

Colorimetric Enzyme-Linked Immunosorbent Assay for the Identification of Strains of *Rhizobium* in Culture and in the Nodules of Lentils

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An indirect enzyme-linked immunosorbent assay has been developed to identify strains of *Rhizobium* in culture and in lentil nodules. The test can be used on cells from both fresh and frozen nodules obtained from plants grown either in a growth chamber or in the field. Test results were confirmed by immunofluorescence. The enzyme-linked immunosorbent assay technique can be used for field studies and requires less antisera than other serological techniques.

Serological techniques provide very specific methods for identifying microorganisms. They have been used extensively to identify rhizobia in culture and in nodules of legumes (10, 5). These methods include tube agglutination (7), microagglutination (3), immunodiffusion (4), and immunofluorescence (IF) (8).

There is a need for serological assays which are rapid and do not require elaborate or expensive equipment. This paper describes a colorimetric method to identify strains of *Rhizobium* in pure culture and in lentil nodules. The test is based on an indirect enzyme-linked immunosorbent assay (ELISA) using strain-specific rabbit antisera and sheep anti-rabbit globulin conjugated to alkaline phosphatase. Bacteria, from culture or nodule, are first coated with a specific rabbit *anti-Rhizobium* serum and then with sheep anti-rabbit antibodies covalently linked to alkaline phosphatase. A chromogenic substrate, p-nitrophenylphosphate, is then applied to the antibody-treated smear, and the color is measured after a period of incubation. The amount of colored product is proportional to the amount of enzyme present, which in turn is indirectly related to the quantity of antigen on the slide. The ELISA is endowed with the specificity of antigen-antibody reactions and the sensitivity of enzyme-catalyzed reactions.

The results obtained with ELISA were confirmed by using the direct IF technique.

MATERIALS AND METHODS

Cultures. *Rhizobium* strains were obtained from the following sources: NZP 5400 (B₁₃), R. M. Greenwood, Applied Biochemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand; Hawaii 5-0 (I₁₁), isolated from a root nodule of a lentil plant grown on Molokai soil on Oahu, Hawaii; and 128A12 (B₈₈), J. C.

Burton, Nitragin Co., Milwaukee, Wis. Cultures of these strains were grown in modified yeast extract mannitol broth (9) for 3 days at 30°C. For the IF test, smears were made directly from the liquid culture. For ELISA, cells were centrifuged and washed three times in distilled water to remove most of the extracellular polysaccharide slime. Suspensions of different strains were made up to the same turbidity (i.e., absorbance at 600nm [*A*₆₀₀]).

Bacteria from root nodules. Two types of preparations were made. For known standards, nodules were pooled from a single plant and crushed in distilled water. The debris was allowed to settle, and the crude cell suspension was decanted. The suspension was adjusted to a turbidity (*A*₆₀₀) of 0.45 to 1.00 in a Spectronic 20 (Bausch and Lomb, Rochester, N.Y.). For unknowns, single nodules were crushed in enough distilled water to give approximately the same turbidity as the standards. These suspensions were used to make slides for both ELISA and IF tests.

Antisera. Rabbit antisera and their fluorescent conjugates were prepared by the method of Schmidt et al. (9). For IF, the 0 to 50% ammonium sulfate fraction of the sera was used; for ELISA, the 30 to 38% ammonium sulfate fraction was used.

SARG. Sheep anti-rabbit globulin (SARG) was initially prepared as the 0 to 35% ammonium sulfate fraction of the serum. Later work used the 7S globulin fraction made by passing the 18% sodium sulfate fraction of the serum through a Sephadex G-200 column and recycling the 7S peak through the column a second time. These preparations were generously provided by A. Benedict and L. Pollard, University of Hawaii.

SARG phosphatase conjugate. The SARG fraction was conjugated to alkaline phosphatase (EC 3.1.3.1, type VII, 1,000 U/mg of protein, Sigma Chemical Co., St. Louis, Mo.), using glutaraldehyde by the procedure of Voller et al. (11).

