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Complementary Methods for the Differentiation of *Rhizobium meliloti* Isolates†

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Because of the scarcity of literature on the successful use of serological methods for differentiation of *Rhizobium meliloti* isolates, the objectives of this study were to provide a rationale for selecting isolates to which antisera could be raised and to appraise the suitability of published methods of preparing *R. meliloti* antigens for the serological identification of field isolates. We used one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis to develop protein profiles of eight field isolates and one commercial inoculant strain of *R. meliloti* in order to choose candidates that were either identical or distinctly different from each other for the production of antisera. The serological methods of tube agglutination and gel immunodiffusion complemented the sodium dodecyl sulfate-polyacrylamide gel electrophoresis method of identification. On the basis of their agglutination titers and gel immunodiffusion analysis, the isolates were placed in five serogroups which were identical to the groupings based on protein profiles. Antigenic characteristics of gel immunodiffusion antigens were influenced by the composition of the growth medium, sonication of whole-cell antigens, and the addition of Formalin. We recommend that careful attention be given to the effects of varying antigen preparation procedures when analyzing *R. meliloti* so that experimental protocols do not complicate the results. The wide range of homologous-antiserum titers observed for the nine isolates indicates different inherent degrees of immunogenicity of *R. meliloti* which cannot be predicted before serum production. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis method is a useful tool for screening a collection of *R. meliloti* isolates to better ensure that strain-specific antisera representative of different types of organisms will be obtained.

Information on the ecology of *Rhizobium meliloti* is limited and is in contrast with the extensive literature on *Rhizobium trifolii* (33, 35) and *Rhizobium japonicum* (21, 22, 24). This can be attributed in part to the success achieved in developing serological methods of identification for these latter two species. There are few reports on the successful use of serological methods for identifying and differentiating *R. meliloti* isolates, most of them being found in the older literature (4, 8, 19, 20, 28, 30).

Stevens (28) used the tube agglutination serological method to show that a random collection of *R. meliloti* isolates could be placed into two serological groups. This method was refined to further characterize *R. meliloti* isolates (8, 30), and the stability of both the antigenic properties of the isolates and the antisera was observed over a 10-year period (20). Dudman (4) introduced the technique of immunodiffusion as a method of differentiating two *R. meliloti* strains which possessed common antigenic determinants.

In more recent times, several reports have been published which showed various problems with using serological methods for the identification of *R. meliloti*. These problems have been attributed to the preparation of the antigens for serological assay (7, 27), the loss of agglutinating specificity by stock cultures (37) that was attributed to the loss of strain-specific antigens (10), and the variability in the antisera obtained from different rabbits in response to the same antigen (12).

The objectives of this study were to provide a rationale for selecting *R. meliloti* isolates to which antisera could be raised. We speculated that the contrast between success and failure of serological methods for *R. meliloti* identification may be due in part to the seemingly random choice of isolates made by other workers. We used a microslab modification (17) of the method of one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separating cellular proteins as originally described by Noel and Brill (18) to develop protein profile patterns of field and commercial *R. meliloti* isolates. With this method, nine isolates were chosen as candidates to which antisera could be raised and which included organisms that possessed protein profile patterns that were either identical to or distinctly different from each other. Subsequently, we raised antisera to the isolates and determined whether serological methods would complement the SDS-PAGE method of identification.

In preliminary experiments, we encountered problems and anomalous results when attempting to analyze some of these *R. meliloti* isolates by the same serological methods that had given us success with *R. trifolii*. With the stability of strain-specific antigens under question, we conducted additional research to critically appraise the suitability for *R. meliloti* of currently published methods of preparing *Rhizobium* antigens.

MATERIALS AND METHODS

Source of *R. meliloti* isolates. The eight field isolates of *R. meliloti*, strains 8, 10, 17, 18, 21, 27, 31, and 41, came from a larger collection of approximately 100 field isolates that were obtained from nodules formed on 10-week-old plants of *Medicago sativa* L. cv. Anchor. Seeds were surface sterilized by standard methods and sown uninoculated into three replicate plot areas (4.9 by 3.05 m). The soil was a sandy loam of the Deschutes series, a member of the family of coarse loamy, mixed, mesic Xerollic Camborthids. The field site was located at the Central Oregon Experiment Station, Powell Butte, Oreg. All the nodules were taken from each of

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five plants sampled from each of the three replicate plot areas. The nodules were surface sterilized and squashed, and the contents were streaked onto plates of yeast extract-mannitol (YM) agar (34; see below). Isolates were picked to slants of YM agar, grown for 72 h at 30°C, and then stored under 20% (vol/vol) aqueous glycerol at −20°C. The commercial inoculant strain of *R. meliloti*, 102F34, was obtained from J. C. Burton, Nitragin Co., Milwaukee, Wis., and hereafter is referred to as strain 34.

