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Bradyrhizobium japonicum rhizobitoxine genes and putative enzyme functions: Expression requires a translational frameshift

(toxin/symbiosis)

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ABSTRACT **Some strains of *Bradyrhizobium japonicum* produce rhizobitoxine, a phytotoxin that causes foliar chlorosis on susceptible host plants. We have previously obtained Tn*5*-induced rhizobitoxine null mutants of *B. japonicum*. DNA sequence analysis of the region surrounding two Tn*5* insertions identifies two overlapping open reading frames. The first open reading frame (*rtxA*) predicts a 54-kDa protein for which the N-terminal 280 residues have sequence similarity to serine: pyruvate aminotransferase. The sequence homology to aminotransferase is consistent with the involvement of this gene in serinol production, a likely intermediate in rhizobitoxine biosynthesis. Previously, a mutant in this open reading frame was shown not to make serinol. The predicted amino acid sequence of the second open reading frame (*rtxB*) has similarity to yeast *O*-acetylhomoserine sulfhydrolase. This enzyme function is similar to that required for dihydrorhizobitoxine synthase. The DNA sequence shows that the *rtxB* open reading frame overlaps *rtxA*, suggesting that expression of *rtxB* requires a −1 translational frameshift. Protein expression experiments demonstrate production of an RtxAB fusion protein. The ability of the overlapping *rtxA* and *rtxB* sequences to promote a translational frameshift was confirmed in a heterologous expression system. In *Escherichia coli*, this frameshift appears to be unusually efficient, occurring at a frequency of 80–90%.**

During plant–microbe interactions, metabolites are exchanged that can serve as developmental signals, as nutrients, or as toxins. In *Bradyrhizobium*–legume symbiosis, all three types of metabolite exchange occur. The symbiosis is established by release of flavonoids from the host root that induce bacterial nodulation gene expression (1, 2). In turn, the bacterial nodulation gene products synthesize modified oligomers of β-1,4-linked *N*-acetylglucosamine that serve as developmental signals, inducing the root to form a nodule structure (3). Once the nodule is established, the legume host provides the bacteria with carbon and energy, and in turn the bacterial endosymbiont provides the plant with reduced nitrogen.

Bradyrhizobium japonicum is composed of at least two divergent groups of bacteria (4). One of the many distinguishing characteristics of group I and group II *B. japonicum* is that group II *B. japonicum* synthesizes rhizobitoxine, an amino acid phytotoxin that is transported from the root nodule to the shoot where it induces foliar chlorosis (5). Rhizobitoxine is also synthesized by the broad host range plant pathogen *Pseudomonas andropogonis*, where the toxin is responsible for the chlorosis produced during infection (6). Structural analogs of rhizobitoxine, aminoethoxyvinylglycine and methoxyvinylglycine, are synthesized by a *Streptomyces* sp. and by the opportunistic human pathogen *Pseudomonas aeruginosa*, respectively (7). A study of the enzymes and genes involved in rhizobitoxine biosynthesis could, therefore, lead to insights into the biosynthesis of these biochemical molecules and add to our understanding of metabolite exchange during nodulation.

We have isolated (8) mutants of the group II *B. japonicum* USDA61 that were altered in their ability to synthesize rhizobitoxine. Among these mutants, some did not synthesize toxin in culture or *in planta* and are likely to be defective in rhizobitoxine structural genes. For two of these mutants, RX18E and RX17E, the mutated genes have been identified and sequenced.† The DNA sequence suggests two enzymatic activities that are expected to be involved in rhizobitoxine biosynthesis and demonstrates a requirement for a translational frameshift for expression of these genes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Media. *Bradyrhizobium* was grown at 30°C in yeast extract, mannitol, and gluconate medium (9), and *Escherichia coli* strains were grown at 37°C in Luria broth (LB) (10). Antibiotics were added to medium at the following concentrations: kanamycin (Km) at 150 μg/ml, tetracycline at 50 μg/ml, and streptomycin at 200 μg/ml for *Bradyrhizobium*; Km at 30 μg/ml, tetracycline at 20 μg/ml, chloramphenicol at 40 μg/ml, and ampicillin at 80 μg/ml for *E. coli*.

