

Isolation and Characterization of Rhizobitoxine Mutants of *Bradyrhizobium japonicum*†

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To explore the role of rhizobitoxine in *Bradyrhizobium*-legume symbiosis, 11 rhizobitoxine mutants of *B. japonicum* USDA61 were isolated on the basis of their inability to synthesize the toxin in culture. Each mutant is prototrophic and symbiotically effective on soybean, cowpea, siratro, and *Glycine soja*. The rhizobitoxine mutants differ in their chlorosis phenotypes and rhizobitoxine production in planta. As expected, one group of mutants fails to make toxin in planta, resulting in the absence of chlorosis. Another group of mutants causes severe chlorosis on all cultivars of soybean tested. Surprisingly, this group of mutants makes more rhizobitoxine in soybean nodules than the wild-type strain does. This phenotype is only observed on soybean and not on other hosts such as cowpea, siratro, or *G. soja*. The remaining mutants all produce rhizobitoxine in planta but vary in the amount of toxin they produce and the severity of chlorosis they induce in soybean plants. Biochemical analysis of mutants demonstrates that one mutant is unable to synthesize serinol, a molecule hypothesized to be an intermediate in rhizobitoxine biosynthesis. By using these mutants, it was found that rhizobitoxine plays no apparent role in the nodulation of *rj₁* soybeans. Recently, it was found that inhibition of ethylene biosynthesis allows *Rhizobium meliloti* to overcome nitrate inhibition of nodule formation on alfalfa. Because rhizobitoxine also inhibits ethylene biosynthesis, we tested the ability of mutants which accumulate high levels of toxin in planta to overcome nitrate inhibition of nodule formation on soybean plants and found that the nodule formation induced by the wild type and that induced by mutant strains were equally suppressed in the presence of nitrate.

There are three types of symbiosis: commensalistic, parasitic, and mutualistic. The symbiotic association of *Bradyrhizobium* species with host legumes is considered to be a mutualistic relationship because both symbionts benefit from the interaction. In this mutualistic symbiosis, the bacteria invade the host root, inducing the formation of a nodule by synthesis of specific lipo-oligosaccharides (22, 38). The bacteria infect specific plant nodule cells where they differentiate into nitrogen-reducing bacteroids. The bacteria provide the plant host with reduced nitrogen, and the plant in turn provides a suitable environment and energy source for reduction of atmospheric nitrogen. However, in some instances, this symbiosis takes on characteristics of a parasitic association and is characterized by foliar chlorosis. This parasitic association occurs between certain combinations of bradyrhizobia and cultivars of soybean and is highly dependent upon the host genotype (8, 16). This phenomenon illustrates an interesting situation in which the genotype of the host determines whether the association with the bacteria will be mutualistic or parasitic.

The bradyrhizobia that cause foliar chlorosis synthesize the phytotoxin rhizobitoxine (30, 31). Strains which synthesize the toxin are a distinct group of *Bradyrhizobium japonicum* on the basis of DNA sequence homology, exopolysaccharide composition, antibiotic sensitivity, and hydrogenase activity (4, 14, 19, 24). Despite the potentially deleterious effects on plants, rhizobitoxine-producing strains are common among the *Bradyrhizobium* species, with 56% of 93 strains from diverse geographical areas testing positive for rhizobitoxine production (20).

The mechanism by which rhizobitoxine causes chlorosis in plants has not been fully explored. As a structural analog of cystathionine, rhizobitoxine has been shown in vitro to inhibit β -cystathionase, an enzyme involved in the methionine biosynthetic pathway in both plants and bacteria (11, 29, 34), and this activity may be the cause of chlorosis. However, another possible cause could be inhibition of an enzyme directly involved in chlorophyll biosynthesis.

One metabolic activity of rhizobitoxine which makes it particularly interesting for study in symbiosis is its ability to inhibit 1-aminocyclopropyl-1-carboxylic acid synthase which catalyzes the rate-limiting step in ethylene biosynthesis (41). Ethylene has long been associated with the induction of plant defense responses and is deleterious to nodule formation and function (5, 12, 13). There have also been reports that inhibition of ethylene biosynthesis can result in a significant increase in nodule number on both wild-type and mutant plants (10, 32, 42). In addition, an ethylene biosynthesis inhibitor structurally similar to rhizobitoxine allowed some suppression of the nitrate inhibition of nodule formation on alfalfa (23). Therefore, synthesis of an ethylene biosynthesis inhibitor may benefit this symbiotic interaction.