Antiserum production. Antisera to isolates 10, 18, 34, and 41 and to isolates 8, 17, 21, 27, and 31 were raised on two separate occasions. Procedures for antiserum production were similar for all isolates except as noted. *R. meliloti* isolates were grown at 30°C in a glutamate-mannitol (GM) defined medium (see below) on a horizontal orbital shaker at 150 rpm for 48 h. Cells were harvested by centrifugation at 7,840 × *g* for 10 min, washed three times in 0.15 M phosphate-buffered saline (PBS; pH 7.0) and resuspended to 2×10^9 cells per ml in 0.15 M sterile NaCl. Formalin (Baker) was added in a 0.3% (vol/vol) concentration to isolates 8, 17, 21, 27, and 31 but not to isolates 10, 18, 34, or 41. All isolates were heat treated at 100°C for 30 min. The antigen suspensions were emulsified in equal volumes of Freund complete adjuvant (Difco Laboratories). One milliliter (10^9 cells) of each antigen was injected intramuscularly into the rear thigh of New Zealand White male rabbits 10 weeks of age; three replicate rabbits received each antigen. Four weeks later, rabbits were given a second injection of cells prepared as described above, except the antigen suspensions were emulsified in Freund incomplete adjuvant (Difco). Ten days after the second injection, 5 ml of blood was collected aseptically from the marginal ear vein of each rabbit, and the sera were assayed by tube agglutination for specific antibody titers. Sufficient titers of isolates 8, 10, 17, 21, 27, 31, and 41 were observed. Subsequently, 60 to 90 ml of blood was collected aseptically from each rabbit by cardiac puncture 14 and 16 days after the secondary immunization. Rabbits immunized with isolates 18 and 34 did not exhibit acceptable titers in response to two injections and were given two additional injections in Freund incomplete adjuvant at 8 and 12 weeks. The antigens used for injection at 8 weeks were unamended with Formalin, whereas the antigens at 12 weeks contained 0.3% (vol/vol) Formalin. Sufficient homologous titers were observed after the fourth immunization, and 60 to 90 ml of blood was collected by cardiac puncture at 14 and 16 days after the final booster. It should be noted that homologous titers of antisera to isolates 10 and 34 dropped to 1:10 in all subsequent assays after exsanguination of the rabbits. Efforts to reproduce the original higher titers were unsuccessful (see Results). Blood was allowed to clot at 23°C overnight, and sera were obtained by centrifugation at 582 × *g*. The sera were heat inactivated at 56°C for 30 min and stored at −20°C in 5-ml portions until required.

SDS-PAGE. Cell extracts were prepared from the nine *R. meliloti* isolates grown either in YM (pH 7.0), which was composed of the following (in grams per liter): mannitol, 10; yeast extract (Difco), 0.4; $K_2HPO_4 \cdot 3H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; and NaCl, 0.1, or in GM defined medium (pH 7.0), which was composed of the following (in grams per liter): mannitol, 10; sodium glutamate, 1; $K_2HPO_4 \cdot 3H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; calcium pantothenate, 1×10^{-2}; thiamine hydrochloride, 1×10^{-2}; biotin, 3×10^{-4}; and 10 ml of the trace element mixture of Evans (6). Each isolate was grown at 30°C as described previously. Cells were washed twice in 0.15 M PBS (pH 7.0) and then once in 10 mM Tris hydrochloride (pH 7.6). Cells were resuspended in 0.5 ml of the same Tris buffer and sonicated in an ice bath three times for 30-s intervals at an output of 40 W with a Branson Sonifier 200 equipped with a double-step, 1/8-in (ca. 0.32-cm) microtip. Sonication for three 30-s intervals was determined to be adequate, because no difference in breakage was observed for preparations sonicated three, six, and nine times. After sonication, 0.5 ml of a sample buffer (14) was added and mixed well, and extracts were stored at −20°C. Immediately before use, cell extracts were thawed and centrifuged at 8,740 × *g* in a Beckman microfuge, and the supernatants were used for analysis.

Each acrylamide gel (8 by 6 cm) was cast 0.8 mm thick in single concentrations of either 9 or 11% (wt/vol) acrylamide by the formulation of Laemmli (14). After polymerization of gels, a stacking gel was cast 1.5 cm high on top of the resolving gel, also by the formulation of Laemmli (14). The gels were placed in a home-made electrophoresis apparatus similar to that described by Matsudaira and Burgess (17). Ten microliters of cell extract supernatant containing 30 to 60 μg of protein was loaded into each well.

The gels were electrophoresed for 2 to 3 h at 4°C with an initial current of 0.0125 A and 80 V. After electrophoresis, gels were stained and destained by the rapid procedure of Matsudaira and Burgess (17). After being photographed on Kodak Technical Pan film, the gels were dried on cellulose dialysis film (BioRad Laboratories) for storage.

Tube agglutination. Agglutination titers of homologous and heterologous reactions were determined by reacting serial dilutions of antisera with antigens containing 10^9 cells per ml in 0.086 M NaCl with 0.5% (vol/vol) Formalin added. Cells were grown for 48 h in GM broth supplemented with 0.5 mM $CaCl_2$ and washed three times in 0.15 M PBS (pH 7.0). Serial twofold dilutions of antisera were made, ranging from 1/10 to 1/5,120 in 0.15 M NaCl, in a total volume of 0.4 ml per tube. An equal volume of antigen suspension was added and mixed well. After incubation in a 52°C water bath for 4 h, reactions were recorded, and the reciprocal of the last positive original serum dilution was designated as the titer.

