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Project Title: Structure and Function of *Frankia* Vesicles in Dinitrogen Fixing Actinorhizal Plants

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ABSTRACT

Structure and function of *Frankia* vesicles in dinitrogen fixation by actinorhizal plants. John G. Torrey, Harvard Forest, Harvard University. - *Frankia*, a filamentous bacterium which induces N_2-fixing root nodules on the roots of a wide range of woody dicotyledonous plants, is the first known actinomycete which fixes dinitrogen when growing in free-living culture. The nitrogenase enzyme is induced in many strains of this organism by withholding fixed nitrogen compounds from its nutrient medium. Terminal swellings of the bacterial filaments develop rapidly and acetylene reduction activity (= nitrogenase) increases in proportion to the number of terminal vesicles formed. The induction of vesicles and establishment of acetylene reduction occurs under aerobic conditions and the evidence is accumulating which demonstrates the existence of a lipid multilaminate vesicle envelope which serves as a physical barrier protecting the oxygen-labile nitrogenase from denaturation. Our studies have concerned the physiology, biochemistry and structural development of the N_2-fixing apparatus in *Frankia* grown *in vitro* and in root nodules of host plants. Diverse strains of *Frankia* were isolated and cultured from different host plants and vesicle form and function were studied. Two strains were studied, especially HFPArI3, an isolate from nodules of the red alder *Alnus rubra* and HFPCcI3 isolated from root nodules of the tropical tree *Casuarina cunninghamiana*. The goal was to understand the structure and function which leads to optimum effectiveness for dinitrogen fixation.

ACCOMPLISHMENTS OF THE PAST YEAR 04/01/88 - 12/31/89.

One of the first plants studied in my research on actinorhizal plants beginning in 1973 was *Casuarina* of the family Casuarinaceae. This important tropical tree from Australia and its close relatives are among the most important trees for fuel wood throughout the developing countries of the tropical and sub-tropical world. Plants whose roots are nodulated by the soil bacterium *Frankia* of the Actinomycetales fix dinitrogen at rates equivalent to nodulated legumes and contribute significantly to the nitrogen availability in a wide range of ecosystems.

The filamentous bacterium *Frankia*, growing in pure culture in N-deficient medium in the laboratory, will form terminal swollen vesicles and fix dinitrogen from the atmosphere catalyzed by the enzyme nitrogenase localized in the vesicles. These vesicles possess multi-layered lipid laminae that are believed to exclude molecular 0_2 and thereby protect nitrogenase from denaturation. In symbiotic association with roots of actinorhizal plants, *Frankia* forms root nodules and within the nodules the infected cells contain differentiated *Frankia* which may form terminal vesicles, intrahyphal or terminal sporangia or only hyphae. In most species of actinorhizal plants (in excess of 200 species have been reported) root nodules active in dinitrogen fixation can be seen to possess *Frankia* with terminal vesicles of different forms - spherical, pear-shaped or club-shaped. Nitrogenase has been demonstrated to occur within these vesicles. Members of the Casuarinaceae are exceptional in that dinitrogen-fixing nodules show no symbiotic vesicles. *Frankia* remains filamentous.

A series of questions have arisen. In *Casuarina* root nodules how is the enzyme nitrogenase within N_2-fixing root nodules protected from denaturation by molecular 0? Under what circumstances is nitrogenase activity optimized? Our studies over the past number of years have been directed toward attempting to understand vesicle structure and function in a range of actinorhizal species and the behavior of *Frankia* that allows it to establish effective dinitrogen-fixing associations with a number of diverse woody species of plants.

In the family Casuarinaceae there are four genera with a total of nearly 100 species. *Casuarina* and *Allocasuarina* have their origins in Australia. *Gymnostoma* and *Ceuthostoma* originate in the South Pacific Islands. We collected seed of *Gymnostoma papuanum* from near Hilo, Hawaii as well as having seed sent from Papua New Guinea. Racette and Torrey (1989a, 1989b) and Torrey and Racette (1989) have published a series of papers on the nodulation and nodule structure of *Gymnostoma papuanum*, especially in comparison to *Casuarina* and to members outside the family Casuarinaceae (especially the Elaeagnaceae). A *Frankia* strain HFPGpI1 (catalog no. HFP021801) was isolated from root nodules of *G. papuanum* plants grown in the greenhouse and was used to inoculate seedlings to study nodulation and symbiotic nitrogen fixation. HFPGpI1 produced effective nodules that were unusual among species of Casuarinaceae in that the endosymbiont formed vesicle-like structures within the nodules. Ultrastructural studies (Newcomb *et al.* 1990) showed the vesicles to be of reduced size compared to *Elaeagnus* species and with limited septa and relatively slight lipid lamination, paralleling reduced rates of acetylene-reducing activity. Infection of *G. papuanum* roots was by root hair infection. Similar studies

(Racette and Torrey 1989b) of *Shepherdia argentea* of the Elaeagnaceae showed that the *Frankia* strain HFPGpI1 infected by direct epidermal penetration, typical of that host family.

