ABSTRACT. *Aeschynomene* is a tropical or semitropical legume, some species of which form nitrogen-fixing stem nodules. It is sometimes used as a green manure in rice fields. Workers at the Charles F. Kettering Research Laboratory had found that endophytes from stem nodules collected in India contain bacteriochlorophyll and photosynthetic reaction centers, and that *Aeschynomene* Rhizobium BTAI 1, which had been isolated by A. R. J. Eaglesham at the Boyce Thompson Institute, will also form the photosynthetic system when grown on suitable carbon sources under cyclic illumination in the presence of oxygen.

Formation of the photosynthetic system in BTAI 1 requires illumination with red or near-infrared light near the end of exponential growth, suggesting that expression of the photosynthetic system may be subject to catabolite repression. Formation of the photosynthetic system is inhibited by blue light.

BTAI 1 cells in which pigmentation has been induced contain a single predominant soluble cytochrome and at least one high-potential and one low-potential membrane-bound cytochrome. Photosynthetic reaction centers from BTAI 1 membranes were partially purified.

Southern hybridization of BTAI 1 DNA with DNA containing genes for the L and M subunits of the photosynthetic reaction center of *Rhodobacter sphaeroides* suggested that this Rhizobium possesses similar genes.

Acetylene reduction by BTAI 1-containing stem nodules was accelerated when the nodules were illuminated with near-infrared light (which can drive electron transport in the bacteroids but not in plant chloroplasts), suggesting that the bacteroids can use sunlight directly to drive nitrogen fixation.

A. Introduction. Most of the familiar legumes form root nodules after inoculation with appropriate strains of rhizobia. Some species of the tropical and subtropical legumes *Aeschynomene*, *Sesbania* and *Neptunia* also form nitrogen-fixing nodules on their stems. *Sesbania*, and to a lesser extent *Aeschynomene*, have been used as green manures in rice fields of India and other tropical countries. This has succeeded in part because nitrogen fixation by the stem-nodulated plants is resistant to flooding. Several years ago Dr. N. S. Subba Rao, of the Indian Agricultural Research Institute, believed that he had seen two different types of endophytes in stem nodules collected from *Aeschynomene indica* plants in India. He brought a few of the nodules to the Charles F. Kettering Research Laboratory and requested that the ultrastructure of the nodules be examined with the electron microscope. Dr. Harry Calvert discovered that some of the nodules contained what appeared to be ordinary rhizobium bacteroids, while others contained a coccoid endophyte possessing intracytoplasmic membranous vesicles resembling the chromatophores of purple photosynthetic bacteria. A few nodules contained both types of endophyte, but in different sections of the nodule. Endophytes isolated from stem nodules subsequently collected in India and brought to the Kettering Laboratory by Dr. Subba Rao and by Dr. S. Shanmugasundaram of Madurai Kamaraj University were found to contain bacteriochlorophyll and photochemically-active photosynthetic reaction centers resembling those of purple photosynthetic bacteria. At that point I was convinced that the stem nodules contained two types of endophyte: a Rhizobium and a purple...
photosynthetic bacterium which had somehow acquired the ability to nodulate Aeschynomene. However, I was unable to culture the endophyte in the light in ordinary photosynthetic bacteria medium in the absence of oxygen.

Several years earlier, Dr. A. R. J. Eaglesham, of the Boyce Thompson Institute at Cornell, had isolated a Rhizobium, which he named BTAi 1, from an A. indica stem nodule which had formed spontaneously on a plant growing at Ithaca in sand obtained from Virginia (1). He gave a culture of BTAi 1 to the Kettering group. W. R. Evans and I. Miller noticed that cultures of BTAi 1 growing on certain media on agar plates turned pink if they were exposed to normal room lighting. Further study revealed that the pink cells contained bacteriochlorophyll A and photosynthetic reaction centers. Dr. Evans and P. Pyati determined that pigmentation developed when cells were grown in media containing fructose, succinate or low concentrations of malate, but not arabinose, as the carbon source. They also learned that pigmentation developed in cultures exposed to cyclic lighting (e.g., 16 hours light:8 hours dark), but not in cells subjected to continuous light or continuous darkness. The cultures would not grow or become pigmented in the absence of oxygen, even if they were illuminated. Perhaps the most significant conclusion to be drawn from their studies was that the Aeschynomene endophyte is not a purple photosynthetic bacterium (Rhodospirillaceae), but instead shares many of the properties of the so-called aerobic photosynthetic bacteria which were discovered about a decade ago by Japanese workers and have been the subject of intensive study in Japan. These bacteria grow, form the photosynthetic system and (in the instances so far examined) perform photosynthetic electron transport only in the presence of oxygen. They do not oxidize water or evolve oxygen. Aerobic photosynthetic bacteria have been the subject of a recent monograph (2).