Antigen production for gel immunodiffusion. The isolates were grown in liquid media on a horizontal orbital shaker at 150 rpm for 48 h at 30°C. Three growth media were utilized, depending on the treatment to be studied (see below). Cells were collected by centrifugation at 8,000 × *g* and washed three times at 10°C in 0.15 M PBS (pH 7.0). Suspensions of ca. 37 mg of cells (dry wt) per ml were made in 0.15 M PBS (pH 7.0) on the basis of packed cell volume, which correlated highly with determinations of dry weight ($r = 0.93$). Formalin was added to 0.5% (vol/vol) as indicated. The antigen suspensions were used as either whole cells or sonicated preparations.

The three variables used in antigen production of four *R. meliloti* isolates were (i) type of growth medium, (ii) presence or absence of Formalin, and (iii) sonicated or whole-cell preparations.

(i) Type of growth medium. Two defined growth media (GM and GM–0.5 mM $CaCl_2$) and one complex growth medium, (YM) as described above, were used for cell growth.

(ii) Presence or absence of Formalin. Formalin (Baker) was added in a final concentration of 0.5% (vol/vol) to the antigen suspension after the cells were washed but before sonication.

(iii) Sonicated or whole-cell preparations. Antigens were sonicated uniformly as described above.

A treatment array of four strains, three media, the presence or absence of Formalin, and sonicated or whole-cell preparations gave 48 possible combinations. Each treatment combination of antigens was tested for performance in gel immunodiffusion. Cell concentration of the antigen and age of the cultures were standard for all tests. During trials of the cultures were standard for all tests. During trials of the effects of both Formalin and sonication, cells were prepared from the same broth culture and were divided into experimental samples immediately before exposure to the treatment being tested.

Gel immunodiffusion. Gels contained 7 ml of a 0.8% (wt/vol) agarose solution (Sigma type IV), with 0.025% (wt/vol) NaN_3 in 0.15 M PBS (pH 7.0), and were poured to a depth of 4 mm in plastic petri dishes (50 by 9 mm; Falcon no. 1006). Antisera and antigen wells were cut with steel cork borers 4 and 5 mm in diameter, respectively, with reacting wells spaced 9 mm apart. The agarose plugs were removed with a vacuum pump assembly. Wells held 85 μl of antigen and 42 μl of antiserum. In the initial experiment, all antigens were tested with homologous serum and the three heterologous sera in a hexagonal pattern around the center antigen well, with the standard sera in every other well. After results were confirmed as repeatable, selected antigens were tested in simplified patterns for clarity of presentation. Plates were incubated at 25°C in a humidity chamber. Results were initially read and recorded photographically at 48 h and read again at 72 h to ensure that no further development of lines had occurred. Photographs were taken with a Minolta 35-mm camera equipped with a 50-mm macrolens and 2× teleconverter, with high-contrast Kodak Technical Pan film. The light source was a box with dark background and indirect illumination underneath the gels.

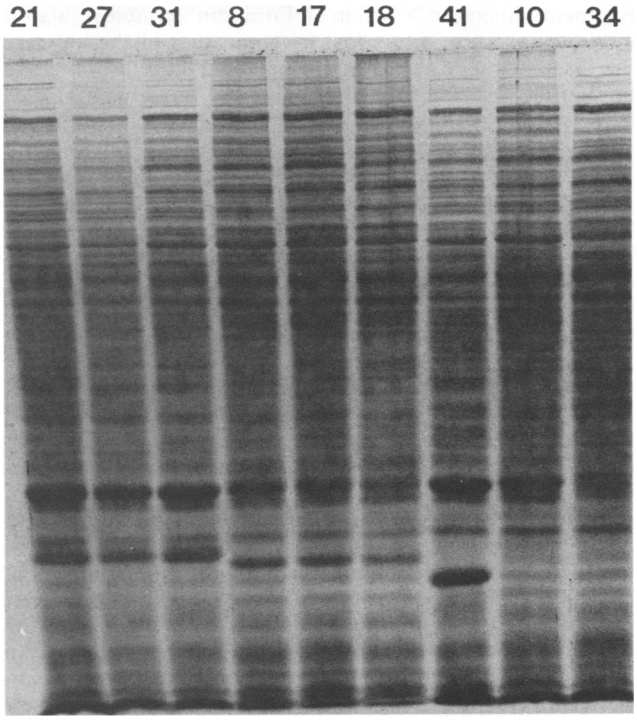

FIG. 1. Protein profile patterns of eight field isolates (21, 27, 31, 8, 17, 18, 41, and 10) and one commercial inoculant strain (34) of *R. meliloti* with SDS-PAGE. The gel is a single concentration of 11% (wt/vol) acrylamide.

TABLE 1. Agglutination titers of eight field isolates and one commercial strain of *R. meliloti*

Antigen	Titer obtained with following antiserum[a]:								
	21	27	31	8	17	18	10	41	34
21	320	1,280	1,280						
27	320	2,560	1,280						
31	320	1,280	1,280						
8				160	80	40			
17				160	160	40			
18				160	80	40			
10							10		
41	2,560	1,280	10	10	80	640	40	1,280	320
34									10

[a] Titers are expressed as the reciprocals of the highest antiserum dilution (not including volume contributed by antigen suspension) showing a positive reaction.

