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Bradyrhizobium elkanii rtxC Gene Is Required for Expression of Symbiotic Phenotypes in the Final Step of Rhizobitoxine Biosynthesis

Shin Okazaki, Masayuki Sugawara, and Kiwamu Minamisawa*

Graduate School of Life Sciences, Tohoku University, Katahira, Aoba-ku, Sendai 980-8577, Japan

Received 28 August 2003/Accepted 10 October 2003

We disrupted the *rtxC* gene on the chromosome of *Bradyrhizobium elkanii* USDA94 by insertion of a nonpolar *aph* cartridge. The *rtxC* mutant, designated Δ*rtxC*, produced serinol and dihydrorhizobitoxine but no rhizobitoxine, both in culture and in planta. The introduction of cosmids harboring the *rtxC* gene into the Δ*rtxC* mutant complemented rhizobitoxine production, suggesting that *rtxC* is involved in the final step of rhizobitoxine biosynthesis in *B. elkanii* USDA94. *Glycine max* cv. Lee inoculated with Δ*rtxC* or with a null mutant, Δ*rtx*::Ω1, showed no foliar chlorosis, whereas the wild-type strain USDA94 caused severe foliar chlorosis. The two mutants showed significantly less nodulation competitiveness than the wild-type strain on *Macroptilium atropurpureum*. These results indicate that dihydrorhizobitoxine, the immediate precursor of the oxidative form of rhizobitoxine, has no distinct effect on nodulation phenotype in these legumes. Thus, desaturation of dihydrorhizobitoxine by *rtxC*-encoded protein is essential for the bacterium to show rhizobitoxine phenotypes in planta. In addition, complementation analysis of *rtxC* by cosmids differing in *rtxC* transcription levels suggested that rhizobitoxine production correlates with the amount of *rtxC* transcript.

Recent studies have suggested that rhizobia adopt at least two strategies to reduce the amount of ethylene synthesized by their host legumes and thus decrease the negative effect of ethylene on nodulation (15). 1-Aminocyclopropane-1-carboxylate (ACC) deaminase, which degrades ACC (an immediate precursor of ethylene) into ammonia and α-ketobutyrate, has been found and well characterized in plant growth-promoting rhizobacteria (9, 36). The ACC deaminase of plant growth-promoting rhizobacteria reduces the amount of ACC in associated roots and promotes root elongation via the reduction of ethylene production (8). Genes encoding ACC deaminase have also been found in some rhizobia, including *Rhizobium leguminosarum*, *Mesorhizobium loti*, and *Bradyrhizobium japonicum* (17). In *R. leguminosarum* bv. *viciae*, ACC deaminase has been confirmed to enhance nodulation of *Pisum sativum* (16).

Another strategy used by some strains of rhizobia to lower ethylene is the production of rhizobitoxine [2-amino-4-(2-amino-3-hydropropoxy)-*trans*-but-3-enoic acid] (27). Thus far, *Bradyrhizobium elkanii* and the plant pathogen *Burkholderia andropogonis* are known as rhizobitoxine-producing bacteria (18, 22). The biochemical action of rhizobitoxine is to inhibit ACC synthase (39) and β-cystathionase (31). Rhizobitoxine is regarded as a phytotoxin because it induces chlorosis in a variety of plants (13, 25). However, recent studies have shown that it has a positive role in the symbiosis between *B. elkanii* strains and their host legumes. Enhanced nodulation and competitiveness by rhizobitoxine production in *B. elkanii* have been reported so far for three legumes: *Amphicarpaea edgeworthii* (28), *Vigna radiata* (4), and *Macroptilium atropurpureum* (41).

The biosynthetic genes of rhizobitoxine have been identified for *B. elkanii* USDA61 (32–34) and USDA94 (38). For a previous paper (38), members of our laboratory cloned and sequenced the genetic locus involved in rhizobitoxine biosynthesis and suggested that at least *rtxA* and *rtxC* are necessary for rhizobitoxine production in *B. elkanii* USDA94. A large-deletion mutant of *B. elkanii*, USDA94Δ*rtx*::Ω1, which lacks *rtxA*, *rtxC*, and two other open reading frames (ORFs), did not produce rhizobitoxine, dihydrorhizobitoxine, or serinol. A cosmid containing a kanamycin resistance cassette within the *rtxC* gene complemented the production of serinol and dihydrorhizobitoxine but not of rhizobitoxine. The deduced amino acid sequence of *rtxC* showed 31% similarity to *Pseudomonas* fatty acid desaturase. From these results, we proposed that *rtxC* protein is involved in desaturation of dihydrorhizobitoxine, although possible polar effects of the kanamycin resistance cassette and cosmid instability may have complicated this interpretation.

A large amount of dihydrorhizobitoxine, an oxidative form of rhizobitoxine, is generally coproduced with rhizobitoxine (20, 26). Biochemical analysis indicates that the ability of dihydrorhizobitoxine to inhibit ACC synthase and β-cystathionase is approximately 99% less than that of rhizobitoxine (39). However, no conclusive data have been reported on the biological effects of dihydrorhizobitoxine.

