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Enhanced Nodulation and Nitrogen Fixation by a Revertant of a Nodulation-Defective *Bradyrhizobium japonicum* Tryptophan Auxotroph

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In greenhouse studies, the symbiotic properties of a prototrophic revertant (TA11 NOD$^+$) of a nodulation defective tryptophan auxotroph of *Bradyrhizobium japonicum* were compared with those of the normally nodulating wild-type strain, *B. japonicum* I-110 ARS. Strain I-110 ARS was the parent of auxotrophic mutant TA11. Plants inoculated with TA11 NOD$^+$ contained significantly more nitrogen per plant than did plants inoculated with wild-type bacteria (275.9 ± 35 versus 184 ± 18 mg). Also, plants that received the revertant were larger, averaging 8.4 ± 0.9 g (dry weight) versus 6.4 ± 0.6 g for those that received the wild-type bacterial strain. Additionally, plants that received the NOD$^+$ strain had 56% more nodules and 41% more nodule mass than did control plants. With both inocula, average nodule size and amount of nitrogen fixed per gram of nodule were about the same. These data indicated that the improvement in nitrogen fixation observed with the TA11 NOD$^+$ resulted from an increase in the overall nodule number. The physiological basis for this increase in nodulation is not known, but enhanced tryptophan catabolism does not appear to be involved.

A number of investigators have studied the symbiotic properties of *Bradyrhizobium japonicum* mutants with altered tryptophan metabolism. Wells and Kuykendall (13) isolated a series of tryptophan-requiring mutants of *B. japonicum* I-110. These mutants fell into five classes, each based on a defective enzyme within the tryptophan biosynthetic pathway. Only mutants that were defective in tryptophan synthetase, the last enzyme of the pathway, were capable of forming nodules from which auxotrophic bacteria could be isolated (8). Other classes of mutants, with defects earlier in the pathway, did not form effective nodules as auxotrophs but did form effective nodules as prototrophic revertants. Also, Hunter (3, 4) has isolated and studied *B. japonicum* I-110 mutants resistant to 5-methyltryptophan. These mutants constitutively overproduced tryptophan and tryptophan products, such as 3-indoleacetic acid (IAA). Nodules formed by these bacteria contained bacteroids with enhanced IAA-producing capability (4) and consequently contained much larger amounts of IAA than did nodules from plants inoculated with wild-type bacteria (3). Often, however, these 5-methyltryptophan-resistant bacteria were poor nodulators and poor nitrogen fixers.

Bradyrhizobia with enhanced tryptophan catabolism have also been studied, and there have been reports (6; T. Kaneshiro, U.S. patent 4,711,656, December 1987) of improved symbiotic properties with catabolic variants of *B. japonicum* L-259 (USDA strain 26). These catabolic variants characteristically degrade tryptophan rapidly and accumulate both large amounts of indole compounds and a tan degradation product (6, 7; Kaneshiro, patent).

The objective of the present study was to compare the symbiotic properties of a prototrophic revertant (TA11 NOD$^+$) of a tryptophan auxotroph (TA11) with those of the wild-type parental strain, *B. japonicum* I-110 ARS.

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MATERIALS AND METHODS

Bacterial cultures. The wild-type strain, *B. japonicum* I-110 ARS, is an azide-, rifampin-, and streptomycin-resistant substrain of USDA 110 (10, 11). Strain TA11 NOD$^+$ does not require tryptophan for growth and is a spontaneous prototrophic revertant of a tryptophan-requiring auxotroph, strain TA11 (8). Strain TA11, derived from the wild-type I-110 ARS strain by nitrous acid mutagenesis, is deficient in a single enzyme of the tryptophan biosynthetic pathway, indole glycerol phosphate synthetase (13). TA11 is defective in nodulation, since it does not form normal nodules from which auxotrophs can be isolated (8). The TA11 auxotroph and the TA11 NOD$^+$ revertant are on deposit at the Agriculture Research Service Culture Collection, Peoria, Ill., as B-18465 and B-18466, respectively.