DNA Manipulations. Isolation of plasmid DNA, restriction enzyme digestion, DNA ligation, and bacterial transformation were performed as described (10). Tn*5*-containing *Eco*RI fragments were cloned from total genomic DNA isolated from strains RX18E and RX17E into pUC119 by selection on agar plates containing Km and ampicillin, resulting in plasmids pRX18E and pRX17E, respectively. The corresponding wild-type gene was cloned from a USDA61 genomic library constructed in λGEM-11 (Promega). Recombinant phage containing rhizobitoxine genes were identified by plaque hybridization, and a 4.3-kb *Eco*RI DNA fragment encompassing the two Tn*5* insertions was cloned into pUC119, resulting in plasmid pRX4.3.

Bacterial Matings. Tn*5* insertions from pRX18E and pRX17E were cloned into pSUP202 (11), resulting in plasmids pSUP18E and pSUP17E. Tn*5* insertions were reintroduced into *B. japonicum* USDA61 by triparental mating using the helper plasmid pRK2013 (12). Total DNAs were isolated as described (13) from rhizobitoxine-negative strains (14) and analyzed on Southern blots. Gene replacements were identified by hybridization with pSUP18E and pSUP17E.

DNA Sequencing. The DNA sequence of the portion of pRX4.3 encompassing the Tn*5* insertions was determined completely for both strands by dideoxynucleotide sequencing. Between bp 1650 and 1950 (see Fig. 1), the DNA

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Abbreviation: Km, kanamycin.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L01470).

sequence was also determined using pRX18E and pRX17E as templates. DNA sequence analysis was performed using DNA Strider (15). DNA and protein database searches (GenBank Release 72) were performed using FASTA (16) and BLAST (17). Multiple sequence alignments were performed using CLUSTAL V (18).

Protein Expression. Protein expression was analyzed using the T7 expression system as described (19). To facilitate protein isolation, the plasmid pET3b was modified to contain six histidine codons by replacing the sequence between the *Ndè* I and *Bam*HI sites with the sequence 5′-TATG-CATCACCATCACCATCACCTCGAGACCATG. This plasmid, pET3-His, was kindly provided by B. Chen and T. Hai (Ohio State Biotechnology Center).

To place the complete open reading frames under control of T7 RNA polymerase, plasmids pRtxAB/BglII and pRtxAB were constructed (see Fig. 1). An *Alw*NI–*Bgl* II fragment from bp 449 to 2139 of pRX4.3 was made blunt-ended first by filling in the *Bgl* II site with the Klenow fragment of DNA polymerase I and then treating the *Alw*NI site with T4 DNA polymerase. The open reading frame of *rtxA* was placed in-phase by cloning it into the *Nco* I site of pET3-His that had been filled-in with the Klenow fragment to produce pRtxAB/BglII. A plasmid containing both complete open reading frames, pRtxAB, was constructed by replacing the *Hin*dIII–*Eco*RI fragment of pRtxAB/BglII with the large *Hin*dIII–*Eco*RI fragment from pRX4.3.

Plasmids pET3-His, pRtxAB/BglII, and pRtxAB were introduced into *E. coli* BL21(DE3)pLysS *hsdS gal*(λ*cIts857 indI Sam7 nin5 lacUV5*-T7 gene *1*) (19). For induction of T7 RNA polymerase-dependent gene expression, overnight cultures were diluted 1:10 into LB containing ampicillin and chloramphenicol. When cultures reached an OD_{600} of 0.6–1.0, isopropyl β-D-thiogalactoside was added to a final concentration of 2 mM. Cultures were incubated for an additional 3–5 h, and cells from 1 ml of culture were collected by centrifugation. Cells were resuspended in 100 μl of 2× loading buffer [400 mM Tris·HCl/20% (vol/vol) glycerol/2% (wt/vol) SDS/5% (vol/vol) 2-mercaptoethanol/0.0025% bromophenol blue] and heated at 100°C for 5 min. Proteins were separated by SDS/PAGE on 12% polyacrylamide gels and visualized by Coomassie blue staining (10).