The first objective of this work was to verify the involvement of *rtxC* in rhizobitoxine biosynthesis by chromosomal nonpolar mutation and complementation analysis. Our second objective was to assess the biological effect of dihydrorhizobitoxine on host legumes, separately from that of rhizobitoxine, in terms of enhancement of nodulation competitiveness and development

* Corresponding author. Mailing address: Graduate School of Life Sciences, Tohoku University, Katahira, Aoba-ku, Sendai 980-8577, Japan. Phone: 81-22-217-5684. Fax: 81-22-263-9845. E-mail: kiwamu@ige.tohoku.ac.jp.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or Source
Bacterial strains		
B. elkanii		
USDA94	Wild-type strain producing high concentrations of rhizobitoxine in culture, Tc^r Km^s	Keyser[b]
MA941	*gusA*-marked USDA94	40
Δ*rtxC*	*rtxC*::*aph* derivative of USDA94, Sm^r Sp^r	This study
Δ*rtx*::Ω1	USDA94, *nodPQ rtxA rtxC* (ORF1), ORF2, ORF3::del/ins Ω cassette, Sm^r Sp^r	38
USDA61	Wild-type strain producing rhizobitoxine in culture, Km^s	32
RX18E	Tn*5* mutant derivative of USDA61, rhizobitoxine production deficient, Km^r	32
E. coli		
JM109	*recA*, cloning strain	Toyobo[c]
DH5α	*recA*, cloning strain	Toyobo[c]
Plasmids		
pRK2013	ColE1 replicon carrying RK2 transfer genes, Km^r	6
pBluescript II (SK+)	Cloning vector, Ap^r	Takara[d]
pSUP202	pBR325 carrying *oriT* from RP4, Ap^r Cm^r Tc^r	37
pUC4-KIXX	Plasmid carrying 1.6-kb *aph* cassette, Ap^r Km^r	Pharmacia
pCR2.1	Cloning vector, Ap^r Km^r	Invitrogen
pHP45Ω	Plasmid carrying 2.1-kb *aph* cassette, Sm^r Sp^r Ap^r	5
pBS-3.4	pBluescript II SK(+) containing 3.4-kb *Bgl*II fragment	This study
pSUP-*rtxC*::*aph*	pSUP202 carrying a 4-kb fragment, *rtxC*::*aph*	This study
pLAFR1	Broad-host-range cosmid, IncP, Tc^r	7
pRTF1	pLAFR1 containing *rtxA* and flanking regions from *B. elkanii* USDA94	38
pLAFRSS	pLAFR1 carrying 1.1-kb *aad*, Sm^r Sp^r Tc^r	This study
pLAFRSS-*rtxC*	pLAFRSS containing 1.9-kb *rtxC* and flanking regions from *B. elkanii* USDA94	This study
pLAFRSS-P_{aph}*rtxC*	pLAFRSS carrying *aph* promoter-fused *rtxC*	This study

[a] Ap^r, ampicillin resistant; Tc^r, tetracycline resistant; Km^r, kanamycin resistant; Km^s, kanamycin sensitive; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Cm^r, chloramphenicol resistant; *aph*, aminoglycosidase-3′- *O*-phosphotransferase gene; *aad*, aminoglycoside adenyltransferase gene.
[b] H. H. Keyser, U.S. Department of Agriculture, Beltsville, Md.
[c] Toyobo Inc., Tokyo, Japan.
[d] Takara Shuzo Co., Kusatsu, Japan.

of chlorosis symptoms, by using wild-type and *rtxC* mutants of *B. elkanii* USDA94.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *B. elkanii* cultures were grown aerobically at 30°C in HM medium (3) supplemented with 0.1% arabinose and 0.025% yeast extract (Difco, Detroit, Mich.) or in Tris-YMRT medium (21). *Escherichia coli* cells were grown at 37°C in Luria-Bertani medium (35). The following antibiotics were added to media at the indicated concentrations: for *B. elkanii*, kanamycin at 150 mg liter^{-1} and spectinomycin and streptomycin at 250 mg liter^{-1}; for *E. coli*, tetracycline at 12.5 mg liter^{-1}, ampicillin at 100 mg liter^{-1}, and kanamycin at 50 mg liter^{-1}.

DNA manipulations. Isolation of plasmid DNA, restriction enzyme digestion, DNA ligation, bacterial transformation of *E. coli*, and Southern blot hybridization were performed as described by Sambrook et al. (35). DNA sequencing was performed by using dye primer technology and a model 373A sequencer (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom).

Construction of nonpolar *rtxC* mutants of *B. elkanii*. For the construction of the nonpolar *rtxC* mutant, a 3.4-kb *Bgl*II fragment carrying the *rtxC* ORF and flanking region from cosmid pRTF1 (38) was cloned into the *Bam*HI site of pBluescript II SK(+). The resultant plasmid, pBS-3.4, was digested by *Bam*HI to remove the 0.5-kb fragment within the *rtxC* ORF, and a 5.9-kb fragment was extracted from an agarose gel. The resulting fragment was ligated with a nonpolar aminoglycoside 3′-phosphotransferase (*aph*) cartridge generated by PCR as follows. Two primers derived from the *aph* sequences aph-1 (5′-TTCATAGGATCCAAGCCAGTCCGAAGAAACG-3′) and aph-2 (5′-TTATGGATCCTCAGAAGAACTCGTCAAG-3′), containing the newly created *Bam*HI sites (underlined), were used to amplify the *aph* gene without the transcription terminator by PCR from template pUC4-KIXX (Amersham-Pharmacia Biotech, Uppsala, Sweden). The amplified fragment (nonpolar *aph* cartridge) was digested by *Bam*HI and then ligated with the 5.9-kb fragment described above, yielding pBS-*rtxC*::*aph*. Plasmid pBS-*rtxC*::*aph* was digested with *Spe*I and *Hin*dIII, and a 4-kb fragment containing *rtxC*::*aph* was blunted and recloned into the *Eco*RV site of a pSUP202 suicide vector (37), yielding pSUP-*rtxC*::*aph* (Fig. 1). Plasmid pSUP-*rtxC*::*aph* was introduced into *B. elkanii* USDA94 by triparental mating, with pRK2013 as a helper plasmid (6). Crossover mutants were selected on HM medium containing 150 mg of kanamycin per liter, and a double-crossover mutant was identified by Southern blot hybridization with the 3.4-kb *Bgl*II fragment as a probe.

