

## Interim Technical Report

"Study of Nodulation of *Aeschynomene* by an Unusual Endophyte"

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## A. Introduction

The objective of this project is to characterize the symbiotic association between the tropical legume *Aeschynomene* and an unusual endophyte which we now know to be capable of photosynthetic electron transport. Earlier experiments have demonstrated that under appropriate conditions the bacterium, which is able to form nitrogen-fixing nodules on the stems and roots of *A. indica*, forms bacteriochlorophyll and functional photosynthetic reaction centers. Preliminary experiments indicate that the bacteroids in stem nodules formed by the bacterium are capable of light-driven acetylene reduction. Acetylene reduction is considered to be an indicator of nitrogenase activity. Most of the experiments performed thus far under this grant have employed a "Rhizobium" isolated from an *A. indica* stem nodule at Ithaca, New York by A.R.J. Eaglesham of the Boyce Thompson Institute at Cornell University and named BTA11.

During the past four months Dr. Sambandam Shanmugasundaram and Dr. Suguna Shanmugasundaram of Madurai Kamaraj University, Madurai, India have worked on the project as consultants. Dr. Sambandam Shanmugasundaram had been included as a Principal Investigator of this project when the proposal was originally submitted from the Charles F. Kettering Research Laboratory. Their principal objectives have been to prepare a gene library from total BTA11 DNA, to identify photosynthesis and nodulation genes in the BTA11 DNA and eventually to isolate, clone and sequence several of the genes.

The rationales for their experiments were the following. I had originally believed the unusual endophyte to be a purple photosynthetic bacterium which had somehow acquired the ability to nodulate *Aeschynomene*. We hoped to compare nodulation genes of the unusual endophyte with those from "normal" *A. indica* rhizobium to determine whether the unusual endophyte might have acquired the genes from the normal endophyte, perhaps on a plasmid. *E. coli* clones containing *nod* genes from *Rhizobium meliloti* were obtained from Sharon Long of Stanford University, to serve as hybridization probes. We now believe that the photosynthetic endophyte is the normal *A. indica* Rhizobium and that it has retained the photosynthetic capability of the purple bacteria which were its presumed ancestors, probably because the ability to use sunlight as an energy source provides a selective advantage for a rhizobium which occupies light-exposed stem nodules. Isolation of the nodulation genes is nevertheless potentially important. There are said to be nearly two hundred species of *Aeschynomene*, varying widely in their habitats, characteristics and agronomic value. Thus far photosynthetic endophytes have been found only in association with *A. indica*. Transfer of host specificity genes might allow other, more useful species of *Aeschynomene* to form associations with photosynthetic

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rhizobia.

BTA11 was isolated a number of years ago, but its ability to form the photosynthetic apparatus was discovered only recently when it was fortuitously grown under conditions which allowed expression of the photosynthesis genes. I suspect that there are other bacteria which form symbiotic associations with plants and have undiscovered photosynthetic potential. Rather than perform laborious variations of growth conditions in order to induce the formation of photosynthetic proteins in candidate bacteria, we plan to search for photosynthesis genes by Southern blotting and hybridization with the highly-conserved bacterial photosynthetic reaction center protein genes. Plasmids containing genes for the L and M subunits of the *Rhodospirillum rubrum* reaction center and for the alpha and beta light-harvesting proteins of *R. rubrum* were obtained from Aiji Takahashi and Colin Wraight of the University of Illinois.

A major unanswered question is whether the *Aeschynomene* *Rhizobium* can fix carbon photosynthetically. It is possible that the function of its photosynthetic apparatus is only to allow photophosphorylation and photosynthetic nitrogen fixation. Efforts to culture the bacterium anaerobically in the light have been unsuccessful. However, other "aerobic photosynthetic bacteria" which will not grow anaerobically in the light have been shown to fix carbon photosynthetically. We are approaching the problem directly by measuring  $^{14}\text{C}$  incorporation by BTA11 cells which are incubated in the light in the presence of  $[\text{C}^{14}]$  bicarbonate. An alternative approach is to search for enzymes specifically involved in carbon fixation. One of these is ribulose-bis-phosphate carboxylase, the enzyme that catalyzes the incorporation of carbon dioxide into sugar. An *E. coli* clone containing the gene for the large subunit of this enzyme (from the cyanobacterium *Anabaena*) was obtained from Susan Golden of the University of Colorado. It is being used as a hybridization probe to search for the gene in BTA11.