So that we could compare the tryptophan-catabolic properties of the TA11 NOD$^+$ revertant of *B. japonicum* I-110 with those of the tan variants of *B. japonicum* L-259 (USDA strain 26), the L-259 strain and the variant strains B-14075 (tan 20d) and B-14077 (tan 4b) derived from L-259 were obtained from the culture collection in Peoria.

Media and culture conditions. A0E nutrient solution contained 0.65% (wt/vol) HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.55% MES [2-(*N*-morpholino)ethanesulfonic acid], 0.0067% $FeCl_3 \cdot 6H_2O$, 0.18% $MgSO_4$, 0.013% $CaCl_2 \cdot H_2O$, 0.25% Na_2SO_4, 0.32% NH_4Cl, 0.125% Na_2HPO_4, and 0.1% L-arabinose (1). The pH was adjusted to 6.8 with NaOH. Arabinose was filter sterilized and added to the autoclaved basal medium. A0E was supplemented with yeast extract (1 g/liter) to make A1E medium (9). For solid media, 1.5% (wt/vol) agar was added. Incubations were at 30°C, and broths were shaken at 130 rpm. Unless otherwise indicated, we used A1E medium for growth and maintenance of all bacteria.

Greenhouse nodulation and nitrogen fixation studies. Soybean (*Glycine max* (L.) Merr. cv. Tracy M) seeds were treated with ethanol and sodium hypochlorite to eliminate

TABLE 1. Effect of inoculum on plant dry weight and carbon and nitrogen contents

Inoculum	Avg ± SE dry wt (g)		Avg ± SE amt (g) in shoots		Avg ± SE amt (g) in roots	
	Shoots	Roots	C	N	C	N
None	1.3 ± 0.1[a]	0.8 ± 0.1	0.408 ± 0.044	0.015 ± 0.001	0.277 ± 0.029	0.012 ± 0.001
Wild type	5.2 ± 0.6	1.2 ± 0.1	2.11 ± 0.22	0.169 ± 0.016	0.433 ± 0.033	0.023 ± 0.002
TA11 NOD$^+$	6.8 ± 0.8	1.7 ± 0.2[b]	2.80 ± 0.33	0.242 ± 0.032	0.632 ± 0.065[b]	0.035 ± 0.004[b]

[a] The values shown are averages of 6 to 10 replicates. All wild-type and TA11 NOD$^+$ values differ significantly from their corresponding uninoculated-control values.
[b] Differs from the wild-type value in the same column at the 0.05 probability level.

viable rhizobia and bradyrhizobia from the seed surfaces. The seeds were then placed in plates containing autoclaved 1.5% (wt/vol) agar in tap water and incubated in the dark at 30°C for 4 days. On day 4, germinated seedlings were inoculated with wild-type or TA11 NOD$^+$ bacteria. Seedlings were incubated for 4 or 5 h more and then transferred to foil-covered, 8-liter hydroponic pots in a greenhouse. Initially, each pot received three seedlings. Each seedling was placed into one of three upright sections of polyvinyl chloride pipe cemented into holes drilled in the lid of the pot. Pipe sections were about 3.5 cm long and had an inside diameter of about 1.25 cm. Seedlings were prevented from falling into the nutrient solution by a wooden dowel (2 mm in diameter and 3 cm long) that passed perpendicularly through the PVC pipe 5 to 10 mm from its lower end. Seedlings were positioned so that the roots extended into the nutrient solution. After 2 weeks of growth, one plant (the smallest) was removed from each pot and discarded. Nutrient solution was one-fifth-strength Hoagland medium modified as indicated by replacing nitrate salts with chloride salts (2). At first, plants were placed in a nutrient solution that contained 0.38 mM nitrate. After 2 weeks, this nutrient solution was replaced with a nitrogen-free solution. Afterwards, plants received fresh solutions, once in week 2, once in week 3, and three times in week 4 of growth. Plants were harvested after 35 days in the greenhouse. Additional details concerning treatment of seeds and greenhouse conditions have been described elsewhere (2).