Translational Frameshift in a Heterologous System. DNA sequences between bp 1654 and 1928 of pRX4.3 (see Fig. 4) were amplified using the PCR. Primers were constructed from sequences homologous to (5′-CCGGATCCCGAACTTGATGGCGAT) and complementary to (5′-GCGGATCCCGCTTCAGCGTGTTAT) the sequences underlined in Fig. 4. For each oligonucleotide, 8 additional bases were included at the 5′ end of the primer sequences to introduce a *Bam*HI site, underlined in the primer sequence above. Upon amplification and digestion with *Bam*HI, the 281-bp fragment was cloned into *Bam*HI-digested pORF1/BamHI (see Fig. 6). Plasmids containing the insert in both orientations were identified by restriction enzyme analysis and confirmed by DNA sequencing. Recombinant plasmids were introduced into *E. coli* MH3000 *araD139* Δ*(ara, leu)7697* Δ*(lac)X74 galU galK rpsL (str^R) ompR101* (20).

RESULTS

Isolation and Characterization of Rhizobitoxine Structural Genes. Rhizobitoxine mutant strains RX18E and RX17E do not make rhizobitoxine *in planta* or in culture (8) and each was determined to contain a single Tn*5* insertion by Southern blot analysis (data not shown). Tn*5*-containing *Eco*RI fragments from each of these mutants were cloned by virtue of the Tn*5* Km-resistant phenotype, resulting in plasmids pRX18E and pRX17E. Restriction enzyme analysis of these clones revealed that the two Tn*5* insertions are in the same *Eco*RI fragment and that their insertion sites are located ≈1.9 kb apart (Fig. 1).

To confirm that the Tn*5* insertions are the primary mutation in these rhizobitoxine mutants, we sought to reconstruct these mutations by using gene replacement. For Tn*5*-17E, gene replacement events were observed in 4 of 17 Km-resistant strains. The reconstructed RX17E mutants were tested for rhizobitoxine production in culture (14) and chlorosis phenotype on soybean cultivar Lee. No chlorosis was observed on plants inoculated with these reconstructed mutants, whereas wild-type USDA61 caused chlorosis. These results confirm the Tn*5*-17E insertion as the primary mutation in RX17E. For the Tn*5*-18E mating, only cointegration events and no gene replacement events were observed in 30 Km-resistant strains. Although we were unable to confirm the Tn*5*-18E insertion as the primary mutation in RX18E by gene replacement, its insertion near that of Tn*5*-17E suggests that it is likely to be the primary mutation in RX18E.

The DNA sequence was determined for ≈3 kb of DNA surrounding the two Tn*5* insertions. Analysis of the DNA sequence revealed two overlapping open reading frames as shown in Fig. 1. Each of these open reading frames is interrupted by one of the Tn*5* insertions. The open reading frame interrupted by Tn*5*-18E is designated *rtxA*, and the open reading frame interrupted by Tn*5*-17E is designated *rtxB*.