B. Regulation of the development of the photosynthetic system in BTAi 1. We have examined the influence of a variety of carbon sources on the development of pigmentation in cultures of BTAi 1. In these experiments the cultures were grown under an alternating light-dark regimen. The best pigmentation was obtained with glutamate (suggested to us by Dr. Eaglesham), alanine and asparagine; intermediate levels were obtained with fructose, malate or succinate; little pigmentation was obtained with glucose, citrate, glutamine or methionine.

Illumination with white light for a single day near the end of exponential growth was as effective in inducing pigmentation as was growth under a light-dark cycle (Fig. 1. The figure is somewhat misleading since the cells were harvested before pigmentation was complete. In most experiments illumination on days 5, 6 and 7 is quite effective). This observation, along with the sensitivity of pigment development to the carbon source employed, lead us to suggest that pigment development may be subject to catabolite repression. Only when the energy source is nearly depleted do the bacteria form the photosynthetic system to allow them to use light as an energy source. Maximum pigmentation requires eight hours of illumination with white light. After the cultures are transferred to the dark, pigmentation begins after a lag of about 6 hours and is complete in about two days. Experiments using a series of long and short pass filters indicate that the photoreceptor responsible for the light induction of pigmentation absorbs maximally at wavelengths between 650 and 830 nm (in these experiments the cultures were illuminated for one day and then transferred to the dark to allow pigment to develop). The ineffectiveness of light beyond 830 nm indicates that photosynthetic electron transport is not involved, since electron transport is presumably driven by light absorbed in the 870 nm antenna bacteriochlorophyll protein absorption band.
BTAi 1 cultures are able to form pigment when grown under continuous red light (e.g., tungsten light filtered through a Corning 2-64 filter). Presumably cultures do not form pigment when grown under continuous white light because development of pigmentation is suppressed, as well as triggered, by illumination. Cultures were subjected to continuous illumination with tungsten light filtered through a series of long-pass filters (these filters transmit the inducing infrared light as well as shorter wavelength light). Pigmentation begins to diminish as the filters begin to transmit light of wavelength below 550 nm, suggesting that the photoreceptor which mediates inhibition of pigmentation absorbs light in the 400-550 nm region. Interestingly, it has been reported recently that blue light suppresses pigmentation development in aerobic and purple photosynthetic bacteria. In summary, pigment development is reciprocally regulated by infrared and blue light. Free-living cells should be able to develop pigment at night after having been illuminated during the day. In stem nodules, the bacteroids are shaded by chloroplasts in the outer nodule cortex. Chloroplasts absorb strongly in the blue region, but transmit light beyond about 700 nm, so pigment is permitted to develop in the nodule.

C. Electron transport components in pigmented BTAi 1 cells. Pigmented BTAi 1 cells contain a single predominant soluble cytochrome whose alpha, beta and gamma absorption bands (in the reduced form) are at 552, 521 and 416 nm, respectively. It is not readily autoxidized. The membranes contain at least one high potential (reducible by ascorbate) and one low potential (reducible by dithionite but not by ascorbate)
cytochrome. The alpha and beta bands of the reduced form of the high potential cytochrome are at about 551 and 522 nm, respectively, and the maxima and minima of the Soret band reduced-minus-oxidized spectrum are at about 416 nm and 408 nm, respectively. The Soret band maxima and minima of the reduced-minus-oxidized spectrum of the low potential cytochrome are at about 431 nm and 408 nm, respectively.

Photosynthetic reaction centers were isolated and partially purified from BTAi 1 membranes using a combination of solubilization with Triton X-100, flotation from an ammonium sulfate solution and density gradient centrifugation. Isolation of reaction centers from membranes containing colored carotenoids using non-ionic detergents such as Triton X-100 is often not possible and ionic or zwitterionic detergents such as lauryldimethylamine-N-oxide (LDAO) is usually necessary. Perhaps the membrane proteins in BTAi 1 are unusually loosely associated. The absorption spectrum of the reaction centers (Fig. 2) resembles those of reaction centers of aerobic and purple photosynthetic bacteria. In future experiments we hope to analyze the electron transport components in the isolated reaction centers, by extraction and electron paramagnetic resonance spectroscopy, in the hope of learning something of how the rhizobium is related to other photosynthetic bacteria and perhaps of why the bacterium is unable to grow photoautotrophically in the absence of oxygen.

I was unable to detect a light-induced EPR signal from the reaction center cation radical in BTAi 1 membranes at liquid nitrogen temperature. This could be because the membranes contain a very low concentration of reaction centers or because the primary electron acceptor is already reduced in the dark in unaerated membranes.

