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The first photosynthetic N_2-fixing *Rhizobium*: Characteristics

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Introduction

Biological N_2 fixation requires large amounts of energy based on either *in vitro* biochemical studies of nitrogenase or *in vivo* studies of root nodulated legume plants. For example, calorimetric experiments with effectively root nodulated soybeans suggest that the aerobic metabolism of the equivalent of about 12 g of carbohydrate to CO_2 and H_2O is required to fix 1 g of N_2 by the rhizobial-legume process (11). This carbohydrate is provided through foliar photosynthesis by the legume macrosymbiont and is used by the rhizobial microsymbiont. A photosynthetic rhizobium that could meet directly its energy needs for biological N_2 fixation could be advantageous since the legume plant might use the saved fixed carbon to produce additional harvestable plant product.

Although photosynthetic N_2-fixing bacteria and blue-green algae are well known, no example of a photosynthetic rhizobium has been described prior to that reported here. Since most rhizobia occur in the relative darkness of soil, either as free-living organisms or symbionts in root nodules, there was little reason for scientists to seek photosynthetic rhizobia. Possible exceptions were the rhizobia that form stem nodules.

Over sixty years ago Hagerup observed nodules on submerged stems of the legume *Aeschynomene aspera*, along the shores of the Niger River. He remarked on the green color of the nodules and the presence of large amounts of starch grains and suggested that a very efficient photosynthesis was occurring (8). Stem nodules have been found and studied on other *Aeschynomene* and on *Sesbania* and *Neptunia* (2, 4, 5, 16, 17, 21) including a report in this 8th International Congress on Nitrogen Fixation (Ladha, JK) on the use of stem-nodulated legumes as green manure. The same rhizobia that produce stem nodules on the above legumes will produce root nodules on them under appropriate cultural conditions.

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Investigations from the early 1980's to the present at the Battelle-C.F. Kettering Laboratory and the Boyce Thompson Institute led to the discovery that an *Aeschynomene indica* stem-nodulating rhizobium identified as BTAi 1 appears to be photosynthetic while that of *Azorhizobium caulinodans* strain BTSr 3 that stem-nodulates *Sesbania rostrata* is not photosynthetic.

Rhizobial strain BTAi 1 was isolated at Boyce Thompson Institute from sand used for plant cultures (16). The sand originated in West Virginia near the Potomac and was strip-mined with some soil contamination; the fact that *Aeschynomene virginica* is indigenous to fresh and brackish tidal shores from New Jersey to Virginia (7) may explain the presence of BTAi 1. Stem nodules have not been reported on *A. virginica*, although it seems fitting that a rhizobium that can utilize light energy can also form nodules on illuminated parts of its legume host. It is possible that photosynthetic capabilities confer competitive advantage *ex planta* in aquatic C-limited conditions.

Stem nodules, but not root nodules, formed by BTAi 1 on *Aeschynomene* were found to maintain N_2-fixing activity for several days following defoliation (6) supporting Hagerup's early suggestion of efficient photosynthesis of the stem nodules. In contrast N_2-fixing activity of both stem and root nodules formed by BTSr 3 declined following defoliation. These observations suggested a possible photosynthetic uniqueness of BTAi 1.

Independent and parallel studies at the Battelle-C.F. Kettering Laboratory of endophyte(s) from *A. indica* stem nodules collected in India revealed an elaborate internal membrane system, resembling that of purple photosynthetic bacteria, as well as bacteriochlorophyll and photosynthetic reaction centers. A similar ultrastructure has been seen in *A. indica* stem nodules collected in Mississippi (19). However, the endophyte could not be cultured. The Battelle-C.F. Kettering Laboratory followed up studies on this/these endophyte(s) with experiments on BTAi 1. Evidence from the Battelle-C.F. Kettering Laboratory and Boyce Thompson Institute which support the photosynthetic and N_2-fixing activities of BTAi 1 but not BTSr 3 are summarized in the Results section.