Construction of cosmids for complementation of *rtxC*. Because *B. elkanii* strains possess high levels of resistance to tetracycline, a pLAFR1 cosmid (7) with a tetracycline selection marker was not available for transconjugation.

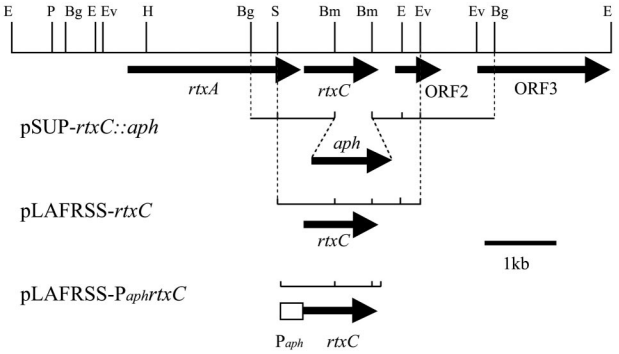

FIG. 1. Physical and restriction enzyme map of the *rtxC* gene and its flanking region in *B. elkanii* USDA94. Arrows denote ORFs. The internal 396-bp *Bam*HI fragment of *rtxC* was removed and replaced with the *aph* gene in pSUP-*rtxC*::*aph*. For complementation analysis, pLAFRSS-*rtxC* and pLAFRSS-P_{aph}*rtxC* were constructed. About 350 bp of the *aph* promoter region was fused with *rtxC* in pLAFRSS-P_{aph}*rtxC*. Abbreviations: Bm, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Ev, *Eco*RV; P, *Pst*I; S, *Sma*I.

Therefore, we conferred streptomycin and spectinomycin resistance on pLAFR1 by introducing the aminoglycoside adenyltransferase (*aad*) gene. The *aad* gene was amplified by PCR from plasmid pHP45Ω (5) with the primers aad-F (5′-TCATAGGATCCGAATTCATAAGCCTGTTC-3′; the restriction sites of *Bam*HI and *Eco*RI are underlined and double underlined, respectively) and aad-R (5′-TTATGGATCCTTATTTGCCG-3′; the *Bam*HI restriction site is underlined). The 1.1-kb PCR products obtained were digested with *Bam*HI, blunted, and cloned into the *Eco*RI site of pLAFR1, yielding cosmid pLAFRSS.

For complementation analysis of *rtxC*, two cosmids were constructed as follows. pBS3.4 was digested with *Sma*I and *Eco*RV, and a 2.0-kb fragment containing the *rtxC* ORF was isolated, blunted with the Klenow fragment, and ligated with pLAFRSS, which was then digested with *Eco*RI followed by blunting with the Klenow fragment, generating pLAFRSS-*rtxC* (Fig. 1).

For assembly of *aph* promoter-fused *rtxC*, we used the overlap-extension PCR technique (2). First, the *aph* promoter region (fragment 1) and *rtxC* gene (fragment 2) were separately amplified with the following primers, which were designed to create complementarity between the 3′ end of fragment 1 and the 5′ end of fragment 2. Fragment 1 was amplified with the primers prtxC-1 (5′-GCCCTTCGAGTCCATCTAGAAAGCCAGTCCGC-3′; the *Xba*I restriction site is underlined) and prtxC-2 (5′-GCGTCTGCCTGATTCATGCGAAACGATCC-3′), using pUC4-KIXX as a template. Fragment 2 was amplified with the primers prtxC-3 (5′-GAGGATCGTTTCGCATGAATCAGGCAGACGC-3′) and prtxC-4 (5′-CAATCTAGTTATCACTGCAGTCACGCCACGTGCCCC-3′; the *Pst*I restriction site is underlined), using pBS-3.4 as a template. Both fragments 1 and 2 were purified from an agarose gel with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany). Second, fragments 1 and 2 were mixed and subjected to template annealing and extension reactions, as described elsewhere (2). Finally, hybrid DNA of fragments 1 and 2 (*aph* promoter-fused *rtxC*) was amplified by using the products of the second step as a template, with primers prtxC-1 and prtxC-4. The amplified fragments were cloned and sequenced in pBluescript II SK(+), followed by cloning into the *Eco*RI site of pLAFRSS, generating pLAFRSS-P_{aph}*rtxC* (Fig. 1).

RNA isolation and reverse transcription (RT)-PCR analysis. For RNA isolation, *B. elkanii* strains were cultured in 15 ml of Tris-YMRT medium at 30°C for 7 days, yielding late-log-phase cells (optical density at 600 nm, 0.5 to 0.6). Total RNA of *B. elkanii* cells was prepared by use of RNAWIZ (Ambion Inc., Austin, Texas) according to the manufacturer's instructions. To remove intact DNA completely, the resultant total RNA extract was treated with DNase I (Takara, Tokyo, Japan) for 30 min at 37°C, followed by purification with an RNeasy mini kit (Qiagen). The resultant total RNA was resuspended in RNase-free water and then quantified by its A_{260} with a spectrophotometer.

