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Light-stimulated $^{14}CO_2$ uptake and acetylene reduction by bacteriochlorophyll containing stem nodule isolate BTAi 1

Mariangela Hungria[1], Joan M. Ellis[2], Ralph W.F. Hardy[2], and Allan R.J. Eaglesham[2]

[1]EMBRAPA-CNPSoja, Cx. Postal 1061, CEP 86001, 970, Londrina, PR Brazil
[2]Boyce Thompson Institute for Plant Research at Cornell University, Tower Road, Ithaca, NY, 14853, USA

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Summary. Bradyrhizobial strain BTAi 1 nodulates both stems and roots of *Aeschynomene* spp. Previous work has shown that it contains bacteriochlorophyll *a* and forms photosynthetic reaction centers, and has provided indirect evidence of photosynthesis by bacteroids within stem nodules. Here we report physiological and biochemical characteristics of BTAi 1 *ex planta*, which also suggest the presence of photosynthetic activity. Light-stimulated uptake of $^{14}CO_2$ by BTAi 1 was detected at all stages of growth. Inhibitors of photosynthesis, 1,10-orthophenanthroline and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and the uncoupler NH_4Cl, immediately suppressed light-driven $^{14}CO_2$ uptake and increased O_2 uptake. BTAi 1 is strictly aerobic and was unable to grow without organic C even in the light; also, it was unable to grow chemoautotrophically in an atmosphere enriched with H_2 and CO_2. In micro-aerobic conditions, strain BTAi 1 expressed acetylene reducing activity *ex planta* in an N-free medium. The highest rates of light-stimulated $^{14}CO_2$ uptake and acetylene-reducing activity occurred during the exponential and early stationary phases of growth. Acetylene-reducing rates at a low glucose concentration were increased following a light-dark cycle in comparison with continuous dark conditions.

Key words: Nitrogen fixation – Photosynthetic bacteria – Stem nodules – *Aeschynomene scabra* – *Sesbania rostrata* – *Azorhizobium caulinodans* – *Erythrobacter* sp. – *Roseobacter denitrificans*

It has been known for many years that legumes of the genus *Aeschynomene* can form nodules on stems as well as on roots when growing under flooded conditions (Hagerup 1928; von Sussenguth and Beyerle 1936; Arora 1954). Another legume that tolerates waterlogged soil, *Sesbania rostrata*, was recently found to form stem nodules (Dreyfus and Dommergues 1981). Rhizobia cultured from stem nodules of *Aeschynomene* spp. and *S. rostrata* are now available for study (Dreyfus et al. 1983; Eaglesham and Szalay 1983; Stowers and Eaglesham 1983; Chakrabarti et al. 1986; Dreyfus et al. 1988; Alazard 1990).

Rhizobia able to nodulate the stems of *S. rostrata* have been classified as the new genus and species *Azorhizobium caulinodans* (Dreyfus et al. 1988). A characteristic of *Azorhizobium caulinodans* is the ability to express nitrogenase activity *ex planta* at high rates in an N-free medium (Dreyfus et al. 1988). Recent work has shown that stem-nodule isolates from *Aeschynomene afraspera* share this characteristic whereas root-nodule isolates do not (Alazard 1990).

The stem-nodule isolate from *Aeschynomene indica* (strain BTAi 1) originated from sand used routinely for the experimental culture of legumes at the Boyce Thompson Institute, and was found to be unusual in having characteristics in common with both fast- and slow-growing rhizobia (Stowers and Eaglesham 1983). Genetic differences found in DNA-homology studies with a stem isolate from *Aeschynomene aspera* indicated that it could be a new *Rhizobium* sp. (Chakrabarti et al. 1986). However, recent studies based on sequencing of a 16 S ribosomal ribonucleic (rRNA) gene segment have shown that BTAi 1 falls in the center of the *Bradyrhizobium* cluster (Young et al. 1991); further study of the taxonomy of these bacteria is called for.

Strain BTAi 1 is now known to produce bacteriochlorophyll *a* and to form photosynthetic reaction centers resembling those of purple photosynthetic bacteria (Evans et al. 1990). There is also indirect evidence of photosynthesis by bacteroids within stem nodules (Evans et al. 1990; Hungria et al. 1992). A summary of the unique characteristics of BTAi 1 was presented recently (Eaglesham et al. 1990). The present paper describes photosynthetic and N_2-fixing characteristics of BTAi 1 based on physiological experiments with the organism cultured *ex planta*.

