PN-ABK-237
75202

FINAL REPORT

Covering Period: October 1987 to October 1990

Submitted to the Office of the Science Advisor

U.S. Agency for International Development

Molecular Identification of Frankiae Using Cross Inoculation Group-Specific DNA Sequences

Principal Investigators and Grantee Institutions:

Dwight D. Baker	Nantakorn Boonkerd
Yale University	BNF Resource Centre
School of Forestry &	for South East Asia
Environmental Studies	Department of Agriculture

Project Number: 7.202

Grant Number: DPE-5542-G-SS-7077-00

A.I.D. Grant Project Officer: Lloyd Frederick/Charles Sloger

Project Duration: October 1987 to October 1990

cc. BUSTID
PPC/CDIE

Table of Contents

I. Executive Summary 3

II. Research Objectives 4

III. Methods and Results 6

IV. Impact, Relevance and Technology Transfer 8

V. Project Activities/Outputs 9

VI. Project Productivity 10

VII. Future Work 10

VIII. Literature cited 11

IX. Publications arising from project (appended)

I. Executive Summary

This collaborative project between Dr. Dwight Baker at Yale University School of Forestry and Environmental Studies and Dr. Nantakorn Boonkerd at the Biological Nitrogen Fixation Resource Centre for Southeast Asia (Bangkok, Thailand) initiated studies of symbiotic nitrogen fixation associated with the fast-growing tree, Casuarina, and its root nodulating bacterium, Frankia. The basic objectives of the overall project were 1) to transfer the technology for isolating and culturing these nitrogen fixing bacteria to the Thai institute, 2) to develop a molecular biological method for identifying newly isolated Frankia strains that would be useful for inoculating Casuarina, and 3) to demonstrate the benefit of inoculating tree seedlings with pure cultures of Frankia. All of the objectives were accomplished over the three year project life. Numerous Frankia strains were isolated from Thai sources and these were characterized. A molecular biological method based on differences in restriction fragment length polymorphisms (RFLPs) of Frankia DNA was developed to provide a simple and rapid screening method to identify the most appropriate strains for the purposes of inoculating Casuarina tree seedlings. A demonstration trial of inoculated seedlings was established in northeastern Thailand which proved the importance of inoculating the trees before plantation. The BNF Resource Centre in Thailand is now better equipped to conduct research on actinorhizal trees and the symbiotic root bacterium Frankia. The Thailand project will serve as a model for similar collaborative projects in the future.

II. Research Objectives

A. Introduction

Actinorhizal trees, especially species of the genera Casuarina and Alnus, are widely planted in developing countries for fuelwood, timber, pulp and for the benefits to soil fertility arising from symbiotic nitrogen fixation. If actinorhizal trees are introduced into an area where they were not present before, inoculation of the plants with the symbiotic bacterium Frankia will usually result in increased growth and/or improved performance, especially on poor sites. Improvement arises for two possible reasons. The first reason is that the inoculation provides bacteria which are not present indigenously in the soil. A second reason is that the bacteria used for inoculation may be superior to those found indigenously. Such inoculation procedures are widely advocated or used for legume species, plants similar to actinorhizal species in their ability to fix dinitrogen. However, inoculation programs for actinorhizal plants are few and thus most actinorhizal plants are distributed without inoculation.

In Thailand, extensive use is made of the actinorhizal species, Casuarina junghuhniana, for fuelwood and pole production. Plants are produced in large numbers by the Royal Thai Forest Department by clonal propagation in nurseries throughout the country. Although the species is promoted as a nitrogen-fixing tree, inoculation with an appropriate Frankia inoculum is not undertaken. Optimum performance of these trees may not be obtained. Poor performance of the trees has been noted in the northeast section of the country and one reason may be the absence of indigenous frankiae and the lack of nodulation of the trees in the plantations. A project to isolate indigenous Frankia for Casuarina in Thailand was desired.

A particular problem with isolating infective and effective Frankia strains from Casuarina for inoculum purposes has been noted by several laboratories (Gauthier et al. 1981, Baker 1987, Torrey and Racette 1989). Frankia strains commonly can be isolated which, upon testing, do not have the ability to infect this host. Testing of new isolates by the conventional method of cross inoculation is time-consuming and tedious. Therefore, a more rapid, simple screening method would be beneficial to inoculum development programs in Thailand as well as other developing countries where actinorhizal trees are used.