RT-PCR was performed with a One-Step RT-PCR kit (Qiagen) according to the manufacturer's instructions. We used 50 ng of total RNA as the template in a total reaction volume of 50 μl. The primers rtxC-7 (5′-GCGGTACGCCTTCAATATCA-3′) and rtxC-8 (5′-TCCGATTCCTGGTTAGATA-3′) were used in the PCR to amplify a 331-bp product from the *rtxC* transcript. As controls, aad-1 (5′-GCGAGATTCTCCGCGCTGTA-3′) and aad-2 (5′-AGCTTCAAGTATGACGGG-3′) were used to amplify a 445-bp product from the *aad* gene in the vector pLAFRSS.

Plant assays. The host legumes used were *Glycine max* cv. Lee, *V. radiata* (mung bean), and *A. edgeworthii*. Mung bean and soybean seeds were surface sterilized with 0.5% hydrogen peroxide for 1 min and then washed 10 times with sterile distilled water. The surface-sterilized seeds were sown in a 300-ml plant box (CUL-JAR300; Iwaki, Tokyo, Japan) containing sterile vermiculite and watered with a nitrogen-free plant nutrient solution (1). Seeds of *A. edgeworthii* were scarified, surface sterilized by immersion in concentrated sulfuric acid for 38 min, rinsed 10 times with sterile water, and then planted in sterile growth pouches (Northrup King, Minneapolis, Minn.). Rhizobial inocula were cultured for 7 days at 30°C in HM medium containing 0.1% arabinose, 0.025% yeast extract, and either 150 mg of kanamycin per liter (for Δ*rtxC*) or 250 mg of spectinomycin per liter (for Δ*rtx*::Ω1). The cells were collected by centrifugation at 5,000 × *g* for 10 min at room temperature and then were washed twice with sterile water. The number of cells was adjusted to 10^7 ml^{-1} in sterile water by direct counting with a Thoma hemocytometer (Kayagaki Irika Kogyo Co. Ltd., Tokyo, Japan), and the cells were inoculated onto the seeds at 10^7 cells per seed. Plants were grown in a plant growth cabinet (LH300; NK Systems Co. Ltd., Osaka, Japan) under a light-dark cycle of 16 h of light and 8 h of dark at 25°C.

Competitive nodulation assay. The nodulation competitiveness of *B. elkanii* mutants was evaluated on *Macroptilium atropurpureum* Urb. cv. Siratro by using *B. elkanii* MA941, a *gusA*-marked strain of USDA94, as a reference strain. Seeds obtained from Yukijirushi Shubyo Co. (Hokkaido, Japan) were surface sterilized with 70% ethanol for 5 min and then with 3% hydrogen peroxide for 1 min; they were washed 10 times with sterile distilled water after each treatment. The surface-sterilized seeds were sown in a 300-ml plant box (CUL-JAR300; Iwaki) containing sterile vermiculite and were watered with a nitrogen-free plant nutrient solution (1). Strain USDA94, Δ*rtxC*, or Δ*rtx*::Ω1 was mixed with strain MA941 at a concentration of 10^7 cells ml^{-1} for each strain (cell ratio, 1:1). We then inoculated 1 ml of each mixture onto the Siratro seeds. Plants were grown in a plant growth cabinet as described above. After 21 days of cultivation, all nodules of >1 mm in diameter were collected, and the nodule occupancy ratios of USDA94, Δ*rtxC*, and Δ*rtx*::Ω1 to MA941 were determined by a β-glucuronidase assay, with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt; Wako Pure Chemical Industries Ltd., Osaka, Japan) as a substrate, as described previously (40). Approximately 100 nodules from three or four plants were examined by β-glucuronidase assay for each inoculation treatment. The chi-square test was used for statistical analysis at a confidence level of 0.05.

Determination of serinol, dihydrorhizobitoxine, and rhizobitoxine contents in culture and plants. Serinol, dihydrorhizobitoxine, and rhizobitoxine were isolated from the cultures as described previously (38). *B. elkanii* strains were grown in 15 ml of Tris-YMRT medium at 30°C for 21 days. Culture supernatants were collected by centrifugation and loaded on a Dowex 50 column (H^+ type; resin size, 50/100-mesh; column volume, 5 ml) (Muromachi Chemicals, Tokyo, Japan). The column was washed with 10 column volumes of deionized water. Serinol, dihydrorhizobitoxine, and rhizobitoxine were eluted with three column volumes of 2 M NH_4OH and dried in a vacuum. Pellets were dissolved in 500-μl aliquots of deionized water.

Serinol, dihydrorhizobitoxine, and rhizobitoxine contents in *G. max* cv. Lee inoculated with *B. elkanii* strains were determined. These compounds were extracted from plant materials as described by Minamisawa and Watanabe (19). Thirty days after sowing and inoculation, nodules and upper shoots (from the third trifoliate leaves, where the chlorosis symptoms were severe) were harvested and weighed, and then the extracts were purified through a Dowex 50 column (20, 21). Purified serinol, dihydrorhizobitoxine, and rhizobitoxine were assayed by liquid chromatography-mass spectrometry as described previously (38).