Correspondence to: M. Hungria

Materials and methods

Bacteria

Initially, it was essential to verify that BTAi 1 was not contaminated with a photosynthetic organism. Culture purity and Koch's postulates were verified as follows. A yeast-mannitol broth culture (Vincent 1970) of BTAi 1 was serially diluted in yeast-mannitol broth containing Tween 80 (0.01%, w/v) and low dilutions were checked with a light microscope to verify that the cells were not aggregated in pairs or clumps. High dilutions were plated on yeast-fructose agar, and cultured at 27 °C in a light-dark cycle (34 μmol m^{-2} s^{-1}, 16:8 h, light:dark). The colonies were regular in form and well separated, and therefore were probably derived from single cells; all were pink. Six colonies were individually cultured in yeast-mannitol broth, and inoculated onto the submerged parts of surface-sterilized stem cuttings of *Aeschynomene scabra* growing in 500-ml Erlenmeyer flasks containing sterilized 1/3 strength N-free nutrient solution (Eaglesham et al. 1983). The apices of the cuttings protruded from the flasks, and the bottles were sealed with sterile cotton plugs. All of the single-colony-derived cultures induced stem nodules within 14 days, whereas uninoculated cuttings remained free of nodules. Six nodules were selected at random, and the endophytes were plated on yeast-fructose agar to provide single colonies; all colonies were pink. One of these single colonies was selected and cultured to provide the source for all subsequent work with BTAi 1.

Cultures of two strains from root nodules of *Aeschynomene* spp. were obtained from other collections, and were tested for the capacity to form stem nodules. Although the production of pink pigment was not observed in either of the strains, all tests for stem nodulation were negative; therefore an isolate from a stem nodule of *S. rostrata* was used as a non-pigmented stem-nodulating comparison; the isolation of *Azorhizobium caulinodans* BTSr 3 had been described previously (Hungria et al. 1992).

The best growth conditions for BTAi 1 and BTSr 3 were obtained using yeast-extract broth (Vincent 1970) containing 9.5 g l^{-1} of sodium glutamate for BTAi 1 (YEG), or 10 g l^{-1} of sodium succinate for BTSr 3 (YES). The bacteria were grown as starter cultures in 50-ml medium, on a shaker, in a growth chamber with incadescent lights at a flux density of 34 μmol m^{-2} s^{-1}, a 16-h photoperiod, and a constant broth temperature of 27 °C. After 6 days, these cultures were used as inocula for broths of larger volume.

The bacteria were invariably grown aerobically; neither strain grows under anaerobic conditions, even with light-dark cycling. Both strains were tested for chemoautotrophic growth (Hanus et al. 1979).

As a comparison in determining photosynthetic properties, we used the first aerobic photosynthetic bacterium to be reported in the literature. *Erythrobacter* sp. strain OCh 114 (Harashima et al. 1982; Shiba 1984), now called *Roseobacter denitrificans* (Shiba 1991). The bacterium was provided by Dr. T. Shiba (Otsuchi Marine Research Center, Tokyo) and was grown using culture media and light conditions described previously (Shiba 1984). The results obtained for growth, O_2 uptake, and $^{14}CO_2$ fixation with this strain are not shown here because they have been published previously (Harashima et al. 1982; Shiba 1984).

Growth

Growth curves were obtained for BTAi 1 and BTSr 3 cultured under a light-dark cycle, or in continuous dark conditions, as described above. Darkness in this evaluation and all others described here was achieved by wrapping the culture flasks in aluminum foil and placing them in a sealed cardboard box. Viable cell counts were made by the drop-plate method (Miles and Misra 1938); three replicate determinations were made at intervals of up to 21 days.

Light-stimulated $^{14}CO_2$ uptake

Broth cultures (500 ml) of BTAi 1 and of BTSr 3 were prepared, centrifuged (7000 g for 20 min) after 5 days, washed twice with buffered salt solution (salts of the yeast-extract broth), and resuspended in the buffered salt solution to make a final volume of 20 ml. One-milliliter aliquots were distributed into each of eight 10-ml scintillation vials. The vials were kept in the dark for 1 h to allow the cells to equilibrate, and were then fitted with rubber serum-stoppers. The assays were initiated by the injection of 1 ml of a gas mixture containing 10 μCi $^{14}CO_2$. The vials were incubated in the light (34 μmol m^{-2} s^{-1}), or in the dark, and sampled at intervals of 1–60 min. Reactions were stopped by the addition of 0.5 ml 10% acetic acid solution (v/v), followed by 2 ml 50% ethanol (v/v). The samples were subjected to ultrasound for 5 min, then extracted for 20 min at 45 °C, and dried under a hood with ventilation at 35 °C for 48 h; bacterial counts were made in 10 ml scintillation fluid. Appropriate $^{14}CO_2$ standards were used to calibrate the scintillation counter and autoclaved cells were used to determine background counts. Protein contents were determined according to Bradford (1976).

