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Influence of 5-Methyltryptophan-Resistant *Bradyrhizobium japonicum* on Soybean Root Nodule Indole-3-Acetic Acid Content†

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***Bradyrhizobium japonicum* mutants resistant to 5-methyltryptophan were isolated. Some of these mutants were found to accumulate indole-3-acetic acid (IAA) and tryptophan in culture. In greenhouse studies, nodules from control plants inoculated with wild-type bradyrhizobia contained 0.04, 0.10, and 0.58 μg of free, ester-linked, and peptidyl IAA g (fresh weight) of nodules^{-1}, respectively. Nodules from plants inoculated with 5-methyltryptophan-resistant bradyrhizobia contained 0.94, 1.30, and 10.6 μg of free, ester-linked, and peptidyl IAA g (fresh weight) of nodules^{-1}, respectively. This manyfold increase in nodule IAA content indicates that the *Bradyrhizobium* inoculum can have a considerable influence on the endogenous IAA level of the nodule. Further, these data imply that much of the IAA that accumulated in the high-IAA-containing nodules was of bacterial rather than plant origin. These high-IAA-producing 5-methyltryptophan-resistant bacteria were poor symbiotic nitrogen fixers. Plants inoculated with these bacteria had a lower nodule mass and fixed less nitrogen per gram of nodule than did plants inoculated with wild-type bacteria.**

It has been known for some time that the root nodules of many legumes contain more auxin than do the roots (28) and that the main auxin in root nodules is indole-3-acetic acid (IAA) (9). Some of this IAA is even transported from the nodule to other parts of the plant (3, 5, 31). Where the IAA within the nodule comes from and why such large amounts accumulate in some nodules are largely unknown. Some of the IAA present in the nodule is clearly of plant origin. Dullaart has shown that plant cells within the nodule have the enzymatic capacity to produce IAA from tryptophan (9), and tracer studies have shown that IAA formed in the plant top is transported into the nodule (1).

Both rhizobia and bradyrhizobia grown in culture can produce IAA, as well as a number of IAA precursors (2, 11, 18, 24), and there has been speculation that the bacterial endophyte may be partially responsible for the large amounts of IAA sometimes present within the nodule (4, 9, 23). Dullaart has suggested that the bacterial endophyte, rather than producing IAA itself, stimulates the host plant cells to produce IAA and that the bulk of the IAA present in the nodule is formed by the plant (9). However, there is little direct evidence that the nodule endophyte can produce or influence the production of IAA in the nodule.

In many plant tissues only a portion of the IAA present is free IAA. Often, much of the IAA is bound or conjugated, by ester or peptidyl bonds, to other compounds (6). Little information exists on the bound IAA content of legume root nodules.

Is the bacterial endophyte responsible for the high level of IAA in the nodule? In this study the influence of the *Bradyrhizobium japonicum* inoculum on the amount of bound and free IAA present in soybean root nodules was examined. To evaluate the effect of the inoculum on nodular IAA levels, *B. japonicum* clones that produce different amounts of IAA were needed. Often, bacterial isolates resistant to the toxic tryptophan analog 5-methyltryptophan (5-MT) constitutively overproduce tryptophan pathway products (12, 27). Since many rhizobia produce IAA from tryptophan (10, 11, 25), it was felt that bradyrhizobia resistant to 5-MT might produce increased amounts of IAA. For this reason, several clones of *B. japonicum* resistant to 5-MT were isolated and evaluated for their ability to accumulate IAA in culture and for their influence on nodular IAA levels. Also, the symbiotic performance of these clones was examined because there has been a report (17) of increased symbiotic nitrogen fixation by a high-IAA-producing mutant of *B. japonicum*.

MATERIALS AND METHODS

Plant material. Treatment of seeds, plant growth, and greenhouse conditions have been described in detail elsewhere (13). Soybean (*Glycine max* (L.) Merr. cv. Tracy) seeds were treated with ethanol and sodium hypochlorite to eliminate viable rhizobia from the seed surface. Seeds were germinated and inoculated on agar plates before being transferred to hydroponic pots in the greenhouse. Inocula were wild-type *B. japonicum* or 5-MT-resistant clones of *B. japonicum* (described below). Greenhouse pots contained 8 liters of one-fifth strength Hoagland medium modified by replacing the nitrate salts with chloride salts. A 0.75 mM nitrate concentration was used unless otherwise indicated.

