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Review

Photosynthetic rhizobia

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Contents

Abbreviations: BChl, bacteriochlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TMAO, trimethylamine-*N*-oxide

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1. Introduction

Many legumes possess root nodules occupied by Gram negative bacteria known as rhizobia, which belong to the family Rhizobiaceae. Rhizobia, commonly found in the soil, exchange chemical signals with suitable host plants. These induce root tissue to differentiate to form nodules, and allow the rhizobia to enter the roots and establish symbiotic associations [1]. Within the nodule, the rhizobia differentiate to form cells known as bacteroids. The bacteroids convert atmospheric nitrogen to ammonium, which they transfer to the plant. The plant in turn provides photosynthetically fixed carbon, usually in the form of dicarboxylic acids, to the bacteroids. The bacteroids use the fixed carbon as a source of electrons for dinitrogen reduction and as a substrate for oxidative phosphorylation. van Rhijn and Vanderleyden [2] recently have reviewed legume–rhizobia symbiosis, including symbioses involving stem nodulation.

In 1928, Hagerup [3] described nodules located on the stems of the legume *Aeschynomene aspera* growing along the banks of the Niger River. Although nodules on the stems of *Aeschynomene* plants had been noticed as early as 1884, Hagerup seems to have been the first to observe that plant cells within the nodules were filled with bacteria. He suggested that the stem nodules might be involved in nitrogen assimilation, since flooding of the roots might preclude nitrogen assimilation by root nodules. In 1981, Dreyfus and Dommergues [4] reported the presence of nitrogen-fixing nodules on the stems of the legume *Sesbania rostrata* in the Sahel region of West Africa. Nitrogen fixation by the *S. rostrata* stem nodules, unlike that by root nodules, proved to be insensitive to fixed nitrogen in the soil as well as to flooding of the roots [5–7]. These characteristics suggested that stem-nodulated legumes might be useful sources of biologically fixed nitrogen—'green manures'—for rice fields. Chemically fixed nitrogen fertilizers, whose synthesis depends on petroleum products, is prohibitively expensive for many small rice farmers in developing countries, so development of such green manures has been a high priority in agricultural laboratories, particularly in India [8,9], the Philippines [10] and Senegal [11].

Rhizobia isolated from *S. rostrata* stem nodules have the unusual ability to fix nitrogen in ex planta culture and to grow with no other source of fixed nitrogen. On this basis, they were assigned to a new

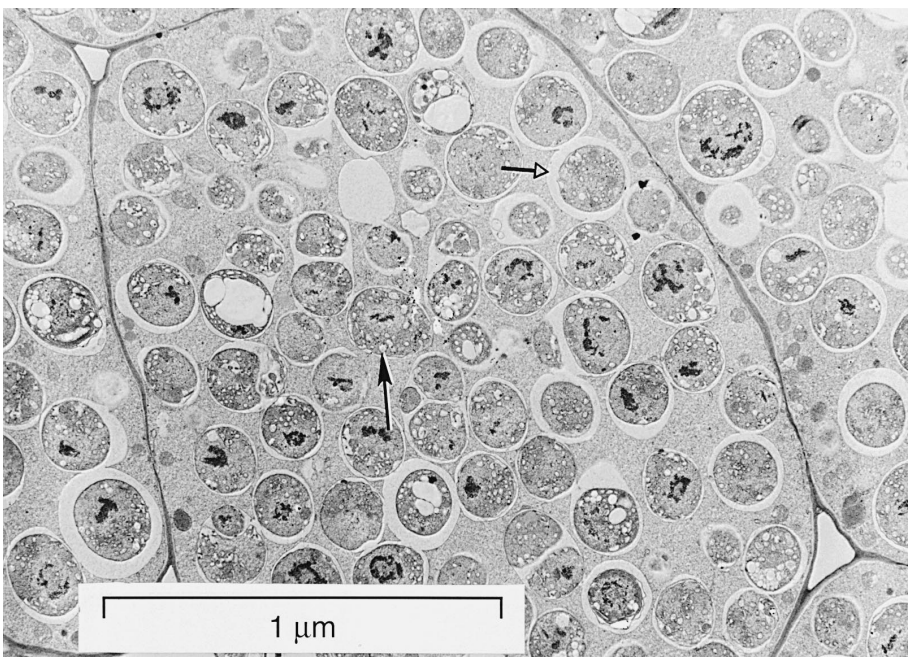

Fig. 1. *A. indica* stem nodule cell containing coccoid bacteroids. Open arrow: peribacteroid membrane; closed arrow: membranous vesicle. Micrograph by Harry M. Calvert.

genus, *Azorhizobium* [12]. Previously, rhizobial species had been divided into two genera, *Rhizobium*, the 'fast-growing' rhizobia, and *Bradyrhizobium*, the 'slow-growing' rhizobia. Eaglesham and Szalay [13] reported in 1981 the isolation of a bacterium from a nodule that had formed spontaneously on the stem of an *Aeschynomene indica* plant at the Boyce Thompson Institute at Cornell. The plant was growing in sand obtained near the headwaters of the Potomic River in the United States. *Aeschynomene virginica*, which is found in this area, may have been the source of the infecting bacterium. The isolate, which was named BTAi 1, was studied in considerable detail, and found to have properties of both fast- and slow-growing rhizobia [14]. Like the *Azorhizobium* isolates (and subsequent *Aeschynomene* stem nodule isolates), BTAi 1 is able to fix nitrogen in ex planta culture and to grow with no other source of fixed nitrogen [15]. As will be discussed subsequently, BTAi 1 was the first rhizobial isolate shown to be capable of developing a photosynthetic system, and has been the object of most of the studies of the photosynthetic properties of rhizobia [16–18].

In the early 1980s, Subba Rao [19] of the Indian Agricultural Research Institute had noticed that stem nodules from *A. indica* plants growing along river banks in New Delhi contained endophytes whose appearance seemed unusual. A collection of *A. indica* nodules was fixed and electron micrographs were prepared by H.E. Calvert at the C.F. Kettering Research Laboratory. The micrographs revealed that some of the nodules contained two types of bacteroids, segregated into different parts of the nodules. One type of bacteroid was coccoid, rather than rod-shaped or pleotropic as are most bacteroids. Minocher Reporter pointed out that the coccoid bacteroids contained intracytoplasmic membranous vesicles which resembled the 'chromatophores' of purple photosynthetic bacteria (Ref. [20], Fig. 1), and proposed that the endophytes were in fact photosynthetic bacteria.

2. Photosynthetic properties of *Aeschynomene* stem nodule endophytes

Fresh endophytes were isolated from a collection of *A. indica* stem nodules collected near Chidambaram in southeastern India. While the amount of material was too small to allow direct detection of pigments, their fluorescence excitation and emission spectra (Fig. 2 and Ref. [21]) resembled those of purple photosynthetic bacteria. The narrowness of the emission spectrum, compared to that of *Rhodobacter capsulatus* (Fig. 2b), suggested that they might, like *Rhodospirillum rubrum*, contain only one type of light-harvesting bacteriochlorophyll protein (see Ref. [22]).

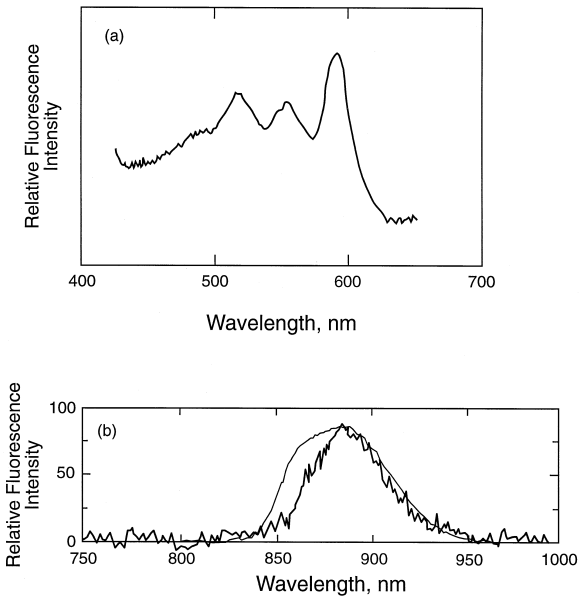

Fig. 2. Fluorescence excitation and emission spectra of endophytes isolated from *A. indica* stem nodules. (a) Excitation spectrum of endophytes. The baseline is placed arbitrarily. (b) Emission spectra of endophytes (heavy line) and of *Rhodobacter capsulatus* cells (light line). The nodules were collected near Chidambaram, in southeastern India.

