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Some aspects of actinorhizal symbiosis

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Recent developments in the field of isolation, culturing, morphology and systematics of *Frankia* and host-microsymbiont interaction including nitrogen fixation have been reviewed. Special emphasis has been laid on the molecular approaches to identification of *Frankia* useing 16S rRNA and *nif* genes.

Key words: *Frankia*, actinorhizal symbiosis, culture, host specificity, identification, 16s rRNA, taxonomy, *nif* genes, *rrn* operon.

Symbiotic associations that develop between micro-organisms and higher plants are receiving considerable attention due to their effect on plant morphogenesis, nutrition and protection against infectious diseases. These associations are distinct cellular recognition processes that arise from specific union between two taxonomically diverse organisms that may exist independently. The symbiotic associations capable of utilizing molecular nitrogen are of prime agronomic 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 definite advantages over others. 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 dinitrogen except possibly *Eriphorum vaginatum*[1]. Presence of high amount of fixed nitrogen in the environment during evolution may not have created any real pressure on the late comers eukaryotes to adopt to dinitrogen utilization. Wherever a need arose, it was made up by entering into an association with prokaryotes, already endowed with the ability to fix nitrogen. Thus, one finds in nature a variety of associations, ranging from colonization of rhizosphere and phyllosphere to the endosymbiotic nitrogen fixing root nodules induced by *Rhizobium* in legumes and *Frankia* in some non-leguminous actinorhizal plants. We shall be discussing here some features of actinorhizal symbiosis.

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Actinorhizal plants

Actinorhizal plants are woody, dicotyledonous, perennial, angiosperms and spread over eight plant families representing about 25 genera[2] including *Rubus* (Table 1). These plants are nodulated by the filamentous bacterium, *Frankia*, belonging the family Frankiaceae. The term 'actinorhiza' is given to root nodules that are formed by *Frankia*.

Unlike *Rhizobium* that nodulates plants belonging to family Leguminosae only, *Frankia* is able to strike an association with plants belonging to several unrelated families. This naturally raises the question. Whether symbiosis originated as a single association long ago and the connecting links have now gone missing, or it had a multiphyletic origin. One wonders how was *Frankia* able to enter into symbiosis with diverse groups of plants. Soltis and coworkers[3] suggested a single origin of the predisposition of the host towards nodulation by *Frankia*. Swensen and Mullin[4] studied the phylogenetic relationship among actinorhizal plants and have concluded that nodulation by *Frankia* occurred independently at least four times during the course of evolution. This view gets support from the studies of Jeong *et al*[5]. It would mean that since *Frankia* has a much wider host range compared to more rigid systems such as *Rhizobium*, it could be a good candidate for creating novel nitrogen fixing symbioses with horticulturally important trees.

The importance of actinorhizal trees in regeneration of nutrient depleted/degraded forest soils is well established. These woody plants fix atmospheric nitrogen by forming symbiotic association with *Frankia* and offer a system for continued supply of fixed nitrogen to the other plant communities[6]. This attribute, if induced is an array of plants,

Table 1. List of actinorhizal plant-genera

Order	Family	Genus	No. of species	*Frankia* isolates reported
Casuarinales	Casuarinaceae	*Allocasuarina*	54	Yes
		Casuarina	16	Yes
		Ceuthostoma	2	No
		Gymnostoma	18	Yes
Fagales	Betulaceae	*Alnus*	47	Yes
Myricales	Myricaceae	*Comptonia*	1	Yes
		Myrica	28	Yes
Protales	Elaeagnaceae	*Elaeagnus*	38	Yes
		Hippophae	2	Yes
		Shepherdia	2	Yes
Ranunculales	Coriariaceae	*Coriaria*	16	Yes
Rhamnales	Rhamnaceae	*Ceanothus*	31	Yes
		Colletia	4	Yes
		Discaria	5	Yes
		Kentrothamnus	1	No
		Retanilla	2	Yes
		Talguenea	1	Yes
		Trevoa	2	Yes
Rosales	Rosaceae	*Cercocarpus*	4	Yes
		Chamaebatia	1	No
		Cowania	1	Yes
		Dryas	3	No
		Purshia	2	Yes
		Rubus?	2	No
Violales	Datiscaceae	*Datisca*	2	Yes

will go a long way in reducing fertilizer dependence in horticultural crops and waste land plantation.

Microsymbiont

The name *Frankia* was given by Brunchorst, in 1886 to honour his mentor, A. B. Frank, who coined the term symbiosis[7]. Nodules of non-legumes were first described by Meyen in 1829 and nodule formation by an endophyte was demonstrated by Woronin in 1866. It was only in 1950s that studies relating to actinorhizal plants began in right earnest. In 1978, the first culture of *Frankia* became available[8]. Since then there have been many reports of endophyte isolation from different host species. For a historical perspective of research on *Frankia*, see Bergersen and Postgate[9].