Rhizobitoxine is also synthesized by a broad-host-range bacterial plant pathogen *Pseudomonas andropogonis* (27, 28). In this symbiotic interaction, rhizobitoxine is the causative agent of the disease symptom of chlorosis and is believed to be a factor in the broad host range of the pathogen. This poses an interesting question regarding the role of the toxin in these two different symbiotic plant-bacterial associations and the evolutionary relationship of the production of rhizobitoxine by these two bacteria.

There have been several studies attempting to characterize the role of rhizobitoxine production in *Bradyrhizobium*-soybean symbiosis. These studies have relied on the comparison of rhizobitoxine-producing and rhizobitoxine-nonproducing strains of *B. japonicum*. The significance of

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TABLE 1. Bacterial strains

| Strain | Relevant phenotype ^a | Source or reference |
|-----------------------|--|---------------------|
| <i>B. japonicum</i> | | |
| USDA110 | Wild type Rtx ⁻ | W. D. Bauer |
| USDA61 | Wild type Km ^s Rtx ⁺ | L. D. Kuykendall |
| RX17E | USDA61 Km ^r Rtx ^{-/-} | This work |
| RX18E | USDA61 Km ^r Rtx ^{-/-} | This work |
| RX24E | USDA61 Km ^r Rtx ^{-/-} | This work |
| RX26F | USDA61 Km ^r Rtx ^{-/-} | This work |
| RX21D | USDA61 Km ^r Rtx ^{-/+} | This work |
| RX28C | USDA61 Km ^r Rtx ^{-/+} | This work |
| RX20A | USDA61 Km ^r Rtx ^{-/+} | This work |
| RX20B | USDA61 Km ^r Rtx ^{-/+} | This work |
| RX17G | USDA61 Km ^r Rtx ^{-/+} | This work |
| RX19E | USDA61 Km ^r Rtx ^{-/+} | This work |
| RX19H | USDA61 Km ^r Rtx ^{-/+} | This work |
| <i>E. coli</i> WA803 | (pGS9::Tn5 Cm ^r Km ^r) | 37 |
| <i>S. typhimurium</i> | | W. C. Swoager |

^a The superscript preceding the slash mark indicates rhizobitoxine production in culture, and the superscript following the slash mark indicates rhizobitoxine production in planta in soybean cultivar Lee.

these studies is weakened by the fact that producing and nonproducing strains are in fact different species with numerous differences as indicated above. A better analysis of the role of rhizobitoxine requires constructing rhizobitoxine mutants whose symbiotic phenotype can be compared with that of the parental strain. To explore the importance of rhizobitoxine in bacterial-plant interactions, we developed a sensitive enzyme assay for the detection of rhizobitoxine (34). In this study, we used the assay to isolate 11 rhizobitoxine mutants of *B. japonicum*, and an initial characterization of these mutants and their symbiotic phenotypes is presented.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* was grown in LB medium (35) and *Salmonella typhimurium* was grown in M9 minimal medium (35) with glucose at 37°C. *B. japonicum* was maintained on and cultured in YEMG medium (32) without calcium, YEMG supplemented with 1% Casamino Acids, or NM minimal medium (9) at 28 to 30°C. Antibiotics were added to media at the following concentrations: 50 µg of kanamycin (Km) per ml and 100 µg of ampicillin (Ap) per ml for *E. coli* and 150 µg of kanamycin per ml and 300 µg of streptomycin (Sm) per ml for *B. japonicum*.

Rhizobitoxine assay. Determination of rhizobitoxine content was done by using an assay based on the inhibition of the *S. typhimurium* β-cystathionase (29) as previously described (34). Briefly, the products of the reaction are homocysteine, ammonia, and pyruvate. Pyruvate can be detected by using dinitrophenylhydrazine to produce a brown reaction product. For rhizobitoxine determinations, all samples were done in triplicate and variation was always less than 10%.

Isolation of rhizobitoxine mutants. Tn5 mutagenesis of *B. japonicum* USDA61 was performed essentially as described previously (33) except that TY agar plates were used for bacterial mating and YEMG medium was used for *B. japonicum* culture. Tn5 was introduced by using pGS9, and *E. coli*

cells were counterselected by using streptomycin. Strain USDA61 Km^r Sm^r colonies were picked and reinoculated on 15-cm petri plates with selective medium in a grid pattern designed for the convenience of transferring and culturing the colonies directly in 96-well microtiter plates. To grow cultures of Tn5-mutagenized *B. japonicum*, each well of a microtiter plate was filled with 100-µl aliquots of the selective medium and inoculated from the collected insertional strains by use of a pronged replica plating device. The inoculated microtiter plates were incubated at 30°C for 6 to 8 days, and bacterial growth was monitored spectroscopically at 600 nm with a microtiter plate reader. Strains unable to produce rhizobitoxine in culture were screened by using the rhizobitoxine enzyme assay as previously described (34). *B. japonicum* USDA61 and USDA110 were used as positive and negative controls for rhizobitoxine production, respectively. Putative mutants from an initial screen were cultured in test tubes and verified as rhizobitoxine-negative (Rtx⁻) mutants by using both bacterial cultures and culture supernatants.