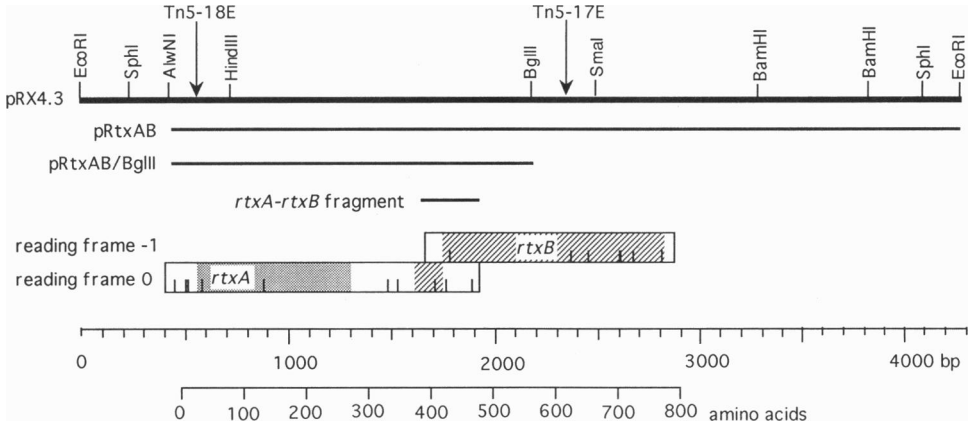

FIG. 1. Restriction enzyme and open reading frame map of pRX4.3. Open reading frames are indicated by rectangles that encompass sequences between stop codons. Possible initiation codons (ATG or GTG) are indicated by short lines within the open reading frame. Portions of open reading frames are similar to rat serine:pyruvate aminotransferase (shaded boxes) or yeast *O*-acetylhomoserine sulfhydrolase (hatched boxes). Plasmid constructs, pRtxAB and pRtxAB/BglII, used in protein expression studies and the *rtxA–rtxB* overlap fragment are also indicated.

The predicted amino acid sequences for *rtxA* and *rtxB* suggested their possible enzymatic functions. The predicted *rtxA* gene product is a 493-amino acid protein with the N-terminal 280 residues having 27% amino acid sequence identity and an additional 40% conserved amino acid substitutions with rat or human serine:pyruvate aminotransferase (refs. 21 and 22; Fig. 2*A*). The program BLAST calculates that