IF staining. Smears from pure cultures and nodule suspensions were stained by the method of Schmidt et al. (9). Gelatin-rhodamine isothiocyanate conjugate (1) was used routinely to control nonspecific staining and autofluorescence.

ELISA test. The procedures used in this study were based on the methods of Voller et al. (11) and

Engvall and Perlman (6), modified for use with an insoluble antigen, the rhizobial cell. The substrate, p-nitrophenyl phosphate, was obtained from Sigma Chemical Co. The method finally adopted is described in the results section.

RESULTS AND DISCUSSION

In preliminary experiments, it was found that the SARG conjugate adsorbed nonspecifically to rhizobial cells. This adsorption was largely eliminated by treatment of the cells with bovine gamma globulin (BGG) as shown in Table 1. This pretreatment is particularly important when visual observations are made. It is possible that other proteins could substitute for BGG.

Three rhizobial strains, I₁₁, B₁₃, and B₈₈, were tested with homologous and nonhomologous antibody by the ELISA system (Table 2). In the first experiment, cells were grown in broth culture, and in the second experiment they were extracted from nodules of laboratory-grown

TABLE 1. *Inhibition of nonspecific adsorption of antiglobulins by BGG^a*

Treatment	I ₁₁ -antiglobulin		B ₁₃ -antiglobulin	
	I ₁₁ cells	B ₁₃ cells	I ₁₁ cells	B ₁₃ cells
Conjugate	0.11	0.17	0.06	0.16
BGG + conjugate	0.06	0.10	0.04	0.07
Antiglobulin + conjugate	0.50	0.30	0.23	0.56
BGG + antiglobulin + conjugate	0.54	0.21	0.16	0.51

^a Rhizobial strains from frozen field nodules suspended to an A_{600} of 0.45. Protein concentrations: BGG, 5.0 mg/ml; antiglobulins, 75 μ g/ml; conjugate, 18.6 μ g/ml. Data represent micromoles of *p*-nitrophenol released in 10 min.

TABLE 2. *Effects of homologous and nonhomologous rabbit antiglobulins on rhizobial strains by ELISA^a*

Expt no.	Rhizobial strain	Antiglobulin		
		B ₁₃	I ₁₁	B ₈₈
1 ^b	B ₁₃	0.57	0.05	0.09
	I ₁₁	0.06	0.39	0.07
	B ₈₈	0.08	0.03	0.79
2 ^c	B ₁₃	0.97	0.07	—
	I ₁₁	0.26	0.45	—
	B ₈₈	0.28	0.11	—

^a Protein concentrations: BGG, 5.0 mg/ml; antiglobulin I₁₁, 39 μ g/ml; B₁₃, B₈₈, 50 μ g/ml.

^b Cell suspensions prepared from broth cultures adjusted to turbidity $A_{600} = 0.75$; conjugate, 18.6 μ g/ml of protein. Values given indicate micromoles of *p*-nitrophenol released in 10 min.

^c Cell suspension prepared from pooled nodules of laboratory-grown plants adjusted to turbidity $A_{600} = 1.0$; conjugate, 10 μ g/ml protein. Values given indicate micromoles of *p*-nitrophenol released in 12 min.

plants. Cells from broth cultures tend to make nonuniform suspensions; those from nodules suspend readily and are free of slime but may contain some plant tissue. Despite these differences, both sets of cells reacted qualitatively in the same manner. The reaction with nonhomologous antisera was weak, whereas that with the homologous antisera was strong. Table 3 shows the results of a blocking experiment in which unconjugated SARG was added to the system before the SARG conjugate. The data indicate that SARG blocks the sites on the rabbit globulins and results in minimal binding of the conjugate.