Results

A brief summary of compositional and functional studies of free-living BTAi 1 and symbiotic BTAi 1 in root and stem nodules of *Aeschynomene* will be presented. Because of the uniqueness of a photosynthetic rhizobium, many different types of measurements were made to support this conclusion. Comparisons will be made with the non-photosynthetic BTSr 3 and other photosynthetic bacteria. Complete information will be published elsewhere by Boyce Thompson Institute and Battelle-C.F. Kettering groups.

Bacteriochlorophyll. In appropriate media such as 0.15% malate and 0.05% yeast extract, colonies of free-living BTAi 1 were pink-red when grown in a light-dark regimen in contrast to white when grown in the dark (16). Both pigmented and white colonies were capable of nodulating *Aeschynomene*.

Spectrophotometric examination of the pigmented cells showed an 870 nm absorption band similar to that of the long-wavelength of Bacteriochlorophyll *a*-containing purple photosynthetic bacteria (18). The endophyte of stem nodules exhibited an absorption spectrum similar to BTAi 1 *in vitro*. BTAi 1 is an aerobic organism; it could not be grown anaerobically in continuous light or dark or a light-dark regimen. The amount of bacteriochlorophyll synthesized aerobically by BTAi 1 is similar to that of aerobic photosynthetic bacteria such as *Erythrobacter* sp. OCh 114 (14) and less than 10% of that of anaerobically-grown *Rhodospirillaceae* (12).

Koch Postulates. The following steps were used to assure that BTAi 1 was a single organism and could produce stem nodules and be reisolated from stem nodules. A broth culture of BTAi 1 was serially diluted in yeast-mannitol broth with 0.01% Tween 80 and low dilutions were checked for absence of cell-aggregation by light microscopy. Cell number was determined using the Petroff Hauser and spread plate methods. All resultant colonies from the spread plate of yeast-fructose agar were pink. Six well separated colonies were cultured in yeast-mannitol broths and inoculated onto the submerged parts of surface-sterilized cuttings of *Aeschynomene*. All of the single colony isolates induced stem nodules whereas uninoculated cuttings remained nodule free. Six nodules were selected randomly and the endophyte plated to provide single colonies. All colonies were pink.

Free-Living Growth. Cultures of each of BTAi 1 and BTSr 3 were grown in 16 h/8 h light/dark, or continuous dark for 20 days. There was no beneficial effect of the light/dark regimen for log phase growth of BTAi 1 and BTSr 3 but the light/dark regimen was able to retain viable cell numbers of BTAi 1 from 10 to 20 days after the exogenous carbohydrate had been utilized while viable cell number declined after 10 days in continuous dark. The viable cell number declined after 10 days for both light/dark and continuous dark regimens for BTSr 3. Light was unable to support log-phase growth of BTAi 1 or BTSr 3 in the absence of carbohydrate in the media. Photosynthesis in BTAi 1 is adequate to maintain steady-state cell numbers but not to support log-phase growth. A similar pattern has been observed for *Erythrobacter* (13).

Free-Living Photosynthetic Functions. Uptake of CO_2 by BTAi 1 was stimulated several fold under light for cells grown in a light/dark regimen versus those under dark and grown in continuous dark. Similar light stimulation of CO_2 uptake was shown for *Erythrobacter* whereas BTSr 3 showed no response to light. Southern-blot hybridization results for BTAi 1 performed by Antonius Suwanto and Samuel Kaplan (University of Illinois, Urbana) provided evidence for the ribulose bisphosphate carboxylase (RBC) gene form I as found in plants, cyanobacteria, and some eubacteria, but not for the RBC gene form II as found in purple photosynthetic bacteria. These hybridization results are consistent with photosynthetic ability in BTAi 1 but are not conclusive proof since some rhizobia can fix CO_2 chemiautotrophically (15).

The rates of O_2 uptake by BTAi 1 and *Erythrobacter* were decreased by 50% or more by illumination. Light had no effect on O_2 uptake by BTSr 3. The decreased need for terminal oxidation to provide energy, a consequence of ATP formation via photosynthesis, has been reported with *Erythrobacter* (9). Inhibitors of photosystem II (DCMU and o-phenanthroline) and an uncoupler of photophosphorylation (NH_4Cl) overcame the O_2-sparing effect of light on BTAi 1 and *Erythrobacter* but had no effect on BTSr 3. Those inhibitors and uncouplers also decreased the light stimulation of CO_2 uptake.