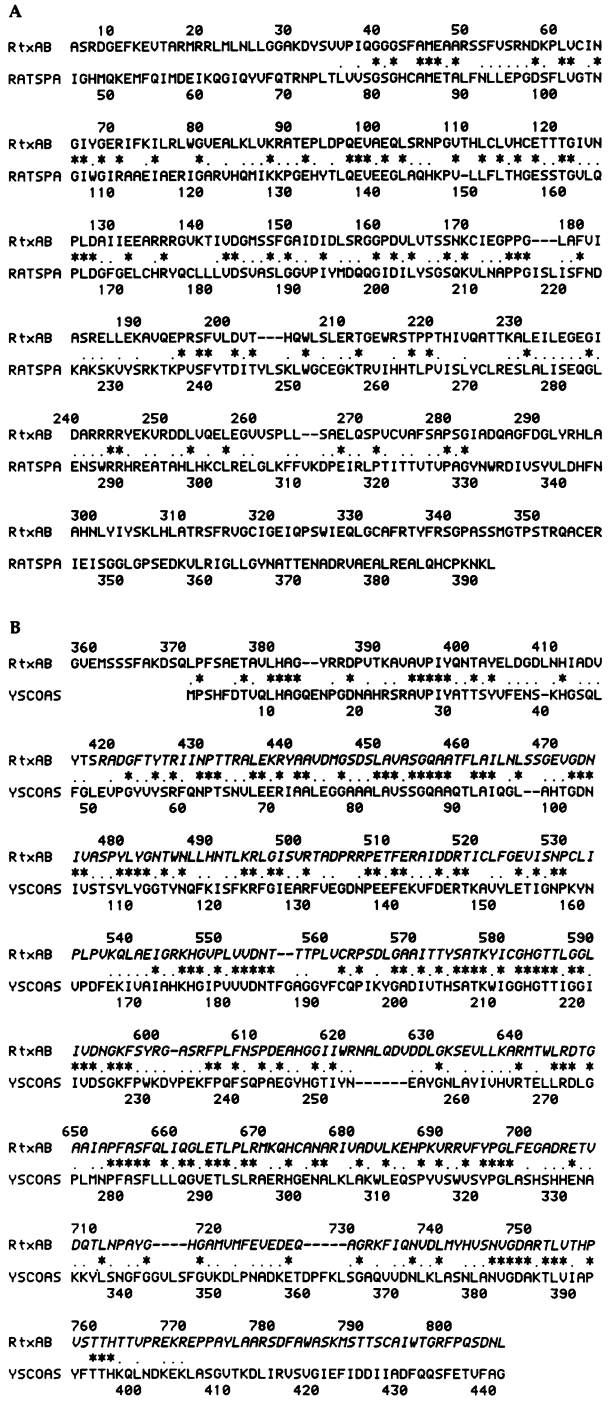

FIG. 2. Protein sequence similarity of RtxAB to known enzymes. (*A*) Comparison of RtxAB to rat serine:pyruvate aminotransferase (RATSPA; GenBank accession no. X06357). (*B*) Comparison of RtxAB with yeast *O*-acetylhomoserine sulfhydrolase (YSCOAS; GenBank accession no. X04493). The italicized amino acids in RtxAB are those from the −1 reading frame. For the purposes of amino acid comparison, the position of the frameshift was determined by the best conservation of amino acids to the sulfhydrolase. Amino acid identities are indicated with an asterisk and conserved substitutions are indicated with a dot.

the observed similarities would occur at random with a probability of 5.1×10^{-6}. An aminotransferase activity is required for the biosynthesis of serinol from dihydroxyacetone in sugarcane (23). Serinol, a proposed precursor of rhizobitoxine (24), is abundant in USDA61-elicited nodules but does not accumulate in RX18E-elicited nodules (8). These data are consistent with the *rtxA* gene product functioning as an aminotransferase in serinol production. The probable positions of serinol and the *rtxA* gene product in the biosynthesis of rhizobitoxine are illustrated in Fig. 3.

The predicted amino acid sequence for the *rtxB* gene product has 38% sequence identity and an additional 38% conserved amino acid substitutions with yeast *O*-acetylhomoserine sulfhydrolase (ref. 25; see Fig. 2*B*). BLAST calculates that the observed similarities would occur at random with a probability of 1.4×10^{-42}. An enzyme activity similar to that of the sulfhydrolase may form dihydrorhizobitoxine, a presumed intermediate in rhizobitoxine biosynthesis (ref. 24; see Fig. 3). The *rtxB* gene product may, therefore, be a dihydrorhizobitoxine synthase, for which the substrates would be serinol and homoserine or derivatives thereof.

A Translational Frameshift Is Required for Expression of Rhizobitoxine Genes. Two observations suggest that a translational frameshift is required for expression of *rtxB*. The sequence similarity of the predicted gene product of *rtxB* to sulfhydrolase extends upstream of the first translation initiation codon in *rtxB* at bp 1776 (Fig. 1). In addition, amino acid sequence similarity to sulfhydrolase was found in amino acids 371–418 of the predicted gene product of *rtxA* (Figs. 1 and 4). This arrangement of open reading frames suggests that a −1 translational frameshift is required for expression of *rtxB* and that the *rtxB* gene product is expressed as a RtxAB fusion protein. This prediction was tested by protein expression experiments.

To evaluate the protein products of *rtxA* and *rtxB*, a full-length template and a truncated template of *rtxA* and *rtxB* were placed under the control of a T7 RNA polymerase promoter in a derivative of plasmid pET3b, pET3-His. For these constructs, protein products contain 11 vector-encoded amino acids at the N terminus. For the full-length template containing plasmid pRtxAB (Fig. 1), two protein products are predicted. Translation of *rtxA* would produce a 55-kDa protein. If there is translational frameshift, a RtxAB fusion