![Absorption spectrum of isolated BTAi 1 photosynthetic reaction centers.](image-url)
A major question which remains unanswered is whether BTAi 1 cells and membranes are able to perform light-driven electron transport under anaerobic conditions. In a preliminary experiment, Dr. Shanmugasundaram detected $^{14}$CO$_2$ assimilation by BTAi 1 cells which were illuminated under air, but not by cells which were illuminated under nitrogen.

D. Light stimulation of acetylene reduction by BTAi 1-containing A. indica stem nodules. Most photosynthetic bacteria are able to use light energy to drive nitrogen fixation. We speculated that the function of the photosynthetic apparatus in stem nodule bacteroids might be to allow them to use light directly to provide energy for nitrogen fixation, thereby diminishing their dependence on imported chloroplast-derived photosynthate. I incubated A. indica stem sections bearing BTAi 1-containing stem nodules with acetylene and oxygen, and compared ethylene formation rates in the presence and absence of illumination. The nodules were illuminated with near-infrared light which can drive electron transport in BTAi 1 bacteroids but not in chloroplasts (A. R. J. Eaglesham had earlier found that white light accelerated acetylene reduction by stem nodules, and attributed the effect to light-driven oxygen evolution by nodule chloroplasts. The near-infrared light used in our experiments is unable to drive oxygen evolution by chloroplasts). In each of about a dozen experiments, I found that illumination of the stem nodules at least doubled the rate of acetylene reduction (Fig. 3). In some experiments the nodules were incubated in the dark until detectable acetylene reduction had stopped; upon illumination it sometimes resumed at nearly the initial rate. In general, my impression is that the predominant effect of illumination is to prolong acetylene reduction, often allowing it to proceed for many hours. Illumination had no effect on acetylene reduction by soybean (Fig. 3) or alfalfa nodules, or on acetylene reduction by nodules collected from a deep part of an Aeschynomene root. These experiments are quite preliminary and must by performed in much greater detail to ascertain whether my results are statistically significant.

Figure 3. Influence of illumination with infrared light on acetylene reduction by BTAi 1-containing A. indica stem nodules (closed circles) and soybean root nodules (open circles). Illumination was begun at arrows.
Whether there are actually two different kinds of stem nodule endophytes seems to remain an open question. Allan Eaglesham had convinced me that the rod-shaped endophytes observed by Dr. Calvert in some nodules were an early stage in the development of bacteroids. He had followed the development of nodules microscopically in BTAi 1-inoculated plants, and observed rod-shaped bacteria in the nodules during early stages of infection. The coccoid bacteroids developed later. The bacteroids in peanut root nodules undergo a similar rod-coccus developmental sequence. However, Iain Miller, who has examined the original fixed nodules in detail with the light and electron microscopes, insists that the rod-shaped endophytes are mature bacteroids representing a distinct organism. The stem nodules which displayed light-stimulated acetylene reduction contained coccoid endophytes. I measured acetylene reduction by single stem nodules from BTAi 1-inoculated A. indica plants. There was a large variation in rates. Nodules displaying the highest rates contained coccoid endophytes. One such nodule was examined with the photoacoustic spectrophotometer and displayed a near-infrared absorption band characteristic of the antenna bacteriochlorophyll of BTAi 1 cells in which pigmentation had been induced (the photoacoustic spectrophotometer seems to be the preferred instrument for detecting bacteriochlorophyll in homogenized nodules, since it can record spectra of highly scattering or opaque samples). These experiments suggest that the coccoid cells are capable of fixing nitrogen and contain bacteriochlorophyll.

The function of the photosynthetic system in the stem nodules may thus be to allow the bacteroids to fix nitrogen photosynthetically. The rhizobia may have retained the photosynthetic capability of the purple bacteria which are their presumed ancestor because it provides a selective advantage for an endophyte which occupies a nodule that is exposed to light (Iain Miller has suggested that the stem nodule rhizobia represent an evolutionary form intermediate between purple photosynthetic bacteria and the more common rhizobia). The ability of the bacteroids to fix nitrogen photosynthetically could be of significant value to the symbiotic association. Photosynthetic carbon fixation by leaf chloroplasts, translocation of the photosynthetic to the roots, and its degradation in the bacteroids to provide ATP by oxidative phosphorylation is an inefficient process. A rough calculation indicates that oxidation of glucose in bacteria can furnish no more than 70% of the free energy required to synthesize glucose in the chloroplasts. This free energy loss could be avoided if ATP were formed by photophosphorylation in the bacteroids themselves. In addition, the bacteroid photosynthetic system can use near-infrared light which is not absorbed or used by the chloroplasts. Both these effects could substantially lessen the competition between carbon and nitrogen fixation for energy. Nitrogen fixation is often limited by the availability of photosynthetic products. Eaglesham has suggested that Aeschynomene stem nodules may be autonomous, i.e., not dependent on imported photosynthetic to support nitrogen fixation, and it has been pointed out that Aeschynomene plants can support an extraordinary number of stem nodules. Thus stem-nodulated plants containing photosynthetic rhizobia may be able to serve as an unusually efficient source of fixed nitrogen.
E. Molecular biology of BTAi 1. Total DNA from BTAi 1 was isolated and subjected to restriction endonuclease digestion. *Hin* I, *Pst* I and *Sal* I could digest it, but *Eco R* I could digest it only partially, if at all. This suggests that the adenosine residues in BTAi 1 may be modified.