The BNF Resource Centre for Southeast Asia, a division of the Department of Agriculture in Thailand, has an ongoing mandate to provide microbial inoculants for the improvement of Thai agriculture. With assistance from the U.S. Agency for International Development during the past decade, an excellent facility has been established for production of rhizobial inoculants. The Centre was well-placed for undertaking programs on frankiae. Technical training and research direction were provided by Dr.

Dwight Baker at Yale University where a large collection of Frankia strains currently exists and where methods could be developed for identifying strains.

B. Objectives of this project

Three objectives were undertaken in this project to address the problems identified above.

1) Our first objective was to transfer the technology for routine isolation and culture of symbiotic Frankia from root nodules and soils to the staff at the BNF Resource Centre in Bangkok.

2) Our second objective was to isolate nod and nif genes as molecular probes to detect symbiotic strains of frankiae.

3) Our third objective was to identify host-specificity group-specific DNA sequences that could be the basis of a simple and rapid screening method for characterizing new Frankia isolates.

4) A fourth objective was added near the end of the project to establish a field trial using the Thai strains in comparison to exotic strains with two host species, C. equisetifolia and C. junghuhniana.

Our approach of trying DNA methods was based on some research on rhizobia which indicated that characterization of strains into host specificity groups might be possible using repetitive DNA sequences associated with sym genes (Kaluza et al. 1985). Few scientists had undertaken genetic studies of frankiae previous to the initiation of our project. However, Dr. Beth Mullin at the University of Tennessee had conducted some preliminary DNA characterization studies and we enlisted her assistance in our project as a technical consultant. Our work on this project was greatly advanced by her advice.

III. Methods and Results

A. Isolation of Thai Frankia strains

Several methods were employed to isolate Frankia strains from root nodules of Casuarina species in Thailand. The sucrose density fractionation method (Baker and O'Keefe 1984) and the filter exclusion method (Benson 1982) were found to be not as useful under the Thai conditions because of equipment limitations or simple lack of success. Numerous isolations were obtained using a modified serial dilution technique (see Baker 1989 for methodological details). Characterizations of these Thai isolates were performed using conventional methods at Yale University by Aphakorn Nittayajarn, a scientist from the Bangkok Centre. The conventional characterizations allowed us to compare these results with those obtained by the DNA techniques developed later in the program.

A summary of the strains isolated from Thailand is provided in Table 1.

Strain number	Trivial name	Source host	Source location	Notes
DAB 020601	1871	C. equisetifolia	Prachin Buri	
DAB 020602	1885	C. equisetifolia	Prachin Buri	ineffective
DAB 020603	1995	C. equisetifolia	Bangkok	
DAB 020604	3952	C. equisetifolia	Rayong	ineffective
DAB 021001	1205	C. junghuhniana	Bangkok	
DAB 021002	1935	C. junghuhniana	Prachin Buri	
DAB 021003	1950	C. junghuhniana	Prachin Buri	
DAB 021004	1960	C. junghuhniana	Prachin Buri	
DAB 021005	2737	C. junghuhniana	Pathum Thani	

As noted in Table 1, we observed differences among strains in the ability to fix nitrogen in association with host plants. This was the first significant observation of ineffectivity in Casuarina-infective Frankia strains. Our results would indicate that some of the indigenous strains in Thailand are not fully effective and that this may be contributing to less than optimal nitrogen fixation by the trees in Thailand. These results provide additional incentive to initiate inoculation programs in Thailand using selected superior strains.

B. Isolation of nif and nod genes and development of a molecular probe for screening new isolates

At the commencement of our project some preliminary research results from several laboratories working on rhizobia and frankiae indicated that selection of repetitive sequences associated with the nif or nod genes would not be a suitable method for development of a molecular probe. Therefore we began a prelimi-

nary set of experiments designed to identify restriction fragment length polymorphisms (RFLPs) in the nif genes which might serve as an approach for screening strains.