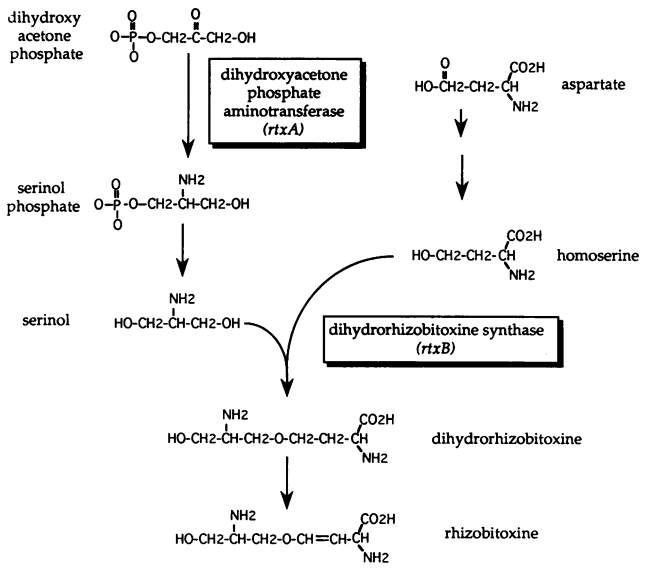

FIG. 3. Proposed biosynthetic pathway of rhizobitoxine. A proposed biosynthetic pathway for rhizobitoxine was adopted from Mitchell and Coddington (24) with additional information from this study and refs. 8 and 23.

```
1621     tgtcgcagtgccgatctatcagaacacggcttacgaacttgatggcgatctgaatcacat
frame-1                                          ***TrpArgSerGluSerHis
frame0    ValAlaValProIleTyrGlnAsnThrAlaTyrGluLeuAspGlyAspLeuAsnHisIle

1681     tgcggacgtctatacgtcaaggctgatggattcacctatacgaggatcatcaacccgacg
frame-1  CysGlyArgLeuTyrValLysAlaAspGlyPheThrTyrThrArgIleIleAsnProThr
frame0    AlaAspValTyrThrSerArgLeuMetAspSerProIleArgGlySerSerThrArgArg

1741     acccgcgcgctggaaaaaaggtatgccgcggtcgacatgggaagcgactcgctcgccgtc
frame-1  ThrArgAlaLeuGluLysArgTyrAlaAlaValAspMetGlySerAspSerLeuAlaVal
frame0    ProAlaArgTrpLysLysGlyMetProArgSerThrTrpGluAlaThrArgSerProSer

1801     gcatcaggtcaagcggcaaccttccttgccatcctcaacctgtcaagcggcgaggtgggg
frame-1  AlaSerGlyGlnAlaAlaThrPheLeuAlaIleLeuAsnLeuSerSerGlyGluValGly
frame0    HisGlnValLysArgGlnProSerLeuProSerSerThrCysGlnAlaAlaArgTrpGly

1861     gacaatatcgtcgcctcaccgtatctctatggcaacacgtggaatctgctccataacacg
frame-1  AspAsnIleValAlaSerProTyrLeuTyrGlyAsnThrTrpAsnLeuLeuHisAsnThr
frame0    ThrIleSerSerProHisArgIleSerMetAlaThrArgGlyIleCysSerIleThrArg

1921     ctgaagcgtcttggtatcagcgtcagaacggcagacccccgaaggcccgagaccttcgaa
frame-1  LeuLysArgLeuGlyIleSerValArgThrAlaAspProArgArgProGluThrPheGlu
frame0    ***
```

FIG. 4. DNA sequence and translation products for overlap region of *rtxA* and *rtxB*. The DNA sequence is presented with translations. Termination codons are indicated by asterisks. Underlined amino acid residues have sequence identity with yeast sulfhydrolase. The reading frame similar to the sulfhydrolase changes between bp 1670 and 1700.

protein of 90 kDa should be produced. Proteins with expected sizes of 55 and 90 kDa are observed in protein extracts of cells expressing the full-length template (Fig. 5). For the *Bgl* II-truncated template pRtxAB/BglII (Fig. 1), the 55-kDa RtxA translation product is unaffected, but the predicted size of the frameshift product is reduced to 64 kDa. Proteins with predicted sizes of 55 and 64 kDa are observed in cells expressing pRtxAB/BglII (Fig. 5). These results confirm that a frameshift fusion protein is made from *rtxAB* in *E. coli*. If the proteins have similar half-lives, then a comparison of the relative staining intensities of the protein bands suggests an exceptionally high translational frameshift efficiency of 80–90%. It is unlikely that either protein species turns over during the expression period because both proteins are sequestered in inclusion bodies.

