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NITROGEN FIXATION BY ACTINORHIZAL SYMBIOSIS

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Abstract: About 288 species of dicotyledonous plants belonging to 25 genera, eight diverse families and seven orders form nitrogen fixing symbiotic association with the actinomycete belonging to genus *Frankia* (family Frankiaceae). This symbiosis has been called as actinorhizal symbiosis. Although presence of *Frankia* in the root nodules of actinorhizal plants was well established by the early part of twentieth century, further study on this microorganism was delayed due to non-availability of its cultures. Attempts to isolate *Frankia* in pure cultures succeeded only in 1978. In pure culture, *Frankia* is microaerophilic and mesophilic. It grows as a dense mass of anastomosing hyphae with terminal or intercalary sporangia. In nitrogen limited conditions it also forms terminal thickenings called as vesicles. Vesicles are the site of nitrogen fixation and have evolved to protect nitrogenase enzyme from oxygen. Studies on origin of actinorhizal symbiosis have indicated multiphyletic origin of this association. Apparently, evolution of this symbiosis has moved towards increased host specificity. Work on molecular diversity in *Frankia*, and genetics and biochemistry of nitrogen fixation are briefly summarised.

5.1 INTRODUCTION

The ability to reduce atmospheric nitrogen to produce ammonia resides only with some prokaryotes. Some of these are able to fix nitrogen independent of a host but others enter into a symbiotic association with host plants. The prokaryotes that can fix atmospheric nitrogen are collectively called as diazotrophs. Free living diazotrophs include *Azotobacter*, *Azospirillum*, etc. Symbiotic associations are formed by *Nostoc*, *Rhizobium*, *Frankia*, etc.

Nitrogen fixation is a highly energy intensive process requiring approximately 18.8 gm of glucose for fixing 1 gm of nitrogen (Gutschick, 1978). When the microsymbiont forms an association with a host, it is assured of an uninterrupted supply of fixed carbon. This enhances the efficiency of nitrogen fixation. In turn the host gets an assured supply of fixed nitrogen. Thus both the host and the microsymbiont play significant part in the process of nitrogen fixation (Verghese and Misra, 2002). For effective improvement in efficiency and capacity of nitrogen fixation superior host and microbe strains need be identified. A superior host-microbe combination could give great boost to the amount of nitrogen fixed in forests.

About 288 species of dicotyledonous plants belonging to 25 genera, eight diverse families and seven orders (Table 5.1) form nitrogen fixing symbiotic association with the actinomycete belonging to genus *Frankia* (family Frankiaceae). This symbiosis has been called as actinorhizal symbiosis. Some important actinorhizal genera found in India are *Casuarina*, *Alnus*, *Myrica*, *Elaeagnus* and *Hippophae*. It is lesser known but extremely important symbiosis fixing large quantities of atmospheric nitrogen, especially in the forest ecosystems. For example, trees of *Casuarina equisetifolia* fix about 260 kg nitrogen/ hectare in one year (Dommergues et al., 1984).

Root nodules in *Alnus* were observed almost 200 hundred years ago. However, initially they were thought to be parasitic in nature. It was in the later part of the nineteenth century that Woronin studied the anatomy of these root nodules and described hyphae passing through the walls from cell to cell. He observed swelling of these hyphae inside the cell to form what he considered as fungal spores. He named this organism as *Schinzia cellulicola*. In late 1880s Brunchorst compared the root nodules of legumes with that of non-legumes and came to conclusion that a novel micro-organism was involved in root nodules of non-legumes. He named this novel microorganism as *Frankia* in honour of his teacher Frank.

Table 5.1 **List of actinorhizal trees (based on Bond, 1983; Torrey and Berg, 1988; Newcomb and Wood, 1987; Baker and Schwintzer, 1990 and Benson and Silvester, 1993).**

Order	Family	Genus	Number of species
Casuarinales	Casuarinaceae	*Allocassuarina*	54
		Casuarina	16
		Ceuthostoma	2
		Gymnostoma	18
Fagales	Betulaceae	*Alnus*	47
Myricales	Myricaceae	*Comptonia*	1
		Myrica	28
Protales	Elaeagnaceae	*Elaeagnus*	38
		Hippophae	2
		Shepherdia	2
Ranunculales	Coriariaceae	*Coriaria*	16
Rhamnales	Rhamnaceae	*Ceanothus*	31
		Colletia	4
		Discaria	5
		Kentrothamnus	1
		Retanilla	2
		Talguenea	1
		Trevoa	2
Roasales	Rosaceae	*Cercocarpus*	4
		Chamaebatia	1
		Cownia	1
		Drayas	3
		Purshia	2
Violales	Datiscaceae	*Datisca*	2

The pioneering work of Hellrigel and Wilfrath established that the microorganism harboured in root nodules of legumes helped in nitrogen fixation. In early twentieth century, Hiltner proved that actinorhizal plants could also utilize atmospheric nitrogen. This led subsequent workers to the conclusion that actinorhizal microsymbionts too were bacteria.