Bacteria. Wild-type cultures of *B. japonicum* I-110, a substrain of USDA 110 (21), were obtained from G. H. Elkan, Department of Microbiology, North Carolina State University, Raleigh. The wild-type cultures were maintained on a yeast extract-mannitol medium (7), supplemented with 1.5% agar when indicated. Cultures were incubated at 28°C and stored at 4°C. Broth cultures were grown in shaken (100 rpm) 125-ml flasks containing 50 ml of medium. Resistant clones were isolated by spreading stationary-phase wild-type cells on yeast extract-mannitol agar plates supplemented with 5 mg of 5-MT ml^{-1}. Before final isolation, each clone was plated onto and isolated from 5-MT plates (5 mg ml^{-1}) three times.

To confirm that each clone was *B. japonicum*, the clones were tested for their ability to form acetylene-reducing

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nodules on soybean plants. These studies were conducted in a plant growth room. Soybean seedlings, surface treated and germinated as described above, were placed into plant growth pouches (one per pouch; American Scientific Products) each containing 100 ml of one-fifth strength Hoagland nitrogen-free medium. Plants were inoculated with 0.2 ml of a late-log-phase culture. Each inoculum was tested on six or more plants. Nutrient solution levels were adjusted daily, and after the first week of growth, the solutions were changed on alternate days. After 21 days of growth, a glass tube was inserted into the pouch, the pouches were sealed with plastic tape and plastic duct sealant, the nutrient solution was removed (by aspiration through the glass tube), the open end of the glass tube was closed with a serum stopper, and 50 ml of a 10% mixture of acetylene and air was injected around the roots. Samples (1 ml) of the pouch atmosphere were taken after 0, 5, 10, and 20 min of incubation. The samples were analyzed for ethylene with a gas chromatograph equipped with a Porapak N column (2 mm by 2 m) and a flame ionization detector. Plants in pouches showing increasing amounts of ethylene with time were marked positive for acetylene reduction. No attempt was made to quantitate the amount of acetylene reduced under these assay conditions. Growth chamber temperatures were 28°C (day) and 23°C (night), and a 16-h photoperiod was used. Light was supplied at a 400-microeinstein m^{-2} s^{-1} photosynthetic photon flux density as previously described (15).

Generation time. A_{660} was used to estimate the generation time of log-phase cells.

Total N determinations. Total N was used as a measure of the relative amount of nitrogen fixed by greenhouse-grown plants. Soybean plants were treated and grown in hydroponic greenhouse pots as described above except that the plants were grown on an N-free medium after the first 2 weeks of growth. Whole plants were analyzed for total N as described by Keeney and Nelson (19).

IAA analysis. IAA in bacterial culture solutions was assayed in the following manner. Culture fluid was centrifuged at 3,000 × *g* for 30 min, 0.5 ml of the supernatant fluid was removed, 10 μg of indole-3-propionic acid was added as an internal standard, and the pH was adjusted to 2.5 with 12 N H_2SO_4. The samples were extracted three times with 0.7- to 0.8-ml volumes of CH_2Cl_2. The CH_2Cl_2 washings were combined, the solvent was evaporated under a stream of dry nitrogen, and the samples were suspended in 0.2 ml of dry CH_2Cl_2 and again dried under nitrogen. Any remaining water was removed by placing the samples, in open vials, in an evacuated desiccator for 4 h. Dried samples were derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA) and analyzed by gas chromatography (15).

The methods used to extract and assay both free and bound nodule IAA and to hydrolyze bound IAA have been described in detail elsewhere (15). Nodules were homogenized with acetone and water, extracted for 24 h, and centrifuged to remove solids. The acetone was evaporated, and samples were suspended in water. For bound IAA determinations, one portion of each sample was hydrolyzed with NaOH. No hydrolysis was required for free IAA determinations. Both bound and free IAA samples were partitioned into ether, into 1 N $NaHCO_3$, and back into ether. The samples were then dried, derivatized with BSTFA, and analyzed by gas chromatography. Indole-3-propionic acid, added during homogenization, was used as an internal standard.