*Pst* I- and *Sal* I-digested BTAi 1 DNA were separated on a 0.7% agarose gel and blotted onto a Nytron membrane. The membrane was probed with DNA fragments containing the puf operon from *Rh. sphaeroides* (obtained from A. Takahashi and C. Wraight of The University of Illinois). The puf operon contains genes for the L and M subunits of the photosynthetic reaction center and for two subunits of the light-harvesting LH I protein, as well as two additional open reading frames and some presumed regulatory elements. Positive hybridization was obtained. The puf operon was further digested with the restriction endonuclease *Sma* I. The blot was probed with *Sma* I the fragment containing the reaction center M subunit and with the fragment containing the reaction center L subunit and the LH I subunits. In both cases positive hybridization was obtained at a moderate degree of stringency, suggesting that the Rhizobium contains genes homologous to these photosynthesis genes (we have seen an unpublished manuscript from another laboratory reporting no hybridization between BTAi 1 DNA and the puf operon. We don't understand the reason for the conflicting observations). We also obtained positive hybridization with the gene for the large subunit of ribulose bisphosphate carboxylase from *Anabaena* 7128. Preliminary attempts to obtain hybridization with Rhizobium *melliloti* nodulation genes, obtained from Dr. Sharon Long of Stanford University, have given ambiguous results.

The ability to detect photosynthesis genes in rhizobia with the use of Southern hybridization will be of use in attempts to isolate photosynthesis genes from these bacteria. Dr. Shanmugasundaram has prepared a BTAi 1 gene library and is attempting to isolate the gene for the reaction center M subunit, which he plans to sequence. A comparison of this protein with the homologous protein from purple bacteria may provide insight into why the Rhizobium will not grow photosynthetically in the absence of oxygen. In addition, Southern hybridization with photosynthesis genes should be useful in efforts to determine whether other rhizobia (e. g. other Aeschynomene Rhizobia and Sesbania Rhizobia) contain latent photosynthesis genes even if conditions for inducing the expression of the genes have not been found.

F. Recent related developments and suggestions for future research. Workers at the International Rice Research Institute have recently reported that stem-nodulating rhizobia are found in much higher numbers on aerial plant parts than are exclusively root-nodulating rhizobia (3). It is possible that the ability to perform photosynthesis allows the rhizobia to persist for longer times in the environment. Persistence in the environment is an especially useful property of rhizobia which are used for inoculation of crop plants.

It has been reported that the rhizobia which form stem nodules on Sesbania share more phenotypic properties with purple photosynthetic bacteria than with other rhizobia. They have have been assigned to a separate genus (4). The photosynthetic system has not yet been detected in this bacterium. The stem-nodulating species, but not the exclusively root-nodulating species, of both Sesbania and Aeschynomene Rhizobia, are capable of nitrogen fixation in pure culture (5).

The observations we have made raise a number of interesting scientific questions, particularly concerning the evolutionary relationships among the photosynthetic bacteria and rhizobia, the regulation of expression of the photosynthetic system, and the interaction between photosynthesis and oxygen in the Rhizobium. From the point of view of USAID objectives, I believe the most important question is whether the photosynthetic
properties of the Rhizobium make the stem-nodulated plants especially good sources of biologically-fixed nitrogen. This should be determined in appropriate green house and field studies in which growth and nitrogen fixation by plants inoculated with photosynthetic and non-photosynthetic rhizobia are compared. There are said to be at least 350 species of Aeschynomene worldwide. It is important to determine how many of these are or can be stem-nodulated by photosynthetic rhizobia. If the photosynthetic properties of the rhizobia do lead to superior symbiotic associations, the various species of host and endophyte should be surveyed to determine which combinations will serve as the most effective green manure crop in specific environments. Simultaneously a search should be made for photosynthetic endophytes of Sesbania and Neptunia, and efforts made to extend the host range of the photosynthetic Aeschynomene Rhizobium to include these plants, since they also can serve as green manures. In the long term, it may be possible by genetic manipulation to extend stem nodulation by photosynthetic rhizobia to food legumes, beginning perhaps with peanut, which is a relative of Aeschynomene.


References