Plant materials and plant tests. *Glycine max* (soybean) varieties used in this study were Lee, Williams, and Harosoy rj₁ SNIC631889. Other hosts used were *Glycine soja* (obtained from D. P. S. Verma, plant introduction unknown), *Vigna unguiculata* (cowpea cultivar California Blackeye), and *Macroptilium atropurpureum* (siratro, obtained from G. Stacey, plant introduction unknown). Seeds were surface sterilized by treatment with 10% sodium hypochlorite for 5 min and a thorough washing in sterile water and were germinated in a moistened tray for 1 to 2 days at 25°C. Germinated seeds were transferred to modified Leonard jars constructed from Magenta boxes (Magenta Co., Chicago, Ill.) containing vermiculite or into plastic growth pouches (Northrup King Seed Co., Minneapolis, Minn.). Plants were watered with sterile Jensen's nitrogen-free solution (40). Bacterial inocula were grown for 5 to 7 days in YEMG medium supplemented with the proper antibiotics to log phase and diluted with sterile distilled water to a density of about 10⁸ cells per ml. Inoculum volumes for plants in Leonard jars and growth pouches were 1.0 and 0.1 ml per plant, respectively. Plants were grown in a plant growth chamber with 400 microeinsteins of light with a light-dark cycle of 16 and 8 h and 28 and 18°C. Nodules were scored visually, and chlorosis induction was scored beginning at 2 weeks postinoculation.

Isolation of bacteria from nodules. Between 10 and 12 nodules were excised with some root tissue from plants inoculated with various *Bradyrhizobium* strains. Excised nodules were washed once with 70% ethanol for 30 s, surface sterilized by treatment with 0.1% mercuric chloride for 3 to 5 min, and washed at least 10 times with sterile distilled water. Nodules were placed individually into microtiter plate wells and crushed by using a glass rod. One hundred microliters of sterile distilled water was added to the crushed tissue to suspend the released bacteroids. The suspension was serially diluted and plated on YEMG agar plates with and without selective antibiotics. No fewer than 15 individual colonies from each reisolation were picked and tested for rhizobitoxine production in culture, and 3 of those colonies were further tested on plants for induction of chlorosis.

Isolation and purification of rhizobitoxine. Isolation and purification of rhizobitoxine from the cultures of *B. japonicum* were done by a modification of the procedure previously described (31). A 100-ml stationary-phase culture grown in YEMG medium was centrifuged, and the resulting supernatant was filtered through a 0.25-µm-pore-size nylon

filter. The filtrate was passed over a Dowex-50W column (2.5 by 30 cm), with the resin in the NH_4^+ form, at a flow rate of 2 ml/min. The column was washed with 10 volumes of distilled water, and rhizobitoxine was eluted with 0.1 M NH_4OH at the same elution rate as that described above. Upon the pH change, 50 ml of eluate was collected and the sample was dried by vacuum distillation. Pellets were resuspended in 5 ml of deionized water for use in the rhizobitoxine enzyme assay. Extraction of rhizobitoxine from fresh nodules formed in association with various *B. japonicum* strains was done by using the rhizobitoxine assay described previously (34). The amount of rhizobitoxine in a 50- μl sample of culture or nodule extract was determined by comparison with a standard curve of authentic rhizobitoxine. There is no direct method to estimate the efficiency of the extraction; however, the amounts estimated from the assay were always proportional to the severity of chlorosis.

TLC. Nodule extracts prepared for rhizobitoxine assays were concentrated an additional 20-fold, spotted onto silica plates, and dried by using a heating plate. For one-dimensional and the first dimension of two-dimensional thin-layer chromatography (TLC), samples were separated by using a mobile phase of a mixture of *n*-propanol-34% ammonia (7:3). For the second dimension, plates were air dried and developed by using a mixture of *n*-butanol-glacial acetic acid-water (12:3:5) at a right angle to the first. Plates were stained for primary amines with 0.3% ninhydrin in 95% ethanol. Amino acid standards were purchased from Sigma Chemical Co., St. Louis, Mo., and authentic rhizobitoxine was a generous gift from Robin Mitchell, Auckland, New Zealand.

