

Use of the Chrome Azurol S Agar Plate Technique To Differentiate Strains and Field Isolates of *Rhizobium leguminosarum* biovar *trifolii*

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Identification of *Rhizobium* and *Bradyrhizobium* strains and especially of indigenous isolates continues to be one of the major difficulties associated with competition studies. Because there is no universally accepted method, the method of choice depends on preference, experience, and equipment. Here, an agar plate technique was used to distinguish strains and field isolates of *Rhizobium leguminosarum* biovar *trifolii* to provide a basis for identifying nodule occupants in further competition studies. A rapid plate technique, based on differential growth characteristics, complements other techniques such as serological reactions, particularly when antisera cross-react with nonhomologous strains. The technique involves culturing strains and isolates on chrome azurol S agar. Although similar responses were observed among some strains, the response was highly reproducible and was considered an ideal complementary technique used in conjunction with serological procedures. Strains with similar responses could often be differentiated by varying media components, such as the source of carbon.

Studies involving effectiveness of legume-*Rhizobium*/*Bradyrhizobium* associations or bacterial strain competition are complicated by the problem of identification of strains present in nodules under both controlled and field conditions. For determinations of effectiveness and competitive ability of strains in mixed inoculum or with indigenous populations there is a need for suitable methods of identifying strains recovered from the nodules. Such methods must be reliable and rapid enough to be applied to a large number of strains.

Various methodologies have been used to differentiate strains of the root nodule bacteria. Among these, serological techniques such as agglutination reactions, immunodiffusion, immunofluorescence, and enzyme-linked immunosorbance have been widely used (1, 6, 7, 10, 11, 17). Since serological techniques rely on reactions with antisera raised against laboratory strains, they do not provide information on strains or isolates that do not react with the antisera available. Therefore, when dealing with large numbers of strains or unknown indigenous populations, serology alone can be limiting. Wide use has also been made of induced and intrinsic antibiotic resistance (2). However, loss of effectiveness (13) and competitive ability (3) has been associated with induced antibiotic markers, and intrinsic antibiotic resistance has been considered unreliable for some *Rhizobium* species (16). Modified intrinsic antibiotic resistance techniques have been applied successfully to a small number of *Rhizobium* strains (4, 14). However, these methods are not suitable for dealing with large numbers of strains or indigenous isolates.

Differentiation of strains based on the separation of cellular proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis has also been used (5, 8, 9, 12). Noel and Brill (12) used polyacrylamide gel electrophoresis to identify

18 and 19 different strains of *Bradyrhizobium japonicum*, respectively, in field-grown soybeans at two locations. Dughri and Bottomley (8) reported both distinct and identical profile patterns for different isolates of *Rhizobium leguminosarum* biovar *trifolii* and concluded that this technique was most valuable when used in conjunction with another method.

Recently, Schwyn and Neilands (15) developed an agar plate technique for determination of microbial siderophore excretion based on chrome azurol S (CAS) medium. Preliminary experimentation with CAS medium in this laboratory has suggested that strains of *R. leguminosarum* bv. *trifolii* respond differently and may be distinguished based on culture characteristics with these media. Strains that are strong siderophore producers are capable of obtaining their iron requirements by chelating the complexed iron. On the blue CAS medium, iron chelation results in a chemically induced color change to bright orange; thus, strains which produce siderophores are easily distinguished.

The detergent component in CAS medium has a tendency to inhibit growth of several microbial species (15), including certain strains of *R. leguminosarum* bv. *trifolii*. Therefore this component acts as a secondary selection mechanism. Within these responses, modifications of carbon source provide additional selection, since some strains that grow on mannitol will not grow on sucrose or fructose.

Since a large number of strains can be applied to each plate 1 liter of medium allows for testing of 600 samples.

The objective of the present study was to assess the suitability of using CAS agar plates as a method of strain identification in competition studies with other strains and field isolates of *R. leguminosarum* bv. *trifolii*.