Plant dry weight and carbon and nitrogen contents. Plant samples were dried for 72 h in a 65°C forced-air oven. The samples were then ground to 200 mesh, and approximately 20 mg of this material was analyzed for carbon and nitrogen contents by automated combustion on a Carlo Erba NA-1500 analyzer (12). Three samples of each plant were analyzed, and the results were averaged.

Tryptophan disappearance studies. Fermentation flasks containing 25 ml of A1E medium supplemented with 39 μmol of tryptophan were inoculated and incubated at 30°C and 130 rpm. Samples were taken at the times indicated and assayed as described below.

Culture fluid tryptophan analysis. Culture fluid was centrifuged at 10,000 × *g* for 20 min to remove bacterial cells, and 20 μl of the resulting supernatant fluid was injected into an isocratic high-pressure liquid chromatograph equipped with a C-18 reverse-phase column (250 by 4.6 mm). The mobile phase buffer was 50 mM acetic acid–50 mM potassium phosphate (pH 4.5) mixed 1:1 (vol/vol) with methanol. Column effluent was monitored at 280 nm. L-Tryptophan (Sigma Chemical Co., St. Louis, Mo.) was used as a calibration standard. Peak identifications were confirmed by coinjection of the L-tryptophan standard.

IAA, ILA, and anthranilic acid analysis. Culture fluid was centrifuged at 10,000 × *g* for 20 min, and a 15-ml sample of supernatant fluid was removed, adjusted to pH 2.5 with 1.2 M HCl, and extracted three times with 5-ml volumes of ethyl acetate. Ethyl acetate fractions were combined, and the solvent was evaporated under a stream of dry nitrogen gas. Dried samples were suspended in 200 μl of methanol, and 20 μl of each was injected into an isocratic high-pressure liquid chromatograph equipped with a C-18 column (250 by 4.6 mm). The mobile phase buffer was 8.5 mM (0.5%) acetic acid in 1:1 methanol-water (vol/vol). IAA and 3-indolelactic acid (ILA) were monitored with a UV detector set at 280 nm and a fluorescence detector set at an excitation wavelength of 285 nm and an emission wavelength of 345 nm. Anthranilic acid was measured with the UV detector at 340 nm and the fluorescence detector at an excitation wavelength of 300 nm and an emission wavelength of 405 nm. IAA, ILA, and anthranilic acid (Sigma) were used as calibration standards. Peak identifications were confirmed by coinjection of the appropriate standard.

RESULTS

Growth on minimal media. We confirmed earlier observations (8) that TA11 NOD$^+$ differs from its parent, the TA11 strain, in that it is able to grow on minimal media containing no tryptophan supplement.

Greenhouse evaluation of TA11 NOD$^+$. In preliminary greenhouse studies (data not shown), plants that received the TA11 NOD$^+$ prototrophic revertant had greener foliage, higher dry weights, and higher nodule masses than did plants inoculated with the wild-type bacterial strain.

More detailed studies were conducted, and the results showed that plants inoculated with TA11 NOD$^+$ were 33% larger and contained 52% more nitrogen and 34% more carbon than did plants inoculated with the wild-type strain (Table 1). Also, plants that received the TA11 NOD$^+$ inoculum had 56% more nodules and 41% more total nodule mass than did plants that received the wild type (Table 2).

The weight of an individual nodule (nodule average weight) did not vary with treatment, indicating that the inoculum a plant received did not influence the sizes of

TABLE 2. Effect of inoculum on nodule number, mass, weight, and activity

Inoculum	Avg ± SE nodule no.	Avg ± SE nodule mass (g)[a]	Avg individual nodule wet wt (g)	Activity[b]
Wild type	134 ± 16	2.9 ± 0.2	0.023	0.043
TA11 NOD$^+$	209 ± 21[c]	4.1 ± 0.5[c]	0.020	0.044

[a] Wet weight of nodules per plant.
[b] Nitrogen fixed per gram of nodule. These values were derived by subtracting the N contents of uninoculated control plants from the N contents of treated plants and dividing by nodule mass.
[c] Differs from the wild-type value in the same column at the 0.05 probability level.

