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A study of the symbiotic relationship between frankia and the genus ceanothus

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genus *Ceanothus*

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A Study of the Symbiotic Relationship Between *Frankia* and
the genus *Ceanothus*

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By

Dadbeh Rouhbakhsh

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ABSTRACT

A STUDY OF THE SYMBIOTIC RELATIONSHIP BETWEEN *FRANKIA* AND THE GENUS *CEANOETHUS*

By Dadbeh Rouhbakhsh

This thesis is a study of *Frankia* and its symbiotic relationship with two members of the genus *Ceanothus*. The actinomycete *Frankia* was isolated from *Ceanothus cuneatus* Esch. and *Ceanothus thrysiflorus* (Hook) Nutt., and was an isolate in broth. The symbiotic relationship between *Frankia* and the two members of genus *Ceanothus* was also studied using scanning electron microscopy and transmission electron microscopy.

It was discovered that the strains of *Frankia* living within *Ceanothus cuneatus* and *Ceanothus thrysiflorus* do not bear spores, or rarely bear spores *in-vivo* or *in-vitro*.

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I would like to thank Dr. Patricia Grilione, my graduate advisor, for her expertise, guidance, encouragement, and endless patience. My sincerest appreciation is also extended to Dr. R. Myatt and Dr. W. Savage for their advice and for taking the time to read my thesis while on their sabbatical leaves. Also, I wish to thank Dr. D. Bruck for his expert advice on the Transmission Electron Microscope, and the material and help and advice when it was needed most.

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LITERATURE REVIEW

Introduction

The genus *Frankia* comprises a group of nitrogen fixing bacteria which form endosymbiotic relationships with a number of woody dicotyledonous plants. This genus was first described by Woronin in 1866 as an imperfect fungus (19). It was later named in honor of A.B. Frank, a Swiss microbiologist, who coined the term symbiosis (19). This microaerophilic prokaryote consists of long, branching, septate filaments that bear spores in grape-like clusters at the terminus of some filaments, *in-vivo* and *in-vitro* (15). However, some strains of *Frankia* only sparsely bear spores *in-vivo*. *Frankia* also infrequently forms spores *in-vitro* as swollen thick-walled cells within the filament (15). Nitrogen fixation occurs in vesicles which are spherical multi-walled structures formed at the ends of the filaments (15).

Studies on the molecular biology of *Frankia* do exist (11,13,20,21,27), but most published research focuses on its isolation or is devoted to the study of the morphology of the organism, both *in-situ* in the host plant nodules and as isolates.

Host Families to *Frankia*

A large number of woody plants belonging to a variety of families are hosts to *Frankia*(6). Among these are the

families *Casuarinaceae*, *Myricaceae*, *Betulaceae*, *Elaeagnaceae*, *Rhamnaceae*, *Coriariaceae*, *Rosaceae*, and *Datisceae*(28). These plants are considered important in forestry because they are able to grow in poor soils and fire devastated areas due to their ability to support *Frankia*, and thus fix nitrogen (1,26).

Classification

Although extensive *in-vivo* and *in-vitro* studies have been done on *Frankia*, difficulties with its isolation, and its slow growth rate of several weeks to several months, have made it difficult to be characterized in pure culture. Therefore, the speciation of this organism remains obscure (15). However, criteria for strain differentiation of *Frankia* have been proposed. These include frequency of sporulation *in-vivo* (29,31), use of nucleotide probes (13), and direct DNA hybridization (27). Of these, frequency of sporulation is the method most often studied. The first study of the spores of *Frankia*, using electron microscopy, was in 1976 (30). The possibility that the organism might have two strains, a spore positive and a spore negative, was suggested by a study of alder nodules (29). The presence of the two strains was later confirmed in *Alnus glutinosa* and *Alnus incana* (31), in *Alnus incana* subspecies *rugosa* (14), and in *Myrica gale* (16).

