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In previous studies, five serotypes of Rhizobium leguminosarum bv. trifolii were identified as root nodule occupants of the annual clover species, subclover (Trifolium subterraneum L. cv. 'Mt. Barker'), and the perennial species, red clover (Trifolium pratense L. cv. 'Florie'), when plants were grown from uninoculated seed in Abiqua soil (Cumulic Ultic Haploxeroll). In the case of red clover, many of the root nodule isolates were either antigenically unrelated to the five serotypes (B1 collection), or were related in a manner of antigenic non-identity to serotype AR21 (B2 collection). Immunofluorescence analysis of root nodule occupants of the annual clover, Trifolium glomeratum L. (PI284286, Spain), grown in Abiqua soil showed that the majority (89%) of nodule occupants were antigenically unrelated to the five serotypes (G collection). A small number of

isolates, antigenically unrelated to the five serotypes, were recovered from the annual clovers, Trifolium parviflorum Ehrh. (PI314397, USSR), Trifolium patens Schreber (PI284286, Spain), and T. glomeratum L. (PI284286, Spain), after seedlings were exposed to an aqueous suspension of Abiqua soil (P collection). Symbiotic effectiveness tests on subclover confirmed that the isolates from the four collections were R. leguminosarum bv. trifolii. No evidence was obtained to support the dogma that isolates recovered from annual clover species are in general more effective on subclover than those recovered from perennial species. Gel immune diffusion analysis of representatives from the G collection showed that the majority reacted against antiserum AR21 in a manner identical with those from the B2 collection. Antisera were raised to isolates AR23, AR16,and AG4 from collections B1, B2, and G, respectively, and to AV13 and AP17 from the P collection. Serological analyses showed that AV13, AR16, and AG4 were antigenically identical, and that AP17 and AR23 were antigenically unique. Nine of the 16 isolates in the G collection, five of the six isolates in the B2 collection and two of four in the P collection were antigenically identical to AG4, AV13, and AR16. Four of five isolates of the B1 collection were identical to AR23. Only six isolates in the four collections did not cross react with any of the six antisera tested. Serotype AG4 occupied a

greater percentage of nodules than each of the other seven serotypes on annual (T. patens, T. parviflorum) and perennial (T. pratense, T. repens) clovers grown together in replicate samples of Abiqua soil. No significant difference was measured in nodule occupancy by serotype AG4 on the four clover species. No evidence was obtained to suggest that annual clover species prefer to nodulate with different serotypes than perennial species.

Characterization of Indigenous Rhizobium leguminosarum bv. trifolii Isolated from Annual and Perennial Species of Trifolium

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CHAPTER I

INTRODUCTION

Nitrogen is the mineral nutrient which limits crop productivity in the majority of soils. Leguminous plants circumvent this problem through their nitrogen-fixing ability which allows them to convert dinitrogen gas into ammonia. Nitrogen fixation is carried out by bacteria of the genera Rhizobium and Bradyrhizobium which form symbiotic associations with members of the Leguminosae. As a consequence, legume crops can be independent of fertilizer nitrogen, and non-nitrogen fixing crops subsequently grown in the same soil should need less input of fertilizer N to achieve maximum economic yields. Fifty years ago it was recognized that the failure of both grain and forage legume crops could be attributed to the absence of the appropriate rhizobia in the soil. This was documented for several agriculturally important legumes including soybean (Erdman, 1943), chickpea (Moodie and Vandecayve, 1943), peanut (Duggar, 1935), clover, and Medicago spp. (Vincent, 1954). Inoculation of legume seed with the appropriate rhizobia remedied this problem. In subsequent years when the same crop was grown, inoculation

responses were not usually observed, thereby showing the capability of rhizobia to persist in soil for long periods of time, even in the absence of the host (Van Rensberg and Strijdom, 1985; Kamicker and Brill, 1986; Brunel et al., 1988).

Although persistence of rhizobia in soil has obvious benefits to legume production, it has also been well documented that many rhizobia recovered from root nodules of field-grown legumes are of sub-optimum nitrogen fixing effectiveness. In the case of forage legumes such as Trifolium and Medicago species, this phenomenon has been well documented throughout the world (Holding and King, 1963; Bergersen, 1970; Holland, 1970; Gibson et al., 1975; Hagedorn 1978; Rys and Bonish, 1981). In the case of the grain legume, soybean (Glycine max), members of serogroups 123 and 31 of Bradyrhizobium japonicum have been shown to dominate root nodules of plants growing in large areas of the north-central (Damirgi et al., 1967) and southeastern states of the U.S.A. (Keyser et al., 1984). Several studies have shown isolates from these two dominant serogroups to be in possession of sub-optimum symbiotic effectiveness characteristics (Caldwell and Vest, 1970; Mullen et al., 1988).

