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Antigenic Differences Between Infective and Noninfective Strains of *Rhizobium trifolii*[1]

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Immunodiffusion and immunoelectrophoresis techniques have revealed the presence of soluble antigens in sonicated preparations of four infective strains of *Rhizobium trifolii* which were absent in similar preparations of related noninfective mutants derived from the infective strains. The soluble antigens unique to the infective strains were cross-reactive with one another.

Rhizobium serology has been useful in the elucidation of the taxonomic relatedness among *Rhizobium* species (16) and in their rapid identification when isolated from nodules from the field (2). In the past, serological techniques have not been useful in elucidating the basis of host specificity in the *Rhizobium*-legume symbiosis. There have been several attempts to detect serological differences between infective and related noninfective strains of *Rhizobium* (7, 12, 14, 15). Studies have indicated no correlation between the ability of *Rhizobium* strains to cross-agglutinate with heterologous antiserum and to nodulate legumes (3, 9, 10). Changes in the legume host range of *Rhizobium* have been reported without detectable changes in the serological properties of the bacteria (4, 8, 13). Considered collectively, these studies have suggested that factors conferring on *Rhizobium* the trait of infectiveness for a particular host are not expressed antigenically. This paper reports antigenic differences between infective and related noninfective strains of *R. trifolii* using a persistent immunization schedule.

MATERIALS AND METHODS

Strains of Rhizobium trifolii. Four combinations of infective and related noninfective strains of *R. trifolii* were employed. Infective strains were capable of producing infection threads in root hairs of *Trifolium repens* L. (white clover) in Fahraeus glass slide assemblies (5). Strains 2S (infective) and 2L (noninfective) were obtained from K. Marshall. Strain 2S-2 (infective) was isolated from a nodule of clover inoculated with the original 2S strain. Strains 0403 (infective) and Bart A (noninfective) were obtained from G. Fahraeus, strains 0435 (infective) and 0435-2 (noninfective) from A. MacGregor, and strains T37 (infective) and Bio-9 (noninfective) from Z. Lorkiewicz.

Growth conditions. All strains were grown on Bergersen's chemically defined medium (1) supplemented with calcium pantothenate (4 mg/liter) and solidified with purified agar (15 g/liter; Difco).

Sonication experiments. Prolonged sonication (20 min) of *Rhizobium* antigens results in a change in their serological reactivity (6). Hence, an experiment was performed to determine the minimal time of exposure of cells to sonication that would release most of the 280-nm absorbing material. *R. trifolii* (2S-2 and 2L) cells were grown on agar plates for 48 h at 22 C and then harvested in phosphate-buffered saline (PBS; 0.05 M K_2HPO_4-KH_2PO_4, 0.14 M NaCl, 0.001 M $MgSO_4$, pH 7.2). The cells were washed twice in PBS and collected by centrifugation at 4 C at 27,000 $\times$ *g* for 15 min. The cells were resuspended in PBS to give an optical density of 0.097 at 520 nm for a 10^{-2} dilution. The suspensions were transferred to an aluminum tube (cold shoulder cell, Branson Heat Systems, Plainview, N.Y.) maintained at 0 to 4 C and sonicated at maximum power output (75 W) with a Branson sonifier (model W140). Sonication proceeded for 600 s at 10-s intervals and 5 s of cooling. Two-milliliter aliquots were removed from the sonicated preparations at various times and filtered through a 0.20-μm membrane filter (TCM-200, Gelman, Ann Arbor, Mich.), and the optical density was read at 280 nm.