Light-driven $^{14}CO_2$ uptake by BTAi 1 and BTSr 3 was also determined as a function of the growth phase, using the procedures described before after 30 min of incubation with $^{14}CO_2$.

To examine the effects of inhibitors of photosynthesis on the assimilation of $^{14}CO_2$, the procedure described above was followed and three treatments were applied, with four replicates, at the following final concentrations: 0.5 μ*M* 3-(3,4-dichlorophenyl)-1,1-dimethylurea [DCMU; dissolved in ethanol for a final concentration of 1.0% ethanol (v/v) in the reaction mixture], 50 μ*M* 1,10-orthophenanthroline, and 500 μ*M* NH_4Cl. The vials were incubated in the light (34 μmol m^{-2} s^{-1}) or in the dark for 30 min, and radioactive incorporation was determined.

Light-decreased O_2 uptake

To determine the rates of O_2 consumption, 0.3-ml aliquots of resuspended cells of 5-day-old cultures (early stationary phase) of BTAi 1 or BTSr 3, in 1.5 ml distilled water, were placed in the reaction vessel of a Gilson 5/6 Oxygraph and monitored using an O_2 electrode. The temperature was maintained at 27 °C by means of a water jacket with an external heater circulator, and light (34 μmol m^{-2} s^{-1}) was turned on and off at intervals of 3–10 min.

Effects of the inhibitors of photosynthesis on O_2 uptake rates were evaluated by adding DCMU, orthophenanthroline, and NH_4Cl at the final concentrations described above.

Bacteriochlorophyll a content

To determine the bacteriochlorophyll *a* content per cell, cultures were centrifuged and washed twice in a buffered salt solution. The cells were then resuspended in 0.25 ml water and extracted with 4.75 ml methanol. The extract was kept in the dark for 30 min, centrifuged again, and assayed for bacteriochlorophyll *a* spectrophotometrically (Cohen-Bazire et al. 1957). Three milliliters of culture were used to determine the cell numbers (Miles and Misra 1938).

Expression of acetylene reducing activity ex planta

To obtain expression of the acetylene-reducing activity *ex planta*, we tested several different media (Kurz and LaRue 1975; Pagan et al. 1975; Bergersen et al. 1976; Dreyfus et al. 1983), O_2 concentrations (0.03–20.9%), micro-aerobic conditions through the addition of agar (2, 3, and 4 g l^{-1} of agar), C sources (glucose, fructose, arabinose, Na-lactate, Na-succinate), and N sources (glutamine, yeast extract, NH_4Cl). Acetylene-reducing activity was detected using the Dreyfus et al. (1983) medium modified by increasing the nicotinic acid concentration from 20 to 40 mg l^{-1}. No acetylene-reducing activity was obtained under a normal atmosphere. A semi-solid medium with 2 g l^{-1} of agar allowed easier manipulation of a large number of test-tubes compared with sparging the individual tubes with gases containing known O_2 concentrations. The C source that maximized the acetylene-reducing activity for both BTAi 1 and BTSr 3 was 10 g l^{-1} of glucose.

To verify the linearity of the acetylene-reducing activity, 4 ml of the medium used for the expression of acetylene-reducing activity, as described above, was placed in 7-ml test-tubes and inoculated with 0.1 ml of resuspended cells (inoculum described under Bacteria). Tubes with 7-day cultures were assayed for acetylene-reducing activity after 5 min to 48 h of incubation under 15% acetylene. Five replicates were used per sampling time and the samples were incubated in the dark at 27 °C, constantly checking for endogenous ethylene formation by the bacteria.

To examine the *ex planta* acetylene-reducing activity profile of BTAi 1 and BTSr 3 during different growth phases, the above procedure for acetylene reducing activity was followed, and on each day for 10 days, 10 tubes of each strain were assayed after 30 min of incubation, in the dark, at 27 °C.

Light effects on acetylene-reducing activity

BTAi 1 and BTSr 3 were inoculated in a semi-solid Dreyfus medium modified, as described above, with 1 or 10 g l^{-1} of glucose. The bacteria were grown in a light-dark cycle (16 h photoperiod) or in the dark as described before. After 8 days of incubation, six replicates of BTAi 1 and BTSr 3 were used to evaluate the acetylene-reducing activity. Another six replicates were used to evaluate $^{14}CO_2$ fixation after 30 min of incubation in the light (34 µmol m^{-2} s^{-1}) or in the dark.