Actinorhizal plants have been used variously by man for centuries. *Casuarina* trees are used as windbreak in coastal areas. They are also used for stabilizing sand dunes in desert areas. Alders yield timber which is used in packaging industry. Timber from *Alnus nitida* found in western Himalayas is used for packaging of apples in Himachal Pradesh. Seabuckthorn (*Hippophae rhmanoides*) is used for making a very useful fruit drink. It is supplied to armed forces as a food supplement in higher reaches of India. It was also used by Russian astronauts during their space missions. Defence Institute of High Altitude Research (DIHAR) of Government of India has taken up ambitious programme of commercialising various food preparations from seabuckthorn. Fruits of *Elaeagnus* and *Myrica* are commercially sold in Meghalaya. *Myrica esculenta* is used in the ayurvedic system for treatment of cough and cold. Because of their ability to fix atmospheric nitrogen, actinorhizal trees are being increasingly used in reclamation of wastelands and mine spoils. They are pioneer trees in disturbed forests and *Alnus nepalensis* grows profusely in areas recently disturbed by landslides.

Although presence of *Frankia* in the root nodules of actinorhizal plants was well established by the early part of twentieth century, further study on this microorganism was delayed due to non-availability of its cultures. Attempts to isolate *Frankia* in pure cultures could not succeed till 1978 when Callaham and co-workers successfully isolated and cultured *Frankia* from root nodules of *Comptonia*.

In pure culture, *Frankia* is microaerophilic and mesophilic. It grows as a dense mass of anastomosing hyphae with terminal or intercalary sporangia. In nitrogen limited conditions it also forms terminal thickenings called as vesicles. The hyphae are branched and septate with the diameter in the range of 0.5 to 1.5 µm. Sporangia are produced readily in culture. They may be induced by low dosages of antibiotics like Ampicillin (2 µm/mL) (Ganesh, 1993). The strains that produce spores *in planta* are called as sp^+ and others that do not, are called as sp. Sporangia are multicellular and contain many spores.

The characteristic feature of *Frankia* is the development of vesicles. Vesicles are the site of nitrogen fixation and have evolved to protect nitrogenase enzyme from oxygen. Mature vesicles are 2 to 4 µm in size and have highly structured laminated lipid layers.

5.2 DIVERSITY OF *FRANKIA*

As stated above *Frankia* is able to nodulate a large number of unrelated genera. *Frankia* seem to have host specific nodulation capacity. Therefore, initially *Frankia* were classified on the basis of the identity of the host plant. Thus *Frankia* nodulating *Alnus* were called as *Frankia alni*. Cross inoculation studies have shown that *Frankia* isolated from *Alnus* could nodulate *Elaeagnus* too (Bosco et al., 1992). Thus it seems that at least for some strains, host specificity is only notional. In this context it has been found that *Frankia* isolated from *Myrica* is most promiscuous, while that isolated from *Casuarina* seem to have greater host specificity. Studies on origin of actinorhizal symbiosis have indicated multiphyletic origin of this association. Apparently, evolution of this symbiosis has moved towards increased host specificity.

Frankia also seem to have diversity with respect to their nitrogenase (see below) activities. In Sikkim Himalayas more efficient nitrogen fixers have been found at altitude around 1000 m, while those present at higher altitudes seem to have lesser nitrogenase activity (Khan et al., 2007). Both host and micro-symbiont play significant role in determining the level of nitrogenase activity in the root nodules. *Frankia* is able to fix nitrogen *ex planta* as well. It has been found that isolates that are superior fixers *ex planta* may not be efficient nitrogen fixers in symbiotic association (Ganesh, 1993). Consequently, selection of superior nitrogen fixers should be done in nodulated form. One may need to conduct an experiment on the lines of combining ability estimates for determining superior host-microbe combination.

Substantial work has been done with respect to molecular diversity in *Frankia*. Ribosomal RNA and *nif* genes have been targeted for this purpose. 16S rRNA gene sequence was used by Hahn et al. (1989) for distinguishing between Ag45/Mut15, AgW1.1 and AgB1.9 strains of *Frankia*. Several workers have since utilized this gene for identification, characterization and distinguishing Frankia infecting different hosts (Nick et al., 1992; Mirza et al., 1994; Murray et al., 1997; Clawson et al., 1998). Lately, nucleotide sequence of this gene has been extensively used for establishing phylogenetic relationship among various *Frankia* isolates as well as sequences obtained directly from nodules (Nazaret et al., 1991; Ganesh et al., 1994). Normand et al. (1996) used 16S rDNA sequences for redefining Frankiaceae.