Tryptophan and anthranilic acid analysis. The tryptophan and anthranilic acid contents of bacterial culture fluids were determined spectrofluorometrically. Samples (1 ml) of culture fluid were centrifuged at 8,000 × *g* for 30 min at 4°C. The supernatant fluid was diluted as necessary and examined fluorometrically at excitation wavelengths of 280 and 340 nm and emission wavelengths of 350 and 385 nm for tryptophan and anthranilic acid, respectively. Additionally, a peak for anthranilic acid appeared on the gas chromatograms used for the IAA analysis described above.

RESULTS

Wild-type *B. japonicum* cells were inoculated onto agar plates containing 5 mg of 5-MT ml^{-1}, and 10 *B. japonicum* clones resistant to 5-MT were isolated. To assure that each isolate was *B. japonicum* and not an unwanted contaminant, nodulation and acetylene reduction studies were conducted with plants grown in plant growth pouches. All clones were found to form nodules capable of reducing acetylene.

Resistance was associated with changes in colony morphology. One clone (no. 4) formed colonies that were wild type in appearance (i.e., mucoid colonies with a circular shape, convex profile, and entire edge). The other clones produced less mucus and formed colonies with an irregular shape, umbonate profile, and undulate edge.

B. japonicum clones resistant to 5-MT were resistant to high levels of L-tryptophan. In the normal medium, all of the 5-MT-resistant clones and the wild type grew with a generation time of about 15 h. The addition of large amounts of L-tryptophan (8 mg ml^{-1}) to this medium had almost no effect on the generation times of most of the 5-MT-resistant clones but increased the generation times of wild-type and clone 4 cells to 41 and 24 h, respectively.

During the study, several of the clones (no. 2, 7, 8, and 9) removed large amounts (80 to 90%) of the tryptophan from the high-L-tryptophan medium. Associated with this loss of tryptophan was an accumulation of 0.6 to 1.0 mg of anthranilic acid ml^{-1}. Although conclusive evidence does not exist (no tracer or enzymatic studies were conducted), these observations are consistent with the formation of anthranilic acid as a degradation product of tryptophan, rather than its accumulation from the biosynthetic pathway. *Rhizobium meliloti* has been observed to convert tryptophan to anthranilic acid by an unknown pathway (16).

Cultures of many of the clones were observed to accumulate IAA, and two clones (no. 3 and 7) were selected for further study. Both tryptophan and IAA accumulated in cultures of these clones grown on the normal medium (Fig. 1). The addition of tryptophan to the medium increased the accumulation of IAA (Fig. 2). No accumulations of either IAA or tryptophan were observed with wild-type or clone 4 cultures (data not shown).

Greenhouse studies, conducted with clones 3 and 7 and the wild type, showed that the inoculum strongly influenced the amount of free and bound IAA present in the nodules (Table 1). Nodules from plants inoculated with clones 3 and 7 had from 12 to 24 times more free IAA and from 18 to 56 times more total bound IAA than did nodules from plants inoculated with wild-type *B. japonicum*.

Although some nitrogen was fixed by the 5-MT-resistant clones, differences from wild-type levels were evident. Plants inoculated with clone 3 or 7 were stressed from lack of nitrogen and had lower dry weights, less total N, and less nodule mass than did plants inoculated with the wild-type control. Clone 3 formed about the same number of nodules as did the wild type, but the clone 3 nodules were smaller.

Clone 7 formed fewer but larger nodules than the wild type did (Table 2).

DISCUSSION

Wild-type *B. japonicum* I-110 was naturally tolerant to high levels of 5-MT. With many other bacteria, only small amounts of 5-MT are required to prevent growth and to select for resistant clones (12, 22, 27). This natural tolerance of wild-type *B. japonicum* may have been due to low tryptophan (and presumably low 5-MT) uptake from the growth medium (30). Large amounts (5 mg ml^{-1}) of 5-MT were required to prevent wild-type growth and to select for resistant clones.

B. japonicum clones 3 and 7, which were resistant to 5-MT, were found to accumulate tryptophan in culture (Fig. 1 and 2), a common feature with bacteria resistant to 5-MT (12, 27) and an indicator that the regulation of the tryptophan biosynthetic pathway had been genetically altered (27). Also, *B. japonicum* clones resistant to 5-MT were resistant to high concentrations of L-tryptophan, and some showed an enhanced ability to catabolize tryptophan.