Isolation and culture

Attempts to isolate and cultivate *Frankia* in pure culture date back to the late 1800s. Numerous workers claim to have successfully isolated this filamentous bacterium but none could demonstrate clearly the consistent re-infection of the host plant. Callaham *et al*.[8] reported first successful isolation of *Frankia* from the actinorhizal genus *Comptonia*. Since then, large number of successful isolations have been reported and at present many *Frankia* strains are held in collections around the world. Despite this rapid advance, isolation of *Frankia* from some hosts has been difficult. This is because of the slow growth, high variability and ill defined growth conditions of the symbiont. All cultivation protocols for *Frankia* employ nitrogen deficient media to select against nondiazotrophs. Isolation methods in are: microdissection[10], serial filtration[11], sucrose density gradient centrifugation[12] and entrapment in alginate beads[13]. We found that the nitrogenase activity of polymer entrapped *Frankia* was not affected by the presence of nitrogen in the culture medium[14]. Not only this, polymer entrapment of *Frankia* colonies enhanced their survival. Sayed *et al*.[15] investigated the relative infectivity of polyacrylamide gel entrapped *Frankia* and found that the infectivity was retained for three months, if the PAG entrapped *Frankia* were stored below 28°C.

Traditionally, *Frankia*-strains are isolated by crushing nodules in suitable culture medium. Since a single nodule lobe may contain several strains[16], this procedure for isolation can not ensure genetic homogeneity of the cultures obtained. Prin *et al*.[17] obtained single spore isolates of strain ORS140102. Lumini and Bosco[18] obtained 22 single spore isolates of

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Elaeagnus compatible *Frankia* strain UFI132715 using solid culture support in petri plates. However, taking advantage of the observation that polymer entrapped *Frankia* spores could germinate on restoration to culture medium[19], we devised a calcium alginate bead based method for genetic purification[13]. This technique has the added advantage of improved handling of spores, not only for genetic purification, but also for other applications, such as transport of cultures and their inoculation in field conditions.

Several studies have concentrated on the optimization of growth conditions for the actinomycete[20-24]. Many culture media have been formulated for the isolation and maintenance of *Frankia*. In general complex media are better compared to defined ones. Microaerophilic strains do not grow at the surface of the liquid medium and can not be maintained on slants. Several isolates were tested for their growth in media containing carbon sources like glucose, succinic acid and propionic acid. Propionic acid was found to be the best[25]. Akkermans *et al*[26] found that strain AvcI1 utilised Tween and fatty acids as carbon sources. Vitamins like biotin, calcium pantothenate and riboflavin stimulated the growth of some *Frankia* isolates[27]. Growth of *Frankia* in cultures was inhibited by phenolics[28] and sodium chloride[29].

Identification

The genus *Frankia* is now recognized for its formation of nitrogen fixing nodules on suitable host species and by its typical morphological and physiological features. *Frankia* are Gram positive actinomycetes, capable of nodulating actinorhizal plants and fixing atmospheric nitrogen (with a few exceptions), They form sporangia, spores and vesicles. They have type III cell wall and have fucose in their cell membrane. The G+C ranges between 66 - 75% and phospholipid pattern belongs to PI group. Presence of sugar 2-O-methylmannose, major menaquinone MK9(H4) and amino acid A2pm are other characteristic features[7,30].

Molecular methods for identification of strains

Modern molecular tools, like DNA sequencing[31] and polymerase chain reaction (PCR)[32], have revolutionized all areas of biology and medicine, especially the field of bacterial phylogeny. Sequencing has enabled an easy, reliable and precise interpretation of phylogenetic data. PCR is a technique that enables amplification of genetic material, which would otherwise be too small to study. The technique involves extraction of nucleic acids of sufficient purity and its amplification 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 rapid screening of large gene libraries. Initial screening of the target gene containing clones by restriction fragment length polymorphism (RFLP) 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 in relation to previously published sequences[33].

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[34,35], cytochrome C gene, *nif* genes in nitrogen fixers, haemoglobin 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[36]. Conserved regions allow identification of universal tracts of sequences in related organisms. Variable domains permit discrimination at different taxonomic levels[37].

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[38]. The genome molecular weight of *Frankia* strains ArI4 and EuI1 as determined by reassociation kinetics analysis, is 8.3 X 10^9 and 6.0 X 10^9 respectively[39]. Analysis of 16S ribosomal RNA genes of two *Frankia* strains established a clear phylogenetic relationship between *Frankia* and the non-symbiotic soil actinomycete *Geodermatophilus*[40,41].

The uniform morphology and growth characteristics of *Frankia* strains in culture gives no hint of the extent of genetic diversity that exists within the genus. Hybridization studies revealed a lot of divergence[42]. On an even smaller geographical scale, total protein patterns[17,43], some enzymes[44], sugars[45], restriction pattern analysis of total

DNA[46] and plasmid profiles[47] have revealed diversity in *Frankia* isolates from single host plants[48] and in some cases from single nodule lobes[49]. We have recently found that even individual cultures, obtained from single nodule lobe, may have mixed genotypes, if not purified through single spore lines[50].

The symbiotic genes of *Frankia* have been studied recently. Structural genes coding for the nitrogenase complex enable to understand the evolutionary sequence of actinomycete. Many other conserved genes like the 16S rRNA, leghemoglobin and *nod* genes are also a subject of intensive study. Several factors have interfered with the understanding of genetics of *Frankia* symbiosis. Mutated symbiolic systems are not available and a transformation system suitable for use with *Frankia* has not been perfected so far. Until these tools are routinely available, progress will continue to be slow.