Since members of indigenous populations are often of mediocre effectiveness, much research has focused on developing strategies to introduce superior nitrogen-fixing strains with the seed such that they represent the

majority of the root nodules and enhance nitrogen fixation. Unfortunately this approach has met with mixed success. The lack of a yield response to inoculation has been widely documented when legume seeds were planted in soils already containing an indigenous soil population of rhizobia (Lynch and Sears, 1952; Johnson et al., 1965; Ham et al., 1971). In the case of soybean, indigenous soil populations of rhizobia form the majority of nodules unless the inoculant strain is applied in quantities so large as to be unachievable with current inoculation technologies (Bohlool and Schmidt, 1973; Weaver & Frederick, 1974).

In the case of forage legumes the situation has been more complex. In some instances, the indigenous populations of Rhizobium leguminosarum bv. trifolii have been a barrier against the introduction of inoculant strains into new seedings of subclover (Ireland and Vincent, 1968; Holland, 1970). There have been reports where inoculant strains were successful at establishment, and yet declined in nodule occupancy in subsequent growing seasons (Dudman and Brockwell, 1968; Roughley et al., 1976). Although there are several reports describing the success of inoculant strains both at establishment and in post-establishment years (Gibson et al., 1976; Brockwell et al., 1977; Brockwell et al., 1982), many of these strains did not perform consistently at different locations (Brockwell, 1981). Researchers have speculated

that the unpredictable competitive behavior of inoculant strains could be related to the variable composition of the indigenous populations at different sites (Roughley et al., 1976; Amarger and Lobreau, 1982). Therefore, it is important to identify and characterize the soil-borne populations of rhizobia if a more comprehensive understanding of inoculant failure and success is to be achieved.

Role of Host Selection in Strain Competition

Different species of Trifolium, and even different cultivars within the same Trifolium species can influence the outcome of competitive nodulation between simple mixtures of two strains of rhizobia (Vincent and Waters, 1953; Robinson, 1969; Jones and Hardarson, 1979). Only recently has this phenomenon been shown to occur in soil-grown Trifolium. Different cultivars of subclover (T. subterraneum L.) were shown to nodulate differentially with four serotypes of a soil-borne population of R. leguminosarum bv. trifolii (Dughri & Bottomley, 1984). Perennial red clover (T. pratense) was shown to nodulate predominantly with different serotypes than had previously been recovered from the annual species, subclover (Valdivia et al, 1988). In this connection, previous studies have shown that annual and perennial clovers planted in the same soil nodulated only with those members of the soil rhizobia population which were symbiotically

effective on that particular host.(Robinson, 1969; Masterson & Sherwood 1974). In those studies the choice of Trifolium species was limited. Trifolium pratense and Trifolium subterraneum were used by Robinson (1969), and T. repens and T. subterraneum were used by Masterson & Sherwood (1974). Weaver et al., (1989) have shown recently that different annual species of clover (T. vesiculosum and T. incarnatum), when planted in the same soil, nodulate with different members of the same soil population. It is, therefore, equivocal whether host selection is related to the annual or perennial nature of the clover species. If a better understanding of this phenomenon is to be achieved, a more comprehensive screening of annual and perennial species is required along with a thorough understanding of the composition of the soil population of rhizobia.

Methods of Identifying Rhizobium

Since there are no reliable media for selectively culturing soil rhizobia, estimates of their total density are made by a most probable number (MPN) technique. This involves the use of host plants inoculated with serially diluted samples of soil (Tsuzimura and Watanabe, 1959; Date and Vincent 1962). Since the MPN method provides no information on the population composition, various methodologies have been developed to discriminate among strains of rhizobia.