Results

Illumination and growth of pigmented rhizobia

The effects of light on the growth cycle of BTAi 1 and BTSr 3 are shown in Fig. 1. Illuminated cells (a photoperiod of 16 h) of BTAi 1 retained viability during the stationary phase, whereas darkened cells went into a death phase (Fig. 1 a). The same patterns for BTAi 1 were obtained with fructose at 1 g l^{-1} and 0.01 g l^{-1} (data not shown). With fructose at 0.01 g l^{-1}, the media pH values at 20 days were 8.3 and 8.2, for light and dark cultures, respectively. Light did not enhance the cell viability of BTSr 3 (Fig. 1 b).

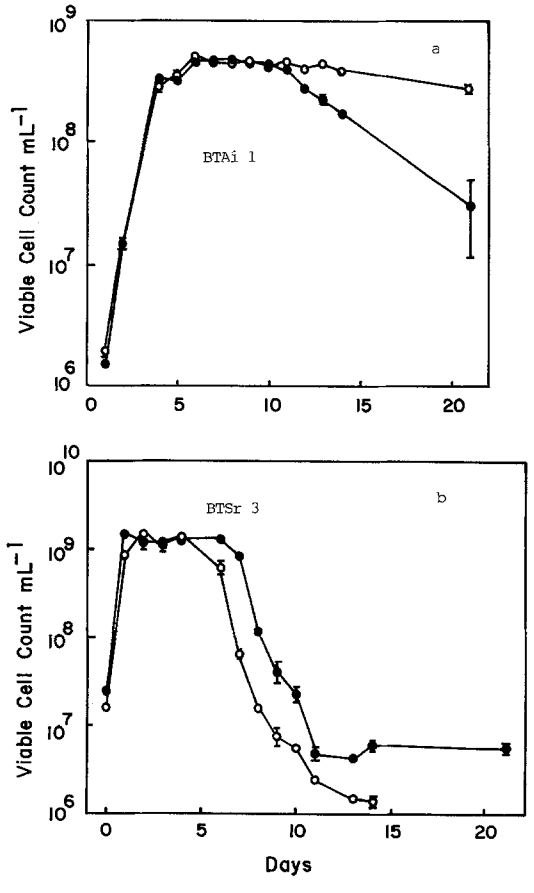

Fig. 1. Growth curves of rhizobial strains BTAi 1 (**a**) and BTSr 3 (**b**) cultured in a light (34 µmol m^{-2} s^{-1})-dark cycle (16–8 h; ○—○) or in the dark (●—●). Bacteria were grown in a medium with 9.5 g l^{-1} of sodium glutamate (BTAi 1) or 10 g l^{-1} of sodium succinate (BTSr 3). Each point represents the average of four replicates ± SE

Light-stimulated $^{14}CO_2$ uptake

$^{14}CO_2$ fixation by strain BTAi 1 was linear with time under both light and dark conditions ($r = 0.99$, 0.91, respectively; $P < 0.01$), and it was stimulated by light at all stages, particularly in the early growth phase (Fig. 2). The rates of $^{14}CO_2$ assimilation in the light by 5-day-old cultures of BTAi 1 (Table 1) and *R. denitrificans* OCh 114 (data not shown) were, respectively, five- and sixfold higher than in the dark. However, even greater increases were observed for illuminated BTAi 1 in other experiments. The rate of dark $^{14}CO_2$ uptake by strain BTSr 3 was similar to that of strain BTAi 1 (Table 1), but it was not stimulated by light at any phase of the growth curve.

DCMU, orthophenanthroline, and NH_4Cl decreased $^{14}CO_2$ uptake by BTAi 1 in the light by 35%, 55%, and 55%, respectively (Table 1), and similar levels of inhibi-

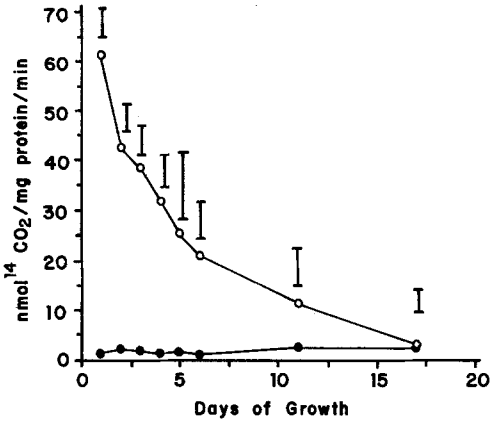

Fig. 2. Rates of $^{14}CO_2$ uptake in the light (34 µmol m^{-2} s^{-1}, ○—○) or in the dark (●—●) during the growth stages of rhizobial strain BTAi 1. BTAi 1 was grown with 9.5 g l^{-1} of sodium glutamate, incubated for 30 min with $^{14}CO_2$, and each point represents the average of four replicates. Vertical bars denote least significant differences between treatments (Tukey's test, $P < 0.01$)