16S rRNA genes as 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 FGPS849 and FGPS1176′ have been designed from this region that enabled the amplification of the required 325 bp fragment in *Frankia*[51]. This fragment was found to be of same length in different *Frankia* strains. Simonet *et al*[52] selected a variable region in the rRNA operon and designed primers FGPS958 and FGPS1093. 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 Bosco *et al*[53], who designed primer FGPS989ac and FGPS989e, 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*[52] 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*[54] studied the rRNA region in many *Coriaria* and *Datisca* isolates and constructed specific primers for identification of such strains. Harry *et al*[55] did a partial sequencing of 16S rRNA gene in several strains and discovered variability in two distinct segments (140 - 220 and 980 - 1060 nt according to the *Escherichia coli* numbering).

The organization of the *rrn* operon itself was studied by Normand *et al*[56], who analyzed the gene in *Frankia*-strains ORS020606 and Ag/mut-15 and compared 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 of 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 present between 16S and 23S genes was 411 nt; and ITS 2, present 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.

Honerlage *et al*[57] studied the 23S region for insertions and discovered that the 23S region was more variable than the 16S region. A large insertion in the domain III was 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 Hahn *et al*[40] attempted a partial nucleotide sequencing of 16SrDNA 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[58]. Subsequently, 16S rRNA genes from a comprehensive set of *Frankia*-strains were completely sequenced[41]. All known *Frankia*-isolates could be delineated into four distinct groups as follows:

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.
4. "Atypical" strains, a group that includes an *Alnus* infective non-nitrogen fixing strain.

Recently, Varghese *et al*[59] used the nucleotide sequence relatedness of the *rrn* operon ITS for determining the evolutionary trend in the alder-*Frankia* symbiosis. They found that the host of closely related *Frankia*-strains, *Alnus nepalensis* and *A. glutinosa*, too have some sequence similarity. They argued that the alder progenitor entered into an association with *Frankia* and evolutionary diversification into

glutinosa and *nepalensis* species may have taken place subsequently.

Nitrogenase genes as taxonomic tools

The *nif* genes provide additional information on phylogenetics. This information is more discriminative as the *nif* genes are present in only nitrogen fixers (Table 2). RFLPs, AFLPs, and DNA sequencing can quickly provide valuable information for strain characterization. In *Frankia*, Nick *et al*[60] used this approach for analyzing the *nif* H-D IGS in *Coriaria* infective isolates and developed specific oligos for their characterization. Jamann *et al*[61] addressed the problem of strain characterization by using the same technique for typing *Elaeagnus* infective *Frankia* strains. Primers FGPD807 and FGPK700′ 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*[62] 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. Fernendez *et al*[42] found a single genomic category for *Casuarina* infective strains.

Complete sequence of *nif* H was determined from *Frankia* strain HRN18a[63]. The open reading frame was found to be 870 bp long that encoded a polypeptide of 270 amino acids. The *nif* H was also sequenced from ArI3[64]. The amino acid sequence similarity between this sequence and the corresponding sequence from *Frankia*-strain HRN18a was found to be 96%. The *nif* H was found to be conserved in many nitrogen fixing organisms. The *nif* D was sequenced in *Frankia*-strain ArI3[65]. The protein coding region was found to be 1458 nt long that encoded 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. 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*[66] studied *nif* D-K IGS in *Frankia* strains ArI3 and ACoN24d and found the region to be 265 nt and 199 nt long respectively. They showed that the IGS had no homology with any known DNA sequence. Alignment of sequences from strains EUNIf, ArI3 and ACoN24d with those of 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. The *nif* K gene sequencing was also done by Hirsch *et al*[67] from *Frankia* strain HFPCcI3. Oh *et al*[68] recently determined the phylogenetic relationship of an *Elaeagnus* compatible *Frankia* strain EuIK1 with *Alnus* infective *Frankia* using *nif*V gene. They found that this strain was close to *Alnus* infective *Frankia* studied by them and the three clustered with the alpha-class of protobacteria. They used this observation to support the hypothesis of vertical descent of *nif*V.

Organization of *nif* genes was also explored by Harriott *et al*[69]. 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.

Table 2: *nif* specific genes and their products based on Dean and Jacobson[9]

Gene	Product and Function
nif H	Fe protein subunit
nif D	MoFe protein a subunit
nif K	MoFe protein b subunit
nif F	Flavodoxin, physiological reductant of the Fe protein
nif J	Pyruvate-flavodoxin -oxidoreductase, couples the oxidation of pyruvate to the reduction of flavodoxin
nif M	Required for activation of the Fe protein
nif U	Appears to be involved in the stabilization of the Fe protein
nif S	Function unknown,
nif V	Probably encodes a homocitrate lyase.
nif E	Required for FeMo cofactor biosynthesis.
nif N	-do-
nif B	-do-
nif Q	-do-
nif W	required for full activity of the MoFe protein
nif Z	-do-
nif A	positive regulatory element
nif L	negative regulatory element
nif X	probably a negative regulatory element
nif T	Function unknown
nif Y	Function unknown

Morphology of *Frankia*

Frankia have typical actinomycete-morphology. They produce a vegetative mycelium composed of septate

filaments. Two types of structures may be produced on the mycelium:

Vesicles: The vesicles are enlarged spherical[26] structures with thick walls[71] attached to the mycelium by a stalk. It may be in completely or sometimes incompletely, divided into compartments by septae. Since fully functional vesicles have been observed without septae, the function of the septum is not clear. Although, vesicles are generally involved in nitrogen fixation[21], some studies indicate that they may also serve as a propagules[22,70]. They are formed on most media by certain strains while others do so on special media[7]. Generally vesicle formation is induced by transfer to nitrogen free medium. However, strain EAN1pec formed vesicles even in medium containing ammonia, while ACN1^{AG} did not in any medium when incubated at 33°C[72]. Some workers[25] have reported formation of super clusters of vesicles. These structures are ball shaped and approximately 0.5 - 1 mM in diameter, consisting of an inner part densely packed with vesicles and a dense outer layer of hyphae.