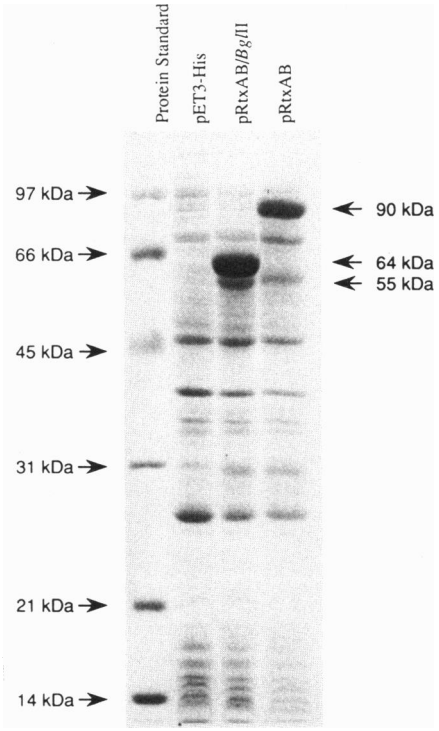

FIG. 5. Protein expression of *rtxA* and *rtxB*. Protein extracts from isopropyl β-D-thiogalactoside-induced *E. coli* BL21(DE3)pLysS cultures carrying pET3-His, pRtxAB/BglII, or pRtxAB were separated by gel electrophoresis and visualized with Coomassie staining. Protein molecular mass markers are indicated.

Gene Overlap Promotes a Translational Frameshift. The DNA sequence and expression of both the *rtxA* and *rtxAB* gene products demonstrate that a −1 frameshift must occur during translation. Two pieces of evidence indicate where the frameshift might occur. (*i*) The similarity to sulfhydrolase changes from reading frame 0 to frame −1 between bp 1670 and 1700 (Fig. 4). (*ii*) The translational frameshift must occur in the overlap region of the two open reading frames as defined by the stop codons at bp 1660 in frame 0 and at bp 1922 in frame −1 (Figs. 1 and 4). To demonstrate that the DNA sequence encompassing the open reading frame overlap promotes a −1 translational frameshift, these sequences were amplified by PCR and cloned into the *Bam*HI site of pORF1/BamHI (Fig. 6). Plasmid pORF1 contains the *E. coli ompF* promoter and a partial *ompF* open reading frame fused out of frame to *E. coli lacZ*, such that production of β-galactosidase activity requires a translational frameshift. Colonies containing pORF1 are white when grown on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) plates as expected. Digestion of pORF1 with *Bam*HI followed by religation places the *ompF* and *lacZ* reading frames in phase, resulting

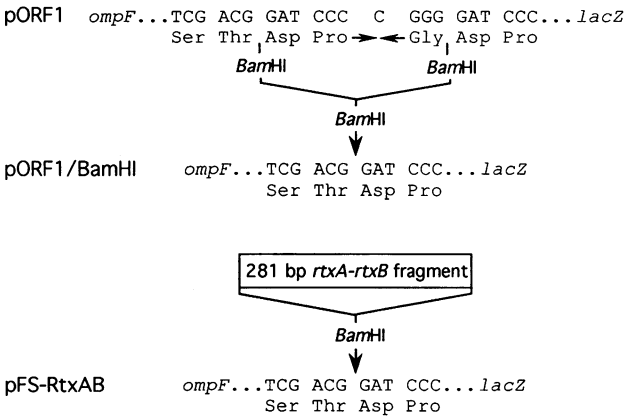

FIG. 6. Plasmid constructs for heterologous frameshift experiment. Plasmid pORF1 contains the *ompF* promoter and a partial *ompF* open reading frame fused out of frame to *E. coli lacZ* such that production of β-galactosidase activity requires a translational frameshift. Plasmid pORF1/BamHI brings the *ompF* and *lacZ* open reading frames into phase. Plasmid pFS-RtxAB contains a 281-bp DNA fragment that includes the *rtxA* and *rtxB* open reading frame overlap (Fig. 4) and requires a −1 frameshift for *lacZ* expression.