Table 1. Effects of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 1,10-orthophenanthroline, and NH_4Cl on the rates of uptake of $^{14}CO_2$ (nmol mg protein^{-1} min^{-1}) in the light (34 µmol m^{-2} s^{-1}) or in the dark by 5-day-old cultures of rhizobial strains BTAi 1 and BTSr 3

Treatment	Light/dark	$^{14}CO_2$ uptake	
		BTAi 1	BTSr 3
Control	Light	33.2a	5.6a
	Dark	6.7d	5.8a
DCMU (0.5 µ*M*)	Light	21.4b	5.6a
	Dark	7.5d	5.8a
Phenanthroline (50 µ*M*)	Light	14.8c	5.0a
	Dark	6.7d	5.0a
NH_4Cl (500 µ*M*)	Light	15.1c	4.8a
	Dark	7.1d	5.7a

Means of four replicates are shown. Values followed by the same letter are not significantly different for each strain (Tukey's test, $P < 0.01$)

tion were found with *R. denitrificans* OCh 114. The two inhibitors and the uncoupler had no effect on the $^{14}CO_2$ uptake by BTSr 3 (Table 1).

Bacteriochlorophyll a content

The maximum bacteriochlorophyll *a* content, of 0.2 pg cell^{-1}, occurred during the early exponential phase, and thereafter was maintained at relatively constant levels (data not shown). Bacteriochlorophyll *a* was not detected in BTSr 3 at any phase of growth, and for *R. denitrificans* a value of 0.25 pg cell^{-1} was found.

Effects of light on O_2 uptake

Illumination slowed the uptake of O_2 by BTAi 1, but had no effect with BTSr 3 (Fig. 3). An average of three experiments with six replicates showed a 43% decrease in O_2 uptake by BTAi 1 exposed to light. With *R. denitrificans* OCh 114, this decrease was, on average, 50% (data not shown).

The addition of DCMU, orthophenanthroline, or NH_4Cl to BTAi 1 eliminated the light-dependent decrease in O_2 uptake (Fig. 3). The results of six additional experiments showed average decreases of BTAi 1 of 36%, 57%, and 53%, respectively, and for *R. denitrificans* OCh 114, 40%, 59%, and 56%, respectively. The addition of these compounds did not affect O_2 uptake rates by BTSr 3 (Fig. 3).

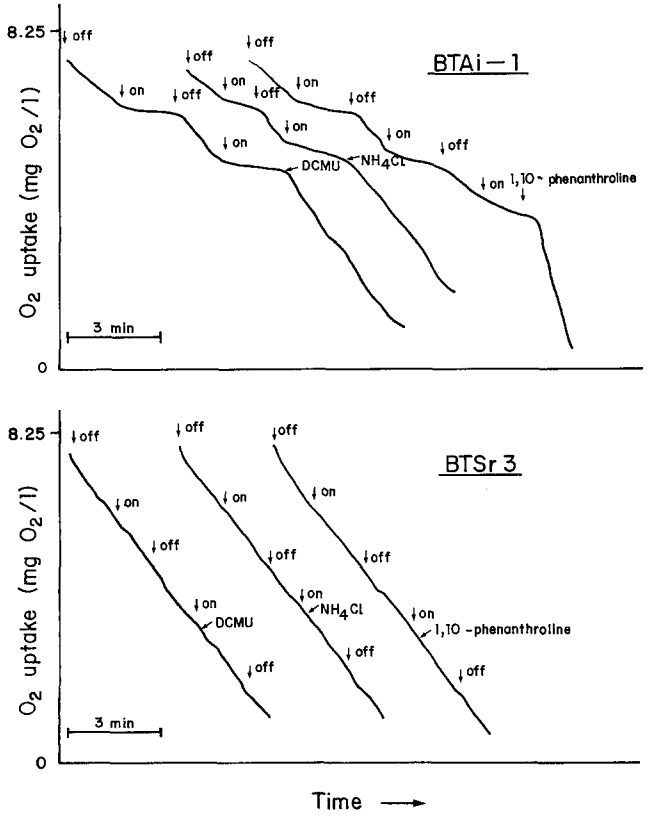

Fig. 3. Uptake of O_2 by 5-day-old rhizobial strains BTAi 1 and BTSr 3 in the light (34 μmol m^{-2} s^{-1}, *on*) or in the dark (*off*), before and after the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 0.5 μ*M*), orthophenanthroline (50 μ*M*), or NH_4Cl (500 μ*M*)