Serological techniques were pioneered several years ago and were originally based on whole-cell agglutination (Stevens 1923; Hughes and Vincent 1942; Vincent, 1942) or gel immune diffusion (Dudman, 1964) characteristics. The serological reactions almost certainly reflect upon fundamental and relatively constant characteristics of the bacterial cell. Serological techniques have been used extensively for Rhizobium strain identification (Vincent, 1941, 1970; Purchase et al., 1951; Koonty & Faber, 1961; Skrdleta, 1965), and have been used to demonstrate that natural populations of rhizobia may be heterogeneous within the same location, and even in nodules formed on the same plant (Hughes & Vincent, 1942; Purchase and Vincent, 1949; Johnson and Means, 1963). Strains of similar antigenic composition have been found in different regions of the same state (Mahler & Bezdicek, 1980; Valdivia et al., 1988), in different states within a country (Vincent, 1954; Johnson & Means, 1963), and on different continents (Vincent, 1954; Dowdle & Bohlool, 1985). The use and interpretation of serological data is restricted by the extent of cross reaction among rhizobia, and by the fact that preparation of strain specific anti-serum for a large number of strains is an unwieldy process. Pre-screening of isolates by other identification methods can reduce the chances of serendipitously selecting cross-reacting strains for generating antisera.

In recent years, workers have attempted to develop alternative methods of strain identification which provide information about the diversity within soil populations of rhizobia. Multi-locus enzyme electrophoresis has recently been used to explore variation within natural populations of bacteria (Hartl and Dykhuizen 1984, Selander et al. 1986). Between six and fifteen enzyme polymorphism patterns were identified within soil populations of Rhizobium leguminosarum bvs. trifolii and viciae, respectively (Young, 1985; Young et al., 1987; Young and Wexler, 1988).

Antibiotic resistant markers have been used to identify inoculant strains in nodules and to distinguish them from members of soil populations (Schwinghamer and Dudman, 1973). Although an antibiotic resistance marker allows selective recovery of an inoculant strain from a collection of nodules occupied both by inoculant strains and native rhizobia, it provides no information about the composition of the indigenous population of rhizobia within the nodules. A different approach which has been used to study the indigenous population of rhizobia in soil involves intrinsic antibiotic resistance characteristics (Beynon and Josey, 1980). Although this method allows the researcher to discriminate between members of a soil population, the highly sensitive nature of some Rhizobium spp. to antibiotics makes this method susceptible to both technical error and to the instability

of antibiotic stock solutions (Valdivia et al., 1988).

Another method which has been used with some success involves the comparison of protein profile patterns after the separation of cellular proteins by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). This method has been used to discriminate between isolates within soil populations of B. japonicum (Noel and Brill, 1980) and R. leguminosarum bv. trifolii (Dughri & Bottomley, 1983). While the method is very sensitive, it is extremely time consuming to carry out if many isolates are to be analyzed.

Plasmid profile analysis is an important method of strain delineation because many genes important to symbiosis establishment, including nodulation (Johnston et al., 1978), nitrogen-fixation (Nuti et al., 1979), host specifity (Johnston et al., 1978), and competitiveness (Brewin et al., 1982, 1983), are encoded by plasmids. Plasmid profiles have been used by investigators to discriminate between isolates from field populations (Gross et al., 1979; Ticky and Lotz, 1981; Glynn et al., 1985; Broughton et al., 1987; Brockman and Bezdicek, 1988; Mozo et al., 1988). In the study by Brockman and Bezdicek (1988), 18 different plasmid profiles were found in a soil population of R. leguminosarum bv. viciae. Nearly all isolates within each plasmid profile group were confined to a particular serogroup.

Rhizobia-specific bacteriophage have been recovered from soil and found to be useful for discriminating between strains (Staniewski, 1970a and 1970b, Lesley, 1982). Bromfield et al. (1986) used phage typing to show extreme diversity within indigenous populations of R. meliloti inhabitating nodules of field-grown Medicago sativa cultivars.

In general, the methods of phage typing (Bromfield et al., 1986), plasmid profiles (Brockman & Bezdicek, 1988) and restriction fragment length polymorphism of symbiotic plasmids (Young & Wexler, 1988) have revealed a more complex picture of population composition in soil rhizobia than obtained from multi-locus enzyme electrophoresis or from serology. There is a need for resolving which of these delineation methods are complementary to each other, and which are providing data of most relevance to understanding soil colonizing and competitive nodulating abilities. A further problem relates to the fact that although many of these methods can identify both the dominant and incompetent nodule occupying types, only immunofluorescence serology can be used to accurately study the different naturalized types in the soil and the rhizosphere phases of their existence (Schmidt et al., 1968).

Objectives

The reseach described in this thesis was conducted with the following objectives in mind: 1) to extend the serological characterization of the population of R. leguminosarum bv. trifolii in Abiqua soil, and 2) to compare nodule occupancy by indigenous serotypes on annual and perennial clover species grown from uninoculated seed in the soil.