A larger number of stem nodules were collected by S. Shanmugasundaram from *A. aspera* plants grow-

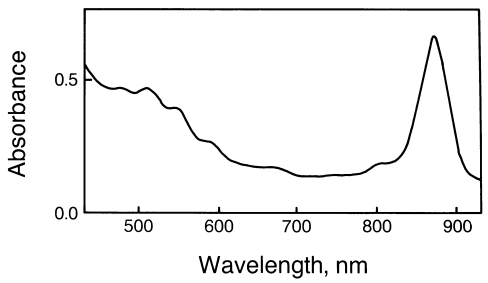

Fig. 3. Absorption spectrum of membranes isolated from *A. aspera* stem nodule endophytes. The nodules were collected near Nagercoil, at the southern tip of India.

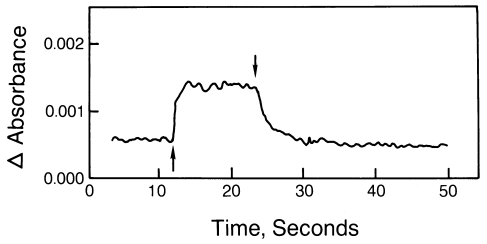

Fig. 4. Light-induced absorbance change, measured as the kinetics of the absorption difference between 905 nm and 870 nm, in membranes isolated from *A. aspera* stem nodule endophytes. Measurements were made as described in Ref. [18].

ing near Nagercoil in southern India. Membranes isolated from endophytes contained in these nodules displayed absorption spectra characteristic of photosynthetic bacteria (Fig. 3), and reversible light-induced absorbance changes (Fig. 4) whose spectrum (Fig. 5) resembled that accompanying oxidation of photosynthetic reaction centers (D. Fleischman and S. Shanmugasundaram, unpublished observations). An acetone–methanol extract of the endophytes had a spectrum resembling that of bacteriochlorophyll *a*; after acidification the spectrum resembled that of bacteriopheophytin *a* (data not shown).

Bacteriochlorophyll has since been found in endophytes isolated from stem nodules of *A. afraspera*, *A. denticulata*, *A. evenia*, *A. nilotica*, *A. pratensis* and *A. sensitiva* [23,24].

3. Stem nodulation

3.1. Diversity of stem-nodulating legumes

Several legume genera include species which bear nitrogen-fixing nodules on above-ground parts of their stems. These include *Aeschynomene*, *Sesbania*, *Neptunia* [25,26] and *Discolobium* [27], as well as the tree legumes *Cassia* and *Parkinsonia* [28]. Most of these plants are found in the tropics or subtropics. Under appropriate conditions, nodules will form on the lower stems of some cultivars of the familiar legumes field bean (*Vicia faba*) [29], peanut (groundnut, *Arachis hypogaea*) [30] and soybean (*Glycine max*) [31]. *Arachis* and *Aeschynomene* belong to the tribe Aeschynomeneae within the subfamily Papilionoideae. Interestingly, *A. hypogeae Bradyrhizobia*, like BTAi 1, form spherical bacteroids [32].

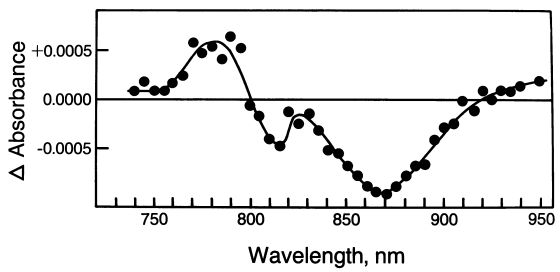

Fig. 5. Light-minus-dark difference spectrum of membranes isolated from *A. aspera* stem nodule endophytes.

Nodulation requires that the rhizobia be able to reach the site of infection and enter the stem. Often the rhizobia enter cracks where adventitious roots or their primordia have broken through the stem epidermis. In *Aeschynomene* species, the primordia form rows which spiral around the stem. Some *Aeschynomene* species have visible primordia on aerial parts of the stem. Aerial parts of the stems of some of these species, e.g., *A. afraspera* and *A. nilotica*, are profusely nodulated, while those of others, e.g., *A. indica*, are more sparsely nodulated. In other species, e.g., *A. elaphroxylon* and *A. fluminensis*, the primordia are not visible, and nodules form only on flooded parts of the stems, where the dormancy of the primordia has been broken and adventitious roots emerge. High humidity as well as flooding often encourages the development of lateral roots from root primordia [33] and may enhance nodule development. The nodulation behavior of *Aeschynomene* species is correlated with their placement in cross-inoculation groups by Alazard [34]. Strains belonging to a single cross-inoculation group form effective (i.e., capable of fixing nitrogen) nodules on a common set of host species. *Aeschynomene* species fall into three cross-inoculation groups. Group I comprises the species which form nodules only on their roots or submerged parts of their stems. Species in group II are profusely stem-nodulated, while species in group III are more sparsely nodulated on aerial parts of their stems.

Ladha et al. [10] have suggested that ‘root nodules’ be defined as nodules formed on above-ground adventitious roots as well as those formed on below-ground roots. The term ‘stem nodules’ would be reserved for nodules ‘whose location prevents contact

with the soil and whose formation is initiated on a primordium and not on an outgrowing adventitious root'. James et al. [35] have made the additional suggestion that only nodules having vascular connections to the stem rather than to the root be considered stem nodules. By the latter criterion, the nodules located at stem nodes of *Neptunia natans* [26] are root nodules, while the nodules located on the stems of *Discolobium pulchellum* [27] and *A. fluminensis* [36] are true stem nodules. Vascular connections between the stem vascular system and stem nodules of *A. indica* plants inoculated with BTAi 1 also have been observed (J.M. Rogers, unpublished observation). When the term 'stem nodule' is used in this review (or elsewhere) the reader should be cautioned that unless the vascular connections have been examined, the nodules may be either root or stem nodules according to the criteria of James et al.

Photosynthetic rhizobia can form nodules on *Aeschynomene* roots and adventitious roots as well as on stems. de Bruijn [37] has speculated, with reference to *S. rostrata*, that 'any undifferentiated meristematic (embryonic) tissue can be induced by the invading rhizobia or azorhizobia to become nodule tissue'. This may well be true of *Aeschynomene*.

Thus far bacteriochlorophyll-containing rhizobia have been found to form stem nodules on only *Aeschynomene* species. However, rhizobia displaying photosynthetic properties have been isolated from root nodules of *Lotononis bainesii* (see Section 5.1). The isolates form nodules on the roots and hypocotyls, but not the stems, of the plants (W. Heumann, personal communication). *Lotononis* belongs to the tribe Crotalarieae within the subfamily Papilionoideae.

All aspects of stem nodulation have been discussed in reviews by de Bruijn [37] and Ladha et al. [23]. Agricultural applications of stem-nodulated legumes have been reviewed by Ladha et al. [10] and Kaladurai and Kannaiyan [9]. The recent review of stem nodulation by Boivin et al. [38] is especially recommended.

3.2. Structure of stem nodules

The most striking feature of *Sesbania* and *Aeschynomene* stem nodules is their deep green color, caused by chloroplasts in cells of their cortex. Eardly and Eaglesham [39] have suggested that the stem nodules of these plants may be autonomous structures, the nodule chloroplasts providing all the fixed carbon needed for nitrogen fixation by the bacteroids. Evidence that this may be true of *Aeschynomene* stem nodules will be discussed in Section 7.

Stem nodules from the profusely nodulated *A. afraspera* [40], the more sparsely nodulated *A. indica* [41], and *A. fluminensis*, which forms only submerged nodules [36], have been described. The general features of the nodules are illustrated in Fig. 6, which is based on a section of a lower stem nodule of an *A. indica* plant which had been inoculated with BTAi 1. Cells in the central part of the nodule are filled with bacteroids (see Fig. 1). The nodules are of the aeschynomenoid type, i.e., there are no uninfected interstitial cells in the infected tissue. The bacteroids may be rod-shaped or spherical, probably depending on the infecting rhizobium species. They are separated from the cytoplasm by peribacteroid membranes which are presumably derived from the plant

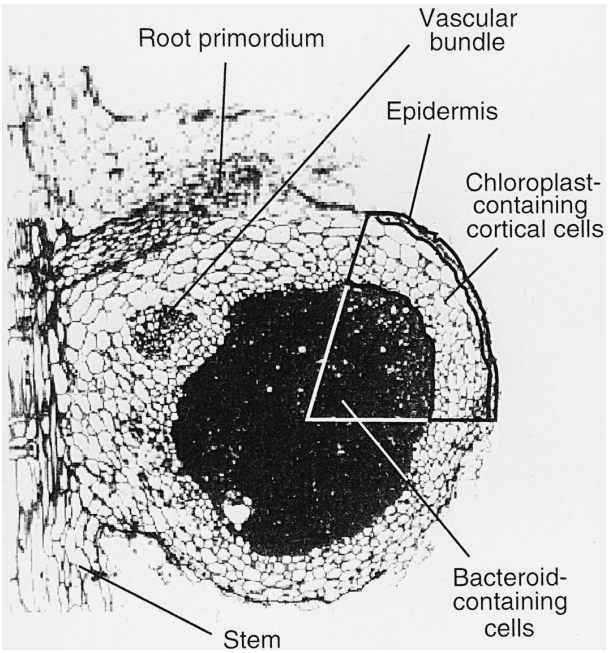

Fig. 6. Structure of an *Aeschynomene* stem nodule. Based on a lower stem nodule of an *A. indica* plant inoculated with BTAi 1. Micrograph by James M. Rogers.

cell plasma membrane (Fig. 1). The infected cells contain nuclei, plastids and mitochondria, but no chloroplasts or vacuoles. The cortical cells surrounding the infected zone contain chloroplasts or plastids. The nodule is surrounded by one or two layers of epidermal cells which do not contain chloroplasts. Vascular bundles are present within the chloroplast-containing cortical tissue. Adventitious roots are often associated with nodules growing on submerged portions of the stem.

