Applied and Environmental Microbiology, Dec. 1993, p. 4371–4373 Vol. 59, No. 12
0099-2240/93/124371-03$02.00/0
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Common Soybean Inoculant Strains in Brazil Are Members of *Bradyrhizobium elkanii*

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Received 12 July 1993/Accepted 24 September 1993

The Brazilian inoculant strains 29W and 587 were found to be members of *Bradyrhizobium elkanii* primarily on the basis of 16S rRNA gene sequences identical to that of *B. elkanii* USDA76 and on the basis of reactivity with antibodies against serogroups 76 and 31, respectively. The agronomic consequences of using strains of *B. elkanii* as soybean inoculants are discussed.

Brazil is second only to the United States in soybean production (21). Despite the importance of soybean production in Brazil, little is known about the taxonomy of the strains used for soybean inoculation in that country. Strains 29W and 587 efficiently nodulate soybean, lack uptake hydrogenase activity, and have been widely used as commercial inoculants for soybean in Brazil (18). In this work, our objective was to determine whether the Brazilian inoculant strains, 29W and 587, were members of *Bradyrhizobium elkanii* or *Bradyrhizobium japonicum.*

Strains of *B. japonicum* were categorized into three DNA homology groups by Hollis et al. (12). Homology group II differs from homology groups I and Ia in host range (5, 6), foliar chlorosis induction (4, 9, 15–17, 19), exopolysaccharide composition (16), colony morphology (9), uptake hydrogenase expression (16), restriction fragment length polymorphism analysis (14), and 16S rRNA gene sequence (26). On the basis of these results, Kuykendall et al. (1, 14) proposed that a new species designation, *B. elkanii*, be used for DNA homology group II strains.

Host range, foliar chlorosis, and intrinsic antibiotic resistance. Strains 29W and 587 were similar to *B. elkanii* USDA61 in that they were capable of limited nodulation of *Glycine max* (L.) Merr. cv. Clark genotype rj_1rj_1, induced foliar chlorosis on Clark soybean, and expressed high levels of intrinsic resistance to streptomycin, spectinomycin, and tetracycline (data not shown). This is in contrast to strains of *B. japonicum*, which do not nodulate soybeans with the rj_1rj_1 genotype, do not induce foliar chlorosis, and are sensitive to those antibiotics.

Southern analysis of common nodulation genes. All DNA isolations and analyses were performed as described previously (2). *Bam*HI fragments of bradyrhizobial total DNA were separated by electrophoresis and transferred to a nitrocellulose filter (23). The filter was probed with the 3.9-kb *Hin*dIII fragment from pMJS18 (20), which contains *nodD₁YABC*, by using the ECL kit from Amersham (Arlington Heights, Ill.). DNA from strain 29W showed a single 6.0-kb hybridization band identical in size to those in DNA from *B. elkanii* USDA61, USDA83, and USDA94 (data not shown). The size of the fragment containing the common *nod* genes in strain 587 was 7.5 kb, which is intermediate between the 6.0- and 8.3-kb fragments in *B. elkanii* USDA61 and *B. japonicum* USDA110, respectively (data not shown).

Serogroup classification. Serotyping of strains 29W and 587 was done with fluorescent antibodies raised against several strains of *Bradyrhizobium* (22). Strain 29W reacted with antibodies raised against USDA76 (data not shown). Fuhrmann and Wollum (10) showed that strain 587 reacts with antibodies raised against USDA31. This places 29W and 587 in serogroups 76 and 31, respectively. Strains USDA76 and USDA31 are both members of *B. elkanii*, with strain USDA76 being the type strain of that species (14). Strain USDA110 did not cross-react with antibodies raised against either USDA76 or USDA31.

Nucleotide sequence of 16S rRNA genes. The internal region of the 16S rRNA gene, which is variable among strains of *Bradyrhizobium* and *Rhizobium*, was sequenced. A 312-bp region of 16S rRNA from each rhizobial strain was amplified by polymerase chain reaction (PCR) and sequenced in a manner similar to that described by Young et al. (26) with the conserved primers Y1 (5′-TGGCTCA GAACGAACGCTGGCGGC-3′) and Y2 (5′-CCCACTGCT GCCTCCCGTAGGAGT-3′). Each 15-μl PCR mixture contained 0.2 mM each deoxynucleoside triphosphate, 0.75 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.5), 20 mM KCl, 0.5 mg of bovine serum albumin per ml, and 20 ng of template DNA. The concentration of each primer in the reaction mixture was approximately 2 μM. The reactions were performed in an Air Thermal Cycler (Idaho Technology, Idaho Falls, Idaho). The profile parameters were 5 min of denaturation at 93°C followed by 35 cycles of 45 s at 93°C, 45 s of annealing at 62°C, 120 s of elongation at 72°C, and a final soak for 5 min at 72°C.