#### B. Effect of carbon sources on pigmentation

Dr. Shanmugasundaram's first attempts to isolate DNA were frustrated by the fact that the cells form large clumps when grown in culture, preventing lysozyme from reaching many of the cells. He therefore explored the effect of varying growth conditions and substrates. Among the more interesting results of these experiments was the discovery that far more pigment than we had previously observed forms when the cells are grown with certain amino acids as the carbon and nitrogen source (Dr. Eaglesham had also told me that growth on glutamate results in enhanced pigment formation). The best pigmentation is obtained with glutamate, alanine or asparagine; intermediate levels are obtained with fructose, malate or succinate; little pigmentation is obtained with glucose, citrate, glutamine or methionine. We are now able to achieve far better pigmentation and far less clumping than before. This will make spectrophotometric studies of electron transport as well as molecular biology far easier.

#### C. Modification of BTA11 DNA

Total DNA from BTA11 was treated with the restriction enzymes Eco R I, Mbo I, Pst I and Sal I. The DNA was poorly digested by Eco R I, suggesting that the BTA11 modification

system may methylate adenine bases. A-methylated DNA is resistant to Eco R I. This finding was disappointing in that it precludes the use of Eco R I fragments for cloning and the preparation of gene libraries. The BTAil DNA was digested satisfactorily by the other three restriction enzymes.

#### D. A plasmid in BTAil

Agarose gel electrophoresis of uncut total BTAil DNA revealed the presence of a DNA band migrating at about 16 or 17 KB. It is presumed to be a plasmid. The intensity of the band varied with the conditions under which the cells had been cultured. Dr. Shanmugasundaram speculates that it may be excised from the chromosomal DNA under conditions such as phosphate starvation. It will receive further study. It is potentially useful as a vector for transferring genes into BTAil, particularly because of its relatively small size. The DNA of the *Aeschynomene americana* Rhizobium and the Sesbania stem and root nodule Rhizobium ORS 571 were examined by similar techniques and no plasmids were detected.

#### E. Southern blotting of BTAil DNA

Cultures of *E. coli* harboring plasmids containing the nod, photosynthetic reaction center and light-harvesting protein, and ribulose-bis-phosphate carboxylase genes were grown. The plasmids were isolated, purified and cut with appropriate restriction endonucleases to remove the desired genes from the vectors. Separate aliquots of total BTAil DNA were treated with the restriction endonucleases Sal I and Pst I. The cut DNA was separated by agarose gel electrophoresis and blotted onto a Nytran membrane. DNA containing the reaction center and light-harvesting bacteriochlorophyll protein genes was separated by agarose gel electrophoresis and eluted from the gel. Next week we plan to end-label the DNA with [<sup>32</sup>P] dCTP and hybridize it with the BTAil DNA on the Nytran membrane in order to locate the photosynthesis genes in the Rhizobium DNA. Similar experiments will be performed with the other genes, and we also hope to probe DNA from other bacteria, beginning with the *A. americana* and Sesbania Rhizobia, with the reaction center genes.

#### F. Preparation of a BTAil gene library

Two gene libraries were prepared, using total BTAil DNA cut with Sal I and with Pst I. The genes were ligated into plasmids which were then used to transform *E. coli* cells. During Dr. Shanmugasundaram's visit next summer the libraries will be used for isolating and cloning the BTAil photosynthetic reaction center protein genes and perhaps other of the genes of interest.

G. Conference presentation. A poster entitled "Photosynthetic Competence in a Rhizobium", by D. Fleischman, W. Evans, S. Shanmugasundaram and S. Shanmugasundaram, was presented at the 1988 Annual Meeting of the American Society of Plant Physiologists.