MATERIALS AND METHODS

***Rhizobium* strains and growth conditions.** Based on previous effectiveness studies conducted in this laboratory (N. Ames-Gottfred, Ph.D. thesis, University of Guelph, 1988), several strains and isolates of *R. leguminosarum* bv. *trifolii*

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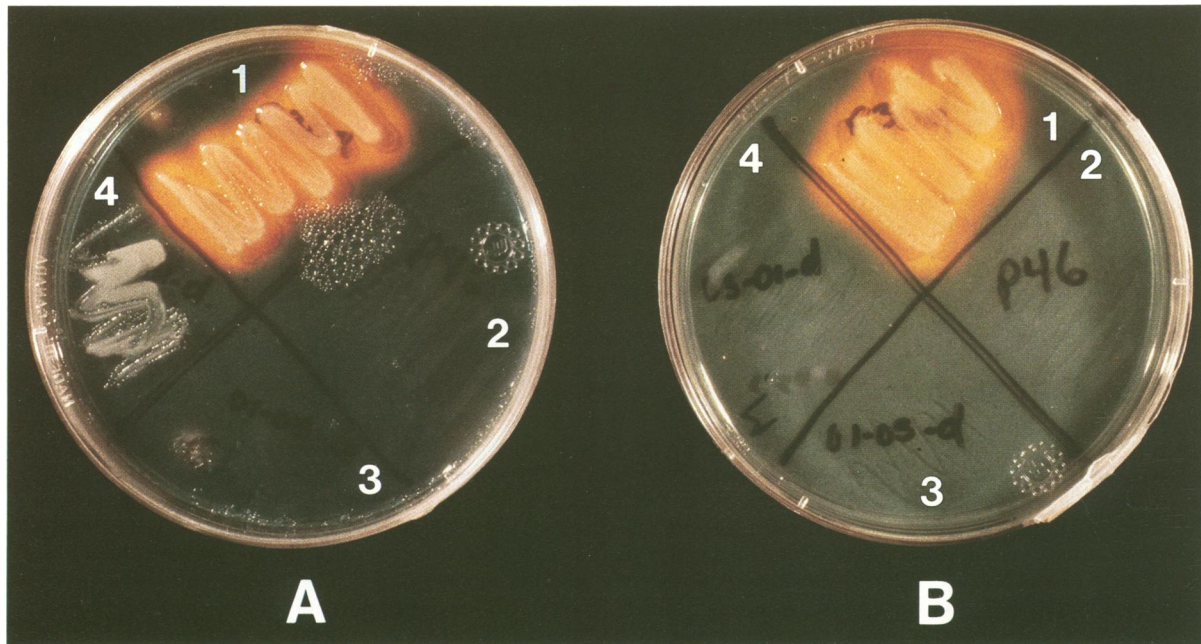


FIG. 1. Response of *R. leguminosarum* bv. *trifolii* strains on CAS agar after 48 h with two carbon sources: fructose (A) and mannitol (B). Growth and large-halo production were evident for strain 162 P30d (smear 1). Strain P46 (smear 2) grew very slowly on CAS; although growth is not evident here, after several days moderate growth and small halos were present on both carbon sources. Strain 01-05-d (smear 3) produced no growth on either plate. Strain 03-01-d (smear 4) grew better on fructose than on mannitol, but no halos were produced.

were selected for future competition studies. Before competition studies could be undertaken, a method of strain differentiation was required. The 12 pure strains (162 P30d, 162 CC1, 162 P17a, 162 P45, 162 P46, 162 P47, 162 P28, 162 S31, 162 P44, 162 E7, 162 P30e, and 162 K13), obtained from Nitragin Co. Inc., varied in effectiveness and were known to be distinct strains. Strain TA1 was obtained from D. C. Jordan (University of Guelph), and strain NG1 (162 CC1) was subcultured from 162 CC1 in this laboratory and observed to have differential colony characteristics in culture. The field isolates (01-05-d, 01-03-d, 03-01-d, 01-20-b, 01-06-d, 10-01-f, 10-01-b, and 02-08-t) were isolated from Ontario soils. Strains were maintained on yeast-mannitol agar (YMA) slants (18), transferred to yeast-mannitol broth (YMB), and grown aerobically at 28°C.