RESULTS

Isolation of rhizobitoxine mutants. To isolate rhizobitoxine mutants of strain USDA61, Tn5 transconjugants were selected as Km^r and Sm^r colonies of USDA61. The transposition frequency of Tn5 was 7×10^{-7} per recipient. Approximately 3×10^3 Km^r and Sm^r colonies were picked and screened for rhizobitoxine production in culture by using the enzyme assay. Putative rhizobitoxine mutants from the initial screening were subcultured and their rhizobitoxine phenotypes were verified by using both bacterial cultures and culture supernatants in the enzyme assay. Eleven mutants were isolated, and results from the enzyme assay performed on these rhizobitoxine mutants are shown in Fig. 1. None of these mutants produced rhizobitoxine at a detectable level in YEMG culture. To ensure that our rhizobitoxine mutants do not make rhizobitoxine at reduced amounts, bacterial culture supernatants were concentrated 20-fold and assayed for rhizobitoxine. The concentrated cultures showed no rhizobitoxine activity. Because each of these strains failed to produce rhizobitoxine in culture, these strains were designated Rtx^- (Table 1).

Auxotrophic phenotype of the Rtx^- mutants. Although the Rtx^- mutants fail to make rhizobitoxine in a rich medium, it is possible that the Rtx^- phenotype is an indirect effect of auxotrophy. Growth of all mutants on the NM medium indicated that each of the Rtx^- mutants is prototrophic.

Chlorosis and rhizobitoxine production phenotypes on a sensitive soybean cultivar. Chlorosis has previously been used as a criterion for determining rhizobitoxine production by bacterial strains (6-8, 30, 31). Some soybean cultivars such as Lee are particularly sensitive to chlorosis. While there can be great variability in the extent of chlorotic symptoms depending on the growth conditions, chlorosis is normally observed on newly-forming leaves at 2 to 6 weeks

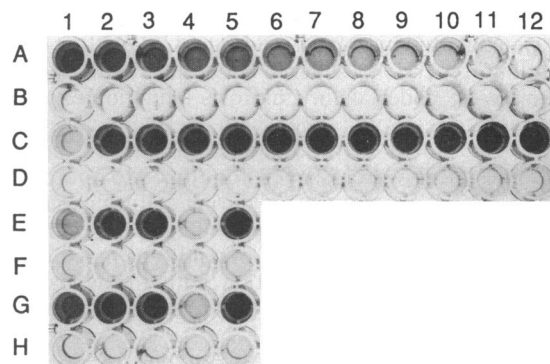


FIG. 1. Measurements of rhizobitoxine in culture supernatants and nodule extracts. Row A, standard inhibition curve with authentic rhizobitoxine in amounts of 0, 0.015, 0.075, 0.15, 0.75, 1.5, 7.5, 15, 75, 150, 750, and 1,500 ng (A1 to A12, respectively); row B, controls without cystathionine substrate corresponding to row A; row C, culture supernatants of USDA61, RX18E, RX17E, RX17G, RX19H, RX19E, RX21D, RX20B, RX20A, RX28C, RX26F, and RX24E (C1 to C12, respectively); row D, control reactions without cystathionine corresponding to the samples in C1 to C12; row E, eluates of extracts from nodules formed on cultivar Lee by strains USDA61, RX18E, RX17E, RX17G, and USDA110 (E1 to E5, respectively); row F, control reactions without cystathionine corresponding to the samples in row E; row G, eluates of extracts from nodules formed on cultivar Williams formed by strains USDA61, RX18E, RX17E, RX17G, and USDA110 (G1 to G5, respectively); row H, controls without cystathionine corresponding to the samples in G1 to G5.

after inoculation, usually on the third trifoliolate leaves (17). Under our experimental conditions, chlorosis appeared on the third trifoliolate leaves of the susceptible soybean cultivar Lee inoculated with strain USDA61. Therefore, each Rtx^- mutant was tested for chlorosis production on soybean cultivar Lee (8). As expected, 4 of the 11 Rtx^- mutants failed to cause chlorosis on cultivar Lee (Table 2). Unexpectedly, the other seven Rtx^- mutants caused chlorotic symptoms equal to or greater than those of the parental strain on

TABLE 2. Chlorosis phenotypes and rhizobitoxine production in soybean

| Strain | Chlorosis ^a | | Rhizobitoxine in nodules ^b ($\mu\text{g/g}$ [fresh wt]) | |
|---------|------------------------|----------|--|-----------------|
| | Lee | Williams | Lee | Williams |
| USDA110 | - | - | 0.01 | 0.06 |
| USDA61 | ++ | - | 96.0 | 15.0 |
| RX17E | - | - | 0.02 | 0.08 |
| RX18E | - | - | 0.03 | 0.09 |
| RX24E | - | - | 0.8 | ND ^c |
| RX26F | - | - | 11.0 | 4.2 |
| RX21D | ++ | - | 104.0 | 6.4 |
| RX28C | ++ | - | 100.0 | 3.6 |
| RX20A | ++ | + | 80.0 | 60.0 |
| RX20B | ++ | + | 52.0 | 80.0 |
| RX17G | +++ | ++ | 200.0 | 128.0 |
| RX19E | +++ | ++ | 160.0 | 112.0 |
| RX19H | +++ | ++ | 208.0 | 104.0 |

^a Degrees of chlorosis symptom scored as follows: -, no chlorosis; +, slight chlorosis; ++, moderate chlorosis; +++, severe chlorosis.