From results of heterologous DNA hybridization published in the scientific literature, we decided to use a nif gene probe, pAS 30, derived from Klebsiella, a free-living nitrogen fixing bacterium. This gene probe in combination with one of several restriction endonucleases allowed us to separate several different Frankia strains into groups that corresponded with known host specificity information. This result was much more promising than we originally anticipated since we expected to have to use a group of restriction endonucleases in combination with a series of probes to characterize the strains.

After our initial success, we undertook a larger screening trial with a large number of strains to assess the utility of this procedure. Our results were very encouraging. In all cases, we were able to predict the host specificity of a Frankia strain by observing the pattern of hybridization of the nif probe to a restriction digest of total DNA. Since DNA isolation can be achieved on a very small amount of bacterial cell biomass, our results would provide the basis for a rapid yet relatively simple technique for screening strains for host specificity.

Our results were published in an international scientific journal (Nittayajarn et al. 1990). These results have been confirmed by DNA studies in other laboratories (Fernandez et al. 1989, Normand et al. 1988) and thus suggest that the RFLP groups which we have identified represent evolutionarily-distinct groups within the genus.

C. Establishment of Casuarina demonstration trial

In May and June of 1989, a field trial of Casuarina equisetifolia and Casuarina junghuhniana was planted near Khon Khaen in northeastern Thailand. This site was chosen because the soil was extremely poor, other trees species planted there had performed poorly, and a nitrogen fixing species was considered to be the best candidate for soil improvement. Seedlings obtained from the Royal Thai Forest Department nurseries in early 1989 were inoculated with a combination of isolated Thai strains, or a combination of exotic strains. Additional plants were left uninoculated as controls. These plants were held at the BNF Resource Centre until June when they were transported to the field site and planted.

Heavy rains occurred soon after planting and seedling mortality was quite high. Young inoculated plants which survived the bad weather grew well whereas those left uninoculated grew extremely poorly. Another field trial will be established in the future so that the comparison studies can be reinstated with a complete statistical design. The follow-up study might be funded in part by funds from the International Atomic Energy Agency.

IV. Impact, Relevance and Technology Transfer

This project has made a significant contribution to the research program at the BNF Resource Centre in Bangkok, Thailand. Expertise in working with actinorhizal plants and the bacterium Frankia has complemented the existing programs on rhizobia, Azolla and other microsymbionts. Some laboratory equipment has been provided to the BNF Centre and this can be utilized for the Frankia work as well as other ongoing work in the Centre. The Bangkok laboratory has forged liaisons with the Forestry Department of Kasetsart University to undertake Casuarina demonstration trials and thus the BNF Resource Centre is viewed as a resource not only for agriculture but also forestry in Thailand.

Larger scale programs of inoculation in government nurseries are now possible and urgently needed. Unfortunately, the Royal Thai Forest Department does not have the funding for this work and the BNF Resource Centre is supported only for work to the agriculture sector. Now that the technology has been developed, it would be appropriate for USAID or another development organization to implement it. This appears not to be possible in the near future.

V. Project Activities/Outputs

A. Meetings attended

1. International Symposium on Frankia and Actinorhizal Plants, University of Connecticut, Storrs, CT, August 1988 (Baker, Boonkerd, Nittayajarn).

2. North American Symbiotic Nitrogen Fixation Meeting and BOSTID-PSTC Workshop, Iowa State University, Ames, IA, August 1989 (Baker, Boonkerd, Nittayajarn).

3. International Workshop on Interactions between Plants and Microorganisms, National University of Singapore, October 1989 (Boonkerd).

4. International Symposium on Nitrogen Fixation, University of Tennessee, Knoxville, TN, May 1990 (Baker, Nittayajarn).

B. Publications arising directly or indirectly from project

Nittayajarn, A. and D. D. Baker. 1989. Methods for the quantification of Frankia cell biomass. Plant and Soil 118: 199-204.

Baker, D. D. 1989. Methods for the isolation, culture and characterization of the Frankiaceae, soil actinomycetes and symbionts of actinorhizal plants. In: Isolation of Biotechnological Organisms from Nature (D. Labeda, ed.). McGraw-Hill, NY. pp. 213-236.

Boonkerd, N. and D. D. Baker. 1989. Interactions between plants and microorganisms in nitrogen-fixing symbioses. In: Interactions Between Plants and Microorganisms (G. Lim and K. Katsuya, eds.). National University of Singapore. pp. 160-169.