Sporangia: The second specialized structure produced by *Frankia* is the sporangium. Sporangia may be produced as terminal appendages of the vegetative mycelium or in some strains may occur as intercalary structures. They are formed by the growth of longitudinally and transversely oriented septae, which compartmentalize the wide hyphae[73]. They are 20 - 60 mM in length and attached to the submerged filaments by thickened sporangiophores[21,74]. Sporangia may be pear or columnar shaped[71]. Spores roughly 1 mM in diameter, spherical to oblong in shape[71] and produced in large numbers within the amorphous sporangia. Spores non-motile and display 1-3 germ tubes for most strains[7]. While Burleigh and Dawson[29] found that aliphatic amino acids increased the frequency of sporulation, Ganesh[23] found that profuse sporulation could be induced by adding ampicilline to the culture medium.

As a symbiont, *Frankia* are filamentous, usually surrounded by a host cell membrane and cell wall like material called capsid. A vegetative mycelium is present in all infected cells. Vesicles are produced in large numbers during active nitrogen fixation but morphology of the vesicles may be significantly modified by the host. Vesicles are absent in *Casuarina*-nodules. Nodules that contain sporangia are referred to as sp$^+$. Other commonly found nodules with few or no sporangia are called as sp$^-$. 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[75-77].

Host - microsymbiont relationship

Host specificaly

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[78]. Current belief among *Frankia* researchers is that host specificity should not be used to define taxa[53] not with standing the strong correlation between host specificity of strains and genetic similarities.

From those *Frankia*-strains that have been isolated in pure culture, four major host specificity groups can be identified: *Alnus*, *Casuarina* and related strains, *Elaeagnus* group, unisolated strains from *Datisca*, *Coriaria* and atypical strains[41]. More host specificity groups may be added to the list as strains from Rhamnaceae and Rosaceae are isolated and characterized.

Factors that lead to symbiosis may be host as well as microsymbiont dependent[79]. Physiology of both the partners is the 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.

Host specificity is the key, which derives 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? Attempts are already afoot in this direction. To arrive at plausible answers, it would be essential to take a recourse to molecular biology. Phylogeny of the host in molecular terms needs to be worked out just as 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 the elucidation of phylogenetic relationships. Bousquet *et al*[80] 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[81]. The *rbc*L gene

sequence comparison has been done for many actinorhizal families[3]. 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%[82].

Many studies on this aspect are still on and large gaps persist. Preliminary reports by Prat[83] revealed that host did have a role in determining the efficiency of symbiosis. Sougoufara *et al*[84] experimented with a series of *Frankia* strains and plant host clones and discovered that a particular *Frankia*-strain was always a better nitrogen fixer, irrespective be the host clone used. The host effect dominated all other effects. Sougoufara *et al*[84] predicted that improvement of nitrogen fixing ability could be achieved by screening for the best host clone and subsequently the *Frankia* strain associated with the best clone. Verghese and Misra[85] used multisite molecular markers strategy to work out the role of the microsymbiont in determining the level of nitrogenase activity in the nodule. They could not find any definite relationship between the microsymbiont genotype and nitrogenase activity in the nodule; and concluded that the genotype of the host was probably the major player in determining the level of nitrogenase activity in nodules. This has been confirmed by Chauhan and Misra[86], who revealed a relationship between the host genotype and the quantum of nitrogenase activity in the actinorhizal root nodules. They used multisite strategy for developing PCR-RFLP based molecular markers for tagging host genotypes and found that out of a total of 100 trees, twelve had a common molecular marker and had low nitrogenase activity. They argued that since these nodules were collected from nature, all the autochthonous *Frankia*-strains had equal opportunity to nodulate the hosts. The fact that these twelve trees had low nitrogen fixing nodules, indicated the role of the host genotype in either harbouring poor *Frankia*-strains, or causing even superior *Frankia* strains harboured by them to have low nitrogenase activity. The first option would be exercised at the time of infection, which may have specificity for poor fixers. The second option would be exercised by the physiological state of the host, thus not providing sufficient support to the microsymbiont for an optimal nitrogenase activity. Wall *et al*[87] found that different regulatory pathways existed for successful infection by invading *Frankia*, depending upon the infection pathway in the host. This might suggest the prevalence of the first option.

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-organism show or 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?

Infection process

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[88] 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* may help soften host cell walls[89]. The invasion by the microbe seems to be delicately controlled in view of the light regulation of the host defence systems. Initiation of nodule development is characterised by development of lateral roots. Phenylacetic acid produced by *Frankia* apparently induces lateral root development[90]. Goetting-Minesky and Mullin[91] isolated a host plant nodule specific cDNA sequence for a nodule specific cysteine proteinase (AgNOD-LP1). They have suggested that this proteinase plays a role in remodelling of tissues in root and nodule tissues.