CHAPTER II

CHARACTERIZATION OF INDIGENOUS *Rhizobium leguminosarum* bv. *trifolii* ISOLATED FROM ANNUAL AND PERENNIAL SPECIES OF *TRIFOLIUM*

INTRODUCTION

Environmental factors and management strategies dictate whether annual or perennial species of clover (Trifolium) are the major legume component of pastures in the temperate world (Taylor, 1985). In the case of perennial species, red (T. pratense L.) and white (T. repens L.) clovers receive most research attention. Amongst annual species, subclover (T. subterraneum L.) has been most extensively studied (McGuire, 1985). Several years ago, perennial clover species were shown to form ineffective symbiotic associations with strains of R. leguminosarum bv. trifolii that are effective on annual species. Strains effective on perennials were ineffective on annuals (Strong, 1937; Vincent, 1945; Purchase and Vincent, 1949). Furthermore, soil-grown plants of red, white, and subclover nodulated only with those members from the soil population which were effective on the particular 'trap' host. In general, isolates recovered from nodules of red or white clover were ineffective when challenged to subclover, and isolates recovered from nodules of subclover were ineffective when evaluated on red or white clover (Robinson, 1969; Masterson & Sherwood, 1974). Valdivia et al. (1988) extended the observations of Robinson (1969) to show that red clover nodulated with serotypes of an indigenous soil population of R. leguminosarum bv. trifolii which were antigenically

distinct from those recovered from nodules of subclover grown in the same soil. Only one study has reported the recovery of R. leguminosarum bv. trifolii by different annual species grown in the same soil. Weaver et al. (1989) showed that arrowleaf clover (T. vesiculosum Savi.) was able to nodulate selectively with effective strains from the same soil in which crimson clover (T. incarnatum L.) nodulated with effective isolates which were ineffective when evaluated on arrowleaf clover.

Based upon previous studies with red clover and subclover, five antigenically distinct serotypes of R. leguminosarum bv. trifolii were identified in Abiqua soil (Dughri and Bottomley, 1983; Valdivia et al., 1988). However, the majority of red clover nodules contained occupants which were either antigenically unrelated to the five serotypes, or were related to one of the serotypes (AR21) by a weak reaction of non-identity (Valdivia et al., 1988).

Our objectives were: 1) to identify and characterize other antigenically distinct members of the indigenous R. leguminosarum bv. trifolii population in the Abiqua soil and (2) to compare the profiles of nodule occupancy of annual clover species with perennial species when both are sown uninoculated into Abiqua soil.

MATERIALS AND METHODS

1. Trifolium species

Seeds of T. glomeratum L. (PI 284286, Spain), T. parviflorum Ehrh. (PI 314397, USSR), and T. patens Schreber (PI 284286, Spain), were obtained from Dr. Gilbert Lovel, U.S. Department of Agriculture, Agricultural Research Service, Regional Plant Introduction Experiment Station, GA 30212. The three species are annual clovers with a natural distribution throughout central, eastern, and southern Europe (Tutin et al., 1968). T. repens L. cv. 'Dutch white', T. subterraneum L. cv. 'Mt. Barker', and T. pratense L. cv. 'Florie' were obtained from the seed certification laboratory, Department of Crop Science, Oregon State University, Corvallis, OR 97331.

2. Rhizobium leguminosarum bv. trifolii

Representative isolates from three collections of R. leguminosarum bv. trifolii, maintained in this laboratory were used. Strains AR8, 9, 15, 22 and 23 were originally recovered from T. pratense and were found to be antigenically unrelated to five serotypes known to be present in Abiqua soil (Valdivia et al., 1988). This collection will hereafter be referred to as the B1 collection. A second group of isolates represented by AR10, 11, 12, 16, 19, and 24 comprise serotype AR21-C. These isolates were recovered from T. pratense grown in Abiqua soil (Valdivia

et al., 1988) and were found to react in a weak, non-identical manner with antiserum AR21 in gel immune diffusion tests. Weak whole-cell agglutination titers, (< 1/20th dilution of antiserum AR21), and negative reactions in immunofluorescence with fluorescent antibody AR21, suggest they belong to a serotype not previously characterized in Abiqua soil. This group is referred to as the B2 collection. The third collection of isolates were obtained from the root nodules of T. glomeratum, T. patens, and T. parviflorum. Seedlings of these species were transferred to test tubes containing mineral salts agar and challenged with a ten-fold dilution series of Abiqua soil. After six weeks of growth, nodules were recovered from the plants, surface sterilized, occupants streaked to purity, and cultures stored on yeast-extract mannitol (YEM) agar slants at 4°C (Vincent, 1970). A preliminary serological characterization of fifty isolates was carried out using the five antisera available in our collection (Dughri and Bottomley, unpubl. observations). Seven isolates AG3, AP3, AP11, AP17, AV4, AV11, and AV13 were antigenically unrelated to the five serotypes and are referred to as the P collection. The composition of these three collections is summarized in Table 1.