RESULTS

Figure 1 shows the one-dimensional protein profile patterns of eight field isolates and one commercial inoculant strain of *R. meliloti* on an 11% (wt/vol) polyacrylamide gel. The isolates were arranged so that similarities and dissimilarities within the profiles were readily apparent. Five different profiles represented the nine isolates. The commercial strain, 34, and two of the field isolates, 10 and 41, possessed unique profiles. Isolates 21, 27, and 31 (group A) possessed identical profiles, as did isolates 8, 17, and 18 (group B). The identities and dissimilarities of the protein profiles were confirmed on polyacrylamide gels of different percentages; four cell-free extracts of each isolate produced from cells grown on four separate occasions; extracts prepared by two methods of cell disruption, i.e., freeze-thaw cycles and ultrasonic oscillation; and extracts prepared from cells grown in either defined (GM) or complex (YM) medium.

Table 1 shows the serological responses of the nine organisms in whole-cell antigen tube agglutination assays when challenged with antisera raised to each of the isolates. Antisera to isolates 21, 27, and 31 cross-reacted reciprocally with each of these isolates and did not cross-react with the other six organisms, with the exception of isolate 41. Similar results were obtained with antisera to isolates 8, 17, and 18, which cross-reacted only with each other and isolate 41. Within this collection of organisms, isolates 10, 34, and 41 behaved serologically in a manner supportive of their unique identities. For example, antiserum to isolate 41 had a high homologous agglutination titer (1:1,280) but did not cross-react with the other antigens, despite the fact that isolate 41 antigen was cross-reactive with the other eight antisera to various titers (1:10 to 1:2,560). Antisera to isolates 10 and 34 showed low homologous titers with no cross-agglutinating activity except with isolate 41. Initial assays with antisera to isolates 10 and 34 collected in test bleeds showed higher titers, yet in all subsequent assays the titers dropped to 1:10. Efforts to reproduce the original higher titers were unsuccessful. These latter observations were made with the antisera obtained from each of the three rabbits immunized with isolate 34, despite the fact that three booster injections of the antigen were administered to each of the rabbits. Several variations in the preparation of antigen for the tube agglutination assays were made in efforts to increase the sensitivity of the assay and hence the titer. These included changes in growth medium composition, inclusion of antigen stabilizers such as Formalin or phenol, variation in the length of heat treatment of the antigen, and variations in saline concentra-

tions and centrifugation procedures. None of these antigen preparation variables were successful in producing increased agglutination titers of homologous antisera to isolates 10 and 34. Despite the cross-reactivity of isolate 41 and the low homologous titers of isolates 10 and 34, results of the tube agglutination assay provided evidence to support the results obtained by the SDS-PAGE method of identification; that is, isolates within group A (21, 27, and 31) and group B (8, 17, and 18) were identical, and isolates 10, 34, and 41 were unique.

Further serological analyses were undertaken with the gel immunodiffusion technique. Despite our lack of success in raising the whole-cell tube agglutination titers by varying the methods of antigen preparation, preliminary experiments showed that the character of precipitin band formation could be influenced by such treatments. Isolates 10, 18, 34, and 41, which displayed differences in protein profile patterns and tube agglutination behavior, were studied in detail in an attempt to develop a standard procedure for antigen preparation which could be used routinely. The variables of three growth media, addition or deletion of Formalin to antigens, and sonication of whole-cell antigens were examined.

Sonicated *R. meliloti* antigens grown in a defined medium produced more precipitin lines with homologous and heterologous antisera than did antigens produced in complex media in seven of eight cases (strain × Formalin variables). Whole-cell antigens grown in defined medium gave more lines in six of eight cases. The effect of growth media on the performance of an antigen in immunodiffusion is illustrated by isolate 18 (Fig. 2A and B). This isolate, grown in defined medium and sonicated, produced additional antigens which were recognized by antisera to antigens 18 and 34. An additional antigen was recognized by homologous antiserum to sonicated antigen 34 prepared from cells grown in a defined medium, both with and without supplemental Ca^{2+}, as compared with a complex medium (Fig. 2C). Previous investigators have stressed the importance of growing organisms in defined medium when they are being used for antibody production (5, 26). Thereafter, it has been suggested that either defined or complex medium can be used for growth of isolates for serological identification. In this study, although antisera were produced to organisms grown in a defined medium, the antigenic characteristics of strains grown for identification purposes in either complex or defined medium were often very different.