Isolation of *Frankia*

Isolation of *Frankia* from root nodules had eluded researchers until relatively recent times. This has been

mainly due to the difficulty in separating contaminating soil bacteria from nodule tissues yielding *Frankia*. The first confirmed isolation was accomplished by Callaham from *Comptonia* in 1978 (7). Since that time a number of other isolates have been reported from several host species, using a variety of isolation techniques. These techniques include culturing whole, individual nodule lobes (7), serial dilution of crushed nodules (7), microdissection of nodules (5), enzymatic digestion of nodule pieces (7), and sucrose density fractionation of crushed nodules (3).

Most of these techniques are very labor intensive and require expensive materials. These extreme steps are required to exclude soil bacteria and fungi, and to expose *Frankia* to the growth medium. It is necessary to take these precautions because the media used for *Frankia* culture are very rich and nonselective in that they do not include chemicals or antibiotics which would inhibit soil microorganisms and not *Frankia*.

Of the methods mentioned above, culturing whole nodules is the least laborious. This technique however results in low yield since only a small quantity of *Frankia* comes in contact with the growth medium.

The symbiosis between *Ceanothus* and *Frankia*

One of the hosts of *Frankia* is the genus *Ceanothus*. This genus consists of between 50 to 60 species of shrubs or small trees, many of which are considered to be of horticultural importance(23). Although *Frankia* has been

successfully isolated from several hosts and studied, surprisingly little published research on the *Frankia*-*Ceanothus* relationship exists. Delwiche studied the nitrogen fixing ability of several un-named species of *Ceanothus* (8). Nitrogen fixation studies have also been reported on *Ceanothus greggii* var. *perplexans* (17), and *Ceanothus velutinus* (22). In addition, one article on the isolation of *Frankia* from *Ceanothus americanus* exists (20).

Objectives of this research

To date, there are no published reports on the ultrastructure of *Ceanothus* nodules containing *Frankia* or on their isolates.

The objectives of this research are: 1. to isolate *Frankia* from two species of *Ceanothus*. 2. study *Frankia in-situ* and as an isolate, with the electron microscope.

In this study, a simple method of isolation, derived from a modification of existing methods, is used to isolate *Frankia* from *Ceanothus cuneatus* Esch. and *Ceanothus thrysiflorus*(Hook)Nutt. In addition, the *Frankia* strains of these two plant species are studied using scanning and transmission electron microscopy, *in-vivo* and *in-vitro*.

MATERIALS AND METHODS

Nodule Collection

Nodules of *Ceanothus cunatus*(Hook)Nutt. were collected from two sites in the Santa Cruz mountains of California (Appendix I). *Ceanothus thrysiflorus* Esch. nodules were collected from a site along the Pacific coast near Santa Cruz, California (Appendix II).

All nodules were excised from the plant roots at the site and were placed in a 4 °C refrigerator within an hour of collection.

Light Microscopy

Fresh field collected nodules from *Ceanothus cuneatus* and *Ceanothus thrysiflorus* were washed in tap water and separated into individual lobes. The cortical cells of the lobes were screened for the presence of *Frankia* by preparing thin sections with a Lafayette cylinder microtome. The sections were then examined as wet mounts using phase contrast microscopy and as stained preparations employing a 0.1% solution of toluidine blue (29) and diluted Fabil's reagent (24).

Isolation

Two media were used for isolation. Medium 1060 (ATCC, Catalogue of Bacteria and Phages, Seventeenth edition. 1989) and yeast extract-dextrose broth (4) were each dispensed in sterile 9ml amounts in 20ml screw-capped test tubes.

The nodule lobes were surface sterilized by submersion in full strength household bleach (5.25% sodium hypochloride) for 15 minutes each with occasional agitation. The lobes were then rinsed with 200ml of sterile distilled water and aseptically crushed with a mortar and pestle. The crushed lobes were aseptically transferred to each medium and incubated at 30 °C. Incubation time ranged from 4 weeks to 2 years for isolates.