Preparation of bacterial antigens. *Rhizobium* cells were grown, harvested, and resuspended as described above. These suspensions were sonicated for 120 s at 15-s intervals with 10 s of cooling at 0 to 4 C and 75 W output. Aliquots of these sonicated preparations containing 9 to 10 mg/ml (dry weight) were distributed in sterile glass tubes, quick frozen in a dry ice-acetone bath, and stored at −70 C for later use in immunization. The remainder of the sonicated preparations was centrifuged at 27,000 $\times$ *g* for 30 min at 4 C. The supernatants were concentrated by dialysis at 4 C against PBS containing 20% (wt/vol) polyethylene glycol (average molecular weight, 15,000 to 20,000; Matheson, Coleman, & Bell, Norwood, Ohio). The protein content of these samples was estimated

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by the method of Lowry et al. (11) and adjusted to 2.0 mg of protein per ml with PBS so as to enable strict comparisons of the soluble antigens from the infective and noninfective strains. The samples were then stored in 0.25-ml quantities at −20 C.

Preparation of antisera. Antigen preparations were thawed at 37 C and then kept at 0 C until emulsified with an equal volume of Freund incomplete adjuvant (Difco) immediately before injection. Antisera to the four infective strains of *R. trifolii* were prepared in 9- to 10-kg female New Zealand white rabbits according to the following immunization schedule. On day 1, 10 ml of normal serum sample was collected, followed by injection of 0.5 ml of antigen-adjuvant mixture in both hind legs (intramuscular) and 0.5 ml of antigen-adjuvant in two areas of the back (subcutaneous); on day 7, 1 ml of antigen alone (intravenous); on day 30, 1 ml of antigen-adjuvant given as intramuscular and subcutaneous injections. Beginning with day 37, and weekly thereafter, test bleedings (10 ml) were obtained from the central ear artery, followed by subcutaneous and intramuscular injections of antigen-adjuvant until the desired reactive antiserum was obtained. The immunization schedule was completed within 6 months. Animals were finally anesthetized with sodium pentobarbital and exsanguinated by cardiac puncture. The blood was clotted at 37 C for 2 h and then held at 4 C for 4 h. The clot was removed, the serum was centrifuged at 620 × *g*, and the supernatant was stored at −20 C.

Immunodiffusion and immunoelectrophoresis. Immunodiffusion plates were prepared with PBS in 0.7% agarose (Calbiochem, La Jolla, Calif.) preserved with merthiolate (0.1 mg/ml). Wells were cut with stainless-steel tubing through a Plexiglas template so that each well was 4 mm deep, 3 mm in diameter, and 5 mm apart. Each well was filled with 15 μl of antigen or antiserum. Antigens from strains 2S-2 and 2L (4 μl, 2 mg of protein per ml) were separated by electrophoresis for 1 h at 22 C on microscope slides layered with 2.5 ml of 0.7% agarose in 0.05 M sodium barbital (pH 8.6) at a current of 10 mA/slide. After electrophoresis, the trenches were filled with 50 μl of anti-2S-2 serum. Slides and plates were incubated at 22 C in moist chambers for 48 h and then examined.

Photography. Precipitin lines in agarose were illuminated by a Quebec colony counter (lid off), photographed on High-Contrast Copy film (Eastman Kodac, Rochester, N.Y.), and printed on Afga no. 6 high-contrast paper (Afga-Gevaert, Leverkusen, Germany).

RESULTS AND DISCUSSION

The kinetics of release of 280-nm absorbing material from *R. trifolii* (2S-2 and 2L) into a 0.2-μm filterable phase during sonication is presented in Fig. 1. The data were essentially identical for both the infective (2S-2) and noninfective (2L) strains. Sonication caused a rapid release of 280-nm absorbing material for the first 120 s, followed by a minimal increment between 120 and 600 s.

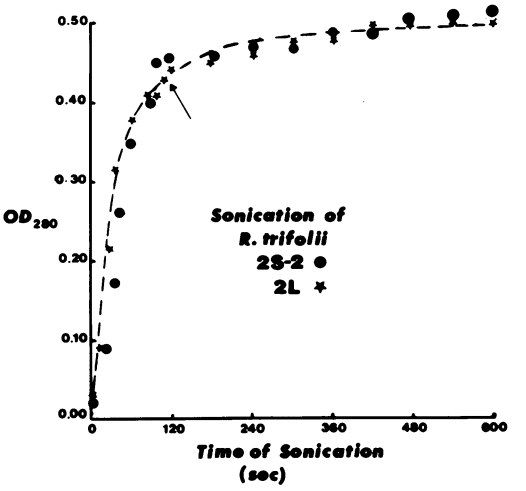

FIG. 1. *Release of 280-nm absorbing material from R. trifolii (2S-2, infective, and 2L, noninfective) into a 0.2-μm filterable phase during sonication.*