^b Rhizobitoxine measured in eluates of nodule extracts.

^c ND, not determined.

cultivar Lee. To ensure that these results were not the result of cross-contamination from wild-type USDA61 or the result of mutant reversion, several additional experiments and controls were undertaken. Chlorosis induction experiments were repeated several times with fresh inocula prepared from frozen stocks of the mutants each time. Inocula for each experiment were assayed for rhizobitoxine to ensure that reversion had not occurred during culturing. One repeat of this experiment was performed in a growth chamber in a separate building in the absence of wild-type inoculum. In all of these experiments, inoculation with the wild-type and mutant strains resulted in chlorosis of soybean cultivar Lee as indicated in Table 2.

To ensure that chlorosis was not due to reversion in planta, bacteroids were isolated from nodules and their *in vitro* phenotypes were assayed. Bacteroids from nodules were plated on selective and nonselective media, with equal numbers of colonies forming on each indicating that the Tn5 Km^r marker had not been lost during nodule formation. No fewer than 15 reisolates for each strain were tested for rhizobitoxine production in culture. Reisolated wild-type strain USDA61 produced rhizobitoxine in culture, while the reisolated mutants did not produce rhizobitoxine. Inoculation of these reisolated strains onto soybean cultivar Lee induced the expected chlorosis pattern. Therefore, the phenotype is stable and not the result of cross-contamination or reversion.

Rhizobitoxine production in planta. Because chlorosis had always been correlated with the presence of rhizobitoxine, this finding suggested that some of the mutants which do not make toxin in culture were competent to make rhizobitoxine in planta. We therefore sought to correlate the severity of chlorosis with the level of rhizobitoxine found in nodule extracts. The amount of toxin in nodule extracts from these plants was determined by using the enzyme assay and was found to be in proportion to the severity of chlorosis (Table 2).

To confirm that rhizobitoxine and not some as-yet-identified toxin was causing foliar chlorosis and inhibiting β -cystathionase, we identified rhizobitoxine from nodule extracts of strains which caused the most severe symptoms by using two-dimensional TLC. Unlike most amines which stain purple or blue with ninhydrin, rhizobitoxine stains a characteristic yellow with ninhydrin (31). A yellow staining spot comigrated with authentic rhizobitoxine in two-dimensional TLC, and the spot was of an intensity expected on the basis of the enzyme assay (data not shown). These strains produce rhizobitoxine in planta on cultivar Lee and are therefore designated Rtx^{-/-}.

Strains RX18E and RX17E did not cause chlorosis, and, as evidenced from the enzyme assay, these strains have completely lost their ability to produce rhizobitoxine in nodules. Therefore, the phenotype of these two strains is Rtx^{-/+}. Mutants RX24E and RX26F always had some activity in our enzyme assay but never induced chlorosis on soybean cultivar Lee. Grouping of these mutants must await more detailed biochemical characterization.

Chlorosis and rhizobitoxine production phenotypes on a resistant soybean cultivar. Some soybean cultivars appear resistant to the toxin as they show no chlorotic symptoms upon inoculation with rhizobitoxine-producing *B. japonicum* strains. Previously, toxin has not been isolated from nodules of resistant cultivars, and, from grafting experiments, it is known that the resistance is imparted by the root and not the shoot (15). This cultivar-dependent production of toxin is illustrated by comparing the levels of toxin synthesized by

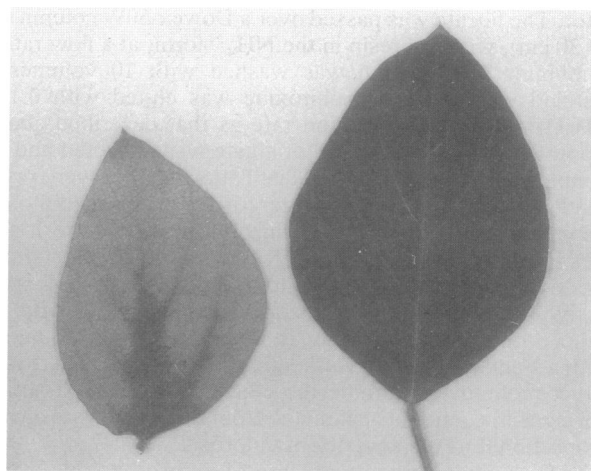


FIG. 2. Chlorosis symptom induced by RX17G on a resistant cultivar. These are leaves from the second (right) and third (left) trifoliolate leaves of a resistant soybean plant inoculated with strain RX17G.

wild-type USDA61 in nodules of sensitive cultivar Lee with those of resistant cultivar Williams (Table 2).