Double-stranded PCR products were purified by using the Magic PCR Preps system (Promega, Madison, Wis.). The purified DNA was denatured by boiling for 40 s followed by rapid cooling in a dry ice-methanol bath. Dideoxy sequencing was carried out with Sequenase (U.S. Biochemical, Cleveland, Ohio). The reactions were run with 120 ng of

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              1                                                50
B sp 2257   GCGGGCgTAG CAATAcGTCA GCGGCAGACG GGTGAGTAAC GCGTGGGAAC
B j 3407    GCGGGCgTAG CAATAcGTCA GCGGCAGACG GGTGAGTAAC GCGTGGGAAC
B j 110     GCGGGCgTAG CAATAcGTCA GCGGCAGACG GGTGAGTAAC GCGTGGGAAC
B j 59      GCGGGCgTAG CAATAcGTCA GCGGCAGACG GGTGAGTAAC GCGTGGGAAC
B e 31      GCGGGCaTAG CAATAtGTCA GCGGCAGACG GGTGAGTAAC GCGTGGGAAC
B e 76      GCGGGCaTAG CAATAtGTCA GCGGCAGACG GGTGAGTAAC GCGTGGGAAC
B e 29w     GGGGGCaTAG CAATAtGTCA GCGGCAGACG GGTGAGTAAC GCGTGGGAAC
B e 587     GCGGGCaTAG CAATAtGTCA GCGGCAGACG GGTGAGTAAC GCGTGGGAAC
Consensus   GCGGGC-TAG CAATA-GTCA GCGGCAGACG GGTGAGTAAC GCGTGGGAAC

              51                                              100
B sp 2257   aTACCTTTTG GTTCGGAACA ACacAGGGAA ACTTgtGCTA ATACCGGATA
B j 3407    gTACCTTTTG GTTCGGAACA ACacAGGGAA ACTTgtGCTA ATACCGGATA
B j 110     gTACCTTTTG GTTCGGAACA ACacAGGGAA ACTTgtGCTA ATACCGGATA
B j 59      gTACCTTTTG GTTCGGAACA ACacAGGGAA ACTTgtGCTA ATACCGGATA
B e 31      gTACCTTTTG GTTCGGAACA ACtgAGGGAA ACTTcaGCTA ATACCGGATA
B e 76      gTACCTTTTG GTTCGGAACA ACtgAGGGAA ACTTcaGCTA ATACCGGATA
B e 29w     gTACCTTTTG GTTCGGAACA ACtgAGGGAA ACTTcaGCTA ATACCGGATA
B e 587     gTACCTTTTG GTTCGGAACA ACtgAGGGAA ACTTcaGCTA ATACCGGATA
Consensus   -TACCTTTTG GTTCGGAACA AC--AGGGAA ACTT--GCTA ATACCGGATA

              101                                             150
B sp 2257   AGCCCTTACG GGGAAAGATT TATCGCCGAA AGATtGGCCC GCGTCTGATT
B j 3407    AGCCCTTACG GGGAAAGATT TATCGCCGAA AGATcGGCCC GCGTCTGATT
B j 110     AGCCCTTACG GGGAAAGATT TATCGCCGAA AGATcGGCCC GCGTCTGATT

B j 59      AGCCCTTACG GGGAAAGATT TATCGCCGAA AGATcGGCCC GCGTCTGATT
B e 31      AGCCCTTACG GGGAAAGATT TATCGCCGAA AGATcGGCCC GCGTCTGATT
B 3 76      AGCCCTTACG GGGAAAGATT TATCGCCGAA AGATcGGCCC GCGTCTGATT
B e 29w     AGCCCTTACG GGGAAAGATT TATCGCCGAA AGATcGGCCC GCGTCTGATT
B e 587     AGCCCTTACG GGGAAAGATT TATCGCCGAA AGATcGGCCC GCGTCTGATT
Consensus   AGCCCTTACG GGGAAAGATT TATCGCCGAA AGAT-GGCCC GCGTCTGATT