The concentrations of antiglobulins and conjugate were varied in an experiment to determine the optimal conditions for the ELISA test (Fig. 1 and 2). The cell densities were held constant ($A_{600} = 0.45$); this value was typical of that obtained from single small lentil nodules. In principle, the conjugate should always be used in excess; in fact, clearest results were obtained with these particular sera at 75 μ g/ml of antirhizobial globulin and 18.6 μ g/ml of SARG conjugate. These concentrations were used in most of the subsequent experiments.

The specificity of the ELISA test was not lost when nodules, together with their bacterial content, were stored frozen, or when the cell suspensions obtained from the frozen nodules were placed in a boiling water bath for 5 min (Fig. 3 and 4). Although the shapes of the antiglobulin saturation curves vary with the fresh and frozen nodules, the total effect is the same. The data suggest that a small loss of activity results from freezing but that this loss is restored by heat treatment, a process which may expose additional antigenic sites. IF techniques have been used to detect specific rhizobial strains in the field (2). The ELISA test was compared with the IF test in a series of blind experiments to type individual nodules from laboratory-grown and field-grown plants under different conditions (Table 4). In every

TABLE 3. *Blocking effect of adding unlabeled 7S SARG on the ELISA reaction^a*

Rhizobial strain	Homologous anti-globulin (2 h) + buffer (1 h) + conjugate (1 h)	Homologous antiglobulin (2 h) + 7S-SARG (1 h) + conjugate (1 h)
I ₁₁	0.55	0.06
B ₁₃	0.61	0.09

^a Rhizobial strains from frozen field nodules suspended to a turbidity at $A_{600} = 0.45$. Protein concentration: antiglobulin, 75 μ g/ml; SARG, 20 μ g/ml; conjugate, 18.6 μ g/ml. Data reported indicate micromoles of *p*-nitrophenol released in 10 min.

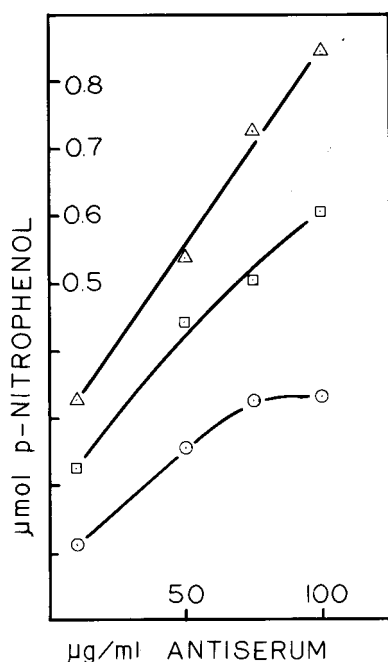


FIG. 1. Effect on the ELISA test of varying the concentration of B_{13} antiglobulins. B_{13} cells were from frozen field nodules prepared to turbidity $A_{600} = 0.45$. The conjugate was used at 46.5 $\mu\text{g/ml}$ (Δ), 18.6 $\mu\text{g/ml}$ (\square), and 9 $\mu\text{g/ml}$ (\circ). Cells were not pretreated with BGG. Results are expressed as micromoles of *p*-nitrophenol released by the conjugate in 10 min.

case, complete agreement was obtained between the ELISA and IF tests.

The following details the procedures used in the ELISA test. Tests were done in glass agglutination slides which contained 12 depression wells. A 0.05-ml amount of cell suspension was deposited in each well and spread evenly over the bottom half surface. The smears were dried under warm air and heat fixed. Slides were used immediately or were stored at room temperature in vacuo for periods up to 1 week. The following steps were done at room temperature (approximately 22°C). The smears were washed twice for 5 min with PBST, a phosphate-buffered saline-Tween 20 solution (11) containing 0.001 M MgCl_2 (6). After removing excess buffer, 0.05 ml of BGG (5 mg/ml) was added to each depression, and the slides were incubated in a moist chamber for 30 min. Slides were washed three times for 5 min with PBST, and excess moisture was removed. A 0.05-ml amount of a dilution of rabbit anti-rhizobium globulin was added to each well, and the slides were reincubated in a moist chamber for 2 h. After washing three times with PBST as above, 0.05 ml of a dilution of SARG conjugate was applied to each well and incubation resumed in the moist chamber for