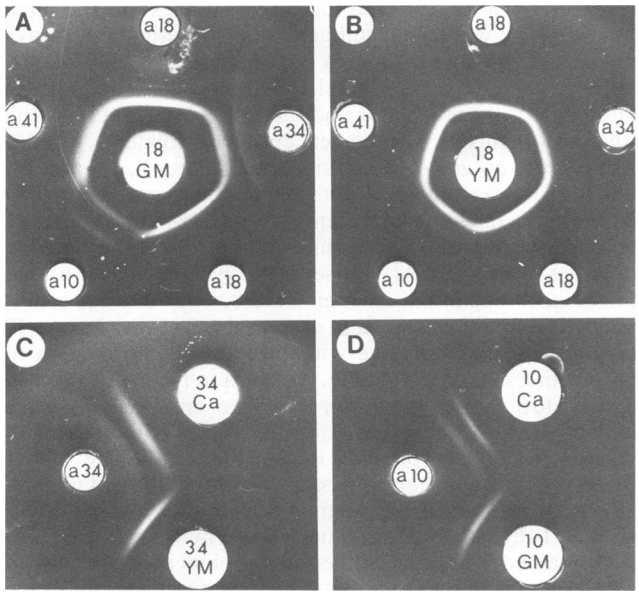

FIG. 2. Immunodiffusion patterns of selected *R. meliloti* isolates illustrating the effect of medium for growth of cells on precipitin line formation. (A) Isolate 18 grown in GM defined medium, sonicated; (B) isolate 18 grown in YM complex medium, sonicated; (C) isolate 34 grown either in GM medium supplemented with 0.5 mM $CaCl_2$ (Ca) or YM medium, sonicated; (D) isolate 10 grown either in GM medium or GM medium supplemented with 0.5 mM $CaCl_2$, whole cells. a, Antiserum.

Evidence was also obtained to show that the composition of the defined medium could affect the antigenic characteristics of one of the isolates. When isolate 10 was grown in defined medium without a calcium supplement, the density and resolution of a second antigenic determinant present in cells grown in calcium-supplemented medium was drastically reduced (Fig. 2D). This result is in contrast with those of previous studies on *R. trifolii*, in which increased numbers of antigens were observed in gel immunodiffusion with cells grown in the absence of supplemental calcium (9, 36). The presence of calcium in defined medium had no effect on the numbers of antigens expressed by isolates 18, 34, and 41, but we repeatedly observed heavier precipitin bands and clearer resolution of lines when supplemental calcium was added to the growth medium. Physicochemical reasons for these differences cannot be overlooked, since Verbruggen (29) discusses the sharpening of precipitin lines in many antigen-antibody systems with the presence of calcium ions in diffusion or electrophoretic medium.

Considering all three media tested, in the presence or absence of Formalin, we observed additional precipitin lines in the majority of cases when whole cells were broken by sonication. Considering only the two defined media, we observed that sonicated antigens gave extra lines in 11 of 16 treatments (medium × strain × Formalin variables), and in only one case was a line lost by sonication of the antigen. The disappearance of the line was largely due, however, to the detrimental effect that Formalin had on antigen 41 (see below), rather than directly to a sonication effect. Additional immunodiffusion precipitin bands were observed with sonicated *R. meliloti* preparations by Sinha and Peterson (27), Dudman (4), and Humphrey and Vincent (10). Results of this study substantiate those observations.

The effect of Formalin treatment on antigenic behavior of *R. meliloti* isolates in gel immunodiffusion assays was investigated after observations made during previous studies with the addition of Formalin to *R. meliloti* tube agglutination antigens. Problems encountered in obtaining reproducible homologous agglutination titers with these particular *R. meliloti* isolates provided evidence for the instability and variability of whole-cell antigens, regardless of the conditions of antigen preparation. Phenol added to a final concentration of 0.5% for the purpose of preservation, stabilization, and killing of cells worked well in assays with *R. trifolii* but was found to be unsatisfactory for these *R. meliloti* isolates. The addition of Formalin to gel immunodiffusion antigens of the four isolates used in this study gave variable results. Approximately half of the treatments benefitted from the addition of Formalin and half showed detrimental effects. An example of the former was the detection of an extra precipitin band of isolate 10 reacting against its homologous antiserum (Fig. 3A and B). Adverse effects were due mainly to one particular isolate, 41. Whole-cell antigens of isolate 41 grown in a complex medium did not produce any strain-specific lines when treated with Formalin (Fig. 3C), whereas strain-

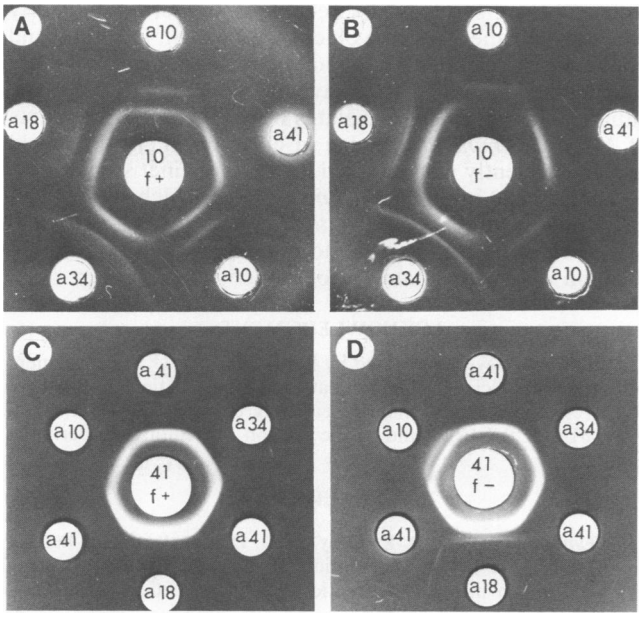

FIG. 3. Immunodiffusion patterns of selected *R. meliloti* isolates illustrating the effect of Formalin addition on precipitin line formation. (A) Isolate 10 with 0.5% (vol/vol) Formalin added (f+), grown in GM defined medium with 0.5 mM $CaCl_2$, sonicated; (B) isolate 10 with no Formalin added (f−), grown in GM medium with 0.5 mM $CaCl_2$, sonicated; (C) isolate 41 with 0.5% (vol/vol) Formalin added, grown in YM medium, whole cells; (D) isolate 41 with no Formalin added, grown in YM medium, whole cells. a, Antiserum.