Soil

Surface samples (0-10 cm) of a silty clay loam of the Abiqua series (Cumulic Ultic Haploxeroll) were used. The

soil is under a permanent pasture composed of grasses and subterranean clover (Trifolium subterraneum L.) and is located on the Department of Animal Science cattle ranch facility at Soap Creek, Corvallis, Oregon. The chemical and physical characteristics of the soil have been described elsewhere (Almendras and Bottomley, 1987; Bottomley and Dughri, 1989).

Soil Experiment 1. To Evaluate the Composition of Nodule Occupants on T. parviflorum, T. patens, T. pratense, and T. glomeratum sown uninoculated into Abiqua soil

A composite sample of soil (pH 5.8) was thoroughly mixed, sieved < 2 mm, and amended with sodium molybdate to provide 1 mg Mo kg^{-1} of oven dry soil. Portions (800 g) of soil were placed in plastic pots, and surface sterilized seeds of each of the four Trifolium species were planted together in each of three replicate pots. After two weeks, seedlings were thinned to two of each species per pot and the soil surface covered with sterile paraffinized sand to prevent aerial contamination (Vincent, 1970).

After eleven weeks of growth under the greenhouse conditions described elsewhere (Bottomley and Jenkins, 1983), plants were recovered and root systems were washed in distilled water containing 0.1% w/v Tween 80, followed by four washes in distilled water. Nodules were recovered from the roots, counted, and subsamples of approximately

forty nodules per species per replicate were squashed individually in distilled water. Six portions (30 μl) of the contents of each nodule were spotted onto separate microscope slides, air dried, heat fixed, stained with flouorescein-labeled immunoglobulin conjugates previously prepared to five serotypes (AS6, AS16, AR21, AS27, and AS36) and the occupancy of the nodules evaluated by immunofluorescence. The preparation of the conjugates and the staining procedures are described elsewhere (Demezas and Bottomley, 1986).

Recovery of Isolates of R. leguminosarum bv. trifolii from T. glomeratum

Since immunofluorescence analysis revealed that the majority of isolates from T. glomeratum were antigenically unrelated to any of the known serotypes, a collection of isolates was made from those nodules not utilized in the immunofluorescence studies. Thirty-two isolates were recovered from surface-sterilized nodules and maintained on YEM agar slants. These isolates are referred to as the G collection.

Evaluation of Symbiotic Effectiveness of the Isolates of R. leguminosarum bv. trifolii

Seeds of subclover were surface sterilized, germinated for 48 h on water-agar plates, and transferred to nitrogen-free mineral salts agar in large (30 x 3 cm) test tubes (Bottomley and Jenkins, 1983). Four replicate

seedlings were inoculated with 1 ml portions of a culture of each isolate grown to late exponential phase in yeast-extract mannitol broth at 27°C. Non-inoculated tubes served as controls and other noninoculated tubes were each supplemented with 3 ml of 18 mM KNO_3 at 14 and 21 d after inoculation. The seedlings were arranged in a completely randomized design under greenhouse conditions previously described. Thirty-five days after inoculation, plants were assessed for nodulation, harvested, and shoot dry weights determined after herbage was dried at 60°C for one week. Symbiotic effectiveness of isolates was evaluated by comparing the shoot dry weights of inoculated plants with those of the nitrate supplemented controls. Tukey's Honest Significant Difference procedure was used for statistical analysis of the data.

In the case of the isolates chosen for raising anti-sera, a more stringent test of symbiotic effectiveness was conducted. Large test tubes (30 x 3 cm) were filled with 11 g of a mixture of vermiculite and perlite (1:1 v/v). The rooting matrix within each tube was supplemented with 60 ml of a one-quarter strength plant nutrient solution lacking mineral nitrogen (Busse and Bottomley, 1989). Surface sterilized seeds of subclover cv. 'Mt. Barker' were sown into each tube. Seven days later seedlings were thinned to one per tube and four replicate tubes were inoculated with each of the five isolates. The tubes were arranged in a randomized complete block design under

greenhouse conditions. During the growth period of the plants, nutrient solution was replenished when necessary and after eight weeks of growth, plants were harvested and shoot dry weights determined. Analysis of variance was carried out on the data and the symbiotic effectiveness of the isolates was compared using Tukey's Honest Significant Difference procedure.