Nodule formation and development

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[92,93] are some of the host genes inductively expressed during the period of nodule formation.

In the endosymbiotic state, the microsymbiont is transformed into a bacteriod which perform and various functions in 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

hydrogenase and specific cytochromes and genes responsible for changes in the outer membrane[94]. 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. The microsymbiont thus evolves as a nitrogen fixing organelle, leading ultimately to a naturally dependent association.

Nitrogen fixation

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 'a' proteins (50000 daltons each), two 'b' 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 'a' subunits (32000 daltons each). The molecular weights of the subunits may vary in different organisms[95]. The reduction of nitrogen involves combination of component 1 and 2, with Mg^{++} ATP and a source of reducing equivalents. In addition to nitrogen, nitrogenase can also reduce acetylene, methyl isocyanide, azide, N_3^-, N_2O, HCN and a host of other substrates. The organization of *nif* genes in *Frankia* is understood to be similar to that in *Klebsiella pneumoniae*[64].

Future research needs

Recently, genome sequencing of *Frankia* has been initiated (Normand, personal communication). This will give a boost to further work and better understanding of this symbiosis. The future research on following aspects may forther improve our understanding about actinorhizal symbiosis and its exploitation for behalf of moment and the envirement.

- Means of reducing doubling time for *Frankia*
- Isolation and study of *Frankia*- strains from new actinorhizal trees.
- Identification, isolation and characterisation of host genes involved in initiation and maintenance of actinorhizal symbiosis.
- Introduction of these genes in trees for initiating nitrogen fixing associations between economically important trees and *Frankia*.

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References

1. Chapin III FS, Moilanen L & Kielland K (1993) Preferential use of organic nitrogen for growth by a non-mycorhizal arctic sedge. Nature 361:150-154.
2. Lechevalier MP (1994) Taxonomy of the genus *Frankia* (Actinomyceteles). Int J Syst Bacteriol 44:1-8.
3. Soltis DE, Soltis PS, Moegan DR, Swenson SM, Mullin BC, Dowd M & Martin PG (1995) Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc Natl Acad Sci (USA) 92:2647-2651.
4. Swenson SM & Mullin BC (1997) Phylogenetic relationship among actinorhizal plants: The impact of molecular systematics and implcations for the evolution of actinorhizal symbioses. Physiol Plantarum 99:565-573.
5. Jeong SC, Ritchie NJ & Myrold DD (1999) Molecular phylogenies of plants and *Frankia* support multiple origins of actinorhizal symbioses. Mol Phylogenet Evol 13:493-503.
6. Carpenter PL & Hensley DL (1979) Utilizing N_2 fixing woody plant sp. for distressed soils and the effect of lime on survival. Bot Gaz 140:76.
7. Lechevalier MP and Lechevalier HA (1989) Genus *Frankia* Brunchorst 1886, 174 AL. In: Bergey's Manual of Systematic Bacteriology (Williams ST, Sharp ME & Holt JG eds). Williams & Wilkins, Baltimore, USA, pp 2410-2417.
8. Callaham D, Tredici P Del & Torrey JG (1978) Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. Science 199:899-902.
9. Bergersen FJ & Postgate JR (1987) A Century of Nitrogen Fixation Research: Present Status and Future Prospects. The Royal Society, London.
10. Diem H & Dommergues, YR (1983) The isolation of *Frankia* from nodules of *Casuarina*. Can J Bot 61:2822-2826.
11. Benson DR (1982) Isolation of *Frankia* strains from alder actinorhizal root nodules, Appl Environ Microbiol 44:461-465.
12. Baker DD & O'Keefe D (1984) A modified sucrose fractionation procedure for the isolation of *Frankiae* from actinorhizal root nodules and soil samples. Plant Soil 78:23-28.
13. Sarma G, Sen A, Varghese R & Misra AK (1998) A novel technique for isolation of *Frankia* and generation of single spore cultures. Canadian J Microbiol 44:490-492.
14. Borthakur M, Sen A & Misra AK (1997) Exogenous nitrogen sources do not drastically reduce nitrogenase activity in

polymer entrapped *Frankia*. Indian J Exp Biol 35:173-175.