Antigenic differences between the infective and noninfective strains were found. Normal preimmune sera produced no precipitin bands with the antigen preparations. The infective and noninfective strains shared common antigens (Fig. 2–5). However, antisera against the infective strains produced one to three unique precipitin bands near the wells containing antigen preparations from the homologous infective strains. These unique bands were absent in the immunodiffusion patterns of antigen preparations from the noninfective strains (Fig. 2–4). Only the antigen preparations of strains 2S-2 and 2L had viscosities sufficiently low to permit accurate examination by immunoelectrophoresis. A precipitin arc (arrow) was produced by antigen(s) from 2S-2 but not by 2L (Fig. 5). In another test (Fig. 6), antiserum against T37 (infective) was reacted with three combinations of infective and noninfective *R. trifolii* strains (2S-2, 2L; 0403, Bart A; 0435, 0435-2). All the strains shared several antigens in common, but an additional precipitin band was formed near the antigen wells of the infective strains only. Antisera against two noninfective strains (2L, 0435-2) prepared as before revealed only reactions of identity when reacted against the respective infective and noninfective strains. Antigens unique to the noninfective strains were not found.

These results clearly indicate that antigenic differences between infective and noninfective strains of *R. trifolii* exist. All the infective strains studied produce at least one common

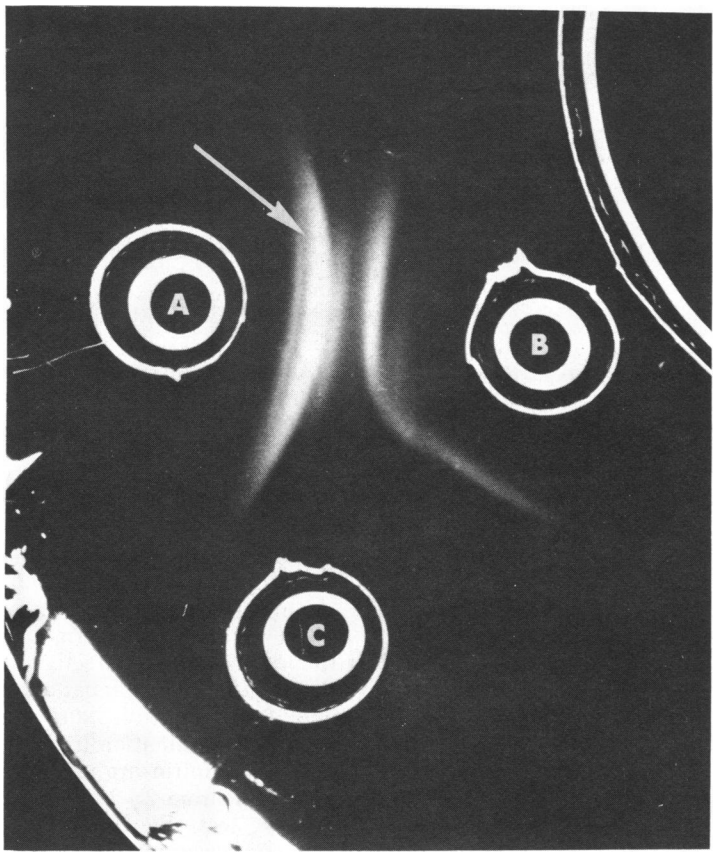

FIG. 2. *Immunodiffusion plate showing antigenic differences between R. trifolii T37 (infective, well A) and Bio 9 (noninfective, well C). Well B, Anti-T37. Precipitin bands close to the antigen well A are present (arrow).*