The Internal Transcribed Spacers (ITSs) found in the *rrn* operon harbour larger variability. Consequently, these have been targeted by many workers for determining the level of diversity in *Frankia*. The ITS regions have also been targeted for developing molecular markers based on PCR-RFLP (Rouvier et al., 1996; Ritchie and Myrold, 1999; Varghese et al., 2003). The same region has also been targeted for the study of molecular ecology and molecular evolution of *Frankia*.

Three important structural genes of nitrogenase enzyme (*nifH, nifD and nifK*) have also been used for determining level of molecular diversity among different *Frankia* isolates. Jamann et al. (1993) used *nifD-nifK* ITS for developing PCR-RFLP typing for determining genetic divergence. The other significant genes studied by various workers include $tRNA^{pro}$, *glnA*, *glnII* and *sodF*.

5.3 BIOCHEMISTRY OF NITROGEN FIXATION

The enzyme nitrogenase brings about nitrogen fixation in all known diazotrophs. All known nitrogenases are oxygen sensitive iron(Fe)-sulphur(S) proteins. However, an oxygen insensitive nitrogenase has been reported from *Streptomyces thermoautotrophicus*. Four genetically distinct nitrogenases have been identified so far. The most common enzyme is a Molybdenum (Mo) and Fe containing enzyme called as Mo-nitrogenase. The second enzyme contains Vanadium (V) in place of molybdenum and is called as V-nitrogenase. Third enzyme lacks both Mo and V and is called as Fe-nitrogenase.

The Mo-nitrogenase comprises of two components. Component 1 also called as FeMo-protein or dinitrogenase is a complex of two α polypeptides (50 kDa each) and two β polypeptides (60 kDa each), 30Fe, 2Mo and 32S. Component 2 also called as Fe-protein or the dinitrogenase reductase comprises of two γ polypeptides each with a molecular mass of 30 kDa and 4Fe and 4S. Molecular mass of γ polypeptide may vary among different organisms. *Frankia* seems to have the Mo-nitrogenase.

The V-nitrogenase also comprises of two components. Component 1 or VFe protein comprises of two units each of α (50 kDa), β (60 kDa) and δ (14 kDa) polypeptides, 2V, 17Fe and 20S. The component 2 is similar to component 2 of Mo-nitrogenase.

Component 1 of the Fe-nitrogenase is similar to component 1 of V-nitrogenase except that it lacks V. Component 2 is similar to other nitrogenases.

In addition to these a markedly distinct nitrogenase has been identified in *S. thermoautotrophicus* (see Ribbe et al., 1997). The St1 protein of this nitrogenase is also an MoFe protein with a molecular mass of 144 kDa. The St2 protein of this nitrogenase on the other hand is a manganese superoxide oxidoreductase with molecular mass of 48 kDa. It does not contain Fe. However, like other nitrogenases, it also requires MgATP.

The reduction of nitrogen requires both the components of the nitrogenase enzyme, Mg^{++}ATP and a source of reducing equivalents. The process may be generalised in the following equation:

$$N_2+8H^{+}+8e^{-}+16ATP=2NH_3+H_2+16MgADP+16Pi$$

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

1. Reduction of Fe protein–The Fe protein is reduced by a Ferredoxin.
2. Activation of Fe Protein by ATP–MgATP binds to the γ subunit of the Fe protein and makes it a more reactive reductant.
3. This is followed by electron transfer between the two components.
4. Binding of N occurs after this.
5. This triggers the formation of the entire enzyme complex.
6. Now the electron is transferred to the substrate.
7. Reduced substrate (N), ADP and phosphate are released.

Along with the reduction of N_2, H_2 is also released. This process is unidirectional and wasteful of energy and reducing equivalents. Some systems posses an uptake hydrogenase (*hup*) which scavenges the H_2 and offsets the energy and reductant otherwise wasted (Dean and Jacobson, 1992).

5.4 GENETICS OF NITROGEN FIXATION

There are at least two sets of genes in the microsymbiont that play roles in effective nodulation and nitrogen fixation. While the genes involved in nitrogen fixation are called as *nif* genes, the genes involved in nodulation *per se* are called as *nod* genes.

5.4.1 The nif genes in Frankia

The primary products of the genes encoding the structural components of nitrogenease enzyme are not catalytically active. The immature components of nitrogenase are processed by the action of other genes involved in the process of nitrogen fixation. These genes are collectively called as *nif* genes. Specific regulatory elements control the expression of *nif* genes under appropriate physiological conditions. About twenty different *nif* genes have been identified in *Klebsiella* (Table 5.2). These genes seem to be organised into 7 transcriptional units.