Given that some of the mutants consistently made high levels of toxin in the nodules of the sensitive cultivar Lee, we wanted to know the phenotype of the mutants on a resistant cultivar such as Williams. Rhizobitoxine mutants were therefore analyzed for their ability to cause chlorosis and synthesize rhizobitoxine on the resistant soybean cultivar Williams. The strains that had caused severe chlorosis on cultivar Lee (RX17G, RX19E, and RX19H) also caused chlorosis on cultivar Williams (Fig. 2). Rhizobitoxine was assayed in nodule extracts of these strains and found to be in proportion to the severity of chlorosis observed.

Those four mutants which caused chlorosis similar to that of the wild-type strain on cultivar Lee showed a differential phenotype on cultivar Williams. Two of the mutants, RX21D and RX28C, did not cause chlorosis, and assayed levels of rhizobitoxine were low and would not be expected to cause chlorosis (Table 2). In contrast, mutants RX20A and RX20B did cause slight chlorosis on cultivar Williams, and assayed levels of rhizobitoxine are consistent with the severity of chlorosis observed (Table 2).

As on cultivar Lee, strains RX18E and RX17E failed to cause chlorotic symptoms and produced no rhizobitoxine on cultivar Williams (Table 2).

Chlorosis and rhizobitoxine production phenotypes on alternate hosts. The above results demonstrate genotype-specific interactions between soybeans and the *B. japonicum* strains which effect rhizobitoxine production. We therefore wished to investigate the phenotype of these mutants on other host species. Inoculation of the wild type and selected rhizobitoxine mutants onto host plants *G. soja*, cowpea, and siratro resulted in no chlorosis being observed after inoculation of any of the strains examined. In addition, much lower levels of rhizobitoxine were measured in nodule extracts from these plants than in soybean nodule extracts for the same mutant (Table 3).

Nodulation phenotype of the rhizobitoxine mutants. The nodulation phenotype of the Rtx⁻ mutants was tested on various cultivars of soybeans and other hosts of USDA61 in various growth conditions. Although all mutant strains were tested on soybeans, only representative strains are pre-

TABLE 3. Chlorosis phenotypes and rhizobitoxine production in cowpea and siratro

| Strain | Chlorosis ^a | | Rhizobitoxine in nodules ^b (µg/g [fresh wt]) | |
|---------|------------------------|---------|--|-----------------|
| | Cowpea | Siratro | Cowpea | Siratro |
| USDA110 | — | — | 0.05 | 0.05 |
| USDA61 | — | — | 3.2 | 1.6 |
| RX18E | — | — | 0.04 | 0.01 |
| RX17G | — | — | 3.6 | ND ^c |

^a Chlorosis scored as described in Table 2, footnote a.

^b Rhizobitoxine measured in eluates of nodule extracts.

^c ND, not determined.

sented for simplicity. Inoculation of soybean cultivars Lee and Williams in pots or Leonard jars resulted in effective symbiosis with no statistically significant differences in nodule number per plant. As there were no differences observed in final nodule number or plant growth among these mutants and the wild-type strain, a more detailed experiment on the kinetics of nodulation was performed. The kinetics of nodule formation by wild-type and several Rtx⁻ strains were tested on soybean by using growth pouches, and no statistically significant differences were observed (Fig. 3). Similar nodulation patterns also occurred when cowpea and siratro were used as the hosts (data not shown).

Nodulation of *rj*₁ soybeans. Soybean plants homozygous for *rj*₁ are unable to form nodules in the field but are able to form a few nodules under greenhouse or growth chamber

