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Plasmid Contents of Commercial *Rhizobium leguminosarum* biovar *phaseoli* Strains

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Bacteria of the genera *Rhizobium* and *Bradyrhizobium* nodulate leguminous plants and convert atmospheric dinitrogen into ammonia for plant use. *Rhizobium leguminosarum* biovar *phaseoli* strains fix dinitrogen in symbiotic associations with *Phaseolus* beans, and rhizobial preparations specifically intended for these agriculturally important legumes are supplied by the legume inoculant industry. The *R. leguminosarum* bv. *phaseoli* strains used in commercial inoculants have been chosen because of superior symbiotic properties identified by plant-inoculation tests, and molecular characterizations of the strains are generally lacking.

Rhizobia often contain large indigenous plasmids (2). Moreover, because nitrogen-fixation (*nif*) genes appear to be highly conserved (11), it has been possible to use DNA-DNA hybridization to locate *nif* genes on large plasmids of fast-growing rhizobia, including *R. leguminosarum* bv. *phaseoli* (formerly *R. phaseoli*) (6,7,9,10). Among the eight *R. leguminosarum* bv. *phaseoli* strains studied previously, all contained two to five plasmids, and *nif* probes hybridized with only one 150- to 250-megadalton (Mdal) plasmid per strain.

The present study was performed to examine the diversity of plasmids in eight commercial strains of *R. leguminosarum* bv. *phaseoli*. Although the plasmid content of each strain was unique, only one plasmid per strain hybridized with the *nif* probe.

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MATERIALS AND METHODS

Rhizobia. Commercial *R. leguminosarum* bv. *phaseoli* strains 127K12, 127K17, 127K35, 127K44, 127K80, and 127K81 were provided by R.S. Smith, The Nitragin Co., Milwaukee, Wis.; strains RP132-4 and RP132-50 were obtained from T.J. Wacek, Kalo Agricultural Chemicals, Inc., Columbus, Ohio. Strain DB1 (*R. phaseoli* ATCC 14482) was purchased from the American Type Culture Collection, Rockville, Md. Rhizobia were grown in mannitol-yeast extract (MYE) broth (8) and maintained on MYE agar slants.

Isolation of rhizobial plasmids. A modified Eckhardt (4) procedure was used to isolate rhizobial plasmids; the lysozyme, SDS, and overlay mixtures were those described for gram-negative bacteria, except that Tris-borate buffer was 89 mM Tris, 2.5 mM disodium EDTA, and 89 mM boric acid. Each strain was grown, without shaking, in YE broth (MYE broth lacking mannitol) for 24 h at 30°C (turbidity of 45-50 Klett units; red filter). Cells contained in 10 ml of the culture were then pelleted by centrifugation, resuspended in 1 ml of 0.1% sarkosyl in pH 8.0 Tris-EDTA buffer (50 mM Tris, 20 mM disodium EDTA), and recentrifuged. The final cell pellet was thoroughly drained, vigorously resuspended in 100 µl of 20% ficoll 400,000 (Sigma Chemical Co., St.

Louis, Mo.) in Tris-borate buffer, and placed in an ice bath. Fifty microliters of the cell suspension was then briefly mixed with 25 µl of lysozyme mixture in a microtube, and 25 µl of the resulting suspension was immediately added to 50 µl of SDS mixture contained in a well (3×8 mm; 1.5 cm deep) of a 0.7% agarose (type I, low EEO; Sigma) vertical gel. After 100 µl of overlay mixture was added to the well, the well was sealed with agarose. Following electrophoresis at 5 mA for 1 h and then 40 mA for 16-18 h at 4°C, DNA in the gel was stained with ethidium bromide (0.5 µg/ml) and photographed with a Polaroid MP-4 camera equipped with Wratten 2B and 25 filters, using a 302-nm transilluminator as the light source. Plasmid sizes were estimated using the 166- and 248-Mdal plasmids of strain DB1 as standards (7; K.L. Levine, M.S. thesis, North Dakota State University, Fargo, 1980).