Further studies in the Casuarinaceae led to the isolation of a strain of *Frankia* HFPCgI4 from root nodules of *Casuarina* glauca collected in Egypt (Mansour *et al.* 1990). This *Frankia* strain infects by root hair invasion and produces effective nodulation with high rates of acetylene reduction activity. The strain is unusual in that it shows high frequency of sporulation when grown in N-free nutrient media and spontaneous spore release. The strain should prove useful as inoculant for *Casuarina* species sensu stricta.

Vesicle structure has been one of the main thrusts of our studies over the years. In 1982 Torrey and Callaham reported from evidence derived from freeze-fracture studies of cultured *Frankia* cells that vesicles developed an envelope that extended down the stem attachment and was made of thin laminations probably of lipid. Later it was shown by other workers that the laminae were in fact lipid and that the number of laminae varied directly in relation to the concentration of oxygen in the atmosphere surrounding the vesicles. It has been inferred that the laminar layer is impenetrable to molecular O_2 and provides the protection required to prevent denaturation of the nitrogenase present within the vesicles.

More recently our own studies have shown that vesicles within nodulated plants also show lipid laminations that can be visualized through freeze-fracture electron microscopy (Newcomb *et al.* 1987, Abeysekera *et al.* 1990, Newcomb *et al.* 1990).

Torrey and Racette (1989) made extensive tests of the cross-inoculation capacities of a number of pure cultured strains from host plants in the Casuarinaceae on host species in the family. They found that there existed a fairly high degree of specificity among the host-microsymbiont combinations. Some *Frankia* strains were able to nodulate three different genera in the family (e.g., CcI3 or AllI1 will nodulate species in *Casuarina, Allocasuarina* and *Gymnostoma*) but other strains will only infect the host of origin (e.g. GpI1 nodulates *Gymnostoma*, but not *Casuarina* or *Allocasuarina*). Thus, there does not seem to be a universally effective *Frankia* strain for host species in the family Casuarinaceae.

Cross-inoculation studies across the wide spectrum of actinorhizal plants have been reviewed by Torrey (1990) and continue to be studied by our research group (cf. Baker and Torrey 1990 and Racette and Torrey 1990).

Recent studies (unpublished) by R. H. Berg of Memphis State University have lead to the isolation of a series of *Frankia* strains that are characterized by the property of spontaneous spore release when grown in nutrient culture. *Frankia* strains UFGCeI5 and UFGCgI1 have proved of particular interest in this respect. Using UFGCeI5 Tzean and Torrey (1989) studied nutrient conditions that would allow germination of filtered spore populations spread in known numbers on agar nutrient plates. Single spore colonies can be prepared from such germinating populations. It was shown that spore germination under favorable conditions occurs in 1-2 days, that colonies derived from such spores began to form sporangia and vesicles

within 6 days of germination and began to show spore release as early as 12 days, completing the cycle.

Using the same strain UFGCeI5, Burleigh and Torrey (1990) have compared the infective capacities of pure hyphal preparations and pure spore suspensions prepared in different ways. It was shown that dessicated hyphal inocula were not infective while dessicated inocula of spores retained their capacity to infect seedlings of appropriate host plants. These trials have implications for the development of inocula for mass-production of actinorhizal plants for reforestation.

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REVIEW OF RESEARCH PUBLICATIONS SINCE 1984

A detailed accounting of progress achieved under this grant has been submitted each year in the annual progress reports. As accomplished research is best reflected in publications, I have listed below publications beginning in 1984 resulting from the current grant and an earlier grant as noted.

The following publications were supported by Department of Energy grants to John G. Torrey.

Published beginning in 1984 with support from Research Grant DE-AC02-82ER 12036:

Murry, M. A., M. S. Fontaine and J. G. Torrey. 1984. A comparison of cultural characteristics and infectivity of *Frankia* sp HFPArI3 grown in batch culture. Plant and Soil 78: 61-78.

Zhang, Z., M. F. Lopez and J. G. Torrey. 1984. A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. Plant and Soil 78: 79-90.

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Zhang, Z. and J. G. Torrey. 1985. Studies of an effective strain of *Frankia* from *Allocasuarina lehmanniana* of the Casuarinaceae. Plant and Soil 87: 1-16.

Murry, M. A., Z. Zhang and J. G. Torrey. 1985. Effect of O_2 on vesicle formation, acetylene reduction and O_2-uptake kinetics in *Frankia* sp. HFPCcI3 isolated from *Casuarina cunninghamiana*. Can. J. Microbiol. 31: 804-809.

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Torrey, J. G. 1985. The site of nitrogense in *Frankia* in free-living culture and in symbiosis. *In:* Nitrogen fixation research progress. H. J. Evans, P. J. Bottomley and W. E. Newton (Eds.). Martinus Nijhoff Publ., Dordrecht, The Netherlands, Pp. 293-299.

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Lopez, M. F., P. H. Young and J. G. Torrey. 1986. A comparison of carbon source utilization for growth and nitrogenase activity in two *Frankia* isolates. Can. J. Microbiol. 32: 353-358.

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Lamont, H. C., W. B. Silvester and J. G. Torrey. 1988. Nile red fluorescence demonstrates lipid in the envelope of vesicles from N_2-fixing cultures of *Frankia*. Can. J. Microbiol. 34: 656:660.

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