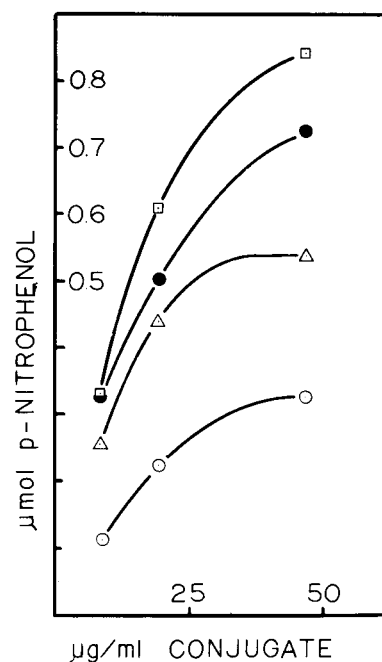


FIG. 2. Effect on the ELISA test of varying the concentration of immunoglobulin-enzyme conjugate. B_{13} cells were prepared from frozen field nodules to turbidity $A_{600} = 0.45$. B_{13} antiglobulins were used at 100 $\mu\text{g/ml}$ (\square), 75 $\mu\text{g/ml}$ (\bullet), 50 $\mu\text{g/ml}$ (Δ), and 10 $\mu\text{g/ml}$ (\circ). Cells were not pretreated with BGG. Results are expressed as micromoles of *p*-nitrophenol released by the conjugate in 10 min.

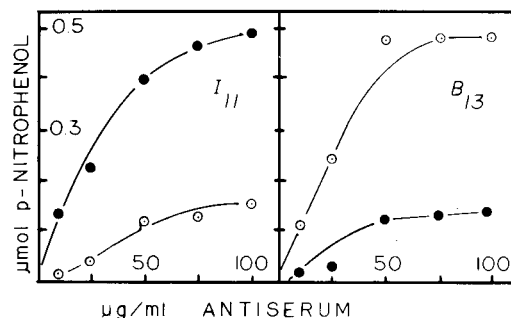


FIG. 3. Effect on the ELISA test of varying the concentration of rabbit antiglobulins, I_{11} on the left, B_{13} on the right. Cells of B_{13} (\circ) and I_{11} (\bullet) were from fresh nodules excised from laboratory plants and made up to turbidity $A_{600} = 0.45$. Results are expressed as micromoles of *p*-nitrophenol released by the conjugate in 15 min.

another hour. Slides were washed 3 times with PBST as above, and 0.05 ml of *p*-nitrophenyl-phosphate (1 mg/ml; Sigma Chemical Co.) in diethanolamine buffer (11) was added to each sample. The slides were reincubated in the moist chamber for 10 to 20 min until sufficient product

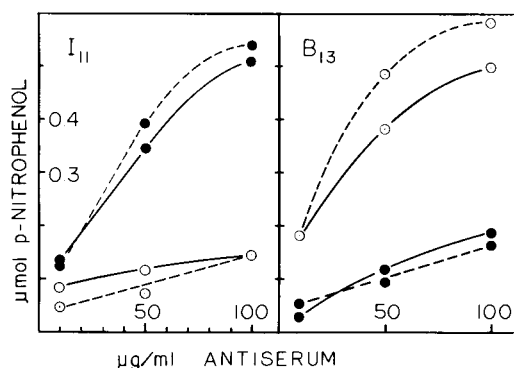


FIG. 4. Effect on the ELISA test of varying the concentration of rabbit antiserum, I_{11} on the left and B_{13} on the right. Cells of B_{13} (○) and I_{11} (●) were from frozen field nodules suspended to $A_{600} = 0.45$. Dotted lines indicate cell suspensions placed in boiling water for 5 min. Results are expressed as micromoles of *p*-nitrophenol released in 15 min.

was produced in the positive controls to be estimated visually and/or measured colorimetrically. A 0.05-ml amount of 3 M NaOH was added to each well to stop the reaction, and the slides were swirled gently to effect mixing. For quantitative measurements, the samples were transferred immediately to tubes containing 3 ml of 0.2 M NaOH. The *p*-nitrophenol released from the substrate was measured at A_{400} ; the amount, in micromoles, was computed from a standard curve.