RESULTS

Construction and characterization of nonpolar *rtxC* mutant of *B. elkanii* USDA94. Members of our laboratory had proposed previously that the *rtxC* gene was responsible for desaturation of dihydrorhizobitoxine, on the basis of sequence homology to fatty acid desaturase and insertional mutagenesis achieved by inserting a cosmid with a kanamycin resistance cassette into *rtxC* (38). However, cosmid pLAFR1 is not always stable in rhizobial cells in planta, and the possible polar effects of the kanamycin resistance cassette restricted the interpretation. To verify the function of *rtxC* more directly, we chromosomally inserted a nonpolar *aph* cartridge into the *Bgl*II site of *rtxC* in the same transcriptional orientation (Fig. 1), generating a nonpolar *rtxC* mutant.

The nonpolar *rtxC* mutant, designated Δ*rtxC*, was tested for production of rhizobitoxine, dihydrorhizobitoxine, and serinol in Tris-YMRT culture supernatants (Table 2). As expected, Δ*rtxC* produced serinol and dihydrorhizobitoxine, but no rhizobitoxine. This result clearly demonstrates that *rtxC* encodes dihydrorhizobitoxine desaturase as a final step of rhizobitoxine biosynthesis.

Complementation analysis of *rtxC*. The newly constructed cosmid pLAFRSS, derived from pLAFR1, was introduced into *B. elkanii* USDA94 and mutant Δ*rtxC* by triparental mating, followed by selection with 250 mg of spectinomycin and streptomycin per liter. The resultant bacterial colonies were grown in HM liquid medium with 250 mg of spectinomycin per liter at 30°C for 7 days, and cell lysates were prepared for PCR amplification of the *aad* gene with aad-F and aad-R primers (data not shown). As a result, all transconjugants tested carried cosmid pLAFRSS, indicating that this cosmid could be main-

TABLE 2. Serinol, dihydrorhizobitoxine, and rhizobitoxine production by *B. elkanii* USDA94 and rhizobitoxine-deficient mutants in culture and in nodules of *G. max* cv. Lee

B. elkanii strain	Production in culture supernatant (μM)[b]			Production in nodule extract (μmol/g of fresh wt)[b]		
	Serinol	DRT[a]	RT[a]	Serinol	DRT	RT
USDA94	221 ± 15	106 ± 11	9.1 ± 3.7	14.9 ± 1.7	15.8 ± 0.8	1.3 ± 0.3
Δ*rtxC*	182 ± 21	48 ± 6	<0.1	17.6 ± 1.8	11.2 ± 0.6	<0.05
Δ*rtx*::Ω1	<3.2	<2.7	<0.1	<0.07	<0.5	<0.05
Δ*rtxC*(pLAFRSS)	88 ± 28	62 ± 11	<0.1	ND	ND	ND
Δ*rtxC*(pLAFRSS-*rtxC*)	107 ± 29	33 ± 9	1.6 ± 1.1	ND	ND	ND
Δ*rtxC*(pLAFRSS-P_{aph}*rtxC*)	134 ± 34	27 ± 13	3.5 ± 1.5	ND	ND	ND

[a] DRT, dihydrorhizobitoxine; RT, rhizobitoxine.
[b] Data are means ± SD; ND, not determined.

tained in *B. elkanii* cells and that transconjugants could be selected by spectinomycin and streptomycin.

Two cosmids, pLAFRSS-*rtxC* and pLAFRSS-P_{aph}*rtxC*, were constructed for *rtxC* complementation and were introduced into Δ*rtxC*. The validity of cosmid constructs was checked by PCR, and *rtxC* transcripts were detected by RT-PCR (Fig. 2; see below). After complementation with either cosmid, transcripts of *rtxC* were recovered from cells cultured for 7 days in Tris-YMRT medium.

The rhizobitoxine content of Tris-YMRT culture supernatants was examined after 21 days of cultivation. Rhizobitoxine production was restored in Δ*rtxC* strains complemented with pLAFRSS-*rtxC* or pLAFRSS-P_{aph}*rtxC* (Table 2). The amounts of rhizobitoxine produced in the supernatants differed between the complemented cosmids (pLAFRSS-*rtxC*, 1.6 μM; pLAFRSS-P_{aph}*rtxC*, 3.5 μM), and both amounts were lower than that of the wild-type strain.

Transcriptional analysis of complemented strains. Total RNA was isolated from Δ*rtxC* strains complemented with pLAFRSS, pLAFRSS-*rtxC*, and pLAFRSS-P_{aph}*rtxC*, and the expression of *rtxC* in these strains was tested by RT-PCR using the *rtxC*-specific primers rtxC-7 and rtxC-8. The same template RNA samples were also subjected to RT-PCR using primers for the *aad* gene to confirm the integrity of the RNA samples used. The amount of *rtxC* transcript in Δ*rtxC*(pLAFRSS-P_{aph}*rtxC*) was higher than that in Δ*rtxC*(pLAFRSS-*rtxC*), whereas transcription levels of *aad* were similar in both strains (Fig. 2). The lower level of *rtxC* transcripts presumably reflected intrinsic transcription from the cosmid vector.