specific lines to antiserum to antigens 10 and 18 were observed in the absence of Formalin treatment (Fig. 3D). For strains 18, 10 and 34, Formalin was in many cases advantageous for precipitin line formation and in most cases not a deterrent. These results indicate the importance of pretesting the effect of Formalin on all isolates used in gel immunodiffusion assays to determine whether it will have a beneficial or a detrimental effect. Although the exact nature of the effect of Formalin on somatic antigens of rhizobia need to be understood more clearly, the addition of 0.5% (vol/vol) Formalin to *R. meliloti* agglutination antigens has contributed to a more consistent recognition of strain-specific antigens of many field isolates of *R. meliloti* in this laboratory.

In this study, a method for gel immunodiffusion of *R. meliloti* which routinely includes growth in a calcium-supplemented, defined medium, sonication of antigens, and testing for a beneficial or detrimental effect of Formalin was developed. This method worked well in our laboratory with field isolates heretofore unstudied. This standard antigen preparation protocol was used in all subsequent analyses by gel immunodiffusion of the other isolates in this study, with confidence that antigen preparation procedures would not complicate the results.

Figure 4 shows the selected reactions between antisera and antigens from groups A and B. Isolates 27 and 31 reacted identically to antiserum 27 (Fig. 4A). The reciprocal reactions of the same antigens with antiserum 31 were also identical, as were reactions involving isolate 21 (data not shown). Isolates 8 and 17 reacted identically against antiserum 17 (Fig. 4B). The reciprocal reactions between either antiserum 8 or 18 and the two isolates (8 and 18) were also identical (data not shown). In contrast, gel immunodiffusion reactions between two isolates representing each of the two groups showed further evidence for their nonidentity. Isolates 17 and 27 reacted to antiserum 17 in different ways, and similar results were evident from the interactions of the same antigens with antiserum 27 (data not shown). Despite the lack of cross-agglutination between the two groups, the isolates possessed some common antigenic determinants. The data obtained from gel immunodiffusion analyses confirmed the serological identities of the isolates from within each of the groups and the heterogeneity between the two groups.

DISCUSSION

The data reported in this paper describe the use of one-dimensional microslab SDS-PAGE for differentiating field isolates of *R. meliloti*. Reports on the success of this method for differentiating *Rhizobium* species and strains have appeared in the literature (15, 18, 23). We wish to discuss the significance of our findings in the context of *R. meliloti* per se and in view of methods for identifying *R. meliloti* in ecological studies.

Observations documented in the older literature emphasize that serological characteristics of *Rhizobium* strains are stable, in contrast with other phenotypic characteristics, such as symbiotic effectiveness (19, 20, 31, 32). In recent years, however, workers have attempted to develop alternative methods of strain identification which are inexpensive

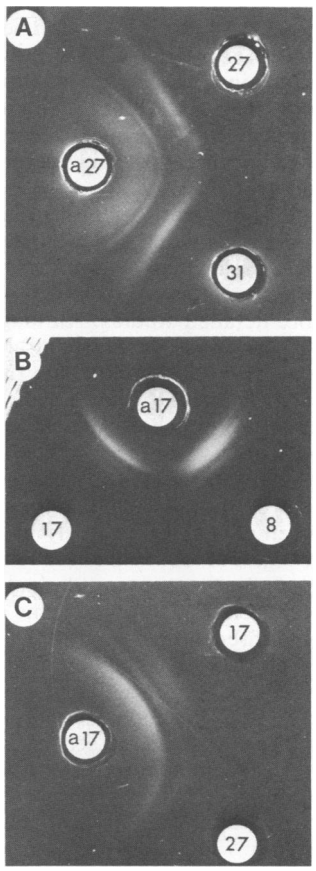

FIG. 4. Immunodiffusion patterns of selected *R. meliloti* isolates illustrating serological identity (A and B) and nonidentity (C). (A) Antiserum to isolate 27 reacting similarly with isolates 27 and 31; (B) antiserum to isolate 17 reacting similarly with isolates 17 and 8; (C) antiserum to isolate 17 reacting differently with isolates 17 and 27. a, Antiserum.

and rapid and with which large numbers of isolates can be handled. The methods of intrinsic levels of antibiotic resistance (1, 11) and antibiotic resistance markers (2, 25) and phage typing (13, 16) have been used in an attempt to circumvent time-consuming and expensive serological methods. Although justifiable criticism of each of these methods has been raised (23), serological methods remain the most useful for direct observation of rhizobia within soil and in nodules, particularly in situations in which the indigenous *Rhizobium* populations are heterogenous. Serological methods have been used widely with *R. trifolii* (33) and *R. japonicum* (3, 21, 22, 24). This can be attributed in part to the high degree of strain specificity of the antigenic constituents of these species. In the case of *R. meliloti*, the preliminary analysis of the isolates by SDS-PAGE and subsequent serological analysis of the isolates has provided important information.