Southern blot hybridizations of BTAi 1 by Suwanto and Kaplan using probes from the purple non-sulfur photosynthetic bacteria gave no positives for various structural genes including *pufBALMX* and *pucBA* for light harvesting proteins and reaction centers L and M polypeptides, *puhA* for reaction center H polypeptide and *cycA* for cytochrome c_2. A positive response was found for *pufBALMX* with *Erythrobacter*. Two strong positive hybridizations and a weak one were obtained for *hemA* for genes for aminolevulinic acid synthesis in BTAi 1. The probes were from *Rhodobacter sphaeroides*.

Free-Living N_2 Fixation Functions. Various media used to obtain free-living N_2 fixation by different rhizobia were evaluated with BTAi 1. A modified Dreyfus *et al.* medium (3) in which the C source was changed to 10 $g \cdot L^{-1}$ of glucose and 2.0 $g \cdot L^{-1}$ of agar was added to provide a suitable microaerobic zone, supported free-living N_2 fixation by BTAi 1 as well as BTSr 3. The specific N_2-fixing activity (measured as C_2H_2 reduction) of BTAi 1 was 10-20% of that of BTSr 3 and was more variable. Free-living N_2 fixation was found for BTAi 1 from one day after inoculation to ten days with maximum activity from two to six days.

Several experiments were performed to define the effect of light, if any, on N_2 fixation by BTAi 1. The glucose C-source was reduced to 1 $g \cdot L^{-1}$ for these tests. There was no short-term (one hour) effect of light or dark on N_2 fixation. Fixation occurred in the light coincident with photosynthesis. There was a positive long-term effect of light on N_2 fixation. BTAi 1 grown for two days on 16 h/8 h light/dark regimen had 2-5 times as much N_2-fixing activity as those grown for two days in continuous dark. Several hours of darkness were required to reduce the activity of the light/dark grown cultures to that of the continuous dark grown ones. A stimulatory effect of light on N_2 fixation occurred, but the slow time-response suggested that it is indirect.

The protein content of BTAi 1 was increased about 10% for light/dark-grown over continuous dark-grown cells.

Symbiotic Functions. Characteristics of stem and root nodules of *A. scabra* formed by inoculation with BTAi 1 and of *S. rostrata* formed by inoculation with BTSr 3 were determined. The starch and soluble carbohydrate contents were greater in stem than root nodules for both *A. scabra* and *S. rostrata* with those for *A. scabra* stem nodules the larger. Light increased the CO_2 uptake

rate of excised stem, but not root, nodules and the rate of *A. scabra* stem nodules was about three times that of *S. rostrata* stem nodules. Possibly of greatest interest is the specific N_2-fixing activity of nodules expressed as μmol $C_2H_4 \cdot g^{-1}$ nodule$\cdot hr^{-1}$. The specific activities of the root nodules were similar while that of *A. scabra* stem nodules was about four times that of *S. rostrata* stem nodules. Transmission electron micrographs of bacteroids of BTAi 1 in Aeschynomene stem nodules show numerous vesicles believed to be chromatophores.

For several parameters stem nodules of *A. scabra* functioned more efficiently than did those of *S. rostrata*. These data are consistent with the photosynthetic ability of BTAi 1. More direct evidence for the positive effect of light on symbiotic N_2 fixation by BTAi 1 is the doubling of acetylene reduction produced by illumination of stem nodules with light in the spectral region beyond 730 nm.