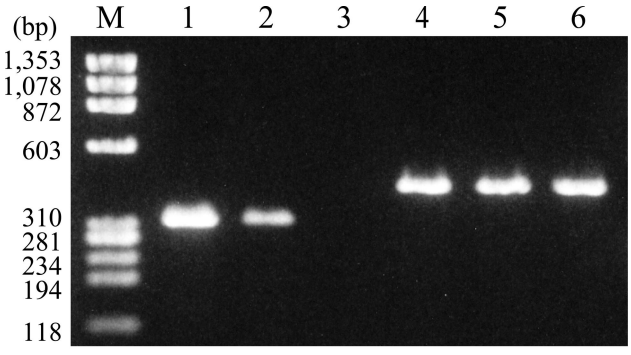

FIG. 2. Determination of *rtxC* gene expression by RT-PCR. RNA was isolated from the *rtxC*-disrupted mutant Δ*rtxC* complemented with pLAFRSS-P_{aph}*rtxC* (lanes 1 and 4), pLAFRSS-*rtxC* (lanes 2 and 5), or pLAFRSS (lanes 3 and 6). RT-PCR was carried out with gene-specific primers for *rtxC* (lanes 1 to 3) and *aad* (lanes 4 to 6), as described in Materials and Methods. M, size marker. Three independent experiments showed similar results.

Chlorosis and rhizobitoxine productivity on *G. max* cv. Lee. *G. max* cv. Lee is a soybean cultivar that is susceptible to chlorosis caused by rhizobitoxine (12). Plants inoculated with USDA94 showed foliar chlorosis on newly forming leaves from 20 days after inoculation, and by 30 days after inoculation these symptoms had worsened (Fig. 3). Inoculation with the null mutant Δ*rtx*::Ω1, which does not produce serinol, dihydrorhizobitoxine, or rhizobitoxine (Table 2), produced no symptoms of chlorosis 30 days after inoculation. Similarly, plants inoculated with Δ*rtxC* showed no chlorosis symptoms. Other parameters, such as number of nodules, nodule weight, and plant fresh weight, were not significantly different between plants inoculated with Δ*rtx*::Ω1 and Δ*rtxC* (data not shown).

We determined the rhizobitoxine, dihydrorhizobitoxine, and serinol contents of nodules and upper shoots of plants grown for 30 days after inoculation. As observed in the culture supernatants, Δ*rtxC* did not accumulate rhizobitoxine in the nodules or upper shoots, whereas USDA94 accumulated large amounts of rhizobitoxine in both the nodules and upper shoots (Table 2; also, see legend to Fig. 3). Plants inoculated with Δ*rtxC* accumulated similar amounts of dihydrorhizobitoxine to those in plants inoculated with USDA94. These results clearly indicated that chlorosis symptoms in *G. max* cv. Lee are caused, not by dihydrorhizobitoxine, but by rhizobitoxine.

Nodulation competitiveness of *B. elkanii* rhizobitoxine mutants on *Macroptilium atropurpureum*. It was recently found that rhizobitoxine is a strong positive promoter of competitive nodulation in association with *Macroptilium atropurpureum* (24, 41). Thus, we compared the nodulation competitiveness of wild-type *B. elkanii* USDA94, Δ*rtxC*, and Δ*rtx*::Ω1 on *Macroptilium atropurpureum* cv. Siratro by using MA941 as a reference strain (Fig. 4). When *B. elkanii* USDA94 and MA941 were mixed in the inoculum at a 1:1 ratio (10^7 cells of each), both strains formed similar numbers of nodules (USDA94, 47.3%; MA941, 52.7%) (Fig. 4). This indicates that *gusA* insertion in MA941 did not affect the nodulation competitiveness of the parent strain, USDA94. However, when Δ*rtxC* was coinoculated with reference strain MA941 onto Siratro roots at a 1:1 ratio, the nodule occupancy rate of Δ*rtxC* decreased to 25.7% (Fig. 4). Similarly, the null mutant Δ*rtx*::Ω1 occupied 24.2% of the nodules. These nodule occupancy rates of rhizobitoxine mutants were significantly different from that of the wild-type strain, at a confidence level of 0.05. Δ*rtxC* produced serinol and