The data from the serological analyses complemented that from the protein profile analyses. The magnitudes of the agglutination titers covered a wide range when the nine isolates were considered as a whole and indicate that *R. meliloti* strains might possess different inherent degrees of immunogenicity. Alternatively, different methods for antigen preparation and immunization may be required to optimize the immune response. A preliminary screening of isolates by SDS-PAGE will, at the least, allow a researcher to make a judicious choice from a collection before the expensive and time-consuming process of antiserum preparation. The absence of cross-agglutination between group A and group B isolates is in contrast with the partial cross-reactivity observed in the immunoprecipitin reactions. These observations are in agreement with those of previous reports showing that *R. meliloti* strains can possess common somatic antigenic determinants despite marginal or no cross-agglutinating ability (4).

The behavior in tube agglutination of isolates 10, 34, and 41 and their corresponding antisera was supportive of the uniqueness of these isolates as determined by SDS-PAGE. However, questions can be raised with regard to their suitability for more-detailed studies. Although antiserum to isolate 34 showed evidence of being strain specific, our inability to raise the titer of the antiserum through repeated immunization would preclude this isolate from being an organism of choice. Although isolate 41 exhibited strain-specific antiserum activity, its cross-agglutination behavior would make it unsuitable for, e.g., competition studies. Isolates which showed serological behavior similar to that of isolate 41 have been reported previously by Purchase et al. (20). These investigators observed that three *R. meliloti* isolates from their collection cross-agglutinated with 14 of 15 antisera, yet the antisera raised to the same isolates reacted only with the same three antigens.

In conclusion, the data reported herein support the need for caution in the use of serological methods as previously documented (10, 27, 37). We recognize this need and encourage others to scrutinize the general serological performance of any new isolates used in the future to ensure that methods of antigen preparation do not complicate the results. We believe, furthermore, that SDS-PAGE should be considered a potential tool with which to screen a collection of *R. meliloti* isolates to provide a better guarantee of obtaining antisera representative of different types of organisms from the collection, and with the possibility that some of these sera may possess specific agglutination and immunodiffusion characteristics.

The need for a preliminary analysis of collections of field isolates before serological screening is supported indirectly by data in a paper from the older literature (8). Diversity and homogeneity within two populations of *R. meliloti* were found and yet would not have been predicted from the situations. Six *R. meliloti* isolates were taken from six plants of *Medicago minima* growing in close proximity in a field and subsequently were classed into five distinct serogroups. In contrast, 10 of 12 *R. meliloti* isolates which were taken from plants of *Medicago sativa*, *Medicago hispida*, and *Melilotus alba* growing in close proximity in a different location were of the same serogroup.

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LITERATURE CITED

1. **Beynon, J. L., and D. P. Josey.** 1980. Demonstration of heterogeneity in a natural population of *Rhizobium phaseoli* using variation in intrinsic antibiotic resistance. J. Gen. Microbiol. **118**:437–442.
2. **Danso, S. K. A., and M. Alexander.** 1974. Survival of two strains of *Rhizobium* in soil. Soil Sci. Soc. Am. Proc. **38**:86–89.
3. **Date, R. A., and A. M. Decker.** 1965. Minimal antigenic constitution of 28 strains of *Rhizobium japonicum*. Can. J. Microbiol. **11**:1–8.
4. **Dudman, W. F.** 1964. Immune diffusion analysis of the extracellular soluble antigens of two strains of *Rhizobium meliloti*. J. Bacteriol. **88**:782–794.
5. **Dudman, W. F.** 1977. Serological methods and their application to dinitrogen-fixing organisms, p. 487–508. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation. Section IV. Agronomy and ecology. John Wiley & Sons, Inc., New York.
6. **Evans, H. J.** 1974. Symbiotic nitrogen fixation in legume nodules, p. 417–426. *In* T. C. Moore (ed.), Research experiences in plant physiology, a laboratory manual. Springer-Verlag, Berlin.
7. **Gibbins, L. N.** 1967. The preparation of antigens of *Rhizobium meliloti* by ultrasonic disruption: an anomaly. Can. J. Microbiol. **13**:1375–1378.
8. **Hughes, D. Q., and J. M. Vincent.** 1942. Serological studies of root-nodule bacteria. III. Tests of neighboring strains of the same species. Proc. Linn. Soc. N.S.W. **67**:142–152.
9. **Humphrey, B. A., and J. M. Vincent.** 1965. The effect of calcium nutrition on the production of diffusable antigens by *Rhizobium trifolii*. J. Gen. Microbiol. **41**:109–118.
10. **Humphrey, B. A., and J. M. Vincent.** 1975. Specific and shared antigens in strains of *Rhizobium meliloti*. Microbios **13**:71–76.
11. **Josey, D. P., J. L. Beynon, A. W. B. Johnston, and J. E. Beringer.** 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. J. Appl. Bacteriol. **46**:343–350.
12. **Kishinevsky, B., and D. Gurfel.** 1980. Evaluation of enzyme-linked immunosorbent assay (ELISA) for serological identification of different *Rhizobium* strains. J. Appl. Bacteriol. **49**:517–526.
13. **Kowalski, M., G. E. Ham, L. R. Frederick, and I. C. Anderson.** 1974. Relationship between strains of *Rhizobium japonicum* and their bacteriophages from soil and nodules of field-grown soybeans. Soil Sci. **118**:221–228.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Leps, W. T., G. P. Roberts, and W. J. Brill.** 1980. Use of two-dimensional polyacrylamide electrophoresis to demonstrate that putative *Rhizobium* cross-inoculation mutants actually are contaminants. Appl. Environ. Microbiol. **39**:460–462.
16. **Lesley, S. M.** 1982. A bacteriophage typing system for *Rhizobium meliloti*. Can. J. Microbiol. **28**:180–189.