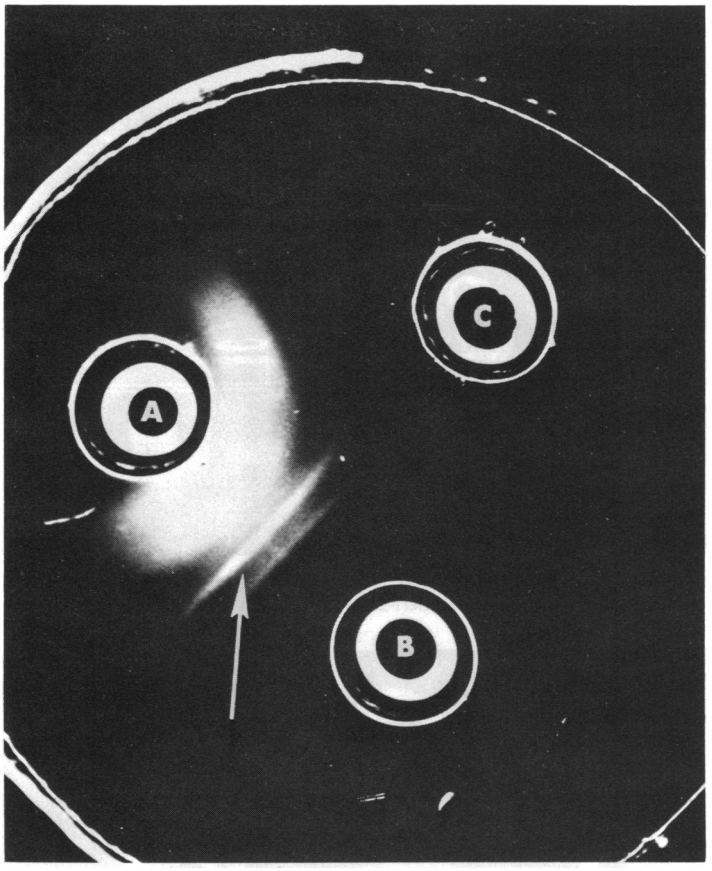

FIG. 3. *Immunodiffusion plate showing antigenic differences between R. trifolii 0435 (infective, well B) and 0435-2 (noninfective, well C). Well A, Anti-0435. Precipitin bands close to the antigen well B are present (arrow).*

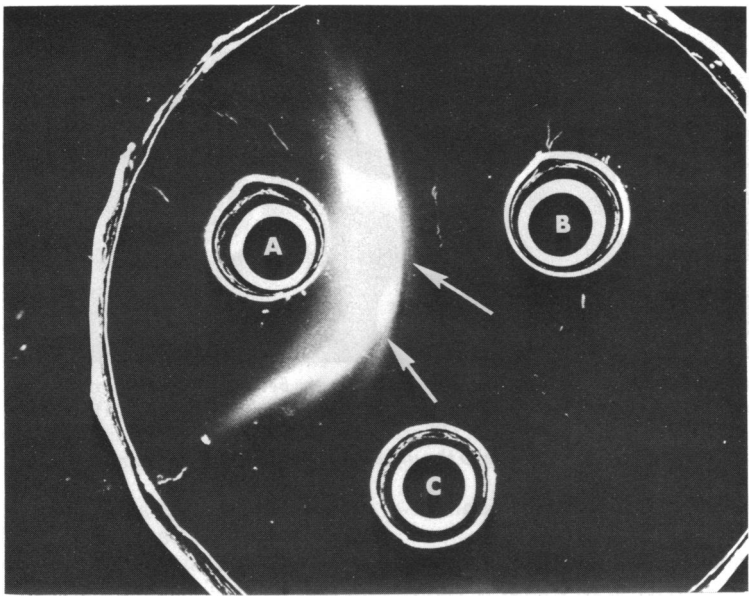

FIG. 4. *Immunodiffusion plate showing antigenic differences between R. trifolii 0403 (infective, well B) and Bart A (noninfective, well C). Well A, Anti-0403. Precipitin bands close to antigen well B are present (arrows).*