CAS. CAS agar was prepared as described by Schwyn and Neilands (15) with some modifications (Ames-Gottfred, Ph.D. thesis). Carbon sources included mannitol, fructose, and sucrose, and nitrogen sources included glutamic acid and yeast extract. Plates were divided into sections and inoculated with 2-day-old YMB cultures. Each strain treatment was replicated eight times with one replicate equivalent to one plate. Plates were incubated in the dark at 28°C for 48 h and examined for growth and production of orange halos surrounding the colonies. Degrees of growth and halo production were assessed as three levels. Heavy growth refers to that which is normally observed on YMA, minimal growth refers to growth less than that normally observed on YMA, and negative refers to no growth. For halo production, large refers to halos of >0.5 cm surrounding colonies, and small refers to halos of <0.5 cm.

RESULTS AND DISCUSSION

When strains or isolates were incubated on CAS agar plates the following responses were observed: no growth,

growth but no halos surrounding colonies, and growth and small to large orange halos surrounding the colonies. Results were visually distinct in terms of halo production, because there was a contrast of orange halos against the blue medium. An example of different strain response is shown in Fig. 1. Response was consistent over all replicates when identical media were used, indicating that this technique is extremely reliable.

Strain isolates inoculated onto CAS agar plates containing mannitol as the carbon source and L-glutamic acid as a nitrogen source showed differential growth and halo production (Table 1). These results suggest that strains can be characterized and differentiated on this medium. Five of the strains produced large halos, presumably due to synthesis and excretion of large amounts of siderophores. Although these five strains were difficult to distinguish from each other, they were clearly different from the other 15 tested. Four of the strains did not grow at all, which may indicate sensitivity to the detergent component in the medium or inability to take up any free iron in the medium. Growth without halo production was observed for five strains, although the degrees of growth differed among these. The remaining six strains had very small halos immediately surrounding the colonies.

Strain response on CAS agar plates where carbon sources varied is shown in Table 2 and Fig. 1. Generally the responses were similar for all carbon sources; however, some strains that grew on CAS with mannitol did not grow on fructose (CC1, P44, P47, E7, 01-06-e, 01-05-d, and 01-03-d) or sucrose (P44, P47, E7, TA1, and 10-01-f). Strain TA1 did not grow on CAS with mannitol or sucrose but did grow without halo production with fructose. Growth accompanied by halo production was consistent regardless of carbon source, although halo production was generally greater with mannitol. The medium used here contained

TABLE 1. *R. leguminosarum* bv. *trifolii* growth response on CAS medium with mannitol as a carbon source^a

Strain or isolate	Growth ^b	Halo formation ^c
162 P30d	++	++
162 P30e	++	++
162 CC1	+	-
NG1 (162 CC1)	++	++
162 P44	+	+
162 P45	++	-
162 P46	++	+
162 P47	-	-
162 P28	-	-
162 S31	++	+
162 P17a	+	+
162 E7	+	-
TA1	-	-
01-06-d	++	++
01-05-d	-	-
01-03-d	+	-
01-20-b	++	+
03-01-d	+	+
10-01-b	++	+
10-01-f	-	-

^a Data represent results of eight replicates, all producing the same response on CAS medium plates with mannitol as a carbon source and L-glutamic acid as a nitrogen source.

^b -, No growth; +, minimal growth; ++, heavy growth.