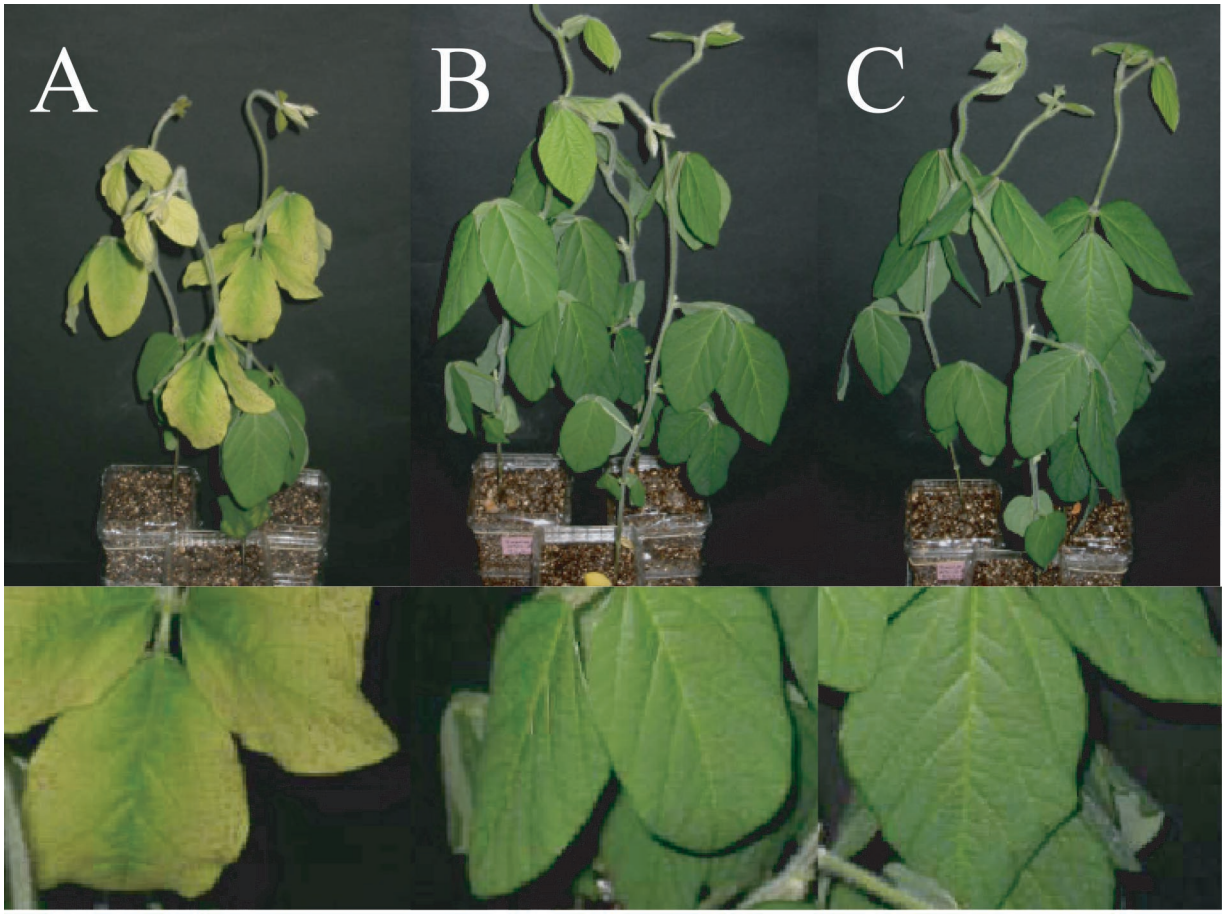

FIG. 3. Foliar chlorosis of *G. max* cv. Lee inoculated with *B. elkanii* USDA94 (A), Δ*rtxC* (B), and Δ*rtx*::Ω1 (C), photographed 30 days after inoculation. The contents of serinol in upper shoots were as follows: with USDA94, 4.5; with Δ*rtxC*, 2.8; with Δ*rtx*::Ω1, <0.1 (nmol/g of fresh weight). The contents of dihydrorhizobitoxine in upper shoots were as follows: with USDA94, 8.4; with Δ*rtxC*, 5.1; with Δ*rtx*::Ω1, <0.2 (nmol/g of fresh weight). The contents of rhizobitoxine in upper shoots were as follows: with USDA94, 3.6; with Δ*rtxC*, <0.1; with Δ*rtx*::Ω1, <0.1 (nmol/g of fresh weight).

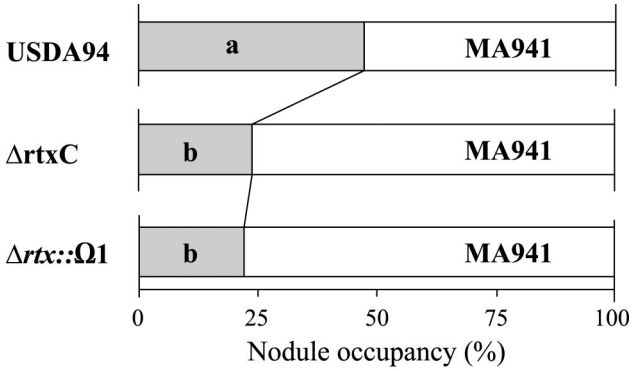

FIG. 4. Nodule occupancy rates of *B. elkanii* strains USDA94, Δ*rtxC*, and Δ*rtx*::Ω1 compared with that of a reference strain, MA941 (*gusA*-marked USDA94), on *Macroptilium atropurpureum* cv. Siratro. Nodule numbers per plant coinoculated with MA941 or USDA94, Δ*rtxC*, or Δ*rtx*::Ω1 were 15.7 ± 4.8, 14.3 ± 4.5, and 15.2 ± 4.9 (mean ± SD), respectively. Different letters denote significant differences in nodule occupancy values between MA941 and USDA94, Δ*rtxC*, or Δ*rtx*::Ω1 by the chi-square test ($P = 0.05$).

dihydrorhizobitoxine at similar levels to those of USDA94 in soybean nodules (Table 2); therefore, the lower nodule occupancy rate of Δ*rtxC* was probably due to lack of rhizobitoxine productivity.

Nodulation profiles of *B. elkanii* rhizobitoxine-deficient mutants on *V. radiata* and *A. edgeworthii*. We also examined the nodulation phenotypes of Δ*rtxC* and Δ*rtx*::Ω1 on two other host legumes, *V. radiata* (mung bean) and *A. edgeworthii*. Nodulation enhancement by rhizobitoxine production has been reported for these species (4, 28). In our experiments, however, no significant differences were observed between inoculation with the wild-type strain USDA94 and inoculation with rhizobitoxine-deficient mutants in terms of nodule numbers, nodule weight, plant growth, or plant fresh weight (data not shown). Nodule numbers per plant on mung beans inoculated with USDA61, RX18E (a rhizobitoxine-deficient mutant of USDA61), and Δ*rtx*::Ω1 (a rhizobitoxine-deficient mutant of USDA94) were 13.4 ± 1.7, 3.0 ± 1.8, and 27.3 ± 1.1 (mean ± standard deviation [SD]), respectively. This suggests that strain USDA94 lacking the ability to produce rhizobitoxine is already compatible with mung bean nodulation, which is probably due to the different strain background from that of strain USDA61 in *B. elkanii*.

