mixed with 5% sodium alginate solution. The mixture was then dropped into 1% $CaCl_2$ solution. This resulted in the formation of alginate beads. The beads after hardening (by keeping them at

10°C) were transferred to fresh sterile medium and the isolates were obtained within two weeks.

To confirm the identity of *Frankia,* nodulation tests, nitrogenase activity studies and PCR with genus specific DNA primers were carried out. The results confirmed the single spore purified cultures to be *Frankia.*

The nitrogenase activity tests showed variation in respect to different single spore cultures. Further it was found that AnpST11^{SSP1} was the best nitrogen fixer among the strains purified as above.

Variations with respect to amplification profiles using total 16S rRNA gene, distal part of 16S rRNA gene, 16S-23S *rrn* ITS, *nif* D-H IGS and *nif* D-K IGS were found both between the single spore cultures obtained from the same mother culture and those obtained from different mother cultures. Restriction Fragment Length Patterns were used to detect variation between different single spore purified cultures by digesting the amplified product with 4 base cutter enzymes. For 16S-23S ITS, *Nci*1 *and* for 16S rRNA gene *Scr*F1 were used. Prediction of the patterns as well as selection of the enzymes were done using MacVector software and Power Macintosh 6100/66 computer. It was observed that in majority of the cases of the digestion of 16S-23S ITS amplified DNA, three similar bands were observed. However, in case of AnpUS8^{SSP1} there were four bands. Amongst that, one band matched with that of the *Casuarina* compatible strain ORS020606. When the amplified DNA for the distal part of 16S rRNA gene was digested, it was observed that all the other isolates showed similar patterns except AnpUS8^{SSP1}, where an extra band was present. This extra band was also similar to that of *Casuarina* compatible strain ORS020606.

Therefore, it was concluded that:

1. A novel technique for genetic purification of *Frankia* cultures was devised and tested. The genetic purification of mother strains by generation of single spore cultures was successful.

2. These single spore cultures were confirmed to be *Frankia.*

3. Nitrogenase activity assays, Amplification profile studies and PCR-RFLP studies confirmed the presence of variability among the single spore cultures derived from the common mother culture.

   So, the contention that the original cultures were genetically impure was right.

4. PCR-RFLP pattern specific to single spore purified culture AnpUS8^{SSP1} was identified.

   Thus, it is possible to use this approach for developing molecular signatures for genetically purified better performing *Frankia* strains.