Scanning Electron Microscopy

Excised fresh nodules were washed in running tap water. These were placed in 2.5% gluteraldehyde in 0.1% sodium cacodylate buffer (pH 7.4) overnight. The nodules were prepared for scanning electron microscopy as previously described (2). The specimens were observed and photographed by a ISI Super III scanning electron microscope using an accelerating voltage of 10Kv. Photographs were taken with a 4x5 Polaroid Land camera using Polaroid 55 positive/negative film.

Transmission Electron Microscopy

After washing freshly collected nodules from both *Ceanothus* species in running tap water, the nodules were cut into pieces varying from 1-3mm in length with a single edged razor blade. The pieces were fixed for 5 hours at 4 °C in 2.5% gluteraldehyde in 0.05 M sodium cacodylate buffer with

2% tannic acid and 2 mM of calcium chloride, pH 6.8. They were then washed once in cold 0.05M sodium cacodylate buffer and placed in buffer at 4 °C for 48 hours and post fixed in 2% osmium tetroxide in sodium cacodylate buffer for 2 hours at 0 °C. Dehydration was in a graded acetone series of 10%-25%-40%-50%-75%-95%-100%-100%-100% for 2 hours per concentration, with the 50% and the first 100% step for over-night.

At this point two different methods were used to embed *Ceanothus cuneatus* nodules: 1.The nodule pieces were infiltrated with Spurr's resin for 6 weeks and embedded at 70 °C for 48 hours in Beem capsules; 2.The nodule pieces were transferred to propylene oxide (2 washes, 10 minutes each), infiltrated with Epon 812 for 2 weeks and embedded at 65 °C for overnight in Beem capsules. *Ceanothus thraysiflorus* nodule pieces were embedded using method 1 only. Embedded nodules of both species were thin-sectioned with an ultramicrotome and mounted on formvar-coated 300 mesh grids. The sections were stained for 60 minutes in 2% uranyl acetate in methanol, followed by 6 minutes in Reynolds lead citrate. The stained material was observed in a JEOL Jem-100S transmission electron microscope at an accelerating voltage of 80Kv.

RESULTS

Light Microscopy

Examination of the nodule sections using phase contrast microscopy and Fabil staining revealed the cortical cells filled with the bacterial endophyte while the stele was devoid of hyphae. However, no differential staining of structures other than the hyphae of *Frankia* was observed.

Isolation

A white to cream colored mass of growth appeared at the bottom of tubes of both media after an average of four weeks of incubation. The growth seemed to be growing from within the crushed nodules and when examined by phase contrast microscopy, the characteristic hyphal nature of an actinomycete was seen. Both media supported the growth of the isolates well. In some cases, Medium 1020 appeared to provide slightly faster growth. About 5% of of the fifty isolation attempts had soil bacterial contaminants, and were discarded.

Electron microscopy

Ceanothus cuneatus: Scanning electron micrographs in Figure 1 a,b show the actinomycete within the cortical cells of the host nodule. In Figure 2, the filamentous nature of the endosymbiont is seen. Figure 2a shows hyphae extending from an infected cell to a non-infected cell. Figures 3 and 4

demonstrate vesicles of *Frankia* within the host cells surrounded by the host polysaccharide capsule. A possible sporangium bearing the spherical spores is shown by Figure 5.

Transmission electron micrographs (Figure 6 a,b) are of hyphae within the cortical cells surrounded by the host polysaccharide capsule. The individual cells and septa of the actinomycete are seen in Figures 7 and 8.

Ceanothus thrysaflorus: Figure 9 is a low magnification scanning electron micrograph of a nodule showing infected cortex and uninfected stele. In Figure 10, fungal hyphae are seen alongside the symbiont. Figures 11, 12, and 13 are electron micrographs of the ultrastructure of infected cells. Numerous actinomycete hyphal elements can be seen among the host polysaccharide capsular material.