Hybridization with nitrogen-fixation genes. Rhizobial plasmids in agarose gels were transferred to GeneScreen (New England Nuclear Corp., Boston, Mass.) membranes according to the general procedure of Southern (12). Plasmid pRmR2 (11), which contains nitrogen-fixation genes (*nifKDH*) of *R. meliloti*, was isolated by the method of Holmes and Quigley (5), purified by CsCl-ethidium bromide isopycnic centrifugation, and labeled with ³²P to a specific activity of 0.5–1×10⁷ cpm/µg using the nick-translation kit and recommended procedures of New England Nuclear Corp. Prior to hybridization, membranes were incubated, with constant agitation, 6-8 h at 42°C in a prehybridization buffer consisting of Denhardt solution (3), 50% deionized formamide, 10% dextran sulfate, 1 M NaCl, 50 mM Tris•HCl – 0.1% sodium dodecyl sulfate (SDS), 0.1% PP_i, and 500 µg of boiled calf thymus DNA per ml. Then, an equal volume of hybridization buffer (prehybridization buffer lacking NaCl and dextran sulfate, and containing 125 µg of boiled calf thymus DNA per ml) and 10-50 ng of boiled, ³²P-labeled pRmR2 DNA were added, and incubation at 42°C was continued for 24 h. Following hybridization, membranes were subjected to two 5-min washes at room temperature with 0.3 M NaCl-60 mM Tris•HCl (pH 8.0) – 2 mM disodium EDTA, two 30-min washes at 60°C with the same buffer containing 0.5% SDS, and two 30-min washes at room temperature in 3 mM Tris. Autoradiography, using an intensifying screen and Kodak XAR-5 X-ray film, was performed at –70°C for 24 to 48 h. The vector of pRmR2, pACYC184, failed to hybridize with rhizobial plasmids when prepared and used in the same manner as pRmR2.

RESULTS AND DISCUSSION

The plasmid contents of *R. leguminosarum* bv. *phaseoli* DB1 and the eight commercial strains are shown in Figs. 1-3. The in-gel lysis procedure allowed detection of a very large plasmid in strain DB1 not

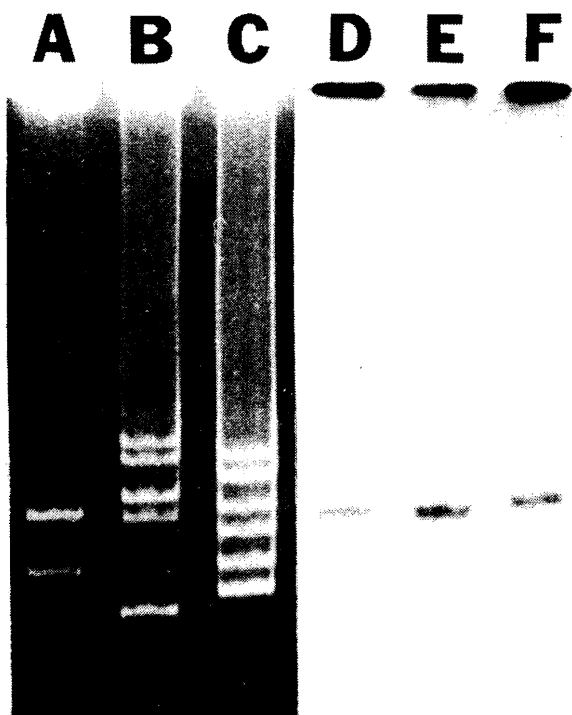


Figure 1. Plasmids (left) and corresponding autoradiograms (right) of strains DB1 (lanes A and D), 127K12 (lanes B and E), and 127K17 (lanes C and F).

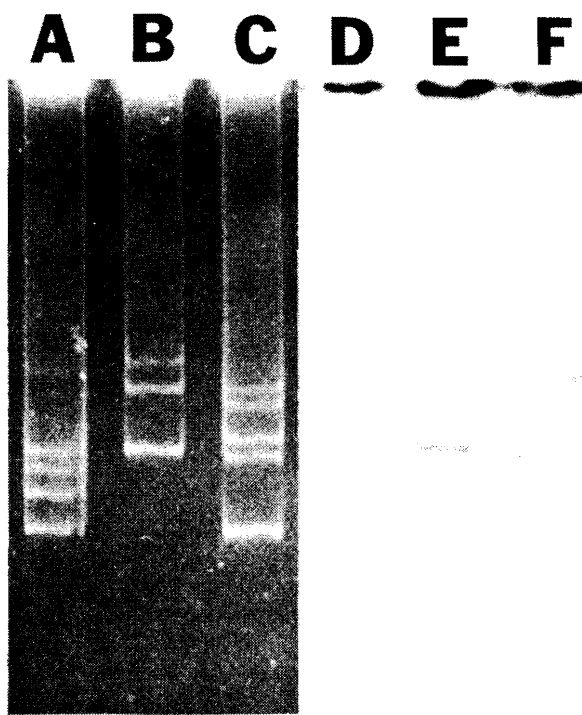


Figure 3. Plasmids (left) and corresponding autoradiograms (right) of strains 127K81 (lanes A and D), RP132-4 (lanes B and E), and RP132-50 (lanes C and F).