TABLE 3. Effect of tryptophan on colony size

Concn (mM) of tryptophan addition	Mean ± SE colony size[a] (mm)	
	Wild type	TA11-NOD$^+$
None	0.93 ± 0.02[b]	0.94 ± 0.03[c]
10	0.80 ± 0.01	0.77 ± 0.01[c]
20	0.60 ± 0.01	0.66 ± 0.01[d]
30	0.38 ± 0.02	0.47 ± 0.01[d]
40	0.13 ± 0.01	0.27 ± 0.00[d]

[a] Colonies were grown at 30°C for 9 days on A1E medium. Measurements were made at a magnification of ×40 with an eyepiece micrometer.
[b] The values shown are means of 10 to 30 measurements.
[c] Does not differ from the wild-type value in the same row at the 0.05 probability level.
[d] Differs from the wild-type value in the same row at the 0.001 probability level.

individual nodules. Also, the inoculum received did not affect nodule activity. With both treatments, the amount of nitrogen that accumulated per gram of nodule was about the same. These data indicate that the improvement in nitrogen fixation observed with the TA11 NOD$^+$ inoculum was due to an increase in overall nodule mass as a result of increased nodule numbers. No differences were observed in the time required for nodules to form nor in their distribution.

Sensitivity of bacterial strains to toxic amounts of tryptophan. To investigate how the wild-type and TA11 NOD$^+$ differ prototrophically and physiologically, we grew the wild-type and TA11 NOD$^+$ strains in the presence of tryptophan. Tryptophan in large amounts inhibits *B. japonicum* growth (3), and in this study, the wild-type strain was more sensitive to the effects on tryptophan than was the TA11 NOD$^+$ strain. Wild-type colonies grown on agar plates supplemented with 20 to 40 mM tryptophan were smaller than colonies of TA11 NOD$^+$ (Table 3). This effect was also seen with broth cultures, although the difference between the two strains was not as great. When incubated for 1 week in A0E medium with 30 mM tryptophan, the turbidity of wild-type cultures was reduced 82% and the turbidity of TA11 NOD$^+$ cultures was reduced 72%.

Tryptophan catabolism. Kaneshiro (patent) and Kaneshiro and Kwolek (5) reported that the tan 4b and 20d variants of *B. japonicum* L-259, with improved nodulation and nitrogen fixation, had enhanced tryptophan catabolism. Since greater resistance to tryptophan toxicity could be due to enhanced catabolism or other changes in tryptophan metabolism, we conducted two studies to determine whether or not the TA11 NOD$^+$ mutant resembles these tan variants.

The first study investigated tryptophan loss and pigment production by bradyrhizobia grown in media supplemented with tryptophan. Both the TA11 NOD$^+$ mutant and wild-type *B. japonicum* I-110, as well as the tan 4b and tan 20d variants and wild-type *B. japonicum* L-259, were included. TA11 NOD$^+$ and the wild-type I-110 strain removed similar amounts of tryptophan from the growth medium (Fig. 1A), and both accumulated only small amounts of pigment (Fig. 1B). There was no evidence suggesting that TA11 NOD$^+$ has enhanced tryptophan catabolism. In contrast, the two tan variants quickly removed large amounts of tryptophan from the medium (Fig. 2A) and rapidly produced large amounts of pigment (Fig. 2B). The L-259 parent of the tan variants removed tryptophan and produced pigment at a slower rate. These data show that the tan variants are different from both the I-110 strains and their wild-type L-259 parent with respect to tryptophan removal and pigment production.