17. **Matsudaira, P. T., and D. R. Burgess.** 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. Anal. Biochem. **87**:386–396.
18. **Noel, K. D., and W. J. Brill.** 1980. Diversity and dynamics of indigenous *Rhizobium japonicum* populations. Appl. Environ. Microbiol. **40**:931–938.
19. **Purchase, H. F., J. M. Vincent, and L. M. Ward.** 1951. The field distribution of strains of nodule bacteria from species of *Medicago*. Aust. J. Agric. Res. **2**:261–272.
20. **Purchase, H. F., J. M. Vincent, and L. M. Ward.** 1951. Serological studies of the root-nodule bacteria. IV. Further analysis of isolates from *Trifolium* and *Medicago*. Proc. Linn. Soc. N.S.W. **76**:1–6.
21. **Reyes, V. G., and E. L. Schmidt.** 1979. Population densities of *Rhizobium japonicum* strain 123 estimated directly in soil and rhizospheres. Appl. Environ. Microbiol. **37**:854–858.
22. **Reyes, V. G., and E. L. Schmidt.** 1981. Populations of *Rhizobium japonicum* associated with the surfaces of soil-grown roots. Plant Soil **61**:71–80.
23. **Roberts, G. P., W. T. Leps, L. E. Silver, and W. J. Brill.** 1980. Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify *Rhizobium* strains. Appl. Environ. Microbiol. **39**:414–422.
24. **Schmidt, E. L.** 1974. Quantitative autecological study of microorganisms in soil by immunofluorescence. Soil Sci. **118**:141–149.
25. **Schwinghamer, E. A.** 1967. Effectiveness of *Rhizobium* as modified by mutation for resistance to antibiotics. Antonie van Leeuwenhoek J. Microbiol. Serol. **33**:121–126.
26. **Schwinghamer, E. A., and W. F. Dudman.** 1980. Methods for identifying strains of diazotrophs, p. 337–365. *In* F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. John Wiley & Sons, Inc., New York.
27. **Sinha, R. C., and E. A. Peterson.** 1980. Homologous serological analysis of *Rhizobium meliloti* strains by immunodiffusion. Can. J. Microbiol. **26**:1157–1161.
28. **Stevens, J. W.** 1923. Can all strains of a specific organism be recognized by agglutination? J. Infect. Dis. **33**:557–566.
29. **Verbruggen, R.** 1975. Quantitative immunoelectrophoretic methods: a literature survey. Clin. Chem. **21**:5–43.
30. **Vincent, J. M.** 1941. Serological studies of the root-nodule bacteria. I. Strains of *Rhizobium meliloti*. Proc. Linn. Soc. N.S.W. **66**:145–154.
31. **Vincent, J. M.** 1944. Variation in the nitrogen-fixing property of *Rhizobium trifolii*. Nature (London) **153**:496–497.
32. **Vincent, J. M.** 1945. Host specificity amongst root-nodule bacteria isolated from several clover species. J. Aust. Inst. Agric. Sci. **11**:121–127.
33. **Vincent, J. M.** 1954. The root-nodule bacteria of pasture legumes. Proc. Linn. Soc. N.S.W. **79**:4–32.
34. **Vincent, J. M.** 1970. A manual for the practical study of the root-nodule bacteria. IBP handbook no. 15. Blackwell Scientific Publications, Ltd., Oxford, United Kingdom.
35. **Vincent, J. M.** 1974. Root-nodule symbiosis with *Rhizobium*, p. 265–341. *In* A. Quispel (ed.), The biology of nitrogen fixation. North-Holland Publishing Co., Amsterdam.
36. **Vincent, J. M., and B. A. Humphrey.** 1968. Modification of the antigenic surface of *R. trifolii* by a deficiency of calcium. J. Gen. Microbiol. **54**:397–405.
37. **Wilson, M. H. M., B. A. Humphrey, and J. M. Vincent.** 1975. Loss of agglutinating specificity in stock cultures of *Rhizobium meliloti*. Arch. Microbiol. **103**:151–154.