Bacteroids require oxygen for oxidative phosphorylation, but nitrogenase is denatured by oxygen at concentrations greater than about 5 mmol m^{-3} [42]. Leghemoglobin within the infected cells of legume nodules allows rapid diffusion of oxygen to the bacteroids while maintaining a low oxygen tension [43]. In addition, nodules may possess a variable diffusion barrier which regulates the flow of oxygen to the infected cells [44]. Cells with interlocking cell walls are found in the inner cortex of *A. fluminensis* stem nodules, and in the surrounding mid-cortex some intercellular spaces are occluded with glycoprotein [36]. One might expect the presence of oxygen-evolving chloroplasts in the cortex of stem nodules to necessitate special protective mechanisms. In *A. fluminensis*, poorly developed chloroplasts with no distinct grana were found in the cells lying between the infected tissue and the presumed barrier created by interlocking cells and occluded intercellular spaces. In *A. indica*, in contrast, fully-developed chloroplasts with prominent grana are present in the layer of cells immediately adjacent to the infected tissue [41].

Nodule structure is affected by light. The more poorly illuminated lower stem nodules of *A. fluminensis* have few well-developed chloroplasts. We have found that shading of BTAi 1-containing *A. indica* stem nodules lowers their content of both chlorophyll *a* and bacteriochlorophyll *a*, and inhibits the formation of photosynthetic vesicles in the bacteroids (J. Rogers and D. Fleischman, unpublished observation).

The structure, development, genetic regulation and physiology of stem nodules has been studied much more extensively in *S. rostrata* [45–47] than in *Aeschynomene* species. The properties of *Sesbania* stem nodules may provide insight into the nature and function of those of *Aeschynomene*, since they appear to be generally similar.

4. Regulation of bacteriochlorophyll accumulation

4.1. Effects of light and oxygen

After photosynthetic systems were found in bacteroids isolated from *Aeschynomene* stem nodules, efforts were made to culture the nodule photosynthetic bacteria using conventional techniques. However, only white, rod-shaped, obligately aerobic bacteria could be obtained. The reason became evident after Eaglesham sent cultures of BTAi 1 to the Kettering Laboratory. There, W.R. Evans noticed that when BTAi 1 cultures were left on the laboratory bench, the colonies became pink. Colonies on plates kept in an incubator were white. The pink colonies contained BChl and carotenoid. Further study revealed that BTAi 1 cultures would accumulate BChl if they were grown under cyclic illumination (as would be found in the laboratory) but not if they were grown in continuous light or continuous darkness [20]. The amount of BChl formed (0.4 nmol BChl mg^{-1} dry weight) was much less than typically found in anaerobically grown purple photosynthetic bacteria (e.g., 13 nmol BChl mg^{-1} dry weight [48]). BTAi 1 would not grow anaerobically even under cyclic illumination. Its behavior resembles that of aerobic photosynthetic bacteria. These (also known as aerobic anoxygenic phototrophs and quasiphotosynthetic bacteria [49]) are a group of obligately aerobic BChl-containing bacteria, most of which have been isolated from aquatic environments or are facultative methylotrophs. They are the subject of a volume edited by Harashima et al. [50] and have been reviewed recently by Shimada [51].

Wettlaufer and Hardy [52] examined the light dependence of pigment formation in BTAi 1, and made the following observations.

(1) BChl accumulates only in the dark, after the bacteria have been illuminated. At least 20 min of illumination were required for detectable BChl to accumulate during a subsequent dark period. Maximal bacteriochlorophyll accumulation followed 20 h of illumination. White light (35 μE s^{-1} m^{-2}) was used in these experiments. This light intensity had been found to cause maximal BChl accumulation when cultures were grown under cyclic illumination (16 h light/8 h dark).

(2) BChl accumulation began about 30 min after illumination ended and continued for 24 h.

(3) BChl accumulation ended immediately if the cells were illuminated again.

(4) A small amount of BChl accumulated in cells kept in continuous darkness. Almost none accumulated in cells that were illuminated continuously.

Wettlaufer and Hardy concluded that light is necessary to initiate bacteriochlorophyll accumulation, but that light has the additional effect of inhibiting BChl accumulation. They [53] reported that the action spectrum for BChl accumulation includes a blue component but not a far red component, excluding the possibility that the photosynthetic light-harvesting system might be the photoreceptor responsible for initiation, since the light-harvesting BChl protein has a major absorption band at 870 nm. We have found that BChl accumulation can be initiated by light whose wavelength is greater than 720 nm, but not by light whose wavelength is greater than 830 nm. It is inhibited only by light whose wavelength is less than 650 nm, demonstrating that initiation and inhibition are mediated by separate photoreceptors. BChl will accumulate in cells grown under continuous red light [18].

BChl accumulation in BTAi 1 requires oxygen and is maximal at atmospheric oxygen concentrations [52]. Thus, BTAi 1 behaves quite differently from typical purple photosynthetic bacteria, in which BChl synthesis is inhibited by oxygen [54]. BChl accumulation is dependent on oxygen in other aerobic photosynthetic bacteria, however [51,55]. In the aerobic photosynthetic bacterium *Roseobacter denitrificans* (formerly *Erythrobacter* sp. OCH 114), the 13^1-oxo group of the isocyclic ring E of BChl is derived from molecular oxygen [56]. Liebetanz et al. [57] and Kortlüke et al. [58] have found that the *puf* Q gene, which is involved in regulation of bacteriochlorophyll synthesis in *Rhodobacter capsulatus* [59] and *Rhodobacter sphaeroides* [60], is absent in *Rsb. denitrificans*. Whether it is also absent in BTAi 1 and other aerobic photosynthetic bacteria, and whether its absence is relevant to their unusual response to oxygen, remain to be determined.

Light strongly inhibits BChl accumulation under aerobic conditions in many aerobic and other purple photosynthetic bacteria in addition to BTAi 1. Evidence has been presented that in *Rhodobacter capsulatus* light destabilizes already-synthesized BChl, rather than inhibiting BChl synthesis [61,62]. In BTAi 1 [52], as well as in *Rsb. denitrificans* [63], light inhibits BChl accumulation when O_2 is replaced by trimethylamine-*N*-oxide (TMAO) as the terminal electron acceptor, indicating that BChl is not destroyed by oxygen-dependent photooxidative degradation. Light also inhibits carotenoid accumulation in these bacteria. A photoreceptor-mediated inhibition of the formation of the entire photosynthetic system would seem more efficient than the destruction of components after they are formed. Nishimura et al. [64] find that light, but not oxygen even at 94% saturation, decreases the level of *pufBALM* mRNA (coding for light-harvesting LHI and reaction center L and M proteins) in *Rsb. denitrificans*. Regulation of the expression of photosynthesis genes is reviewed in Refs. [62,65].

Light initiation of pigment accumulation is more unusual. It occurs in the methylotrophic aerobic photosynthetic bacteria *Methylobacterium rhodesianum* and *Methylobacterium extorquens* AM1 [66,67] as well as in photosynthetic rhizobia, but most strains of aquatic aerobic photosynthetic bacteria have been reported to form BChl even when grown in continuous darkness [51]. The nature of the photoreceptor responsible for initiation in BTAi 1 is unknown. The effectiveness of far-red as well as blue light may suggest the involvement of a tetrapyrrole. The photoreceptors that regulate pigment synthesis in cyanobacteria may provide hints. For example, a phytochrome-like protein has been reconstituted from phycocyanobilin and a protein coded by *Synechocystis* DNA [68]. The ‘red’ form of the protein (i.e., the form present after irradiation with 567 nm light) has an absorption spectrum qualitatively similar to the action spectrum of initiation in BTAi 1, with maxima at 400 and 702 nm (unfortunately, a detailed action spectrum of initiation in BTAi 1 has not been published). The *Synechocystis* protein has homology to bacterial sensory histidine kinases [69]. Photosynthetic rhizobia may be ideal organisms in which to elucidate the mechanism of this sort of photosensory response.