              151                                 188
B sp 2257   AGCTAGTTGG TagGGTAATG GCctACCAAG GCGACGAT
B j 3407    AGCTAGTTGG TagGGTAAcG GCctACCAAG GCGACGAT
B j 110     AGCTAGTTGG TagGGTAAcG GCctACCAAG GCGACGAT
B j 59      AGCTAGTTGG TgaGGTAATG GCtcACCAAG GCGACGAT
B j 31      AGCTAGTTGG TgaGGTAATG GCtcACCAAG GCGACGAT
B j 76      AGCTAGTTGG TgaGGTAATG GCtcACCAAG GCGACGAT
B j 29w     AGCTAGTTGG TgaGGTAATG GCtcACCAAG GCGACGAT
B j 587     AGCTAGTTGG TgaGGTAATG GCtcACCAAG GCGACGAT
Consensus   AGCTAGTTGG T--GGTAATG GC--ACCAAG GCGACGAT
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FIG. 1. Sequence alignment of 16S rRNA genes from eight strains of *Bradyrhizobium*. The consensus sequence is shown at the bottom for those nucleotides which are identical for all eight strains. Those bases which differ are shown as dashes in the consensus sequences and as lowercase letters in the strain sequences. Sequences of strains NZP 2257, RCR 3407, USDA110, USDA59, USDA31, and USDA76 were taken from the work of Young et al. (26). B sp, *Bradyrhizobium* species strain; B j, *B. japonicum*; B e, *B. elkanii.*

template DNA and 336 ng of either primer Y1 or Y2. Multiple sequence alignment and calculation of the consensus sequence were determined with the PRETTY program of the Genetics Computer Group (Madison, Wis.) package (3).

The multiple sequence alignment of the internal variable region of the 16S rRNA gene for several *Bradyrhizobium* strains is shown in Fig. 1. The 188 bases sequenced are identical among strains 29W and 587 and the known sequences from *B. elkanii* USDA31 and USDA76. The *B. elkanii* sequences differ from those of *B. japonicum* USDA59 and USDA110 and *Bradyrhizobium* sp. strain NZP 2257 by 6, 10, and 11 bases, respectively.

Thus, two important inoculum strains from Brazil are members of *B. elkanii*. This conclusion is based on six lines of evidence: (i) the ability of these strains to nodulate soybeans with the rj_1rj_1 genotype; (ii) their ability to induce chlorosis in soybean leaves; (iii) their expression of high levels of intrinsic antibiotic resistance; (iv) their classification as either serogroup 76 or 31, both of which include members of *B. elkanii*; (v) common *nod* gene restriction patterns similar to that of strains of *B. elkanii*; and (vi) 16S rRNA gene sequences identical to that from strains of *B. elkanii*. Also, Neves et al. (18) have shown that strains 29W and 587 lack uptake hydrogenase activity, as is the case with other strains of *B. elkanii*.

The fact that soybean inoculants commonly used in Brazil are *B. elkanii* strains may have important consequences for inoculation practices in that country, given recent agronomic results in the United States and Brazil. Recent studies by Fuhrmann and coworkers (24, 25) suggest that soybeans inoculated with rhizobitoxine-producing *B. elkanii* strains exhibit smaller chlorophyll concentrations in leaves, lower nodule and shoot dry weight, lower total N_2 fixation, and less leaf protein than plants inoculated with non-chlorosis-inducing strains of either *B. elkanii* or *B. japonicum*. Soybeans inoculated with strains 29W and 587 had grain yield production about 30% below that of soybeans inoculated with CB1809, a *B. japonicum* strain used as a commercial inoculant in Australia (18). These yield declines may be caused by both the lack of uptake hydrogenase activity and the ability to produce rhizobitoxine in soybean nodules induced by *B. elkanii* strains (15, 16). The absence of uptake hydrogenase activity reduces the yield of soybean plants (7, 8, 11, 13).

Nucleotide sequence accession numbers. The partial sequences of the 16S rRNA genes from strains 587 and 29W have been assigned accession numbers L20781 and L20867, respectively.

This work was supported by USDA competitive grant 9103443 and by the University of Wisconsin—Madison College of Agricultural and Life Sciences Hatch Project D479 to E.W.T. and by fellowship support of N.G.R. by EMBRAPA/CNPAB and CNPq of Brazil.

REFERENCES

1. **Anonymous.** 1993. Validation of the publication of new names and new combinations previously effectively published outside the IJSB: list no. 45. Int. J. Syst. Bacteriol. **43:**398–399.
2. **Breil, B. T., P. W. Ludden, and E. W. Triplett.** 1993. DNA sequence and mutational analysis of genes involved in the production and resistance of the antibiotic peptide trifolitoxin. J. Bacteriol. **175:**3693–3702.
3. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the Vax. Nucleic Acids Res. **12:**387–395.
4. **Devine, T. E., L. D. Kuykendall, and J. J. O'Neil.** 1988. DNA

homology group and the identity of bradyrhizobial strains producing rhizobitoxine-induced foliar chlorosis on soybean. Crop Sci. **28**:938–941.