Antisera Production

Antisera were raised to somatic antigens of isolates AR23 (collection B1), AR16 (collection B2) AP17 and AV13 (collection P) and AG4 (collection G). Published procedures were followed (Dughri and Bottomley, 1983), with the exception that primary immunizations involved a combination of rear foot pad (0.2 ml each) and subcutaneous (1.2 ml) injections. After three weeks, rabbits immunized with AR16, AP17, AV13, and AG4 possessed serum of adequate titers (> 1:1250th dilution of antisera) as determined by whole-cell agglutination. The rabbit immunized with isolate AR23 required a secondary immunization with a heat-treated cell suspension mixed 1:1 with Freund's incomplete adjuvant (Difco). Once adequate titers were reached, portions of blood (40 ml) were drawn from the marginal ear vein at three day intervals. After clotting, blood cells were separated from antisera by centrifugation. Antisera were supplemented with thimerosal (0.01% w/v) and stored at -60°C until required.

Serological Analysis

Whole-cell somatic agglutination and gel immune diffusion analyses were carried out on isolates of R. leguminosarum bv. trifolii as described elsewhere (Dughri and Bottomley, 1983).

Soil Experiment II. To evaluate the distribution of indigenous serotypes of R. leguminosarum bv. trifolii in root nodules of three annual (T. patens, T. parviflorum, T. glomeratum) and two perennial (T. repens, T. pratense) species of clover grown in Abiqua soil

Soil was sampled from the field, sieved < 2 mm, and supplemented with KH_2PO_4 (55 mg P kg^{-1} soil) and Na_2 MoO_4 (1 mg Mo kg^{-1} soil). Suface sterilized seeds of the five species were pre-germinated and sown into each of three replicate pots, and thinned to three seedlings of each species per pot after two weeks. Six weeks after sowing plants were harvested, roots separated and washed in tap water, and nodule occupants were isolated into pure culture as described earlier. Few nodules were formed on plants of T. glomeratum and even fewer isolates were recovered into culture. As a result, T. glomeratum isolates were not included in the analysis of these data.

Soil Dilution Experimental Protocol

Seeds of T. glomeratum, T. parviflorum, T. patens, T. pratense and T. repens were surface sterilized by standard procedures (Vincent, 1970) and germinated on water agar

plates for 2 days at 27°C. Seedlings were transferred to sterile test tubes (30 x 3 cm) containing mineral salts agar supplemented with 20 mg per liter of KNO_3 (Vincent, 1970). The nitrate was added to stimulate the early growth of the small-seeded species. Ten grams of Abiqua soil were dispensed into 95 ml of distilled water (10^{-1} soil dilution) and successively diluted over the range of 10^{-2} to 10^{-6}. Portions of each dilution (1 ml) were used to inoculate each of five seedlings at each dilution of soil. The seedlings were grown in a completely randomized design in the greenhouse using natural day light supplemented with artificial illumination. Sterile distilled water (5 ml) was added to each seedling 14 days after inoculation. The experiment was terminated after 42 days and the plants were scored for nodulation. Nodules were recovered from seedlings exposed to the 10^{-1} soil dilution, surface sterilized, and isolates recovered as described above. Isolates were analyzed by gel immune diffusion as described elsewhere (Dughri and Bottomley, 1983).

RESULTS

Despite five serotypes of R. leguminosarum bv. trifolii having already been identified in Abiqua soil, immunofluorescence analysis of nodules recovered from T. parviflorum, T. glomeratum, T. patens, and T. pratense revealed a substantial percentage of nodules which were occupied by rhizobia antigenically unrelated to the previously identified serotypes (Table 2). In the case of both T. parviflorum and T. glomeratum, the majority of nodules (66 and 89%, respectively) were occupied by unknown representatives of R. leguminosarum bv. trifolii. Despite this similarity, distinctly different numbers of nodules were formed on plants of the two species. An average of 15 nodules per plant were found on T. glomeratum, whereas 100 per plant were formed on T. parviflorum.