^c -, Absence of halo formation; +, small halos <0.5 cm wide surrounding colonies; ++, large halos >0.5 cm wide surrounding colonies.

yeast extract as a nitrogen source, which may account for some differences observed between Tables 1 and 2. Yeast extract appears to be the preferred nitrogen source and may also contain some free iron that is available for growth. The CAS agar plates used for results shown in Table 1 contained

TABLE 2. Response of *R. leguminosarum* bv. *trifolii* on CAS media with different carbon sources^a

Strain or isolate	Mannitol		Fructose		Sucrose	
	Growth	Halo production	Growth	Halo production	Growth	Halo production
162 P30d	++	++	+	++	+	+
162 P30e	+	++	+	+	+	+
162 CC1	+	-	-	-	+	-
NG1 (162 CC1)	+	++	+	++	+	++
162 P44	+	-	-	-	-	-
162 P45	++	-	+	-	+	-
162 P46	+	+	+	+	+	+
162 P47	+	-	-	-	-	-
162 P28	-	-	+	-	+	-
162 S31	+	+	-	-	++	+
162 P17a	+	+	-	-	+	+
162 E7	+	-	-	-	-	-
TA1	-	-	+	-	-	-
01-06-d	+	+	-	-	+	+
01-05-d	-	-	-	-	+	-
01-03-d	+	-	-	-	+	-
01-20-b	++	+	+	+	+	+
03-01-d	+	-	++	-	+	+
10-01-b	++	++	+	+	+	+
10-01-f	+	-	+	-	-	-
02-08-t	++	+	+	+	++	+
162 K13	-	-	-	-	-	-

^a Data represent results of eight replicates on CAS medium plates with different carbon sources and yeast extract as a nitrogen source. See footnotes *b* and *c* of Table 1.

minimal free iron, since glutamic acid was used as a nitrogen source. It appears that consistency in preparation of the media is critical for reliable results. Any variation in medium preparation that results in changes in final pH or iron concentration may affect strain response. Studies done here suggest that original strain response testing should be done with media prepared in one batch. Plates used for strain identification of nodule isolates or strain mixtures should include test strains for comparison.

The results presented indicate that CAS agar plates can be used as a selective medium for discriminating between strains and isolates of *R. leguminosarum* bv. *trifolii*. Although this method of identification is empirically based, it is a simple plate technique that is inexpensive, rapid, and reliable for a number of *R. leguminosarum* bv. *trifolii* strains and isolates tested in this laboratory. *Rhizobium* response on this medium is a function of the medium contents and the ability to produce siderophores. The responses observed include no growth, growth but no halo production (siderophore excretion), growth plus halo production, and different degrees of the last two responses. Some strains varied within these categories depending on carbon source and nitrogen source. Although not all strains could be distinguished by using CAS agar plates the data obtained will complement and extend the information obtained from other analyses. The major difficulty associated with the CAS agar plate method was medium preparation. However, plates could be stored for 2 to 3 months under refrigeration. We advise testing a maximum of 12 strains per plate to avoid the possibility of overlapping halos.

Results from a related study (Ames-Gottfred, Ph.D. thesis) indicate that when specific strains are combined in inocula the nodule occupants are accurately identified by using CAS agar plates as confirmed by the indirect fluorescent-antibody technique. Combining identification methods allows greater differentiation of strains than would be possible using one technique alone. Serology and CAS selective medium can be used in conjunction for confirmation when strains are known to produce nonspecific cross-reactions. These findings support work by Dughri and Bottomley (8) and Jenkins and Bottomley (9), who advocated multiple methods of identification for the best delineation of nodule contents.

Although the advantage of using differential growth characteristics for strain identification will depend on the *Rhizobium* species and the specific strain, a number of *R. leguminosarum* bv. *trifolii* strains show distinct characteristics on CAS agar plates. CAS medium also can be employed in studies on *Rhizobium* systematics. Recent unpublished data collected by one of us (D.C.J.) indicate that *Rhizobium fredii* strains can be grouped into three categories based on their differential responses in CAS medium containing mannitol, mannitol plus fructose, glucose, or glucose plus fructose.

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