5. Sayed WF, Wheeler CT, el-Sharouny HM, Mohawad SM & Abdel-Karim MM (2002) Effects of storage time and temperature on the infectivity and effectiveness of *Frankia* entrapped in polyacrylamide gel. Folia Microbiol (Praha) 47:545-550.
6. Dobritsa SV & Stupar OS (1989) Genetic heterogeneity among *Frankia* isolates from root nodules of individual actinorhizal plants. FEMS Microbiol Lett 58:287292.
7. Prin Y, Maggia L, Picard B, Diem HG, & Goullet P (1991) Electrophoretic comparison of enzymes from 22 single spore cultures obtained from *Frankia* strain ORS 140102. FEMS Microbiol Lett 77:223-228.
8. Lumini E & Bosco M (1996) PCR-restriction fragment length polymorphism identification and host range of single spore isolates of flexible *Frankia* sp. UFI32715. Appl Environ Microbiol 62:3026-3029.
9. Borthakur M, Sen A & Misra AK (1996) Immobilized *Frankia* spores remained viable on dry storage and restoration to medium regenerated active colonies. Plant Soil 181:227-231.
10. Baker DD & Torrey JG (1979) The isolation and cultivation of actinomycetous root nodule endophytes. In: Symbiotic Nitrogen Fixation in the Management of Temperate Forests. (Gordon JC, Wheeler CT & Perry DA eds) Oregon State University Press, Corvallis, USA, p 38.
11. Baker DD, Torrey JG & Kidd GH (1979) Isolation by sucrose density fractionation and cultivation *in vitro* of actinomycetes from nitrogen fixing root nodules. Nature 281:76-78.
12. Zhongze Z & Torrey JG (1985) Biological and cultural characteristics of the effective *Frankia* strain HFPCcI3 (*Actinomycetales*) from *Casuarina cunninghamiana* (*Casuarinaceae*). Annal Bot 56:367-378.
13. 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. p.
14. Selim S, Delacour S & Schwencke J (1996) Specific long chain fatty acids promote optional growth of *Frankia*: accumulation and intracellular distribution of palmitic and propionic acids. Arch Microbiol 165:252-257.
15. Burgraff AJP & Shipton WA (1983) Studies on the growth of *Frankia* isolates in relation to infectivity and nitrogen fixation (acetylene reduction). Can J Bot 61:2774-2782.
16. Akkermans ADL, Roelofsen W, Blom J, Huss-Danell K & Harkink (1983) Utilization of carbon and nitrogen compounds by *Frankia* in synthetic media and in root nodules of *Alnus glutinosa*, *Hippophae rhamnoides* and *Datisca cannabia*. Can J Bot 61:2793-2800.
17. Shipton WA & Burggraff AJP (1982) A comparison of the requirement of various carbon and nitrogen sources and vitamins in some *Frankia* isolates. Plant Soil 69:149-161.
18. Perradin Y, Mottet MJ & Lalonde M (1983) Influence of phenolics on *in vitro* growth of *Frankia* strains. Can J Bot 61:2807-2814.
29. Burleigh SH & Dawson JO (1991) *In vitro* sporulation of *Frankia* strain HFPCc13 from *Casuarina cunninghamiana*. Can J Microbiol 37: 897. **72**
30. Lechevalier MP (1983) Cataloging *Frankia* strains. Can J Bot 61:2964-2967.
31. Sanger F, Nicklin S & Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci (USA) 74:5463-5467.
32. Mullis K, Faloona F, Scharf S, Saiki R, Horn G & Erlich H (1986) Specific enzymatic amplification of DNA *in vitro*: The polymerase chain reaction. Cold Spring Harbor Symp Quant Biol 51:263-273.
33. Maidak BL, Lars N, McCaughey MJ, Overbeek R, Olsen GJ, Fogel K, Blandy J & Woese CR (1994) The ribosomal database project. Nucl Acids Res 22:3485-3487.
34. Woese C, Gutell R, Gupta R & Noller HF (1983) Detailed analysis of the higher order structure of 16S like rRNAs. Microbiol Rev 47:621-669.
35. Woese C (1987) Bacterial evolution. Microbiol Rev 51:221-271.
36. Gutell RR, Larsen N & Woese CR (1994) Lessons from an evolving rRNA:16S and 23S rRNA structures from a comparative perspective. Microbiol Rev 58:10-26.
37. Varghese R, Chauhan VS & Misra AK (2003). Hypervariable spacer regions are good sites for developing specific PCR-RFLP markers and PCR primers for screening actinorhizal symbionts. J Bioscience 48:437-442.
38. An CS, Riggsby WS & Mullin BC (1985) Relationships of *Frankia* isolates based on deoxyribonucleic acid homology studies. Int J Syst Bacteriol 35:140-146.
39. An CS, Riggsby WS & Mullin BC (1987) DNA relatedness of *Frankia* isolates ArI4 and EuI1 to other actinomycetes of cell wall type III. The Actinomycetes 20:50-59.
40. Hahn D, Lechevalier MP, Fischer A & Stackbrandt E (1988) Evidence for a close phylogenetic relationship between members of the genera *Frankia*, *Geodermatophyllus* and *Blastococcus* and emendation of the family *Frankia*ceae. Syst Appl Microbiol 11:236-242.
41. Normand P, Orso S, Cournoyer B, Jeanin P, Chapelon C, Dawson J, Evtusenko L & Misra AK (1996) Molecular phylogeny of the genus *Frankia* and amendation of family *Frankia*ceae. Int J Syst Bacteriol 46:1-9.
42. Fernandez MP, Meugnier G, Grimont AD & Bardin R (1989) Deoxyribonucleic acid relatedness among members of the genus *Frankia*. Int J Syst Bacteriol 39:783-791.
43. Gardes M & Lalonde M (1987) Identification and subgrouping of *Frankia* strains using sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Physiol Plantarum 70:237-244.
44. Girgis MGZ & Schwencke J (1993) Differentiation of *Frankia* strains by their electrophoretic patterns of intracellular esterase and aminopeptidases. J Gen Microbiol 139:2225-2232.