With soybeans, the free IAA content of the wild-type root nodule was estimated to be 0.04 to 0.06 μg g (fresh weight)$^{-1}$. This is in close agreement with an earlier estimate by Kefford et al. (20). Root nodules of many other plants contain more IAA. Nodules from *Pisum sativum*, *Phaseolus vulgaris*, *Vicia faba*, and *Lupinus luteus* (8, 14, 31) appear to contain several times more free IAA (0.25 to 0.50 μg g [fresh weight]$^{-1}$), and nodules of several leguminous trees (*Erythrina indica*, *Sesbania grandiflora*, and *Pterocarpus sautalinus*) contain almost 1,000 times more free IAA (41 to 65 μg g [fresh weight]$^{-1}$) (4).

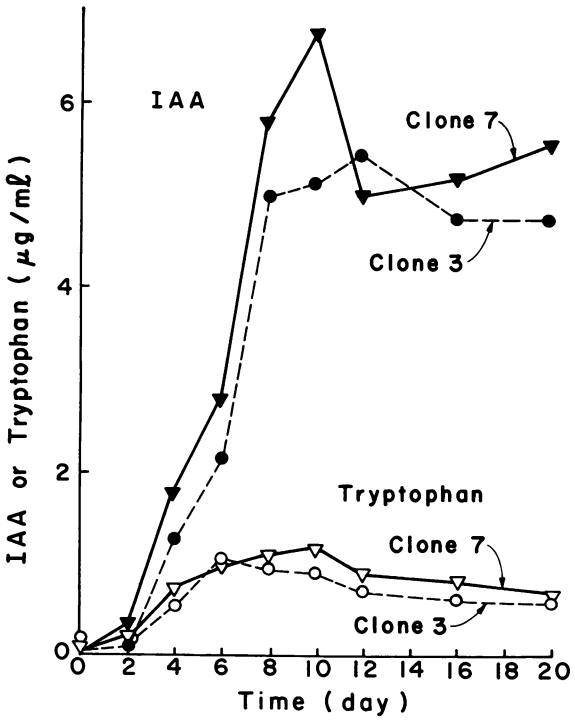

FIG. 1. IAA and tryptophan production by clones 3 and 7 grown on yeast extract-mannitol medium without supplemental tryptophan. The culture and analytical conditions are described in Materials and Methods. Each value is an average for three independent samples.

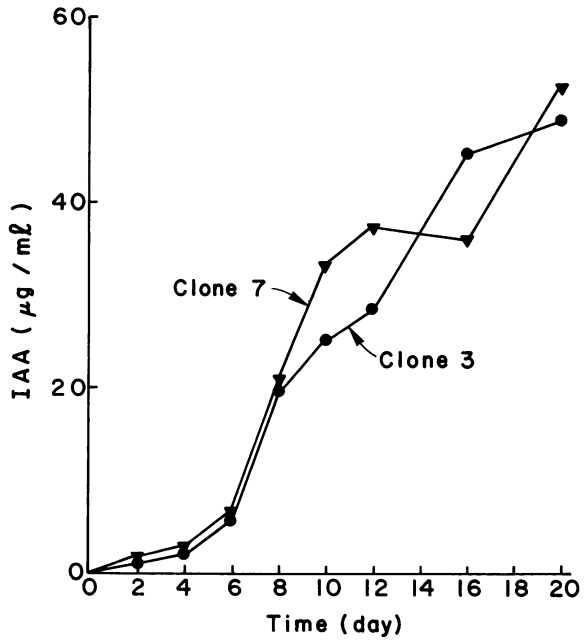

FIG. 2. IAA produced by clones 3 and 7 grown on medium supplemented with 8 mg of tryptophan ml^{-1}. The culture broth was yeast extract-mannitol medium supplemented with 8 mg of L-tryptophan ml^{-1}. Other culture and analytical conditions are described in Materials and Methods. Each value is an average for three independent samples.