The organization of *nif* genes in *Frankia* is believed to be similar to that of *Klebsiella* (Normand et al., 1988). Most of the *Frankia* genes involved in the synthesis and assembly of nitrogenase FeMo cofactor are clustered in contiguity to the *nifHDK* operon except for *nifV* which is found upstream of *nifHDK*. The organization of *Frankia nif* genes from *nifE* to *nifW* is similar to *Anabaena*.

5.4.2 The nod like genes in Frankia

The *nod* genes in *Rhizobium* are responsible for producing proteins (Nod factors) that direct the synthesis of a chitooligosaccharide backbone. This molecule is sensed by dedicated kinases in the host that activate a cascade of events, including Ca spiking, that lead to nodulation of the host roots. Apparently, *nod* genes are expressed in response to flavonoid exudates of host

Table 5.2 **List of *nif* genes with their products and/or functions (based on Dean and Jacobson, 1992)**

Sl No.	Gene	Product/function
1	*nifH*	Fe protein subunit
2	*nifD*	MoFe protein a subunit
3	*nifK*	MoFe protein b subunit
4	*nifF*	Flavodoxin, physiological reductant of the Fe protein
5	*nifJ*	Pyruvate-flavodoxin-oxidoreductase, couples the oxidation of pyruvate to the reduction of flavodoxin
6	*nifM*	Required for activation of the Fe protein
7	*nifU*	Probably involved in the stabilization of Fe protein
8	*nifS*	Function unknown
9	*nifV*	Encodes homocitrate lyase
10	*nifE*	α subunit of NifEN complex required for FeMo cofactor biosynthesis
11	*nifN*	β subunit of NifEN complex required for FeMo cofactor biosynthesis,
12	*nifB*	Synthesis of an iron sulphide entity required for FeMo cofactor biosynthesis
13	*nifQ*	Required for FeMo cofactor biosynthesis
14	*nifW*	Processing of homocitrate
15	*nifZ*	Required for full activity of the MoFe protein
16	*nifA*	Positive regulatory element
17	*nifL*	Negative regulatory element
18	*nifX*	Product binds to NifB-co, probably negative regulatory element
19	*nifT*	Function unknown
20	*nifY*	Function unknown

plants. Nod factors induce cell division in the root cortex thus initiating nodule development. The cell division leads to formation of nodule primordium. A search for *Rhizobium nod* gene homologous sequences in *Frankia* could retrieve sequences with as little as 40% homology. It is therefore likely that the mode of induction of nodulation by *Frankia* is very different from that by *Rhizobium*. Further work on deciphering the exact mode of nodulation induction in actinorhizal symbiosis is still underway. However, Phenylacetic hopanetetrol is found present in Frankia in nodules. It induces plant growth in a manner similar to the one induced by IAA.

5.4.3 *The nodulin genes in hosts*

Host roots react to appropriate signals from the microsymbiont. As discussed above, the root hairs undergo modifications to accommodate the invading microsymbiont. Host's responses to non-symbiotic invaders are different, generally manifested in the necrosis of affected cells or tissues. When *Frankia* invades, the host helps in establishment of the microsymbiont in the cortical region. This is facilitated by the expression of a series of genes in response to

Table 5.3 | **Lists some of the *nodulin* genes identified in actinorhizal symbiosis**

Gene	Host plant	Probable function
ag12	*Alnus glutinosa*	subtilisin-like protease, homologous to *Arabidopsis* gene *ara12*
cg12	*Casuarina glauca*	subtilisin-like protease
Dg93	*Datisca glomerata*	Homologous to *Glycine max* early nodulin gene *GmENOD93*
AgDCAT1	*Alnus glutinosa*	dicarboxylate transporter involved in transport of carbon source to microsymbiont
DgGS1-1	*Datisca glomerata*	Gln synthetase enzyme
Dgrca	*Datisca glomerata*	Rubisco activase
agNt84	*Alnus glutinosa*	Metal binding protein
cgMT1	Casuarina glauca	?
EuNOD-PUB1	*Elaeagnus umbellata*	Codes for polyubiquitin
Agthi1	*Alnus glutinosa*	biosynthesis of the thiamine precursor thiazole
AgNOD-CP1	*Alnus glutinosa*	represents a nodule-specific cysteine proteinase similar to cysteine proteinases of the papain superfamily.

invasion by *Frankia*. Apparently, there are a few genes that express in response to the signals emanating from *Frankia*. Several other genes are then recruited to facilitate formation of functional nodules.

The host genes that are specific to nodule formation have been called as *nodulin* genes.

Some enzymes that express in normal tissues too have been found to express in nodules. For example, Chalcone synthase, an enzyme involved in secondary metabolite pathway of plants, has been found to express in actinorhizal root nodules too. Therefore, it appears that in actinorhizal symbiosis only few initial genes are involved in triggering nodule formation. Other l genes, found even in non-actinorhizal plants, are then recruited for nodule development.

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