All thirty-one isolates of the G collection from T. glomeratum nodulated subclover (T. subterraneum L.), thereby establishing their identity to R. leguminosarum bv. trifolii. However, heterogeneity was confirmed within the collection since three different effectiveness rankings were identified (Table 3). Approximately one-half of the isolates were of suboptimum effectiveness on subclover. Although all isolates from the B1, B2 and P collections nodulated subclover, the majority of isolates from the B2 and P collections were of mediocre effectiveness. In contrast, the majority of isolates from

the B1 collection were of superior effectiveness. There was no trend for isolates recovered from the perennial red clover (B1 and B2 collections) to be predominantly of mediocre effectiveness on the annual subclover, nor for those recovered from the annual clovers (P and G collections) to be of superior effectiveness on subclover.

Since the G collection of isolates which were recovered from T. glomeratum did not originate from the same nodules upon which immunofluorescence analysis had been carried out, a preliminary serological survey of these isolates was conducted against various antisera in our collection. The majority of isolates evaluated (12 of 14) from T. glomeratum cross reacted identically with each other, and in a manner of non-identity with antiserum AR 21 (Table 4). The reaction was determined to be identical to that produced by members of serotype AR21-C originally recovered from red clover (Fig. 1a and b). Coincident with the weak precipitin reaction produced against antiserum AR21, these representatives of the G collection behaved identical to those in the B2 collection in other serological tests with antiserum AR21. They did not cross react with the fluorescent antibody conjugate prepared from AR21 antiserum, and produced only a weak whole-cell agglutination response against whole antiserum AR21. It is understandable, therefore, that such a high percentage of the nodule smears originally examined from T. glomeratum by immunofluorescence showed no positive reaction

against the fluorescent antibody conjugate AR21.

Based upon these findings, antiserum was raised to one representative isolate from each of the B2 and G collections which showed immune diffusion precipitin reactions identical to serotype AR21-C. These isolates are designated AR16 and AG4, respectively. In addition, one and two isolates were chosen from the B1 and P collections, respectively. They were antigenically unrelated to any of the five serotypes in our collection. The B1 isolate was recovered from T. pratense (AR23), and the P isolates were recovered from T. parviflorum (AV13), and T. patens (AP17). Prior to raising antibodies, the symbiotic effectiveness characteristics of the isolates were evaluated under the superior plant growth conditions of an open tube system (Table 5). Although all isolates were effective nitrogen fixers, only isolate AR23 was a superior nitrogen fixer. Antisera with adequate homologous titers were developed (≥ 1:1250 dilution of antisera). Based upon heterologous serological testing, evidence was obtained that AR16, AG4, and AV13 were antigenically identical (Fig. 2a, b, and c), and that antisera AR23 and AP17 were highly specific (Fig. 3a, and b).

When the R. leguminosarum bv. trifolii isolates from the B1, B2, G and P collections were examined, the majority cross reacted with the antisera. Nine of the 16 isolates recovered from the G collection, five of six isolates in the B2 collection, and two of the four

isolates in the P collection reacted in a manner of identity with AG4, AV13, and AR16 antisera. In addition, four of five isolates of the B1 collection cross reacted in a manner of complete identity against antiserum AR23 (Table 6). Only three isolates in the G collection, and one from each of the P, B1, and B2 collections did not cross react with any of the six antisera tested.

When two perennial (T. repens and T. pratense) and two annual (T. patens and T. parviflorum) clover species were grown in the same pots of soil, serotype AG4 occupied a significantly ($P = 0.05$) greater percentage of nodules than any of the other seven serotypes on T. repens and T. pratense (Table 7). Nodule occupancy by serotype AG4 was not significantly different among clover species. Of the 109 isolates analyzed in the experiment, only 12 were found to be antigenically unrelated to the eight known serotypes. In general, < 10% of the isolates analyzed from each replicate of each species were antigenically unrelated to the eight serotypes.

Although serotype AG4 dominated nodule occupancy of the soil-grown plants, preliminary evidence was obtained to suggest that the success of serotype AG4 was influenced by plant growth conditions (Table 8). When a ten-fold dilution of a soil suspension was inoculated onto seedlings of the four clover species, only six of 37 isolates belonged to the AG4 serotype. The nodule

occupants were distributed throughout the other seven serotypes, with only three of 37 isolates not antigenically characterized.