Expression of acetylene-reducing activity ex planta and effects of light

Acetylene-reducing activity was observed for both BTAi 1 and BTSr 3 in a modified semi-solid Dreyfus' medium. In this semi-solid medium, containing 2 g l^{-1} of agar, both strains formed a pellicle below the surface, a growth pattern that is typical of micro-aerophilic N_2-fixing organisms (Gibson et al. 1988). BTSr 3 expressed acetylene-reducing activity with 10 g l^{-1} of either Na-lactate or glucose, but BTAi 1 expressed activity only with glucose as the C source. Inorganic or organic N inhibited activity in both strains. Acetylene-reducing activity was expressed by both BTAi 1 and BTSr 3 without a lag phase, with linear reactions ($r = 0.98$, $P < 0.01$) for 5 min to 48 h under acetylene in cultures from 1 to 9 days old (data not shown). The activity was greatest at 3–6 days of incubation for both BTAi 1 and BTSr 3, but the rates shown by BTSr 3 were threefold higher than those of BTAi 1 (Fig. 4).

A decrease in the C supply from 10 to 1 g l^{-1} of glucose lowered the rates of acetylene-reducing activity of BTSr 3 but not BTAi 1 (Fig. 4, Table 2). Comparisons of light-dark grown with dark grown cultures of BTAi 1 showed that *ex planta* acetylene-reducing rates were higher in the light than in the dark after 3, 6, and 8 days of incubation. Table 2 displays the results obtained with 8-day-old cultures (late stationary phase), when the acetylene-reducing activity was significantly higher in BTAi 1 cultures grown in the light-dark cycle and incubated with acetylene under light. Simultaneous measurements of $^{14}CO_2$ uptake with these cultures confirmed the light-stimulated $^{14}CO_2$ fixation (Table 2).

Discussion

Photosynthetic bacteria have been traditionally thought to synthesize bacteriochlorophyll *a* and to grow using light energy exclusively under anaerobic conditions (Pfening 1978). However, this chlorophyll has been reported in strictly aerobic bacteria, such as *Pseudomonas*

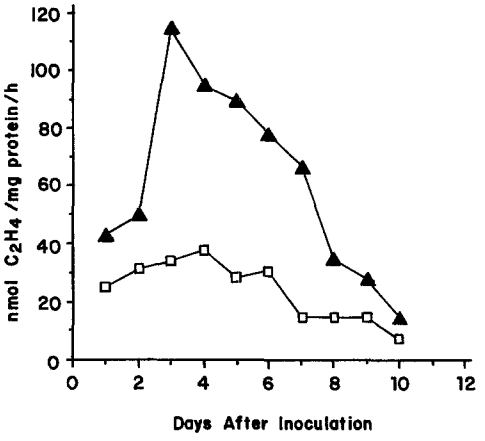

Fig. 4. *Ex planta* acetylene-reducing activities of cultures of rhizobial strains BTSr 3 (▲—▲) and BTAi 1 (□—□) over the growth cycle. Bacteria were grown in an N-free medium with 10 g l^{-1} of glucose. The tubes were kept in the dark during the 30-min incubation with acetylene and each point represents the mean of 10 replicates

Table 2. Effects of light (34 μmol m^{-2} s^{-1}) on $^{14}CO_2$ uptake and acetylene-reducing activity (ARA) of 8-day-old cultures of BTAi 1 and BTSr 3

Rhizobial strain	Light cycle during growth	Light during the assay	$^{14}CO_2$ uptake (nmol CO_2 mg protein^{-1} min^{-1})	ARA (nmol C_2H_4 mg protein^{-1} h^{-1})
BTAi 1	Light – dark	Light	28.2a	24.6a
	Light – dark	Dark	4.2b	16.1b
	Dark	Light	4.5b	11.0c
	Dark	Dark	3.9b	10.6c
BTSr 3	Light – dark	Light	2.7a	31.2a
	Light – dark	Dark	3.0a	28.7a
	Dark	Light	2.8a	26.2a
	Dark	Dark	2.7a	27.4a

Bacteria were grown in C-limited conditions (1 g l^{-1} of glucose). Means of six replicates are given. Values followed by the same letter are not significantly different within each column (Tukey's test, $P<0.01$)