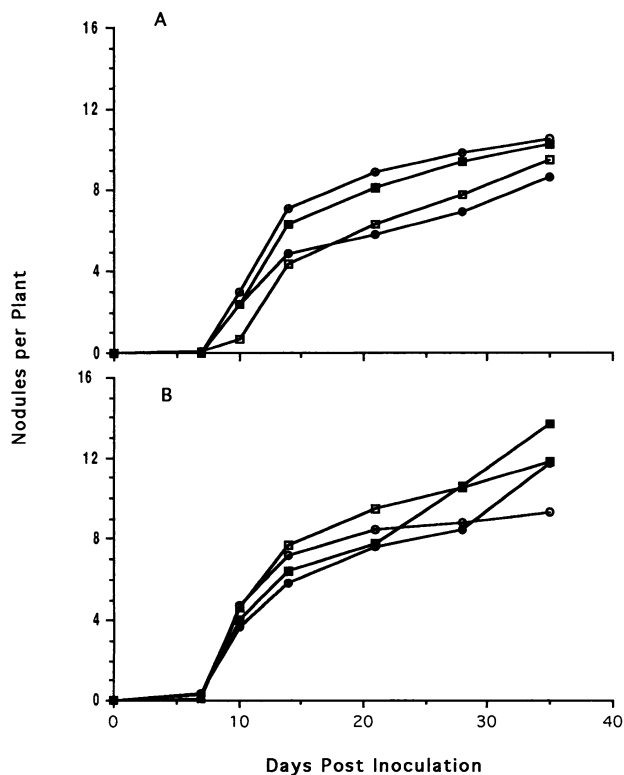


FIG. 3. Kinetic nodulation experiments on soybean cultivars Lee and Williams. Nodule formation was monitored on cultivars Williams (A) and Lee (B) with strains USDA61 (□), RX17G (■), RX18E (●), and USDA110 (○).

TABLE 4. Nodulation of soybean cultivar Harosoy *rj*₁

| Strain | Chlorosis ^a | Nodules per plant ^b |
|---------|------------------------|--------------------------------|
| USDA110 | — | 2.5a |
| USDA61 | — | 10.7b |
| RX17E | — | 9.0b |
| RX18E | — | 8.2b |
| RX17G | ++ | 9.3b |

^a Chlorosis scored as described in Table 2, footnote a.

^b Means followed by a different letter are significantly different (0.01 confidence level) by Duncan's new multiple-range test.

conditions (2). One previously hypothesized role for rhizobitoxine in *Bradyrhizobium*-legume symbiosis was associated with the ability of rhizobitoxine-producing strains to form nodules on *rj*₁ soybeans (3, 21). Previous attempts to evaluate this hypothesis are weakened by the fact that the rhizobitoxine-producing strains and nonproducing strains are effectively different species. By using the mutant strains described in this study, it was possible to more rigorously test the role of rhizobitoxine in nodulation of *rj*₁ soybeans.

As was expected under our growth conditions, nodulation by the rhizobitoxine-producing *B. japonicum* USDA61 is better than that by the nonproducing strain USDA110 (Table 4). It is clear from the nodulation of *rj*₁ soybeans by the two Rtx^{-/-} mutants, RX18E and RX17E, that rhizobitoxine is not required for nodule formation on *rj*₁ plants. In addition, the Rtx^{-/+} mutant RX17G formed similar numbers of nodules on *rj*₁ soybeans as the wild-type strain USDA61 did, demonstrating that rhizobitoxine does not enhance nodulation of *rj*₁ soybeans (Table 4).

Nodulation in the presence of nitrate. Recently it has been shown that nitrate inhibition of nodule formation on alfalfa can be overcome partially by the use of the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (23). Aminoethoxyvinylglycine and rhizobitoxine are structurally similar, and both are inhibitors of ethylene biosynthesis. To test if the Rtx^{-/+} overproducing mutants could similarly overcome nitrate inhibition in soybeans, the mutant strain RX17G and the wild-type strain USDA61 were inoculated onto soybean cultivar Williams and cultured in the presence and absence of 7 mM nitrate. In the presence and absence of nitrate, the wild-type strain formed 30 and 80 nodules per plant, respectively, and the mutant strain RX17G formed 38 and 117 nodules per plant, respectively. By using Duncan's new multiple-range test, there was no significant difference (0.01 confidence level) in nodule number between the wild type and mutant.

Biochemical analysis. During the TLC analysis of nodule extracts to confirm the presence of rhizobitoxine, we noticed that one major spot present in extracts of USDA61 nodules was absent from the nodule extracts of mutant RX18E and wild-type USDA110 (Fig. 4). We discovered that this spot has the same color as that of serinol and comigrated in both one- and two-dimensional TLC with serinol. Serinol is found in nodules formed by group II *Bradyrhizobium* species (25) and is hypothesized to be a precursor of rhizobitoxine (26). Nodule extracts of all other mutants contained serinol. These observations suggest that mutant RX18E is defective in formation of serinol.