in plasmid pORF1/BamHI. Colonies containing pORF1/BamHI are blue when grown on X-gal plates. Introduction of a 281-bp (93 codons plus 2 bp) DNA fragment encompassing the *rtxA–rtxB* overlap region into the *Bam*HI site of pORF1/BamHI creates plasmid pFS-RtxAB and places the *ompF* reading frame out of frame with the *lacZ* open reading frame such that production of β-galactosidase activity requires a −1 translational frameshift. Bacteria containing the pFS-RtxAB plasmid develop blue colonies on X-gal agar plates equal in intensity to those colonies containing pORF1/BamHI. This demonstrates that the *rtxA–rtxB* overlap region is sufficient to promote a −1 translational frameshift.

DISCUSSION

The cloning and characterization of two rhizobitoxine genes has substantiated the presumed biosynthetic pathway for this toxin. Serinol is abundant in nodules formed by group II *Bradyrhizobium* (26, 27), and it has been proposed as a precursor of rhizobitoxine (24). We have shown (8) that mutant RX18E does not accumulate serinol in nodules. The only other demonstrated occurrence of serinol in biological systems is in sugarcane, where it is biosynthesized via amino transfer to dihydroxyacetone phosphate, as depicted in Fig. 3. Our finding that Tn*5*-18E disrupts a gene that has amino acid sequence homology to aminotransferase is consistent with such a biosynthetic scheme and provides further evidence for the role of serinol in rhizobitoxine biosynthesis.

The similarity of the *rtxB* gene product to yeast *O*-acetylhomoserine sulfhydrolase is consistent with the function required of a proposed dihydrorhizobitoxine synthase. Dihydrorhizobitoxine synthase would form dihydrorhizobitoxine from serinol and homoserine or derivatives activated by succinylation, acetylation, or phosphorylation. Confirmation of this role for *rtxB* in rhizobitoxine synthesis should come from studies identifying an absence of dihydrorhizobitoxine in mutants RX18E and RX17E.

The DNA sequence, protein expression data, and ability of the *rtxA–rtxB* overlap region to allow expression of *lacZ* demonstrate that a translational frameshift occurs during expression of *rtxB*. Translational frameshifting has not been shown in rhizobia but has been identified in several systems, including but not limited to *E. coli* RF2 (28), bacteriophage T7 gene *10* (29), IS*1* transposase (30, 31), and human immunodeficiency virus and other retrovirus *gag–pol* genes (32, 33). There are several different mechanisms that promote −1 and +1 frameshifts over a broad range of efficiencies (for review, see ref. 34). Our data are consistent with a −1 frameshift but do not exclude a +2 frameshift or ribosome hopping. The frequency of frameshifting of the *rtxA–rtxB* overlap when expressed in *E. coli* appears to be extremely high, 80–90%, which suggests an efficient molecular mechanism. We do not presently know the frequency of frameshifting for *rtxA–rtxB* in *B. japonicum*, and it is possible that the rate of frameshifting in *B. japonicum* will not be the same as in *E. coli*.

There are a couple of ways in which translational frameshifting may be important in rhizobitoxine biosynthesis. One result of frameshifting is production of a constant ratio of two gene products, RtxA and RtxAB. By balancing the production of enzymes, a balance between serinol and rhizobitoxine production can be maintained. The RtxAB fusion protein carries out an enzymatic step that commits metabolites from common amino acid biosynthesis to rhizobitoxine production and may, therefore, be a key regulatory point. By regulating the frequency of frameshifting, the bacteria may regulate the biosynthesis of toxin.

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