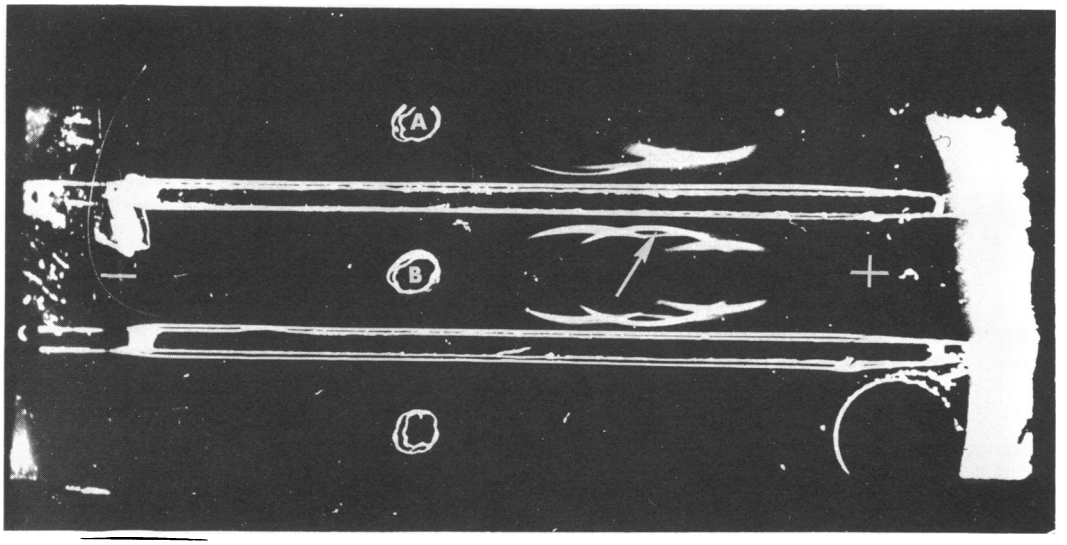

FIG. 5. *Immunoelectrophoresis pattern showing antigenic differences between R. trifolii 2S-2 (infective, well B) and 2L (noninfective, well A). Trenches, Anti-2S-2. Precipitin band unique to 2S-2 is present (arrow).*

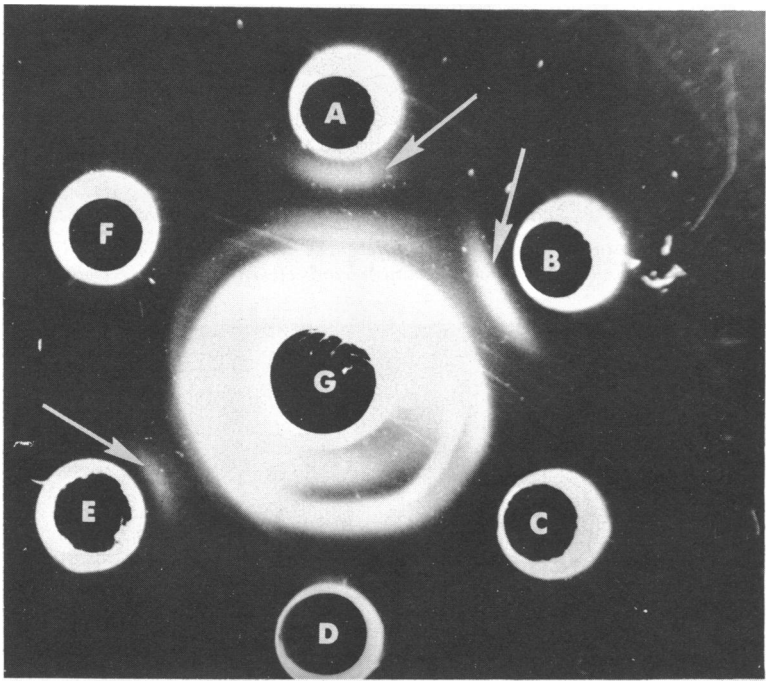

FIG. 6. *Immunodiffusion plate showing antigenic differences between infective and noninfective strains of R. trifolii using anti-T37 (infective, well G). Outer wells had antigens, respectively, (A) strain 0403, infective; (B) strain 0435, infective; (C) 0435-2, noninfective; (D) strain 2L, noninfective; (E) strain 2S-2, infective; (F) strain Bart A, noninfective. Precipitin bands unique to the infective strains are present (arrows.)*

antigen which is not detected in the noninfective strains.