45. St-Laurent L, Bousquet J, Simon L & Lalonde M (1987) Separation of various *Frankia* strains in the *Alnus* and *Elaeagnus* host infectivity groups using sugar analysis. Can J Microbiol 33:764-772.
46. Bloom RA, Mullin BC & Tate RL (1989) DNA restriction patterns and DNA hybridisation studies of *Frankia* isolates from *Myrica pensylvanica* (bayberry). Appl Environ Microbiol 55:2156-2160.
47. Normand P, Simonet P, Butour JL, Rosenberg C, Moiroud A & Lalonde M (1983) Plasmids in *Frankia* sp. J Bacteriol 155:32-35.
48. Simonet P, Thi Le N, Moiroud A & Bardin R (1989) Diversity of *Frankia* isolated from a single alder stand. Plant Soil 118:13-22.
49. Reddell P, & Bowen GD (1985) Do single nodules of *Casuarinaceae* contain more than one *Frankia* strain. Plant Soil 88:275-279.
50. Sarma G & Misra AK (2002) Mixed genotypes in some axenic cultures of *Frankia*. Indian J Microbiology 41:11-14.
51. Nazaret S, Cournoyer B, Normand P & Simonet P (1991) Phylogenetic relationships among *Frankia* genomic species determined by the use of amplified 16S rDNA sequences. J Bacteriol 173:4072-4078.
52. Simonet P, Grosjean MC, Misra AK, Nazaret S, Cournoyer B & Normand P (1991) *Frankia* genus specific characterisation by polymerase chain reaction. Appl Environ Microbiol 57:3278-3286.
53. Bosco M, Fernandez MP, Simonet P, Materassi R & Normand P (1992) Evidence that some *Frankia* sp. strains are able to cross boundaries between *Alnus* and *Elaeagnus* host specificity groups. Appl Environ Microbiol 58:1569-1576.
54. Mirza MS, Hameed S & Akkermans AD (1994) Genetic diversity of *Datisca cannabina*- compatible *Frankia* strains as determined by sequence analysis of the PCR-amplified 16S rRNA gene. Appl Environ Microbiol 60:2371-2376.
55. Harry DE, Yang DC & Dawson JO (1991) Nucleotide sequence and diversity in the 16S ribosomal RNA from *Frankia*. Plant Soil 131:143-146.
56. Normand P, Cournoyer B, Nazaret S & Simonet P (1992) Analysis of a ribosomal RNA operon in actinomycete *Frankia*. Gene 111:119-124.
57. Honerlage W, Hahn D, Zepp K, Zeyer J & Normand P (1994) A hypervariable 23S rRNA region provides a discriminatory target for specific characterisation of uncultured and cultured *Frankia*. Syst Appl Microbiol 17:433-443.
58. Ganesh G, Misra AK, Chapelon C & Normand P (1994) Morphological and molecular characterization of *Frankia* sp. isolates from nodules of *Alnus nepalensis* (Don), Arch Microbiol 161:152-155.
59. Varghese R, Chauhan VS & Misra AK (2003) Evolutionary implications of nucleotide sequence relatedness between *Alnus nepalensis* and *Alnus glutinosa* and between corresponding *Frankia* micro-symbionts. Plant Soil 254:219-227.
60. Nick G, Paget E, Simonet P & Normand P (1992) The nodular endophytes of *Coriaria* spp. form a distinct lineage within the genus *Frankia*. Mol Ecol 1:175-181.
61. Jamann S, Fernandez MP & Normand P (1993) Typing method for N_2 fixing bacteria based on PCR-RFLP - application to the characterization of *Frankia* strains. Mol Ecol 2:17-26.
62. Rouvier C, Prin Y, Reddell P, Normand P, & Simonet P (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.
63. Normand P & Bousquet J (1989) Phylogeny of nitrogenase sequences in *Frankia* and other nitrogen fixing microorganisms. J Mol Evol 29:436-447.
64. Normand P, Simonet P & Bardin R (1988) Conservation of *nif* sequences in *Frankia*. Mol Gen Genet 213:238-246.
65. Normand P, Gouy M, Cournoyer B & Simonet P (1992b) Nucleotide sequence of *nif* D from *Frankia* strain ArI3: Phylogenetic inferences. Mol Biol Evol 9:495-506.
66. Nalin R, Domenach AM & Normand P (1995) Molecular structure of the *Frankia* sp. *nif* D-K intergenic spacer and design of *Frankia* genus compatible primer. Mol Ecol 4:483-491.
67. Hirsch AM, McKhan HI, Reddy A, Liao J, Fang Y & Marshall CR (1995) Assessing horizontal transfer of *nif* HDK genes in eubacteria: Nucelotide sequence of *nif* K from *Frankia* strain HFPCcI3. Mol Biol Evol 12:16-27.
68. Oh CJ, Kim HB & An CS (2003) Molecular cloning and complementation analysis of *nif*V gene from *Frankia* EuIK1 strain. Mol Cells 15(1):27-33.
69. Harriott OT, Hosted TJ & Benson D R (1995) Sequences of *nif* X, *nif* W, *nif* Z, *nif* B and two *nif* ORF in *Frankia* nitrogen fixation gene cluster. Gene 161:63-67.
70. Torrey JG (1985) The site of nitrogenase in *Frankia* in free living culture and in symbiosis. In: Nitrogen fixation research progress (Evans HJ, Bottomley PJ & Newton WE eds). Martinus Nijhoff, The Hague Netherlands, pp 293-318.
71. Tjepkema JD, Ormerod W & Torrey JG (1980) Vesicle formation and acetylene reduction activity in *Frankia* sp. CpI1 cultured in defined nutrient media. Nature 287: 633-635.
72. Tisa, L, McBride M & Ensign JC (1983) Studies on growth and morphology of *Frankia* strains $EAN1_{pec}$, $EuI1_c$, CpI1 and $ACN1^{AG}$. Can J Bot 61-2768-2773.
73. Newcomb W, Callaham D Peterson RL & Torrey JG (1979) Morphogenesis and fine structure of the actinomycetous endophyte of nitrogen fixing root nodules of *Comptonia peregrina*. Bot Gaz 140 (Suppl):22-23.
74. Diem H, Gauthier D & Dommergues YR (1983) An effective strain of *Frankia* from *Casuarina* sp. Can J Bot 61:2815-2821.