In doing the ELISA test, it was found necessary to include on every depression slide both positive and negative controls for the sera employed. The use of depression slides rather than test tubes was found to be preferable because the sequential addition of solutions is more readily controlled. The diethanolamine used as buffer for the substrate in these studies can be replaced with 0.05 M Na_2CO_3 (pH 9.8) (6). This

TABLE 4. Comparison between blind ELISA test and IF test on cells from single nodules

Actual strain identity ^a	ELISA				IF ^d	
	Visual ^b		Colorimetric ^c		Anti- I_{11}	Anti- B_{13}
	Anti- I_{11}	Anti- B_{13}	Anti- I_{11}	Anti- B_{13}		
1. I_{11} ^e	+	—	0.50	0.14	4+	—
2. I_{11} ^e	+	—	0.50	0.28	4+	—
3. Unknown	—	—	0.22	0.14	—	—
4. Unknown	—	—	0.20	0.22	—	—
5. Control I_{11}	+	—	0.57	0.23	4+	—
6. Control B_{13}	—	+	0.17	0.71	—	4+
7. B_{88}	—	—	0.11	0.07	—	—
8. Unknown	—	—	0.08	0.14	—	—
9. Unknown	—	—	0.04	0.04	—	—
10. Mixed infection ^e	—	±	0.09	0.28	—	Some + Some —
11. Control I_{11}	+	—	0.53	0.13	4+	—
12. Control B_{13}	—	+	0.19	0.47	—	4+
13. I_{11}	+	—	0.59	0.18	4+	—
14. B_{13}	—	+	0.16	0.55	—	4+
15. B_{88}	—	—	0.18	0.24	—	—
16. B_{88}	—	—	0.16	0.23	—	—
17. Control I_{11}	+	—	0.31	0.16	4+	—
18. Control B_{13}	—	+	0.11	0.40	—	4+
19. B_{13}	—	+	0.11	0.34	—	4+
20. I_{11}	+	—	0.48	0.11	4+	—
21. B_{88}	—	—	0.11	0.09	—	—
22. Unknown	—	—	0.21	0.15	—	—
23. Control I_{11}	+	—	0.37	0.09	4+	—
24. Control B_{13}	—	+	0.12	0.56	—	4+

^a 1–6, Frozen field nodules; antiglobulins, 50 μ g/ml; conjugate, 46.5 μ g/ml. 7, 11, 12, Frozen field nodules; 8–10, fresh field nodules, no BGG. 13–18, Frozen field nodules—cell suspension placed in boiling water bath for 5 min. 19–24, Fresh laboratory nodules.

^b +, Bright yellow; ±, yellow; —, very pale yellow.

^c Data given as micromoles of *p*-nitrophenol released from substrate in 10 min. BGG, 5.0 mg/ml; anti- I_{11} or B_{13} globulins, 75 μ g/ml; conjugate, 18.6 μ g/ml; substrate, 1.0 mg/ml.

^d 4+, Bright fluorescence; —, no fluorescence.

^e I_{11} or B_{13} cells found in uninoculated control plants grown in adjacent rows with inoculated plants.

solution can also be used as the diluent instead of 0.2 M NaOH.

In cases where only a few strains of rhizobia are to be studied, a direct ELISA may be used. In that test, the specific antirhizobial globulin is conjugated directly to alkaline phosphatase. This procedure would be faster but is less sensitive than the indirect assay. The direct ELISA may still require the use of BGG to reduce nonspecific adsorption.

The advantages of the ELISA test are apparent. No microscopic equipment is necessary; small amounts of antisera are used, and the test can be done away from large central laboratories.

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