Isolates: Figure 14 depicts scanning electron micrographs of a developing sporangium of an isolate. The filamentous nature of the bacterium is obvious. In Figure 15, the septum between two cells of a filament of an isolate is prominent.

DISCUSSION

The modified isolation technique developed as part of the thesis research combines the best of many methods. Since it only requires a few tubes of medium, household bleach and a sterile mortar and pestle, it is inexpensive. Bleach kills contaminating soil microorganisms on the surface of the nodules and the crushing of nodule pieces exposes large quantities of *Frankia* to the isolation medium.

The 95% success rate in isolation clearly demonstrates the efficiency of this method. Considering the large number of microorganisms residing in the soil, and therefore on the surface of the nodule, the 5% contamination rate in the isolation tubes is acceptable.

Examination of nodule sections from both host species with electron and light microscopes revealed selective infection of the cortical cells, and absence of *Frankia* from the cells of the vasculature. This finding is consistent with reports on other host plant species (30,12).

Examination of the nodules from the two *Ceanothus* species revealed the infected cortical cells to be larger than the neighboring noninfected cells. This is presumably due to the pressures exerted on the cell by the extra hyphal mass within it. Nitrogen fixing vesicles of *Frankia* were seen within cells of the nodule. These vesicles were abundant in

cells bearing them and were always partially covered by a thick polysaccharide capsule presumably deposited by the host. The vesicles in both *Ceanothus* species nodules were similar in size, shape, and abundance.

One scanning electron micrograph was obtained of a possible sporangium from a *C. cuneatus* nodule. No other sporangia were seen in any of the other nodules of either species examined. The absence of sporangia in *Ceanothus* has been noted by at least one other researcher (Berry, A., U.C.Davis, personal communication).

Since the nodules collected were one year or older in age, it is assumed the *Frankia* within them were mature enough to have spores. Therefore, the absence of sporangia must be due to reasons other than the age of the nodules. There are four possible reasons that could explain this absence: 1. *Frankia*, regardless of strain, is unable to sporulate in members of the genus *Ceanothus*, 2. spore positive *Frankia* are present in the soil but are unable to infect *Ceanothus* spp., 3. spore positive *Frankia* are absent from the environment in which *Ceanothus* grows, and/or 4. the strain of *Frankia* infecting these two species of *Ceanothus* is a previously undescribed strain which is completely unable to bear spores *in-vivo* and *in-vitro*. Absence of spore positive *Frankia* from the environment is unlikely, even-though the prevalence of one strain of *Frankia* over another in a given type of soil has been documented (14). *Ceanothus. cuneatus* and *C. thrysiflorus* grow in different

environments. It is not likely for both environments to be inhibitory to spore positive *Frankia*. Inability of spore positive *Frankia* to infect the two species of *Ceanothus* studied is another possibility. This could be the result of some anatomical or physiological barrier in the host's root hairs where the infection begins, or infection may be checked as it progresses from the root hair into the cortex of the root. This hypothesis merits further investigation. The third possibility, inability of *Frankia* to bear spores in *Ceanothus*, regardless of strain, must also be considered in future research. This could be due to some chemical interaction between the host and the symbiont. The fourth hypothesis requires further research since the number of samples taken were not high enough to make this a valid hypothesis.

Scanning electron microscopy of *C. thrysiflorus* nodules revealed fungal filaments within one of the nodules examined. This phenomenon was not observed in *C. cuneatus* nodules. The nodule bearing the fungus was several years old and had begun to decay. It is very likely that the fungus present in the nodule was there as a saprobe and not of a mycorrhizal origin.

Preparation of nodules from both plant species for transmission electron microscopy presented a myriad of problems. The dehydration steps had to be extended to a half hour each, as compared to a normal of ten minutes. In addition, the infiltration required a period of 6 to 8

weeks. The difficulties experienced in dehydration and infiltration were thought to be caused by the impermeable barrier presented by the combination of the thick cell walls of the root cells, the host-produced polysaccharide capsule around *Frankia*, and the thick gram-positive cell wall of *Frankia* itself.