Nittayajarn, A., B. C. Mullin and D. D. Baker. 1990. Screening of symbiotic frankiae for host specificity using restriction fragment length polymorphism analysis. Applied and Environmental Microbiology 56: 1172-1174.

C. Training

Aphakorn Nittayajarn, a scientist from the BNF Resource Centre in Bangkok was trained in the Yale University laboratory of Dr. Baker from February 1988 to April 1989. After her return to Bangkok, Dr. Baker visited the Bangkok laboratory for several weeks and jointly the trained Kanittha Khandej. Ms. Khandej is now maintaining the collection of frankiae held at the Bangkok laboratory.

VI. Project Productivity

This project accomplished the overall goals that were set forth in the original proposal. However, as indicated in the Methods and Results section, the approach differed considerably from that originally described. The final method developed is more simple yet accomplishes the goal of rapidly screening newly isolated strains for host specificity.

The BNF Resource Centre for SE Asia is now capable of conducting research on Frankia and actinorhizal species. A modest collection of frankiae for Casuarina has been established there and inoculum could be produced upon demand. A field trial of Casuarina in a challenging region of the country has been established and additional research on actinorhizal plants and frankiae are expected to continue.

VII. Future Work

Drs. Baker and Boonkerd both have adopted the methodologies developed in this project into their ongoing research programs. Dr. Baker has used the RFLP screening technique to study another large set of Frankia strains in a comparative research project on frankiae from the Peoples' Republic of China and similar strains from Europe. Dr. Boonkerd has established a Casuarina field trial in northeast Thailand to demonstrate the usefulness of inoculation.

Dr. Baker and Dr. Boonkerd individually have attempted to obtain support, both financial and philosophical, for the establishment of routine inoculation programs at the Royal Thai Forest Department and in other analagous institutions which produce actinorhizal nursery stock. To date, applications to the U.S. Forest Service and U.S.A.I.D. Forestry Division have been unsuccessful. We feel that we have proven the value of inoculation, established a collection of Frankia strains suitable for inoculum, and developed a simple procedure for screening additional isolated strains in the future. Unfortunately, the application of this technology must be delayed until an enlightened development agency or government institution chooses to support such a program.

VIII. Literature Cited

Baker, D. D. 1987. Relationships among pure cultured strains of Frankia based on host specificity. Physiol. Plant. 70: 245-248.

Baker, D. D. 1989. Methods for the isolation, culture and characterization of the Frankiaceae, soil actinomycetes and symbionts of actinorhizal plants. In: Isolation of Biotechnological Organisms from Nature (D. Labeda, ed.). McGraw-Hill, NY. pp. 213-236.

Baker, D. and D. O'Keefe. 1984. A modified sucrose fractionation procedure for the isolation of frankiae from actinorhizal root nodules and soil samples. Plant and Soil 78: 23-28.

Benson, D. R. 1982. Isolation of Frankia strains from alder actinorhizal root nodules. Appl. Environ. Microbiol. 44: 461-465.

Fernandez, M. P., H. Meugnier, P. A. D. Grimont and R. Bardin. 1989. Deoxyribonucleic acid relatedness in the genus Frankia. Int. J. System. Bacteriol. 39: 424-429.

Gauthier, D. L., H. G. Diem, and Y. R. Dommergues. 1981. Infectivite et effectivite de souches de Frankia isolee de nodules de Casuarina equisetifolia et d'Hippophae rhamnoides. Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences. III. 293: 489-491.

Kaluza, K., M. Hahn and H. Hennecke. 1985. Repeated sequences similar to insertion elements clustered around the nif region of the Rhizobium japonicum genome. J. Bacteriol. 162: 535-542.

Nittayajarn, A., B. C. Mullin and D. D. Baker. 1990. Screening of symbiotic frankiae for host specificity using restriction fragment length polymorphism analysis. Appl. Environ. Microbiol. 56: 1172-1174.

Normand, P., P. Simonet, and R. Bardin. 1988. Conservation of nif sequences in Frankia. Molec. Gen. Genet. 213: 238-246.

Torrey, J. G. and S. Racette. 1989. Specificity among the Casuarinaceae in root nodulation by Frankia. Plant and Soil 118: 157-164.