Discussion

Substantial information has been gathered to support the photosynthetic activity of the rhizobium BTAi 1 (Table 1). In several areas such as the positive but modest bacteriochlorophyll content, aerobic nature, inability to grow under light in the absence of a carbon source, ability for stationary phase growth under light, and light-sparing of O_2 uptake, BTAi 1 is similar to the photosynthetic bacteria, *Erythrobacter*. The effects of inhibitors and uncouplers on O_2 and CO_2 uptake in light further solidify the conclusion of the photosynthetic activity of BTAi 1. In all of the above characteristics BTAi 1 is unlike BTSr 3. However, they both have the somewhat unusual ability to form N_2-fixing stem as well as root nodules on their respective legume hosts. Both BTAi 1 and BTSr 3 fix N_2 asymbiotically under microaerobic conditions although BTSr 3 is more active. The co-occurrence of N_2 fixation and photosynthesis in BTAi 1 is unusual. The source of electrons for photosynthesis in BTAi 1 has not been determined. Many other interesting questions can be asked about BTAi 1.

Although *Rhizobium, Bradyrhizobium, Azorhizobium*, and probably the new genus *Sinorhizobium* (1), are phylogenetically linked to the purple photosynthetic bacteria (4, 10) including *Erythrobacter* (20), these rhizobia have not been found to contain bacteriochlorophyll and/or to be photosynthetic. The relatively low content of bacteriochlorophyll, the inability to grow in the light without exogenous C, and the lack of stimulation of logarithmic growth by illumination in the presence of exogenous C indicate that BTAi 1 is limited in its photosynthetic capability. It may be a primitive form, with features in common with present-day rhizobia, bradyrhizobia, azorhizobia and also the purple photosynthetic bacteria. However, the lack of structural similarity between key photosynthesis genes of BTAi 1 and of *Erythrobacter* and those of *Rhodobacter sphaeroides* may indicate a horizontal rather than vertical route of acquisition of these genes by the aerobic photosynthetic bacteria. The absence of plasmids (> 400 kb) in BTAi 1, based on Suwanto and Kaplan's analysis, would appear to rule out the possibility of an aerobic photosynthetic organism (e.g.

Erythrobacter) having obtained plasmid bearing nodulation genes from *Rhizobium*. Pending full taxonomic description, we propose that BTAi 1 be named *Photorhizobium thompsonianum*.

Nitrogen fixation by the legume-rhizobium symbiosis is important in natural ecosystems and in agriculture. Study of this novel organism and its symbiosis with *Aeschynomene* may shed light, not only on the evolution of the rhizobia,

TABLE 1

Comparison of the Photosynthetic and N_2-Fixing Characteristics of *Photorhizobium thompsonianum* (proposed) with a Stem-Nodulating Bacterium and an Aerobic Photosynthetic Bacterium

Characteristics	*Photorhizobium thompsonianum* (Proposed) Strain BTAi 1	*Azorhizobium caulinodans* Strain BTSr 3	*Erythrobacter sp.* Strain OCh 114
N_2-Fixing Symbiosis			
Root and Stem Nodules	*A. indica* *A. scabra*	*S. rostrata*	N.D.*
Free-Living Bacteria			
Bacteriochlorophyll *a*	+		+
Light/Dark Regimen	+		
Aerobic Conditions	+		
Light-Stimulated CO_2 Uptake	+	—	+
Light-Decreased O_2 Uptake	+	—	+
Activity of Inhibitors of Photosystem 2 and Uncouplers	+	—	
N_2 Fixation	+	+++	—
Light-Stimulated N_2 Fixation	+	N.D.	N.D.
Light Effect on Growth			
Log Phase	None	None	None
Steady State	Maintain	None	Maintain
Symbiotic Functions			
Soluble Carbohydrate and Starch			
Stem Nodules	++++	++	N.D.
Root Nodules	+	+	N.D.
Light-Stimulated CO_2 Uptake			
Stem Nodules	++++	++	N.D.
Root Nodules	—	—	N.D.
Specific N_2-Fixing Activity			
Stem Nodules	++++	++	N.D.
Root Nodules	++	++	N.D.

*N.D. is Not Determined.

but also on the evolution of the infection process and of plant-bacterium interactions in general. Photosynthesis by bacteroids may explain the energy sufficiency of stem nodules on *Aeschynomene* spp. (6). If energy-sufficient nodules could be induced to form on the stems of agriculturally important legumes, e.g., soybean, N_2 would be fixed with less carbon drain on the host plant with the possibility of concomitant yield improvement.

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