5. **Devine, T. E., L. D. Kuykendall, and J. J. O'Neil.** 1990. The Rj4 allele in soybean represses nodulation by chlorosis-inducing bradyrhizobia classified as DNA homology group II by antibiotic resistance profiles. Theor. Appl. Genet. **80**:33–37.
6. **Devine, T. E., and D. F. Weber.** 1977. Genetic specificity of nodulation. Euphytica **26**:527–535.
7. **Evans, H. J., F. J. Hanus, R. A. Haugland, M. A. Cantrell, L. S. Xu, S. A. Russell, G. R. Lambert, and A. R. Harker.** 1985. Hydrogen recycling in nodules affects nitrogen fixation and growth of soybeans. Proc. World Soybean Conf. **3**:935–942.
8. **Evans, H. J., A. R. Harker, H. Papen, S. A. Russell, F. J. Hanus, and M. Zuber.** 1987. Physiology, biochemistry, and genetics of uptake hydrogenase in rhizobia. Annu. Rev. Microbiol. **41**:335–361.
9. **Fuhrmann, J.** 1990. Symbiotic effectiveness of indigenous soybean bradyrhizobia as related to serological, morphological, rhizobitoxine, and hydrogenase phenotypes. Appl. Environ. Microbiol. **56**:224–229.
10. **Fuhrmann, J., and A. G. Wollum II.** 1985. Simplified enzyme-linked immunosorbent assay for routine identification of *Rhizobium japonicum* antigens. Appl. Environ. Microbiol. **49**:1010–1013.
11. **Hanus, F. J., S. L. Albrecht, R. M. Zablotowicz, D. W. Emerich, and S. A. Russell.** 1981. Yield and N content of soybean seed as influenced by *Rhizobium japonicum* inoculants possessing the hydrogenase characteristic. Agron. J. **73**:368–372.
12. **Hollis, A. B., W. E. Kloos, and G. H. Elkan.** 1981. DNA:DNA hybridization studies of *Rhizobium japonicum* and related *Rhizobiaceae*. J. Gen. Microbiol. **123**:215–222.
13. **Hungria, M., M. C. P. Neves, and J. Dobereiner.** 1989. Relative efficiency, ureide transport and harvest index in soybeans inoculated with isogenic HUP mutants of *Bradyrhizobium japonicum*. Biol. Fertil. Soils **7**:325–329.
14. **Kuykendall, L. D., B. Saxena, T. E. Devine, and S. E. Udell.** 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp-nov. Can. J. Microbiol. **38**:501–505.
15. **LaFavre, J. S., and A. R. J. Eaglesham.** 1986. Rhizobitoxine: a phytotoxin of unknown function which is commonly produced by bradyrhizobia. Plant Soil **92**:443–452.
16. **Minamisawa, K.** 1989. Comparison of extracellular polysaccharide composition, rhizobitoxine production, and hydrogenase phenotype among various strains of *B. japonicum*. Plant Cell Physiol. **30**:877–884.
17. **Minamisawa, K., and K. Fukai.** 1991. Production of indole-3-acetic acid by *Bradyrhizobium japonicum*: a correlation with genotype grouping and rhizobitoxine production. Plant Cell Physiol. **32**:1–9.
18. **Neves, M. C. P., A. D. Didonet, F. F. Duque, and J. Dobereiner.** 1985. *Rhizobium* strain effects on nitrogen transport and distribution in soybeans. J. Exp. Bot. **36**:1170–1192.
19. **Owens, L. D., and D. A. Wright.** 1965. Production of the soybean-chlorosis toxin by *Rhizobium japonicum* in pure culture. Plant Physiol. (Bethesda) **40**:931–933.
20. **Sadowsky, M. J., P. B. Cregan, and H. H. Keyser.** 1990. DNA hybridization probe for use in determining restricted nodulation among *Bradyrhizobium japonicum* serocluster 123 field isolates. Appl. Environ. Microbiol. **56**:1768–1774.
21. **Sanford, S.** 1993. Oil crops situation and outlook report. Economic Research Service, U.S. Department of Agriculture, Washington, D.C.
22. **Somasegaran, P., and H. J. Hoben.** 1985. Methods in legume-Rhizobium technology. University of Hawaii NifTAL Project, Maui, Hawaii.
23. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
24. **Teaney, G. B., and J. J. Fuhrmann.** 1992. Soybean response to nodulation by bradyrhizobia differing in rhizobitoxine phenotype. Plant Soil **145**:275–285.
25. **Vasilas, B. L., and J. J. Fuhrmann.** 1993. Field response of soybean to nodulation by a rhizobitoxine-producing strain of *Bradyrhizobium*. Agron. J. **85**:302–305.
26. **Young, J. P. W., H. L. Downer, and B. D. Eardly.** 1991. Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J. Bacteriol. **173**:2271–2277.