AM-1 (Sato 1978) and *Protomonas extorquens* (originally *Protaminobacter ruber* and *Pseudomonas radiora;* Sato 1978; Nishimura et al. 1981), and also with photosynthetic activity (Takamiya and Okamura 1984). Bacteriochlorophyll *a* has also been detected in strictly aerobic bacteria isolated from marine environments (Harashima et al. 1978; Shiba et al. 1979a, b), in particular in *Erythrobacter longus* OCh 101 (Harashima et al. 1978, 1982) and *Erythrobacter* sp. OCh 114 (Harashima et al. 1982) now, classified in the new genus *Roseobacter* (Shiba 1991). The amount of bacteriochlorophyll *a* found in OCh 114 (Shiba et al. 1979a) implied a physiological function in the cell, and photosynthetic properties were subsequently described for this strain (reviewed in Shiba and Harashima 1986). Consequently, we used OCh 114 for comparison as an aerobic photosynthetic bacterium, and as a non-photosynthetic control we used *Azorhizobium caulinodans* strains BTSr 3, which nodulates stems and roots of *Sesbania* sp.

Bacterial growth, synthesis of bacteriochlorophyll *a*, and phototrophic activity in both *Roseobacter* (Harashima et al. 1980, 1982; Shiba 1984; Okamura et al. 1986) and BTAi 1 (Evans et al. 1990) do not occur under anaerobic conditions even in the light. However, under aerobic conditions and a light-dark cycle, bacteriochlorophyll *a* and light-stimulated $^{14}CO_2$ uptake are detected from the exponential to the late stationary phase in cultures of BTAi 1. The bacteriochlorophyll *a* content of BTAi 1 (Evans et al. 1990 and this paper) and of *Roseobacter* (Harashima et al. 1978; Shiba et al. 1979a), although low compared with other anaerobic photosynthetic bacteria (Lascelles 1959), is consistent with the modest levels of light-driven $^{14}CO_2$ uptake by both species (Fig. 2; Shiba 1984) and of photophosphorylation activity in *R. denitrificans* OCh 114 (Okamura et al. 1986). Although *Rhizobium, Bradyrhizobium,* and *Azorhizobium* are phyllogenetically linked to the purple photosynthetic bacteria (Hennecke et al. 1985; Dreyfus et al. 1988) and to *Roseobacter* (Woese 1987), they have not been found to contain bacteriochlorophyll *a* and/or to be photosynthetic.

Both BTAi 1 and *R. denitrificans* OCh 114 show a substantial increase in $^{14}CO_2$ fixation in the light (Fig. 2; Tables 1, 2; Shiba 1984). BTAi 1 does not grow in a medium lacking organic C, either in a normal atmosphere or chemoautotrophically in an atmosphere enriched with H_2 and CO_2; this is similarly observed for *Roseobacter* (Shiba and Simudu 1982). Thus, the physiological significance of photosynthesis in aerobic bacteria is still unclear, although there is strong evidence that the ability to fix $^{14}CO_2$ in the light enhances the survival of BTAi 1 and *R. denitrificans* OCh 114 under C-limited conditions (Fig. 1a; Shiba 1984). Harashima et al. (1982) suggested that the decline in the death rate occurs because a light-driven cyclic electron flow does not require consumption of a substrate, and the bacteria may obtain some energy from light under starvation conditions. In the present study, neither bacteriochlorophyll *a* nor light-stimulated $^{14}CO_2$ assimilation was detected in cultures of BTSr 3 and there was no positive effect on the survival of the cells (Fig. 1b).

The decrease seen in O_2 uptake by aerobically grown BTAi 1 using endogenous substrates resembles the property of anaerobically grown cells of some photosynthetic bacteria, in which a respiration-dependent electron flow can be replaced by a light-driven cyclic electron flow (Thore et al. 1969; Keister 1978); in the present study we confirmed a photoinhibition of O_2 uptake by BTAi 1, but not by BTSr 3 (Fig. 3). Photoinhibition of O_2 uptake has also been observed in cells of *Roseobacter* strains OCh 114 and OCh 101 (Harashima et al. 1982). The effect of high exogenous levels of NH_4Cl on the O_2 uptake rates (Fig. 3) implies that photophosphorylation reactions are present in BTAi 1 but not in BTSr 3. Also, the effects of DCMU and orthophenanthroline, both of which interfere with the binding of quinone in photosystem II and purple bacteria reaction centers (Izawa and Good 1972), suggest photosynthesis in BTAi 1 but not in BTSr 3 (Fig. 3).