Some photosynthetic rhizobia form BChl without preillumination. van Berkum et al. [70] isolated rhizobia capable of nodulating *A. indica* from soil samples collected throughout the world. Of 79 isolates

tested, 38 accumulated BChl and carotenoids during heterotrophic growth. Of these, nine accumulated BChl during growth in continuous darkness.

4.2. Effect of carbon source

Evans et al. [20] found that even under cyclic illumination, BChl accumulation in BTAi 1 was strongly dependent on the carbon source present in the medium. Little pigment formed in the presence of mannitol, a common constituent of rhizobium media. Good pigmentation resulted when the cells were grown with low (1.5%) concentrations of malate, succinate or fructose. The greatest pigmentation has been obtained with glutamate (A. Eaglesham, personal communication). Arabinose was inhibitory, even in the presence of dicarboxylic acids [20]. Wettlaufer and Hardy [71] reported that oxygen uptake by BTAi 1 cultures is faster in the presence of malate than of glutamate. We (unpublished) find that the growth rate of BTAi 1 cultures is greatest with arabinose as the carbon source, much slower with malate, and slowest with glutamate. Thus, the poorest carbon sources seem to lead to the greatest pigment accumulation. When grown with malate, BTAi 1 cultures begin to accumulate BChl just at the end of exponential growth [18]. Their behavior resembles catabolite repression. Perhaps the bacteria form the photosynthetic system to provide a source of energy when adequate sources of reduced carbon are not available.

The conditions under which BTAi 1 accumulates pigment are precisely those to be expected within stem nodules in the field—dicarboxylic acid as the carbon source and diurnal illumination. Even during the day, chloroplasts in the nodule cortex would absorb inhibitory visible light, while initiating far-red light would reach the bacteroids. BChl accumulation would be suppressed during ex planta growth in bright light, minimizing photooxidative damage. Wasteful synthesis of the photosynthetic system would not occur in the unilluminated root nodules. Among isolates that accumulate BChl without preillumination are MKAa 2 and MKAa 3 [72]. These were isolated from stem nodules of *A. aspera* plants growing under permanently flooded conditions. In this environment, the plants form almost no root nodules, perhaps obviating the need to suppress BChl accumulation in the dark.

5. Taxonomy and phylogeny of bacteriochlorophyll-containing rhizobia

5.1. Taxonomic studies

Young et al. [73] asked whether the BChl-containing stem nodule bacteria are related to known photosynthetic bacteria, to known symbiotic bacteria, to both or to neither. To answer the question they sequenced 260- to 264-bp segments of the 16S ribosomal RNA (rRNA) genes of BTAi 1 and of 13 bacteria belonging to the alpha subdivision of the Proteobacteria. The BTAi 1 sequence was found to differ by only one base from that of *B. japonicum* USDA 110 (a *Bradyrhizobium* that nodulates soybeans) and by only six bases from that of *Rhodopseudomonas palustris*. Young et al. state that the correct name for BTAi 1 according to the current nomenclatural system is *Bradyrhizobium* sp. (*Aeschynomene*).

The 16S rRNA study was expanded by So et al. [74] to include four strains of BChl-containing rhizobia isolated in the Philippines and Senegal from *A. indica*, *A. denticulata*, *A. nilotica* and *A. afraspera*. All of the BChl-containing rhizobia fell into the *Bradyrhizobium* cluster. Wong et al. [72] determined nearly complete or short partial 16S rRNA sequences of five strains of BChl-containing rhizobia isolated from stem nodules of *A. indica*, *A. aspera*, *A. afraspera* and *A. nilotica* growing in the United States, India and the Philippines. A phylogenetic tree based on comparison of their sequences with those of other members of the alpha subclass of the Proteobacteria is presented in Fig. 7. The BChl-containing rhizobia IRBG 230, MKAa 2 and BTAi 1 were found to be closely related, and form a cluster with *Bradyrhizobium*, *Blastobacter denitrificans*, *Rp. palustris*, and *Afipia felis*. The surprising association of *Blastobacter* and *Afipia* (a pathogen which causes cat scratch disease) with the *Bradyrhizobium* cluster had been noted previously [75]. *Azorhizobium* branches more deeply. The cluster that includes the *Rhizobium* and *Agrobacterium* species is only distantly related. In spite of their phenotypic similarity to the photosynthetic rhizobia, the aerobic photosyn-

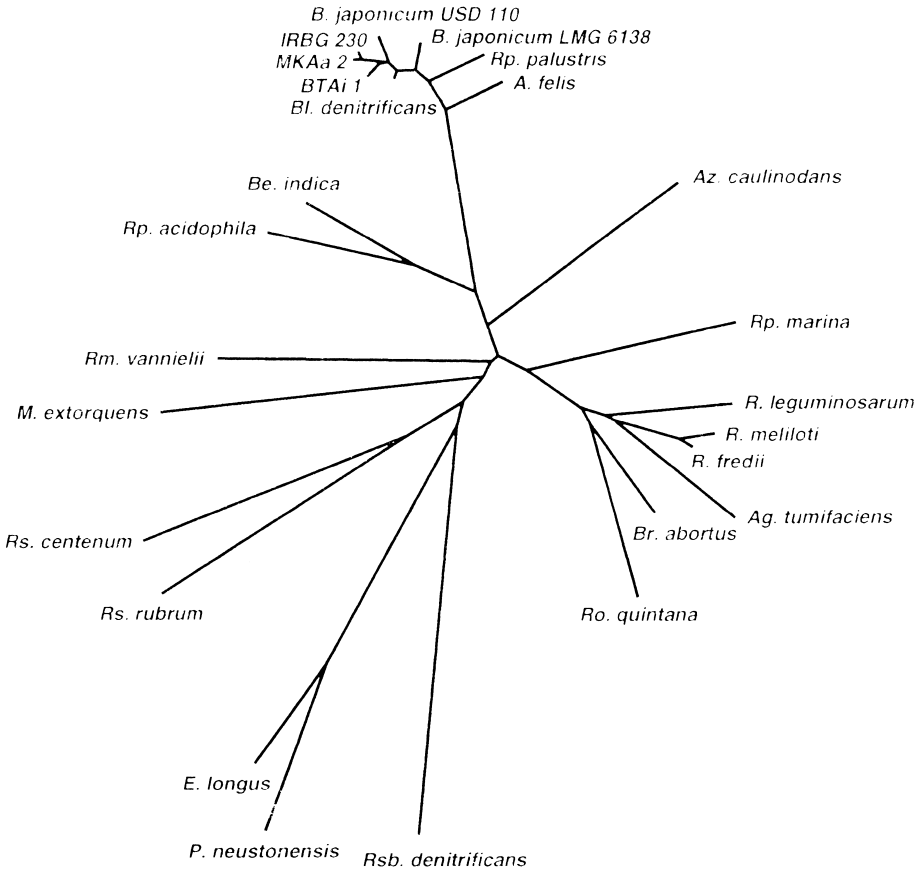

Fig. 7. Unrooted phylogenetic tree based on nearly complete 16S rDNA sequences, showing the relation of selected members of the alpha subclass of the Proteobacteria to bacteriochlorophyll-synthesizing stem-nodulating strains. IRBG 230, MKAa 2 and BTAi 1 are bacteriochlorophyll-containing rhizobia isolated from stem nodules of *A. nilotica*, *A. aspera* and *A. indica*, respectively. Abbreviations: *A.*, *Afipia*; *Ag.*, *Agrobacterium*; *Az.*, *Azorhizobium*; *B.*, *Bradyrhizobium*; *Be.*, *Beijerinckia*; *Bl.*, *Blastobacter*; *E.*, *Erythrobacter*; *M.*, *Methylobacterium*; *P.*, *Porphyrobacter*; *R.*, *Rhizobium*; *Rm.*, *Rhodomicrobium*; *Ro.*, *Rochalimaea*; *Rp.*, *Rhodopseudomonas*; *Rs.*, *Rhodospirillum*; *Rsb.*, *Roseobacter* (from Ref. [72], by permission of the American Society for Microbiology).

thetic bacteria *Erythrobacter longus*, *M. extorquens*, *Porphyrobacter neustonensis* and *Rsb. denitrificans* are scattered through the subclass, as are the other non-sulfur purple photosynthetic bacteria.