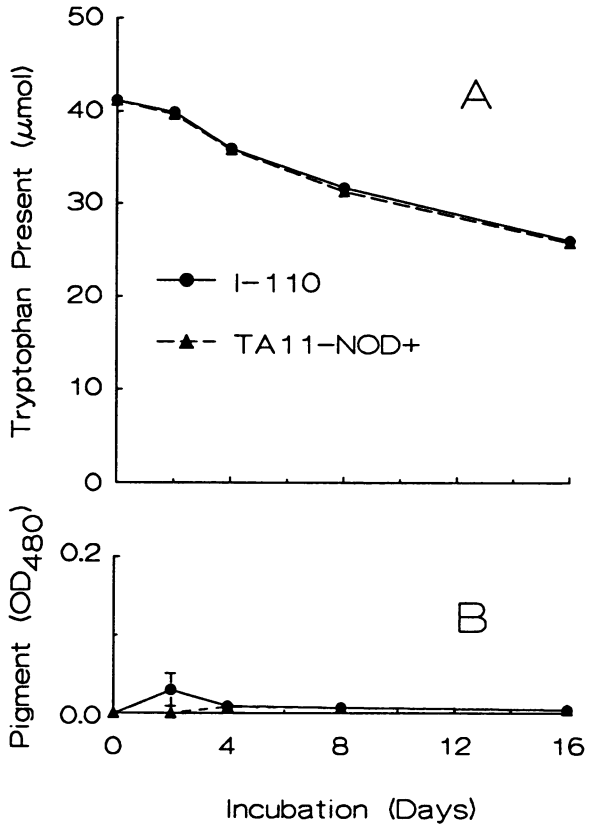

FIG. 1. Tryptophan disappearance (A) and pigment production (B) by wild-type and mutant *B. japonicum* I-110. Tryptophan loss is in micromoles per fermentation flask. Pigment production was estimated by the optical density at 480 nm (OD_{480}) of culture fluid supernatant following centrifugation at 10,000 × *g* for 20 min. The values shown are means of three replicate determinations ± the standard errors.

Also, these data confirm that the two tan strains are tryptophan-catabolic variants of L-259 (7).

Growth data, turbidity at 660 nm (data not shown), indicated that the growth rates of all strains were about the same. Thus, the differences in tryptophan removal and pigment production were not due to differences in growth.

Accumulation of tryptophan degradation products. In the second study, accumulation of IAA, ILA, and anthranilic acid in culture fluid was examined. These studies were conducted with the wild-type I-110 and TA11 NOD$^+$ strains only. The tan variants of L-259 are known to accumulate large amounts of IAA and ILA when grown on tryptophan-containing media (6, 7; Kaneshiro, patent).

In this study, IAA and anthranilic acid did not accumulate in either TA11 NOD$^+$ or wild-type I-110 cultures (Table 4). Levels of these compounds were the same as or lower than those present in the uninoculated control. ILA accumulated in approximately equal amounts in cultures of both strains. These data show that the TA11 NOD$^+$ mutant does not differ from the wild type with respect to these three accumulation products. Also, these data differentiate the TA11 NOD$^+$ strain from the tan mutants derived from L-259.

DISCUSSION

By using the host soybean plant as a means of selecting for nodulating revertants of a nodulation-defective tryptophan