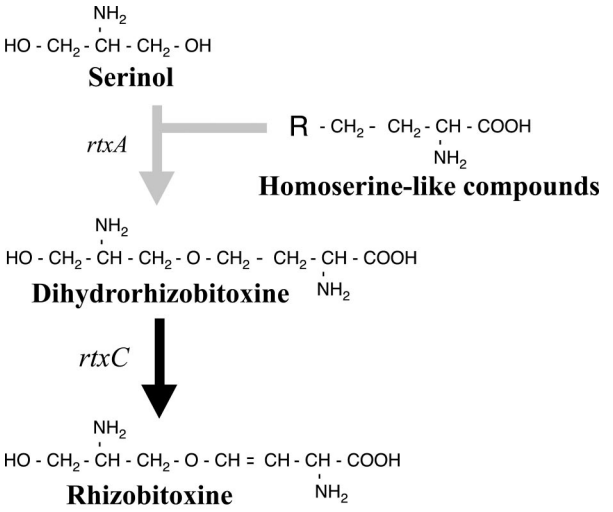

FIG. 5. Structures of rhizobitoxine and relevant compounds in the rhizobitoxine biosynthetic pathway.

DISCUSSION

We confirmed the function of the *rtxC* gene of *B. elkanii* in rhizobitoxine biosynthesis by nonpolar gene disruption on the chromosome to exclude possible polar effects and the effect of cosmid curing. As expected, the resulting mutant, Δ*rtxC*, produced serinol and dihydrorhizobitoxine, but no rhizobitoxine, and introduction of cosmids harboring *rtxC* complemented rhizobitoxine production in this mutant. Additionally, rhizobitoxine production in the complemented strains correlated somewhat with the amount of *rtxC* transcript. Therefore, we conclude that dihydrorhizobitoxine is an immediate precursor of rhizobitoxine and that *rtxC* is involved in the final step of rhizobitoxine biosynthesis (Fig. 5).

Dihydrorhizobitoxine is usually coproduced with rhizobitoxine in culture and in planta at concentrations that are >10 times higher than those of rhizobitoxine (20), but the biological effects of the two substances have not been characterized separately. Use of Δ*rtxC* and the null mutant Δ*rtx*::Ω1 enabled us to separate the biological effects of dihydrorhizobitoxine and rhizobitoxine in symbiosis without the effects of cosmid curing. The accumulation of rhizobitoxine, dihydrorhizobitoxine, and serinol in plants, and the resulting nodulation profiles, provided clear insight into the fact that enhancement of nodulation competitiveness on *Macroptilium atropurpureum* and chlorosis induction on *G. max* were completely dependent on the presence of rhizobitoxine, not dihydrorhizobitoxine. Although the mechanism by which rhizobitoxine induces foliar chlorosis on soybean plants has not been elucidated fully, enhancement of nodulation competitiveness on *Macroptilium atropurpureum* by rhizobitoxine is known to be mediated by the reduction of ethylene evolution from the roots of the host legume (41). An in vitro enzymatic assay has shown dihydrorhizobitoxine to be approximately 99% less potent than rhizobitoxine as an inhibitor of ACC synthase (a key enzyme in plant ethylene biosynthesis) (39). This reduction in the inhibitory effect on ethylene biosynthesis resulted in the lower nodulation competitiveness of Δ*rtxC*. These results indicate that dihydrorhizobitoxine, the oxidative form of rhizobitoxine, has no distinct effect on nodulation phenotype in these legumes, and that introduction of a carbon double bond between C-3 and C-4 by the *rtxC* product is essential for the bacterium to show the rhizobitoxine phenotype in planta.

In contrast, we did not observe a significant difference between the nodulation profiles of rhizobitoxine mutants and the wild-type strain of *B. elkanii* USDA94 when *V. radiata* and *A. edgeworthii* were used as host plants. Other workers have suggested that rhizobitoxine plays a positive role in the establishment of compatible symbiosis between *B. elkanii* USDA61 and these legumes (4, 28). Our results indicate that rhizobitoxine production is not essential for the association between these legumes and USDA94. One possible explanation is that the nodulation ability of USDA94 makes it compatible with these legumes, whereas USDA61 is a less compatible partner; therefore, rhizobitoxine is probably required for effective nodulation by USDA61.

A practical aspect of rhizobitoxine production by *B. elkanii* is its application in the development of rhizobial inoculants. To attain a practical inoculation effect, the rhizobial inoculant has to be superior not only in the ability to fix nitrogen, but also in nodulation competitiveness with indigenous strains. Rhizobitoxine production could improve the inoculant in terms of both nodulation and competitiveness. We estimated previously that rhizobitoxine production by *B. elkanii* USDA94 gave the bacterium a nodulation competitiveness about 10 times higher than that of a non-rhizobitoxine-producing mutant strain on *Macroptilium atropurpureum* (24). Conferring the ability to produce rhizobitoxine and ACC deaminase into rhizobia would be one strategy to improve nodulation competitiveness of inoculants, because ethylene regulates nodulation negatively in various legumes, including *P. sativum* (10, 14), *Trifolium repens* (10), *Medicago sativa* (23, 30), *Vicia sativa* (11), *Medicago truncatula* (29), and *Lotus japonicus* (23). Furthering our understanding of rhizobitoxine biosynthesis will contribute to the construction of novel rhizobitoxine-producing rhizobia by genetic engineering. This in turn should be a very promising strategy for overcoming the competition problem and furthering progress towards sustainable agriculture.

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