Using these procedures, transmission electron micrographs of the actinomycete filaments within the cortical cells were taken but no vesicles were photographed. Presumably, the vesicles with their thick multilayered walls, were not infiltrated sufficiently and thus fell out of the sections during microtoming. It would be recommended to any future researcher to extend the dehydration/infiltration times to as much as twice the amount employed during this research project.

The polysaccharide capsule around *Frankia* appears in transmission electron micrographs as a thick, electron dense membrane. This capsule is thought to be a nonspecific host response to infection (10,18). The capsule is seen in scanning electron micrographs as a layer covering the hyphae and the vesicles of *Frankia*.

In some transmission electron micrographs, cells of *Frankia* growing in the nodules appear to contain a variety of intracellular matter. These are possibly storage granules since no organelles are expected in *Frankia*. This presence of intracellular matter is not seen in micrographs of the isolates. It is possible that the differences in the

environment between the cortical cell and the isolation medium would cause a different response by the bacterium. It is also possible for these intracellular granules to be artifacts created during the dehydration steps. Also, in transmission electron micrographs a clear zone was observed between the polysaccharide capsule of the host and the cells of *Frankia*. This was not seen in the scanning electron micrographs suggesting that its appearance in the transmission electron microscopy sections was an artifact from the dehydration steps. It is possible to minimize these dehydration artifacts by increasing the concentration of dehydrant from 0 to 10% in smaller increments than those mentioned in the materials and methods section. This can be achieved by adding drop-wise, dehydrant to the buffer in which the nodule pieces are suspended. The dehydration steps after 10% can be as outlined in the materials and methods section.

Electron micrographs of the isolates showed typical hyphae of an actinomycete, but no mature sporangia were observed. Early developmental stages of a few sporangia were seen and photographed by scanning electron microscopy. Since the cultures used for the electron microscopy were between 6 months and 24 months of age, the absence of sporangia was unusual. Other researchers have reported abundant mature sporangia *in-vitro* irregardless of the strain (18,25). Of the four hypotheses set forth previously for the absence of sporangia in the nodules, this finding

strengthens the fourth. Since spore negative strains of *Frankia* sporulate readily *in-vitro*, *Frankia* infecting *Ceanothus* spp. must be of a strain separate from the spore positive and the spore negative described previously.

No vesicles were seen in the isolates. This was expected since a source of fixed nitrogen was readily available to the isolates and hence there was no stimulus for them to form the nitrogen fixing structures (9).

The possibility exists that the isolates obtained are not *Frankia*. This however is not likely because of the steps taken to ensure absence of soil contaminants, and because of the conformation of the isolates to the physical characteristics, growth rate, and oxygen requirement of *Frankia*. To be able to absolutely ensure that these isolates are *Frankia*, Koch's Postulates, a classical technique used in microbiology, must be fulfilled. Sterile *Ceanothus* seedlings growing in a sterile environment must be inoculated with these isolates. If nodules begin to appear on the roots of the seedlings then the isolates must be members of the genus *Frankia* since these are the only actinomycetes that nodulate in *Ceanothus*. This procedure remains to be performed.

Figures 1 through 5 are scanning electron micrographs of *Ceanothus cuneatus* nodules.

Fig 1. (a) Enlarged infected cortical cells with *Frankia* (arrows) alongside smaller uninfected cells(235x). (b) A higher magnification of two cells bearing the endophyte (solid arrows) next to an uninfected smaller cell (hollow arrow)(1200x).

Fig 2. (a) Hyphae from a cell bearing *Frankia* extending across to a neighboring uninfected cell (arrows). Part of the host cell wall (w) is still covering the endophyte inside the cell(3360x). (b) A higher magnification showing the hyphae. Some of the host polysaccharide capsular matter is visible (c). Also prominent is a branch in a hypha (arrow) (16800x).