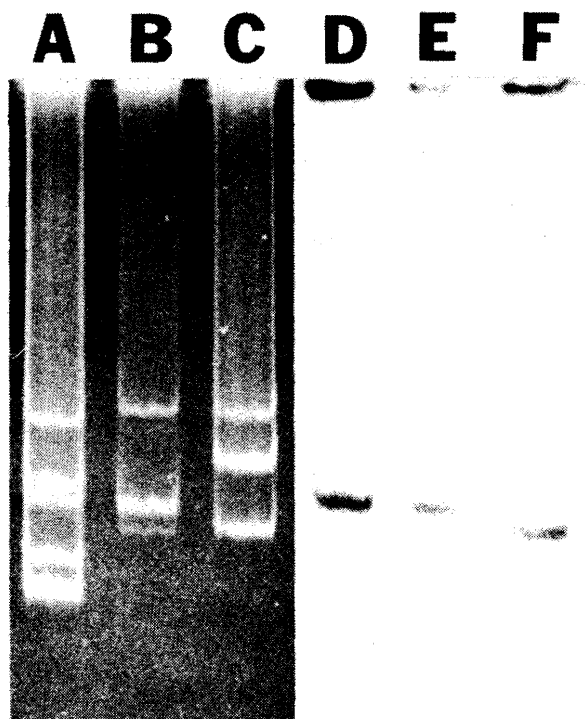


Figure 2. Plasmids (left) and corresponding autoradiograms (right) of strains 127K35 (lanes A and D), 127K44 (lanes B and E), and 127K80 (lanes C and F).

reported by Masterson et al. (7), and three to six large plasmids in each commercial strain. Strain 127K17, with six plasmids, has the largest number of plasmids thus far detected in any *Rhizobium* species. Estimates of plasmid sizes are given in Table 1; the sizes indicated for the largest plasmids are very approximate due to the lack of suitable size standards and the non-logarithmic relationship between very large plasmids and their electrophoretic mobilities (1).

Figs. 1-3 also show the autoradiograms obtained when Southern blots of the rhizobial plasmids were

Table 1. Estimated plasmid sizes and *nif* gene locations

Strain	Plasmid sizes (Mdal)
DB1	166, 248 ^a , 585
127K12	130, 250 ^a , 265, 395, 460
127K17	130, 165, 225, 250 ^a , 340, 395
127K35	120, 155, 250 ^a , 290, 460
127K44	195, 225 ^a , 545
127K80	180 ^a , 315, 460
127K81	130, 165, 210 ^a , 250, 585
RP132-4	250 ^a , 460, 630
RP132-50	120, 225 ^a , 265, 365, 460

^aHybridized with *nif* probe pRmR2.

hybridized with *nif* probe pRmR2. Only one plasmid per strain, often about 250 Mdal in size (Table 1), seems to carry *nif* genes. Our hybridization results confirm those of Masterson et al. (7) with strain DB1, and support observations with seven additional *R. leguminosarum* bv. *phaseoli* strains (6,9,10) that *nif* hybridization is confined to a single 150- to 250-Mdal plasmid per strain.

Among the 16 strains of *R. leguminosarum* bv. *phaseoli* which have been characterized as to plasmid contents, including the nine strains in the present study, no identical plasmid profiles exist. It may be possible to take advantage of this diversity by using plasmid profiles for strain identification. For example, experiments concerned with competitive ability might be facilitated because bean inoculant strains, as well as indigenous rhizobia, could be readily differentiated.

SUMMARY

A modified in-gel lysis procedure was used to isolate large plasmids from eight commercial *R. leguminosarum* bv. *phaseoli* strains. Each strain had a unique plasmid content of three to six plasmids ranging in size from 120 to about 630 Mdal. Only one plasmid per strain, 180 to 250 Mdal in size, hybridized with nitrogen-fixation genes of *R. meliloti*.

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