The problem of deciding how to classify the photosynthetic rhizobia illustrates a dilemma that has faced bacterial taxonomy since the introduction of molecular methods. Bacteria that resemble each other phenotypically, and perhaps even have been assigned to the same genus, may have DNA sequences suggesting that they are only distantly related evolutionarily. Minimal standards for the description of new genera and species of root and stem-nodulating bacteria have been proposed by the International Subcommittee for the Taxonomy of *Rhizobium* and *Agrobacterium* [76] and by Elkan [77]. Both recommend the use of both phenotypic and phylogenetic traits of a large number of isolates. In order to fulfill their recommendations, Ladha and So [24] performed a statistical analysis of 150 phenotypic features of 52 strains of photosynthetic rhizobia isolated from stem and root nodules of nine *Aeschynomene* species, along with 80 reference rhizobial strains. BChl-containing *Aeschynomene* rhizobia were grouped in a single phenon, separate from the phena that included *Bradyrhizobium*, *Azorhizobium* and *Rhizobium* strains. A dendrogram constructed from the data links the photosynthetic rhizobia more closely to *Azorhizobium* and *Rhizobium* than to *Bradyrhizobium*. The level of similarity between the photosynthetic rhizo-

bia and the other rhizobia was low enough to suggest that the photosynthetic rhizobia might comprise a separate genus. Non-photosynthetic *Aeschynomene* rhizobia, including some that formed effective stem nodules, fell within *Bradyrhizobium* phena. The phenotypic grouping of the *Aeschynomene* rhizobia corresponded loosely to the cross-inoculation groups described by Alazard [34].

Parenthetically, the data presented by Ladha and So provide an excellent source of information for researchers about the antibiotic sensitivity, nutrient requirements and other properties of many photosynthetic rhizobial strains.

So et al. [74] examined the relation between photosynthetic rhizobia that nodulate ten *Aeschynomene* species and reference rhizobial strains by analyzing cellular fatty acid methyl esters (FAME). The members of each rhizobial genus produced distinct FAME patterns. The photosynthetic rhizobia formed a subcluster within the *Bradyrhizobium* cluster.

Van Berkum et al. [70] used Southern hybridization analysis to determine the DNA homology of *B. japonicum* USDA 110 and BTAi 1 with 29 *A. indica*-nodulating rhizobial strains obtained from soil samples and several other strains of *Bradyrhizobium*, aerobic photosynthetic bacteria and purple photosynthetic bacteria. The mean DNA homology of BTAi 1 with the other BChl-containing rhizobia was 29%; its homology with the *Bradyrhizobium*, aerobic photosynthetic bacterium and purple photosynthetic bacterium strains was 15% or less. The DNA homology of *B. japonicum* 110 with the BChl-containing rhizobia was 9% or less. It had been proposed that closely related species should have DNA homologies ranging from 20 to 60% [78]. On this basis, most (although not all) of the soil-sample rhizobia are closely related to each other, but not to *B. japonicum*.

Lorquin et al. [79] isolated 126 strains of rhizobia from stem nodues of *A. americana*, *A. elaphroxylon*, *A. pfundii*, *A. schimperi*, *A. uniflora*, *A. afraspera*, *A. nilotica*, *A. ciliata*, *A. indica*, *A. sensitiva* and *A. tambacoudensis*. Of these, 83 formed BChl and carotenoids. The unpigmented strains could form effective nodules only on species belonging to cross-inoculation group I, while the pigmented strains effectively nodulated only species in groups II and III. Lorquin et al. compared the SDS-polyacrylamide gel electrophoresis patterns of whole-cell proteins from the isolates and from reference strains. Analysis of the data indicated that the pigmented *Aeschynomene* isolates belong to three new subclusters within the *Bradyrhizobium* group, while the nonpigmented isolates belong to known subclusters. These workers also tested hybridization between DNA from some of the isolates and rRNA from a *B. japonicum* strain. Again the results suggested that the isolates belong to the *Bradyrhizobium* rRNA cluster.

The consensus of most investigators may have been expressed best by So et al. [74], who concluded that the photosynthetic rhizobia probably do belong in the *Bradyrhizobium* cluster, but may deserve species status. They argue that sequence comparisons are likely to provide the best guide to evolutionary relationships, since phenotypic properties may be variable or unstable in culture, and may arise from convergent or parallel evolution in response to similar selection pressures. Still, they point out, '... sequence data [probably] do not by themselves tell us everything (or anything) that is interesting about the biology of organisms...'.

The taxonomy of the BChl-containing rhizobia isolated from *Lotononis* is less clear. Norris [80] isolated several strains of pigmented rhizobia from effective root nodules of *L. bainesii* in Australia. Others were isolated by W. Heumann, who found that some of his isolates, as well as strain CB376 isolated by Norris, contain bacteriochlorophyll (personal communication). We have found that strains CB376 (4–46 in Heumann's nomenclature) and 4–144, obtained from Prof. Heumann, have absorption spectra resembling those of purple photosynthetic bacteria, possess intracellular membranous vesicles resembling the chromatophores of photosynthetic bacteria and display light-induced far-red optical absorbance changes characteristic of photosynthetic reaction centers (J. Rogers, C. Coleman, I. Forquer and D. Fleischman, unpublished observations). The properties of strain CB376 have been studied by Franke [81]. Early studies [82,83] failed to find a close relation between the pigmented *Lotononis* strains and other rhizobia. Their chromosomal guanosine + cytosine content is 72.1% (W. Heumann, unpublished observation), a value much higher than that typical of rhizobia, but similar to that of many photosynthetic bacteria. The *Lotononis* rhizobia contain a C_{30} carotenoic acid [84]. Similar carotenoic acids, some-

times glycosylated, have been found in several aerobic photosynthetic bacteria [51], but not yet in photosynthetic rhizobia that nodulate *Aeschynomene* [85]. Heumann et al. [86] have stressed the similarity of the *Lotononis* rhizobia to some of the C_{30} carotenoic acid-containing aerobic photosynthetic bacteria, in particular *M. rhodinum* (formerly *Pseudomonas rhodos*). The phylogenetic position of the *Lotononis* isolates may prove to be quite interesting.

With the inclusion of the *Lotononis* strains from Australia, rhizobia that contain BChl or photosynthesis genes have been found on all continents except Antarctica. Sites from which photosynthetic rhizobia have been obtained are summarized in Table 1. Liesack and Stackbrandt [87] and Stackbrandt et al. [88], recognizing that many soil bacteria have not been identified because suitable culture conditions have not been found, used the polymerase chain reaction to amplify 16S rRNA genes directly from samples of Australian soil. The sequences of some of these corresponded closely to that of the BTAi 1 rRNA gene. New strains of photosynthetic rhizobia undoubtedly remain to be discovered.

Table 1
Sources of bacteriochlorophyll-containing rhizobia

Site of origin	Host plant	References
Australia	*L. bainesii*	[80][a]
Brazil	*A. fluminensis*	[36]
France	*A. indica*[b,c]	[70]
India	*A. aspera*	[72]
	A. indica	[70]
Japan	*A. indica*[b,c]	[70]
	A. indica[b]	[70]
Nigeria	*A. indica*[b]	[70]
Philippines	*A. afraspera*	[24]
	A. denticulata	[24]
	A. evenia	[24]
	A. indica	[24]
	A. nilotica	[24]
	A. praetensis	[24]
	A. rudis	[24]
	A. schimperi	[24]
	A. sensitiva	[24]
Senegal	*A. afraspera*	[24,79]
	A. sensitiva	[79]
	A. indica	[79]
Taiwan	*A. indica*[b,c]	[70]
United States		
Florida	*A. indica*[b]	[70]
Lousiana	*A. indica*[b]	[70]
Maryland	*A. indica*[b]	[70]
West Virginia	probably *A. virginica*[b]	[16]

[a]The presence of BChl was discovered by W. Heumann (unpublished observation).
[b]*A. indica* was inoculated with material obtained at the site indicated, and the bacteria were isolated from nodules that formed.
[c]These bacteria did not contain BChl, but their DNA hybridized with DNA of the *Rb. capsulatus puf* operon.