Fig 3. (a) Vesicles (hollow arrow) and hyphae (solid arrow) embedded in the host's capsular matter (c). The infected host cell wall (w) as well as starch granules in a neighboring cell (s) are also visible (4080x). (b) Higher magnification showing hyphae (H), vesicles (solid arrows), and polysaccharide capsule (c). Also prominent are cross sections of hyphae obtained during slicing of the nodule (hollow arrows) (8320x).

Fig 4. (a) Numerous vesicles in a cortical cell(4080x). (b) Higher magnification of the vesicles (V), Hyphae (H), and the host's capsule (C). The elongated club shape of the vesicle is apparent (22400x).

Fig 5. Possible sporangium rising against the background of a cortical cell wall (W). The individual spores (S) can be identified (23500x).

Figures 6 through 8 are TEM shots of a *Ceanothus cuneatus* nodule.

Fig 6. (a) Infected cell filled with the endophyte. The cell walls of the host (W) (14000x). (b) Cell bearing the *Frankia*(16000x).

fig 7. (a) *Frankia* inside a cortical cell (F). The polysaccharide capsule of the host (arrows) is also seen (43200x). (b) Host cell wall (W), cells of the endophyte (solid arrows), and a septum between two cells of *Frankia* (hollow arrow) (36000x).

Fig 8. (a) Cells of the endophyte between two host cortical cell walls (long arrows). Also visible is a septum (short arrow) between two of the bacterial cells (22000x). (b) Cross section of several cortical cells showing a hypha

(arrow) spanning the cell walls of three adjacent cells (8160x).

Figures 9 and 10 are scanning electron micrographs of *Ceanothus thyrsiflorus* nodules.

Fig 9. Cross section of a nodule showing infected cells of the cortex (arrows) as well as the uninfected stele (S) (112x).

Fig 10. (a) The actinomycete (A) inside cortical cells of the nodule. Also visible are two fungal hyphae (F) (4200x). (b) Actinomycete (A) inside cortical cells as well as fungal hyphae (F) (2400x).

Figures 11 through 13 are transmission electron micrographs *Ceanothus thyrsiflorus* nodules.

Fig 11. (a) A cortical cell wall (W) dividing two cells bearing *Frankia* (arrows) (20400x). (b) A cortical cell with the actinomycete (arrow) inside. The cortical cell wall (W) is also visible (14400x).

Fig 12. (a) Cortical cell bearing the actinomycete (11800x). (b) Higher magnification of the endophyte (17100x)

Fig 13. (a) Individual cell of *Frankia* (arrow) and the surrounding polysaccharide capsule (double arrows) (13400x). (b) Higher magnification of *Frankia* showing the individual cells (arrows) and the surrounding host capsule (double arrows) (24000x).

Figures 14 and 15 are electron micrographs of the Isolated actinomycete.

Fig 14. (a) SEM shot of the actinomycete with the beginning of a developing sporangium (s) (14000x). (b) The developing sporangium of an isolate (arrow). The beginning of the cleavage (double arrows) that will result in the production of spores is evident (42000x).

Fig 15. TEM shot of the isolate. The septum (arrow) between two adjacent cells is evident (72000x).

FIGURE 1

b.

FIGURE 2

a.

b.

FIGURE 3

a.

b.

FIGURE 4

a.

b.

FIGURE 5

FIGURE 6

a.

b.

FIGURE 7

a.

b.

FIGURE 8

a.

b.

FIGURE 9

FIGURE 10

b.

FIGURE 11

a.

b.

FIGURE 12

a.

b.

FIGURE 13

a.

b.

FIGURE 14

a.

b.

FIGURE 15

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APPENDIX I

APPENDIX II

MANITRESA
STATE BEACH
Sand Dollar

BAY

MONTEREY

SANTA CRUZ

CAPITOLA

SEACLIFF

APTOS

MONTE TOYON

SCOTTS VALLEY

FOREST OF NISENE

STATE PARK

TRAIL CAMP

Hinkley

Santa Road

1275

1275

1275

1275

1275

1275