75. Normand P & Lalonde M (1982) Evaluation of *Frankia* strains isolated from provenances of two *Alnus species*. Can J Microbiol 28:1133-1142.

76. Torrey JG (1987) Endophyte sporulation in root nodules of actinorhizal plants. Physiol Plantarum 70:279-288.

77. Simonet P, Bosco M, Chapelon C, Moiroud A & Normand P (1994) Molecular characterization of *Frankia* microsymbionts from spore positive and spore negative nodules in a natural alder stand. Appl Environ Microbiol 60:1335-1341.

78. Lechevalier MP & Lechevalier HA (1984) Taxonomy of *Frankia*. In: Biological Biochemical and Biomedical Aspects of Actinomycetes (Ortiz-Ortiz L, Bojaalil LF & Yakoleff V eds). Academic Press, New York, pp 575-582.

79. Verghese SK & Misra AK (2002) *Frankia*-Actinorhizal symbiosis, with special reference to host-microsymbiont relationship. Current Science 83:404-408.

80. Bousquet JS, Strauss H, Doerksen AH & Price RA (1992) Extensive variation in evolutionary rate of *rbc*L gene sequences among seed plants. Proc Natl Acad Sci (USA) 89:7844-7848.

81. Savard L, Michaud M & Bousquet J (1986) Genetic diversity and phylogenetic relationships between birches and alders using ITS, 18S rRNA and *rbc*L gene sequences. Mol Phylogenet Evol 2:112-118.

82. Maggia L & Bousquet J (1994) Molecular phylogeny of the actinorhizal Hamamelidae and relationship with host promiscuity towards *Frankia*. Mol Ecol 3:459-467

83. Prat D (1989) Effect of some pure and mixed *Frankia* strains on seedling growth in different Alnus species. Plant & Soil 113:31-38.

84. Sougoufara B, Maggia L, Duhoux E & Dommergues YR (1992) Nodulation and nitrogen fixation in nine *Casuarina* clone-*Frankia* combinations. Acta Oecol 13:497-503.

85. Verghese SK & Misra AK (2000). PCR-RFLP based screening of *Frankia* genotypes in alder nodules having different levels of nitrogenase activity. Symbiosis 28:337-350.

86. Chauhan VS & Misra AK (2002) Development of molecular markers for screening *Alnus nepalensis* (D. Don) genotypes for the nitrogenase activity of the actinorhizal root nodules. Molecular Genetics Genomics 267:303-312.

87. Wall LG, Valverde C & Huss-Danell K (2003) Regulation of nodulation in the absence of N_2 is different in actinorhizal plants with different infection pathways. J Exp Bot 54:1253-1258.

88. Prin Y & Rougier M (1987) Preinfection events in the establishment of *Alnus-Frankia* symbiosis. Study of the root hair deformation step. Plant Physiol (Life Sci Adv) 6:99-106.

89. Seguin A, & Lalonde M (1989) Detection of pectolytic activity and *pel* homologous sequences in *Frankia*. Plant Soil 118:221-229.

90. Hammad Y, Nalin R, Marechal J, Fiasson K, Pepin R, Berry AM, Normand P & Domenach AM (2003) A possible role for phenylacetic acid (PAA) on *Alnus glutinosa* nodulation by *Frankia*. Plant Soil 254:193-205.

91. Goetting-Minesky MP & Mullin BC (1994) Differential gene expression in an actinorhizal symbiosis : Evidence for a nodule-specific cysteine proteinase. Proc Natl Acad Sci (USA) 91:9891-9895.

92. Ribeiro A, Akkermans AD, van Kammen A, Bisseling T & Pawlowski K (1995) A nodule specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. Plant Cell 7:785-794.

93. Laplaze L, Ribeiro A, Franche C, Duhoux E, Auguy F, Bogusz D & Pawlowski K (2000) Characterisation of a *Casuarina glauca* nodule specific subtilisin-like protease gene, a homolog of *Alnus glutinosa* ag12. Mol Plant Microbe Interact 13:113-117.

94. Sutton WD, Pankhurst CE & Craig AS (1981) The *Rhizobium* bacteriod state. In: Int Rev Cytol Suppl 13 (Giles KL & Atherly AG eds). Academic Press, New York, USA, pp 149-177.

95. Schwintzer CR & Tjepkema JD (eds) (1990) The biology of *Frankia* and Actinorhizal plants. Academic Press, California, USA.

96. Cronquist A (1988) The evolution and classification of flowering plants. New York Botanical Garden, Bronx, NY, USA.

97. Dean DR & Jacobson MR (1992) Biochemical genetics of nitrogenase. In: Biological nitrogen fixation (Stacey G, Burris RH & Evans HJ eds). Chapman and Hall, London, pp. 763-834.

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