5.2. Speculation about evolution

How might *Bradyrhizobium* strains have come to have photosynthetic systems? Woese [89] has postulated that photosynthetic bacteria were the ancestors of the Rhizobiaceae. I.M. Miller (see Ref. [18]) suggested that the photosynthetic rhizobia may represent an intermediate form in the evolution of *Bradyrhizobium*. A possible scenario has been presented by Sprent [90,92] and Sprent and Raven [91]. They imagine that photosynthetic bacteria may first have formed nodules on the stems of plants growing in flooded tropical areas, entering the stems at sites where the epidermis was broken. As the host plants colonized drier areas, stem nodulation would have become more difficult, and the nodulation would have migrated to below ground. The photosynthetic system, no longer necessary, eventually would have been lost. van Berkum et al. [70] have isolated several strains of rhizobia which will nodulate *A. indica* from soils of temperate regions. Some of these strains are unpigmented, yet their DNA (like that of pigmented strains they isolated) will hybridize with DNA of the *Rb. capsulatus puf* operon. These may be strains in which photosynthesis genes have not yet been completely deleted from the genome, even though the photosynthetic system is no longer needed or formed.

The progenitor of *Bradyrhizobium* presumably belonged to the lineage from which *Rp. palustris* had branched. It may or may not have been obligately aerobic; aerobic photosynthetic bacteria appear to have branched from several independent lines of purple photosynthetic bacteria (see Fig. 7 and Ref. [51]), implying that major genetic changes are not required.

Most purple photosynthetic bacteria are capable of fixing nitrogen. A phylogenetic tree based on the sequences of nitrogen fixation (*nif*) genes of rhizobia and purple photosynthetic bacteria nearly parallels

the tree based on 16S rRNA sequences, suggesting that these bacteria inherited their *nif* genes directly from a common ancestor [93].

Nodulation genes may have evolved in these bacteria, or they may have been obtained from *Rhizobium* or *Azorhizobium* species by lateral transfer. *Rhizobium* and *Bradyrhizobium* may have diverged before the emergence of land plants [94]; if so, nodulation genes were not inherited from a common ancestor. There is evidence for lateral transfer of nodulation genes between rhizobia [95]. In *Bradyrhizobium*, the nodulation genes are found on the chromosome, but in *Rhizobium*, they are carried on very large plasmids. The recently published sequence of such a plasmid [96] revealed plasmid transfer genes as well as insertion sequences. Much of the above is speculative. The possibility that *Bradyrhizobium* instead acquired photosynthesis genes by lateral transfer cannot be excluded (see Ref. [97]).

6. The photosynthetic properties of *Bradyrhizobium* strain BTAi 1

6.1. Effects of light on metabolism, growth and survival

Extensive studies have been performed by the group at the Boyce Thompson Institute and their associates to determine whether the photosynthetic system performs useful functions in free-living BTAi 1 cells [98]. The behavior of BTAi 1 was compared to those of the aerobic photosynthetic bacterium *Rsb. denitrificans* and *A. caulinodans* strain BTSr 3, which forms stem nodules on *S. rostrata* but does not contain BChl. Light stimulated the uptake of $^{14}CO_2$ by BTAi 1 at all stages of growth, sometimes more than five-fold. The stimulation was inhibited by orthophenanthroline, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and NH_4Cl. These reagents also decreased the suppression of oxygen uptake by light, an effect that had been described previously by Wettlaufer and Hardy [71]. Similar light stimulation and inhibitor effects were found with *Rsb. denitrificans* but not with BTSr 3. Orthophenanthroline competes with quinone binding at the reaction center Q_B site. DCMU acts at the corresponding site in higher plants, but usually not in purple photosynthetic bacteria. However, it inhibits at the Q_i site (the quinone-reducing site) of the cytochrome bc_1 complex in some purple bacteria [99]. The effects of the phosphorylation uncoupler NH_4Cl suggest that BTAi 1 is capable of photophosphorylation. Using nuclear magnetic resonance to measure nucleotide levels, Ingham [100] found that the ATP concentration in BTAi 1 cells is increased 2-fold by illumination if oxygen is present, again suggesting photophosphorylation.

Although these experiments provide strong evidence that BTAi 1 can fix carbon photosynthetically, illumination did not increase the growth rate of BTAi 1 cultures [98]. However, illumination allowed the cells to remain viable after the cultures reached stationary phase. The viability of dark-grown cells began to decrease quickly a few days after the end of exponential growth. *Rsb. denitrificans* behaved similarly, leading Shiba [101] to suggest that the photosynthetic system allows the bacteria to survive in the absence of energy-yielding substrates. However, subsequent experiments by Harashima et al. [102] demonstrated that bright light would stimulate the growth of *Ro. denitrificans* and lead to higher yields if the cells had first been grown in the dark so that BChl had been allowed to accumulate. The stimulation was especially pronounced when the cells were supplied with a carbon source that supported only slow growth. Yurkov and van Gemerden [103] found that light would also stimulate the growth of *Erythromicrobium hydrolyticum.* Thus the perception that aerobic photosynthetic bacteria are not true phototrophs seems to have been the result of their being grown in the laboratory under artificial conditions which inhibited formation of their photosynthetic systems. Under conditions more closely resembling those in their natural habitat—cyclic illumination with bright light and substrate limitation—their growth may be enhanced by illumination. The BTAi 1 cultures [98] were grown under cyclic illumination, but they may have acquired photosynthetic capability only at the end of exponential phase (see Section 4.2). It would be worthwhile to perform a similar experiment with BTAi 1 using a lower concentration of the carbon source, or to perform the experiment with a strain such as MKAa 2 which forms the photosynthetic system constitutively [72].

Hungria et al. [98] found that illumination also could stimulate acetylene reduction (the common as-

say for nitrogenase activity [104]) of BTAi 1 cells. An enhancement of about 50% was obtained. It was observed only in cells grown under cyclic illumination and was most evident in carbon-limited cells at the end of exponential phase. Glucose was the most effective carbon source; organic or inorganic nitrogen inhibited acetylene reduction. Light did not stimulate acetylene reduction by BTSr 3. In these experiments, an oxygen concentration that would allow both respiration and nitrogenase activity was achieved by growing the cultures in semi-solid medium (2 g l^{-1} agar). The cells then could grow at the depth in the medium where the oxygen concentration was appropriate.

The experiments of Hungria et al. [98] apparently demonstrate that light can provide energy for the fixation of both carbon and nitrogen in free-living BTAi 1 cells, if the photosynthetic system is present.

6.2. The photosynthetic system

Although recent experiments have begun to elucidate the photosynthetic machinery of photosynthetic rhizobia, our knowledge at present remains sketchy, especially compared with that of purple bacteria such as *Rb. spaeroides*. The majority of experiments have been performed on the strain BTAi 1 and reveal that its photosynthetic apparatus most closely resembles those of the aerobic photosynthetic bacteria (see reviews by Shimada [51] and Fleischman et al. [18]).

6.2.1. Light harvesting complexes

BTAi 1 appears to possess a cyclic electron transfer chain similar to those of the purple photosynthetic bacteria (see reviews by Crofts and Wraight [105] and Blankenship [97]) or the aerobic photosynthetic bacteria [51]. As discussed above, BTAi 1 contains bacteriochlorophyll *a* and genes encoding light harvesting complex and/or reaction center proteins. Evans et al. [20] have estimated that the photosynthetic unit of BTAi 1 contains approximately 80 bacteriochlorophyll molecules per reaction center. The fluorescence excitation and emission spectra (Fig. 2 and Ref. [21]) resemble those of purple bacteria with a simple complement of light harvesting complexes, such as that of *Rs. rubrum* (see Ref. [22]). Also consistent with this interpretation is the failure to observe large flash-induced electrochromic shifts of carotenoids in the 450–550 nm range (D. Kramer and D. Fleischman, unpublished observations). This suggests that the LH II complex, which houses the major electrochromically shifted carotenoids [105,106] is missing or altered in BTAi 1. A reduced complement of light harvesting systems is a common feature among the aerobic photosynthetic bacteria and BTAi 1 appears to resemble species of aerobic photosynthetic bacteria such as *E. longus* and *M. radiotolerans* which have been found to contain only RC and LH I complexes [51].

Spirilloxanthin is the main carotenoid found in BTAi 1 and several other light pink strains of photosynthetic rhizobia [85]. In a number of dark pink and orange strains, canthaxanthin (4,4′-diketo-β-carotene) is the main carotenoid. Smaller amounts of spirilloxanthin, echinenone (a canthaxanthin precursor) and other carotenoids are also present. Some strains contain quite high concentrations of canthaxanthin. Lorquin et al. [85] suggest that its primary role may be protection of the cells from damage by oxygen and light, rather than light harvesting. They speculate that, like the main carotenoid in some other aerobic photosynthetic bacteria [51], canthaxanthin might not be bound to the BChl–protein complex. In the fluorescence excitation spectrum of *A. indica* endophytes (Fig. 2a), the presumed carotenoid peaks (between 450 and 575 nm) are smaller than the BChl peak at 590 nm. If the carotenoid absorption peak of the *A. indica* endophytes is higher than the 590 nm BChl absorption peak (as it is in the *A. aspera* endophytes, Fig. 3, and in free-living BTAi 1 cells [18]), this would indicate that excitation transfer from the carotenoids to the BChl in these cells is not very efficient. Verification will require measurement of the absorption and fluorescence emission spectra of bacteroids from plants inoculated with a single pure culture. Thus, light harvesting may not be the major role of the carotenoids in these cells.