Whether or not these unique antigens are involved in the infectivity of *R. trifolii* to clover is unknown. But we have found a 100% correlation with the eight strains tested (detection of unique antigens was always correlated with the ability of these strains to form infection threads in root hairs of clover). These data suggest that serological techniques may aid in the screening

of *Rhizobium* isolates for infectivity in cases where noninfective mutants are sought. Noninfective mutants have acquired the infectivity trait by transformation using deoxyribonucleic acid isolated from infective donors (13). If the transformed infective recombinants possess these antigens normally unique to the infective strains, then their role in infection would be highly suggestive. Experiments using affinity chromatography to isolate and characterize these unique antigens are in progress.

These unique antigens were not recognized using the short immunization schedules employed in earlier studies. Apparently these are poor immunogens which induce the formation of antibody at detectable levels only after repeated, persistent immunization.

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LITERATURE CITED

1. Bergersen, F. 1961. The growth of *Rhizobium* in synthetic media. Aust. J. Biol. Sci. **14**:349-360.
2. Brockwell, J., W. Bryant, and R. Gault. 1972. Ecological studies of root-nodule bacteria introduced into field environments. 3. Persistence of *Rhizobium trifolii* in association with white clover at high elevations. Aust. J. Exp. Agric. Anim. Husb. **12**:407-413.
3. Bushnell, O., and W. Sarles. 1939. Investigations upon the antigenic relationships among the root-nodule bacteria of the soybean, cowpea, and lupine cross-inoculation groups. J. Bacteriol. **38**:401-410.
4. Dart, P. 1974. The infection process, p. 381-430. *In* A. Quispel (ed.), The biology of nitrogen fixation. North-Holland Publishing Co., Amsterdam.
5. Fahraeus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. **16**:374-381.
6. Gibbons, L. 1967. The preparation of antigens of *Rhizobium meliloti* by ultrasonic disruption: an anomaly. Can. J. Microbiol. **13**:1375-1378.
7. Graham, P. 1969. Analytical serology of the *Rhizobiaceae*, p. 353-378. *In* J. Kwapinski (ed.), Analytical serology of microorganisms, vol. 2. Interscience, New York.
8. Higashi, S. 1967. Transfer of clover infectivity of *Rhizobium trifolii* to *Rhizobium phaseoli* as mediated by an episomic factor. J. Gen. Appl. Microbiol. **13**:391-403.
9. Kleczkowski, A., and H. Thornton. 1944. A serological study of root nodule bacteria from pea and clover inoculation groups. J. Bacteriol. **48**:661-673.
10. Koontz, F., and J. Faber. 1961. Somatic antigens of *Rhizobium japonicum*. Soil Sci. **91**:228-232.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
12. MacGregor, A., and M. Alexander. 1972. Comparison of nodulating and non-nodulating strains of *Rhizobium trifolii*. Plant Soil **36**:129-139.
13. Mareckova, H. 1970. Transformation of infectivity in *Rhizobium japonicum*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **125**:594-596.
14. Marshall, K. 1967. Electrophoretic properties of fast and slow-growing species of *Rhizobium*. Aust. J. Biol. Sci. **20**:429-438.
15. Van Rensburg, H., B. Strijdom, and C. Rabie. 1968. Nature of the genetic determinant of infectiveness in strains of *Rhizobium japonicum*. S. Afr. J. Agric. Sci. **11**:623-626.
16. Vincent, J., and B. Humphreys. 1970. Taxonomically significant group antigens in *Rhizobium*. J. Gen. Microbiol. **63**:370-382.