DISCUSSION

The most interesting aspect of toxin production emerging from the mutant analysis is the difference between rhizobi-

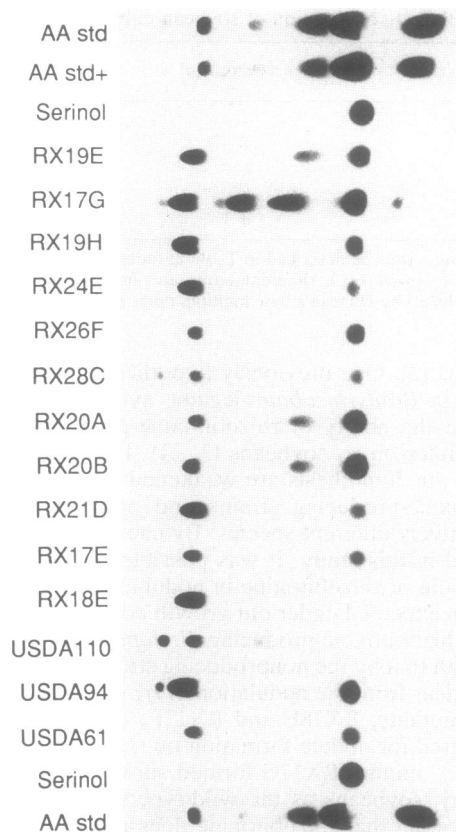


FIG. 4. TLC of nodule extracts. Nodules formed in soybean cultivar Williams induced by wild-type and mutant strains were crushed and extracted, and the extract was analyzed by using TLC. Abbreviations: AA std, an amino acid standard mixture of 17 amino acids; AA std+, the amino acid standard supplemented with serinol.

toxine synthesis in culture and that in planta. We originally expected to find cultivar Lee plants inoculated with the Rtx^- mutants to be free of chlorosis. This was true for only 4 of the 11 mutants, while 7 other mutants unable to synthesize rhizobitoxine in culture synthesized rhizobitoxine in planta. There appear to be two general possible explanations for the observed difference between rhizobitoxine biosynthesis in culture and that in planta. One possibility is that the plant supplies an intermediate to the bacteroids that is not provided by yeast extract in our culture medium. This appears to be the case for heme biosynthesis in nodules formed by bacterial mutants unable to synthesize heme in culture (36). A second possibility is that many of the mutations are in regulatory genes which are required for synthesis of toxin in culture but not in planta.

Of the rhizobitoxine mutants isolated in this study, two clearly fail to make the toxin in culture and in planta. These mutations are most likely in genes which encode enzymes responsible for the biosynthesis of the toxin. The finding that RX18E does not synthesize serinol suggests that the interrupted gene is directly involved in serinol biosynthesis. In sorghum, serinol is synthesized via serinol phosphate from dihydroxyacetone phosphate (1). This gene may therefore be an aminotransferase. The absence of serinol from nodule extracts of RX18E also suggests that the hypothesized involvement of serinol in rhizobitoxine of Mitchell and Coddington (26) is most likely correct. Biochemical information on other intermediates of rhizobitoxine biosynthesis in

P. andropogonis (26, 27) and the mutant strains described here will be helpful in elucidating the biosynthetic pathway of rhizobitoxine in *B. japonicum*.

Soybean cultivars, such as Williams, are considered resistant because bacterial chlorotic symptoms do not appear when the cultivars are inoculated with chlorosis-inducing strains such as USDA61. This is an unfortunate use of terms because, as demonstrated earlier, resistance is not a consequence of the shoot of the plant being less sensitive to rhizobitoxine. Rather, these plants do not show symptoms because the toxin does not accumulate to chlorosis-inducing concentrations in these plants (15). This illustrates a plant genotype-specific interaction with the bacteria which determines expression of toxin. Toxin may not accumulate in these resistant cultivars of soybeans for one of three possible reasons: the plant may suppress rhizobitoxine biosynthesis, it may fail to induce toxin synthesis, or it may detoxify the toxin.

The $Rtx^{-/+}$ mutant, RX17G, which causes chlorosis on cultivar Williams does not cause chlorosis on the alternative host plants *G. soja*, cowpea, or siratro. This implies that, in addition to genotype-specific interactions within a species, there are genotype-specific interactions among host species and the toxin-synthesizing *Bradyrhizobium* species. Similar host genotype interactions have been observed for *Bradyrhizobium* hydrogenase activities (18, 39). The group II *Bradyrhizobium* strains do not exhibit hydrogenase activity on most cultivars of soybeans but do on cowpea. Recently, some introduction lines of soybean have been found to promote hydrogenase activity by the group II *Bradyrhizobium* strains (39).

Mutualistic symbiotic relationships are believed to evolve from commensalistic or parasitic associations. Seldom are there such dramatic examples of a symbiosis which can be transformed from a mutualistic to a parasitic association by single mutations. This bradyrhizobial-induced chlorosis of the host plant is such a symbiotic association, and its study should provide insight into the precarious balance of symbiosis.

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