As pointed out in Section 5.1, C_{30} carotenoic acids are the main carotenoids in the *Lotononis* rhizobia.

6.2.2. Reaction centers

Membranes of BTAi 1 have been shown to have photosynthetic reaction centers by light-induced absorbance spectroscopy [20]. On the basis of the light-induced absorbance spectra [20] and the hybridization of DNA with the *Rb. capsulatus puf* operon [70], the reaction center of BTAi 1 is likely to be similar to those of the anaerobic and aerobic photosynthetic

purple bacteria. Furthermore, as has been previously found in purple bacteria [97], illumination of aerobic BTAi 1 cells or isolated membranes increased the yield of bacteriochlorophyll fluorescence [107], most likely reflecting a reduction of the primary quinone electron acceptor of the reaction center, Q_A, to the semiquinone Q_A^-. Upon flash illumination, relaxation of the high fluorescence state occurred with a half-time of approximately 100 μs (D. Kramer, A. Kanazawa and D. Fleischman, unpublished results), similar to the observed Q_A^- reoxidation time in purple bacterial reaction centers (see Crofts and Wraight [105] for review).

6.2.3. Secondary electron transfer components

There is also good evidence for the existence of other components of a cyclic electron transfer chain. Illumination of intact, aerobic BTAi 1 cells with saturating single-turnover xenon flashes led to a transient oxidation of a *c*-type cytochrome presumably involved in shuttling electrons from a bc_1 complex to the reaction center [107]. The oxidized *c*-type cytochrome was subsequently reduced on the 20 ms time range. Addition of 10 μM myxothiazol slowed the reduction of the *c*-type cytochrome to the seconds time range, suggesting that the quinol oxidase site of a cytochrome bc_1 complex is involved in cytochrome reduction. After addition of 10 μM antimycin A, a single saturating flash resulted in light-induced accumulation of a reduced *b*-type cytochrome with α- and γ-bands at approximately 561 and 430 nm (D. Kramer, A. Kanazawa and D. Fleischman, unpublished results) as well as a small extent of stably-oxidized *c*-type cytochrome. These results are consistent with the existence of a bc_1-type complex catalysing a Q-cycle (i.e., the oxidation of a quinol in an oxidant-induced reduction process) (see Ref. [105] for review). Subsequent flash illumination in the presence of antimycin A resulted in further accumulation of oxidized *c*-type cytochrome and oxidized primary chlorophyll donor, strongly suggesting that the Q-cycle is obligatory for reduction of the reaction center.

Isolated membranes lost the ability to photooxidize the *c*-type cytochrome or to photoreduce *b*-type cytochrome in the presence of antimycin A (D. Kramer, A. Kanazawa and D. Fleischman, unpublished observation). This suggested that the *c*-type cytochrome oxidized upon flash excitation was soluble and lost during the membrane preparation. Interestingly, low concentrations of ferrocene (10 μM) were able to partly replace the soluble *c*-type cytochrome in shuttling electrons from the bc_1 complex to the reaction center, and allowed the accumulation of reduced *b*- and *c*-type cytochromes in the presence of antimycin A. The *c*-type cytochrome was presumably analogous to cytochrome c_1 of the bc_1 complex.

6.2.4. Oxygen dependence of photosynthetic electron transport

As is the case for the aerobic photosynthetic bacteria [51], BTAi 1 cannot grow photosynthetically under anaerobic conditions, but only in the presence of O_2 or alternative electron sinks (see Section 4). The most likely reason for this is that the primary quinone acceptor in the reaction center becomes reduced under anaerobic conditions [107]. Similar conclusions have been drawn for the aerobic photosynthetic bacteria [51]. Electrons can be introduced into or removed from the photosynthetic bacterial electron transfer system by peripheral processes such as oxidation of substrates and reduction of NAD^+ and O_2. Since full reduction of Q_A or full oxidation of P would prevent cyclic turnover, it is essential to have an operational redox 'poising system' to maintain the intermediate carriers in states that allow turnover of the whole cycle (reviewed in Refs. [108]). It appears as though the aerobic photosynthetic bacteria [109] and BTAi 1 [107] are incapable of maintaining Q_A in the oxidized form in the absence of O_2, but it is yet unclear why this is the case. In *R. denitrificans*, Q_A appears to have a more positive midpoint potential ($+35$ mV) than is typical of purple photosynthetic bacteria [109]. If this is true in BTAi 1 as well, the redox equilibrium between Q_A and subsequent acceptors may be such that Q_A is largely reduced even when the quinone pool is only partially filled. However, preliminary results on BTAi 1 show that the rise in BChl fluorescence that occurs after one or two saturating actinic flashes decays nearly completely to the dark level within a few ms (D.K. Kramer and D.E. Fleischman, unpublished observations), indicating that the equilibrium constant for sharing of electrons in the quinone acceptor complex of the reaction center is likely to be strongly in favor of Q_B reduction. Alternatively, BTAi 1 and the aerobic photosyn-

thetic bacteria may maintain the redox state of their quinone pools via oxidative processes involving cytochrome oxidase or alternate oxidases and may lack other acceptor pools present in photosynthetic bacteria that are capable of anaerobic growth. A third possibility is that they may lack a mechanism to assure that the flow of electrons by non-photosynthetic processes into and out of the quinone pool occur at the same rate under anaerobic conditions. Chloroplasts of higher plants may utilize the cytochrome *bf* complex to sense the redox state of the plastoquinone pool and use protein kinases to adjust electron transfer rates appropriately [110]. A similar mechanism might operate in photosynthetic bacteria, which possess cytochrome bc_1 complexes and protein kinases [111]. This might function differently in the aerobes and anaerobes.

7. Interactions between chloroplasts and rhizobia in stem nodules

The photosynthetic bacteroids occupy a truly unique ecological niche within the stem nodules. Here their function is to supply fixed nitrogen, in the form of ammonium, to the plants. Almost nothing is known yet about how the photosynthetic system of the rhizobium adapts to play this unusual role. BTAi 1 cells undergo a striking morphological change as they differentiate to form bacteroids. The long rod-shaped free-living cells give rise to spherical bacteroids. The presumed photosynthetic vesicles are smaller and more numerous in the bacteroids and seem to lack the granular material seen in the lumen of the intracellular vesicles of the free-living cells (compare Figs. 3 and 5 of Ref. [18]). The photosynthetic system seems usually to be expressed in the bacteroids. BChl has been found in bacteroids isolated from stem nodules of plants inoculated with several strains of photosynthetic rhizobia [24]. BChl is found in bacteroids isolated from stem nodules, but not from root nodules, of *A. indica* plants inoculated with BTAi 1 (J. Rogers and D. Fleischman, unpublished observation).

The symbiosis is doubly fascinating because of the presence of chloroplasts in cells of the nodule cortex. The chloroplasts of *S. rostrata* stem nodules have been shown by immunoblotting to contain photosystem II thylakoid membrane proteins [45], suggesting that they are capable of oxygen evolution. Similar experiments have not yet been performed with *Aeschynomene* stem nodule chloroplasts, but it would be surprising if they do not possess photosystem II as well.

Eardly and Eaglesham [39] suggested that the stem nodules of *Aeschynomene* and *Sesbania* may be autonomous structures, the nodule chloroplasts providing all the fixed carbon needed for nitrogen fixation by the bacteroids. To test this hypothesis, they examined the effect of defoliation of the plant on acetylene reduction by nodules of BTSr 3-inoculated *S. rostrata* plants and BTAi 1-inoculated *A. scabra* plants. Acetylene reduction by the root nodules of both plants and by the stem nodules of *S. rostrata* decreased rapidly after defoliation. However, defoliation had no effect on acetylene reduction by *A. scabra* stem nodules, even after several days.