There is little published information on the amount of bound IAA present in legume root nodules. Kefford et al. (20) suggested that IAA conjugates might be present in the nodule. Badenoch-Jones et al. (1, 3) examined *P. sativum* nodules fed [^{3}H]IAA and found that 51 to 57% of the label was recovered as indole-3-acetyl aspartic acid. Only small amounts of label (1.3 to 2.6%) were recovered as free IAA. Another study (14) indicates that about 68% (1.4 μg g [fresh weight]$^{-1}$) of the IAA in *P. vulgaris* nodules and about 74% of the IAA in *P. sativum* nodules were bound. In *G. max*

TABLE 1. Effect of inoculum on nodule IAA level[a]

Inoculum	Amt of IAA (μg g [fresh weight]$^{-1}$)		
	Free	Ester[b]	Peptidyl[c]
Expt 1			
Wild type	0.04 ± 0.02[d]	0.10[e]	0.58 ± 0.13
5-MT resistant (clone 7)	0.94 ± 0.32	1.3 ± 0.1	10.7 ± 0.3
Expt 2			
Wild type	0.06 ± 0.02		0.19 ± 0.03
5-MT resistant			
clone 3	0.94 ± 0.08		10.6 ± 0.5
clone 4	0.12 ± 0.04		0.18 ± 0.01
clone 7	0.72 ± 0.28	2.1 ± 0.1	10.6 ± 0.5

[a] Plants were grown in greenhouse hydroponic pots and harvested at 7 weeks of age.

[b] Ester IAA values = IAA detected after hydrolysis in 1 N NaOH; includes free IAA.

[c] Peptidyl IAA = IAA detected after hydrolysis at 100°C in 7 N NaOH; includes free and ester IAA.

[d] Values are averages of two to six analyses ± standard error. Nodules from two to four plants were combined for each analysis.

[e] Value is from a single analysis.

TABLE 2. Effect of inoculum on plant total N and nodule formation

Inoculum	Plant[a]		Nodule	Avg wt nodule^{-1}[b] (mg)
	Dry wt (g)	Total N (mg)	No.	
Wild type	4.7 ± 0.5[c]	138 ± 14	151 ± 21	14.6
5-MT resistant (clone 7)	3.0 ± 0.5	64 ± 14	21 ± 4	52.5
5-MT resistant (clone 3)	2.6 ± 0.4	53 ± 11	158 ± 27	9.3
None	1.9 ± 0.2	23 ± 1		

[a] Plants were grown in greenhouse hydroponic pots and harvested at 6 weeks of age.
[b] Wet weight.
[c] Values are averages of six to nine analyses ± standard error.

nodules, with wild-type inoculum, the bulk of the IAA (0.13 to 0.54 μg g [fresh weight]$^{-1}$ or 63 to 92% of the total) was present as bound IAA (Table 1). While most of this IAA was present as peptidyl IAA, ester-linked IAA was also present. Thin-layer chromatography has indicated that indole-3-acetyl aspartic acid is one of several IAA conjugates present in *G. max* nodules (14).

The present study demonstrated that the bacterial endophyte plays an important role in determining the amount of IAA, both bound and free, present in the root nodule. The effect that the different *Bradyrhizobium* inocula had on IAA levels within the nodule was dramatic. Inoculation with *B. japonicum* clones that produce increased amounts of IAA in culture resulted in nodules with greatly increased amounts of both bound and free IAA. This correlation between IAA production in culture and IAA formation within the nodule implies that the bacterial endophyte was directly responsible via bacterial pathways for the production of much of the increased IAA. Although this hypothesis seems most probable, there are several other mechanisms by which the *Bradyrhizobium* endophyte might influence nodular IAA levels. As Dullaart (9) suggested, the bacterial endophyte might stimulate plant cells to produce more IAA. Bacterial production of IAA precursors or other compounds that act to induce or activate plant enzymes could stimulate IAA production in the nodule. Also, the nodule endophyte might act in a manner analogous to its close relative *Agrobacterium tumefaciens*. In *A. tumefaciens*, virulence involves the transfer of bacterial DNA into the plant genome (29). Regions of this DNA are involved in IAA biosynthesis (26).

Plants inoculated with clone 3 or 7 were poor nitrogen fixers. These plants had low nodule mass, low nitrogen accumulation per gram of nodule, and, with clone 7, low numbers of nodules (Table 2). These data indicate that nodule initiation and development were altered. These alterations may have been due to the large amounts of IAA present, but other metabolic changes are associated with 5-MT resistance, and one or more of these changes, rather than the amount of IAA present, may have altered the symbiotic performance of these bacteria.

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