The enzyme primarily responsible for fixation of CO_2 in cyanobacteria, other autotrophic prokaryotes, and higher plants is ribulose 1,5-bisphosphate carboxylase (RuBPCase; Stewart 1980). Both BTAi 1 and *R. denitrificans* OCh 114 have DNA restriction fragments that hybridize to RuBPCase genes from *Rhodobacter sphaeroides,* whereas genomic digests from BTSr 3 do not hybridize with these genes (A. Suwanto and S. Kaplan, personal communication). Light-independent RuBPCase activity has been detected in a hydrogenase-positive *Bradyrhizobium* strain, after derepression of hydrogenase in

an atmosphere enriched with H_2 and CO_2 (Simpson et al. 1979). However, rates of $^{14}CO_2$ fixation by *Bradyrhizobium* were usually 1/10 (Simpson et al. 1979) of those recorded by BTAi 1 in the light. Neither BTAi 1 nor *Roseobacter* fix CO_2 under the conditions established by Simpson et al. (1979); therefore RuBPCase is probably related to the photosynthetic activity in these bacteria.

Acetylene-reducing activity *ex planta* has been reported in *Bradyrhizobium* and *Rhizobium leguminosarum* strains (Kurz and LaRue 1975; McComb et al. 1975; Pagan et al. 1975; Bergersen et al. 1976) in media containing combined N. Using an N-free medium, Dreyfus et al. (1983) obtained acetylene-reducing activity *ex planta* in rhizobial strain ORS571, and this was later proposed as a characteristic of the new genus and species *Azorhizobium caulinodans* (Dreyfus' et al. 1988). It is probable that BTSr 3 is the same as ORS571 (Hungria et al. 1992), and this is supported by the high rates of acetylene-reducing activity in a Dreyfus medium (Table 2). BTAi 1 reduces acetylene *ex planta* in N-free media (Fig. 4), in common with recent findings with other stem-nodulating rhizobial isolates from *Aeschynomene* spp. (Alazard 1990). An increase in acetylene-reducing activity in response to light is observed for BTAi 1 but not BTSr 3 (Table 2). The light effects on acetylene-reducing activity are detected in cells grown in a C-limited medium (1 g l^{-1} of glucose) and at the late exponential phase consistent results were obtained (Table 2). This is also the growth phase where BTAi 1 shows advantages in terms of enhanced cell survival (Fig. 1 a).

Bradyrhizobial strain BTAi 1 was isolated from sand originated from Virginia near the Potomac, and was strip-mined with some soil contamination. The fact that *Aeschynomeme virginica* is indigenous to fresh and brackish tidal shores from New Jersey to south Virginia (Fernald 1950) may explain the presence of BTAi 1. It is possible that light-stimulated $^{14}CO_2$ assimilation gives some competitive advantage *ex planta* in aquatic, C-limited conditions. The $^{14}CO_2$ assimilation rates found in BTAi 1 and *R. denitrificans* OCh 114 do not allow growth in a C-free medium, but enhance the survival of both strains. Photofixation of $^{14}CO_2$ probably occurs via RuBPCase, which is detected in both species, *Bradyrhizobium* BTAi 1 and *Roseobacter* OCh 114, – but why these bacteria do not have the ability to grow in the absence of organic C is not known. These properties also seem to occur in the symbiotic stage, since bacteroids of BTAi 1 contain pigmented chromatin bodies and look like purple photosynthetic bacteria (Evans et al. 1990). Furthermore, stem nodules of *Aeschynomene* with BTAi 1 have also shown increased $^{14}CO_2$ fixation rates under light, which appear to contribute to the higher rates of acetylene-reducing activity observed in these nodules (Hungria et al. 1992).

Clearly, BTAi 1 provides a novel source of germplasm for fundamental study, and appears to be the first photosynthetic *Bradyrhizobium* to have been isolated. In addition, preliminary studies with 177 isolates from stem nodules of *Aeschynomene fluminensis* grown in flooded areas of central Brazil have identified 17 rhizobial isolates producing pink pigment, synthesizing bacteriochlorophyll *a* and showing light-decreased O_2 uptake (M. F. Loureiro, M. Hungria, M. J. Sampaio, J. I. Baldani, and A. A. Franco, unpublished data). Further study of BTAi 1 and the Brazilian isolates may lead to new insights into the physiology, genetics, taxonomy, and evolution, not only of the bradyrhizobia, but also of the legume N_2-fixing symbiosis, and may have agronomically useful benefits.

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