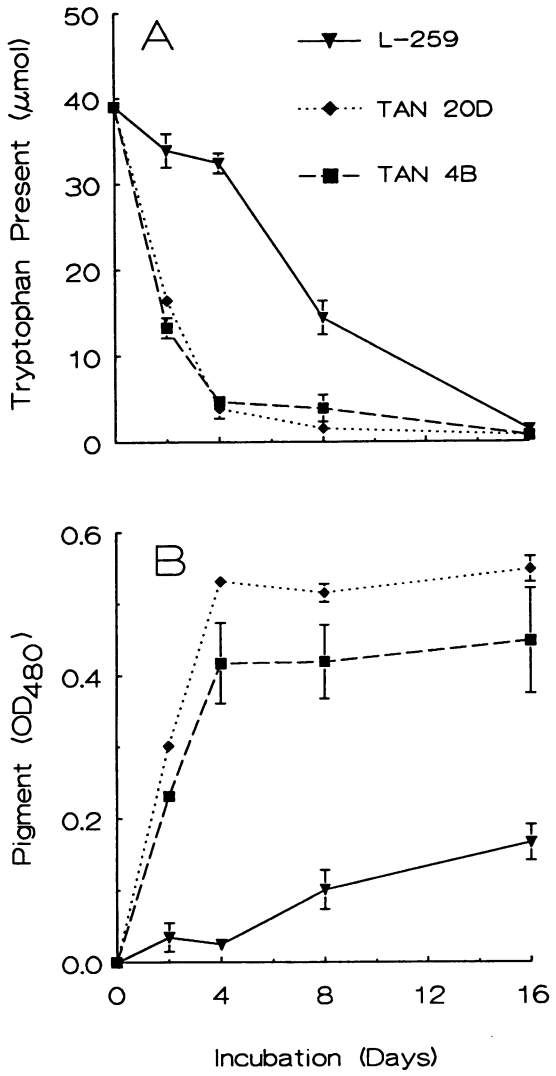

FIG. 2. Tryptophan disappearance (A) and pigment production (B) by wild-type and tan variants of *B. japonicum* L-259. The conditions used were those described in the legend to Fig. 1. OD_{480}, Optical density at 480 nm.

auxotroph, a normally nodulating prototrophic revertant of *B. japonicum* I-110 TA11 was isolated (8). The revertant resembles the wild type in that it does not require tryptophan for growth. However, greenhouse studies with this TA11 NOD^+ revertant showed that it fixed more nitrogen symbiotically than did the original wild-type strain. The increase in nitrogen fixation does not correlate with larger nodule size or greater nodule activity. Rather, it correlates with an increase in nodule mass resulting from a larger number of nodules per plant (Tables 1 and 2).

The physiological basis for this improved symbiosis is not understood, but it is suspected that TA11 NOD^+ differs from the wild type in some aspect of its tryptophan metabolism. There are two reasons for this suspicion. (i) The TA11 strain is a well-defined tryptophan auxotroph known to be defective in a single enzyme of the tryptophan pathway (13). TA11 NOD^+ is a spontaneous prototrophic revertant; therefore, it is extremely unlikely that more than a single mutation was involved in the reversion. The reversion that occurred must involve the tryptophan pathway, as the change corrected for or circumvented the enzyme deficiency that existed in the auxotroph. (ii) TA11 NOD^+ differs from the wild type in its sensitivity to tryptophan at 20 to 40 mM (Table 3).

TABLE 4. Presence of tryptophan degradation products IAA, ILA, and anthranilic acid in cultures of *B. japonicum* strains I-110 and TA11 NOD^+[a]

Strain	Avg[b] ± SE concn (nmol) of:		
	IAA	ILA	Anthranilic acid
Uninoculated control[c]	0.25 ± 0.02	0.14 ± 0.08	0.63 ± 0.13[d]
Wild-type I-110	0.32 ± 0.18	3.07 ± 0.60	0.17 ± 0.08
TA11 NOD^+	0.26 ± 0.03	3.52 ± 0.26	0.09 ± 0.02

[a] Cultures were grown for 3 weeks at 30°C in A0E medium supplemented with 20 mM tryptophan.
[b] The values shown are averages of three or four replicates (unless otherwise indicated).
[c] The uninoculated control provided a measure of the amounts of these compounds that were present as medium contaminants or as a result of natural degradation of tryptophan during incubation.
[d] This value is an average of two replicates.

While it is not clear how TA11 NOD^+ and I-110 differ physiologically, it is easy to distinguish TA11 NOD^+ from the tan variants of L-259. The tan strains are tryptophan-catabolic variants (6, 7; Kaneshiro, patent) that rapidly degrade tryptophan (Fig. 2A) and, in doing so, accumulate large amounts of IAA, ILA, and pigmented degradation products (Fig. 2B). TA11 NOD^+ showed none of these characteristics; rather, it behaved similarly to its wild-type parent in the studies on tryptophan catabolism (Fig. 1A and B), demonstrating that it is not a mutant with enhanced tryptophan catabolism. We speculate that reversion of the TA11 auxotroph resulted in more nodules per plant because of production of more nearly optimum amounts of a metabolic compound required for establishment of effective nodules.

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