The rate of acetylene reduction by *A. scabra* stem nodules increased when they were illuminated and immediately decreased when they were placed in the dark. Eardly and Eaglesham examined the effect of increasing the oxygen supply in the dark. Acetylene reduction by stem nodules of both *A. scabra* and *S. rostrata* was accelerated. Thus illumination of *A. scabra* stem nodules may have increased the acetylene reduction rate by increasing the supply of oxygen to the bacteroids. There is evidence that nitrogen fixation by legume nodules is sometimes oxygen-limited [112]. A variable diffusion barrier may have protected the bacteroid nitrogenase from exposure to excessive oxygen (see Section 3.2). *A. scabra* [113] and *S. rostrata* [114] nodules contain several forms of leghemoglobin. Some forms are present at higher concentrations in stem nodules than in root nodules. Whether these forms provide additional protection against oxygen in the stem nodules remains to be determined. Parsons et al. [115] subsequently found that darkening had little effect on acetylene reduction by *S. rostrata* stem nodules.

The *A. scabra* stem nodules presumably contained photosynthetic BTAi 1 bacteroids as well as chloroplasts. In order to observe the effect of light absorbed only by the bacteroids, Evans et al. [20] illuminated nodule-containing stem and root segments with light in the wavelength range between 730 nm and 950 nm. The quantum efficiency of chloroplast photo-

synthesis diminishes rapidly at wavelengths beyond 700 nm, while BTAi 1 bacteroids have a major light-harvesting bacteriochlorophyll absorption band near 870 nm [21]. The illumination caused an immediate acceleration of acetylene reduction by *A. indica* stem nodules, but not by *A. indica* or soybean (*G. max*) root nodules. In the dark, acetylene reduction by the nodules slowed after a few hours, but the illuminated stem nodules continued to reduce acetylene at a constant rate for many hours.

Wettlaufer et al. (personal communication) performed similar experiments using a flow-through gas exchange system. They followed nitrogenase activity of BTAi 1-containing *A. indica* stem nodules by monitoring hydrogen evolution rather than acetylene reduction (H_2 is formed by nitrogenase as a byproduct of N_2 reduction). Net photosynthesis and respiration were monitored by observing the CO_2 concentration. In this way, they avoided possible inhibition of nitrogenase by acetylene [116], and were able to record the activities continuously. Electron transport in the nodule chloroplasts was driven selectively by 680 nm light, which is absorbed by chloroplasts but falls at an absorbance minimum of the bacteroids, which also are shaded by the chloroplasts. 880 nm light was used to drive electron transport in the bacteroids. H_2 evolution (nitrogenase activity) was found to be stimulated by both 680 and 880 nm light. Saturating white light caused a further several fold increase in H_2 evolution. Replacement of 680 nm illumination by 880 nm illumination produced an immediate, but transient, acceleration of H_2 evolution. These experiments suggest that photosynthetic electron transport in both the chloroplasts and the bacteroids contribute to nitrogen fixation, and that they may act synergistically. Further experiments of this sort should allow their contributions to be sorted out.

We may speculate that light absorbed by the stem nodule chloroplasts increases the availability of oxygen to the bacteroids, while light absorbed by the bacteroids increases their content of ATP and low-potential reductant. Illumination of free-living BTAi 1 cells decreases their rate of consumption of oxygen and presumably of substrate (see Section 6.1). If the bacteroids behave similarly, illumination would allow them to consume the carbon sources available in the nodule more slowly, permitting nitrogen fixation to proceed for a longer time before they are depleted. Reduced carbon compounds would still be needed to furnish the electrons for dinitrogen reduction, but photophosphorylation could replace oxidative phosphorylation as the source of energy.

Hungria et al. [117] conducted a detailed comparison of the physiological properties of *A. scabra* and *S. rostrata* root and stem nodules. Illumination increased the rate of $^{14}CO_2$ assimilation by *S. rostrata* stem nodules about 10-fold and by *A. scabra* stem nodules about 34-fold. Illumination did not affect $^{14}CO_2$ assimilation by root nodules of either plant. The stem nodules of both plants contained substantially more starch and soluble carbohydrates than did the root nodules, probably because of their photosynthetic activity. The specific acetylene reduction rate was higher in the stem nodules of both plants, much higher in *A. scabra* stem nodules (363 μmol g^{-1} nod h^{-1}). The stem nodules of both plants had 2- to 4-fold higher glutamine synthetase and glutamate synthase activity than did the root nodules. These enzymes are responsible for assimilating ammonium. The *A. scabra* nodules had a higher ureide content. Fixed nitrogen is transported mainly as ureides in many tropical legumes.

The observations cited above lead to several tentative conclusions. The stem nodules, but not the root nodules, of both plants are capable of photosynthetic carbon assimilation, and contain high levels of fixed carbon. They are capable of high rates of nitrogen fixation. They contain high levels of the enzymes needed for nitrogen assimilation, probably because high levels of their substrates (ammonium and fixed carbon) are available. The stem nodules of *A. scabra* are probably indeed autonomous structures. Whether the presence of photosynthetic bacteroids in these nodules is responsible for the fact that their acetylene reduction activity is resistant to defoliation, and that they are capable of high rates of light-stimulated $^{14}CO_2$ fixation and acetylene reduction remain to be determined.

8. Speculation about the role of the rhizobium photosynthetic system

The availability of photosynthetically fixed carbon is thought to limit symbiotic nitrogen fixation [118].

At the same time, actively fixing legumes may partition as much as half their total photosynthate to the roots, thereby limiting plant growth [119]. These facts have led to the suggestion that photosynthetic bacteroids may be able to use the energy of sunlight directly to drive nitrogen fixation, and so diminish competition between carbon and nitrogen fixation [16,20]. This idea is supported by the observation that light absorbed by the bacteroids enhances acetylene reduction by stem nodules (Ref. [20]; Wettlaufer, unpublished observation). More energy is required to produce photosynthate in the leaves and transport it to the root nodules than can be regained when it is consumed to drive oxidative phosphorylation in the bacteroids. It would be much more efficient to use the photosynthetic system of the bacteroids to generate ATP and reductant directly within the cells in which dinitrogen is reduced. In addition, the bacteroids are able to use light in the far-red region of the spectrum which otherwise would be wasted. Whether light absorbed by the bacteroids could provide a significant fraction of the energy needed by the plant is not entirely clear, however. Bacteriochlorophyll constitutes only about 20% of the chlorophyll found even in stem nodules [24], and much more chlorophyll is present in leaf chloroplasts. In addition, a much larger fraction of the sun's radiation lies in the visible region of the spectrum than in the 700 nm–900 nm region [120]. The importance of the bacteroid photosynthetic system in the plant's energy economy will probably be clarified when it becomes possible to inoculate plants with non-photosynthetic mutants of the photosynthetic rhizobia.

The high rate of light-driven $^{14}CO_2$ assimilation by BTAi 1-containing *A. scabra* stem nodules [117] suggests that the bacteroids may perform photosynthetic carbon fixation. It seems unlikely that much of the photosynthate produced would be used for cell growth. Perhaps the bacteroids use it to form storage materials which can be used as sources of energy and reductant for nitrogen fixation during the night. It would be worthwhile to examine the storage material content of bacteroids collected at various times of the day.

The observation that illumination enhances the survival of BTAi 1 and other aerobic photosynthetic bacteria after sources of fixed carbon have been depleted [51,98] has led to the suggestion that the function of their photosynthetic systems is to allow them to survive in an ex planta environment in which the supply of fixed carbon is limited. One such environment might be the surface of leaves. Adebayo et al. [121] report that the surfaces of the leaves of *Sesbania* and *Aeschynomene* plants contain substantial populations of rhizobia which are capable of forming profuse stem nodules on aerial parts of the stems of their host plants (e.g., *S. rostrata* and *A. afraspera*). They suggest that the bacteria may inoculate the stems after rains. In such an aerobic environment, with circadian illumination and substrate limitation, the photosynthetic system of the bacteria should be expressed and light-stimulated growth might even be possible. The under surfaces of leaves might be especially hospitable. Here the lower light intensity would be less likely to cause light damage, and the leaves would be expected to transmit the far-red light that can induce BChl formation and drive bacterial photosynthesis. Facilitating ex planta survival may be the only role of the photosynthetic system of the *Lotononis* rhizobia. Their similarity to aerobic photosynthetic bacteria, in particular to facultative methylotrophs, has been mentioned [86]. Many aerobic photosynthetic bacteria have been isolated from leaf surfaces [51], and pigmented methylotrophic bacteria have been reported to comprise a substantial fraction of the bacteria found on white clover leaves [122]. It seems possible that the *Lotononis* rhizobia could even have evolved from phyllospheric methylotrophs.

In summary, the rhizobium photosynthetic system may play two roles. The fact that it is expressed in stem nodules but not in root nodules strongly suggests that it has a useful role there. Light enhancement of acetylene reduction and $^{14}CO_2$ assimilation provide hints about what it may be, but further experiments are necessary. Finally, the photosynthetic system undoubtedly facilitates survival of the rhizobia during life outside the nodule.

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