DISCUSSION

From previous studies with Trifolium subterraneum L., four serotypes of R. leguminosarum bv. trifolii were identified to be present in Abiqua soil (Dughri and Bottomley, 1983; 1984). Subsequent studies with the perennial red clover (T. pratense L.) showed these four serotypes were virtually absent from root nodules of this host (Valdivia et al., 1988). Furthermore, although a new 'serocluster' of isolates (AR21) was identified, two-thirds of the total number of isolates recovered were either antigenically non-reactive against the five available antisera, or, in the case of serotype AR21-C, reacted weakly in immunological tests against antiersum AR21. For all practical purposes, serotype AR21-C was antigenically uncharacterized. As a result of the studies reported here, the major antigens of serotype AR21-C have been more comprehensively characterized. Since AG4, AR16 and AV13 were found to be antigenically identical to each other, and also to all isolates previously placed in serotype AR21-C, the new serotype is named AG-4. As a result of the three additional serotypes having been identified in Abiqua soil (AR23, AG4, and AP17), the total number of serotypes has risen to eight. Considering the diversity of the 'trap' hosts used, and since only a small percentage (< 10%) of isolates were antigenically unidentified, it is reasonable to speculate that the Abiqua soil

population might be composed of nine or 10 serotypes.

These data can be compared with those from other studies where attempts have been made to delineate the qualitative composition of soil populations of rhizobia. In the older literature, serological studies showed nine to 10 serotypes were resolved in collections of isolates of R. meliloti (Hughes and Vincent, 1942), R. leguminosarum bv. trifolii, (Holland, 1966) and Bradyrhizobium japonicum (Johnson and Means, 1963) recovered from root nodules on plants growing in specific locations. In a series of studies using the technique of multi-locus allozyme electrophoresis, Young and co-workers identified between six and 15 electrophoretic types within soil populations of R. leguminosarum bvs. viciae and trifolii at specific locations (Young, 1985; Young et al., 1987; Harrison et al., 1987; Young and Wexler, 1988). At this time there is a need to determine if serotyping and allozyme electrophoresis are complementary and which of them are providing information relevent to the identification of subpopulations with superior symbiosis establishing capabilities.

In this study, serotype AG4 was found to occupy a significantly greater percentage of nodules than the other seven serotypes, regardless of whether the host species was an annual (T. patens, T. parviflorum) or a perennial (T. repens, T. pratense), and independent of the

differences in the root hair infection and nodulating characteristics of these species (Nutman, 1959; 1965, Kumarasinghe et al., 1975). Of considerable interest is the fact that serotypes AR21-a and AS6 occupy the majority of nodules on the annual subclover grown in Abiqua soil, with other serotypes (including AG4) invariably being minor nodule occupants (Dughri and Bottomley, 1984; Leung and Bottomley, unpubl. observations). As a consequence, researchers comparing nodulation and symbiotic effectiveness of annual and perennial clovers should be cautious about comparing data generated with subclover as the representative annual with those obtained from perennial clover species.

Finally, with the exception of serotypes AG4, AR21-a, and AS6, the remaining five serotypes have been consistently recovered as minor nodule occupants regardless of the host species used. In studies of isolates recovered from the same soil population, Young and Wexler (1988) have shown the tremendous variation of symbiotic plasmids which can exist within isolates of R. leguminosarum bv. viciae of similar chromosomal structure, and also the restricted diversity of symbiotic plasmids which can occur in isolates in possession of other chromosomal structures. Given the promiscuous competitive nature of the AG4 serotype, the restricted competitive nature of the AR21-a and AS6 serotypes, and the non-competitive nature of the other five serotypes, it will be

of considerable interest to further probe the chromosomal and symbiotic plasmid structures of this antigenically well-defined soil population of R. leguminosarum bv. trifolii.

Table 1. Serologically uncharacterized isolates of *Rhizobium leguminosarum* bv. *trifolii* recovered from Abiqua soil.

Collection	Clover species from which isolates were recovered	Isolate Code	% of Serologically[+] uncharacterized isolates	No. of isolates
B1	*T. pratense*	AR 8 AR 9 AR 15 AR 22 AR 23	21	5
B2	*T. pratense*	AR 10 AR 11 AR 12 AR 16 AR 19 AR 24	25*	6
P	*T. parviflorum*	AV 4 AV 11 AV 13	19	3
	T. patens	AP 3 AP 11 AP 17	20	3
	T. glomeratum	AG 3	5	1

*: Weak reaction of non-identity against antiserum AR 21

[+]: Percentage values refer to the number of the total isolates in each collection which were antigenically unrelated to the five serotypes of *R. leguminosarum* bv. *trifolii* previously identified in Abiqua soil.

Fig. 1 Gel immune diffusion characteristics illustrating (a) the identical antigenic reactions of different isolates from the G collection (G3 and G4), and the B2 collection (AR16, AR24) against antiserum AR21, and (b) the identical reactions of isolates from the G and B2 collections against antiserum AR21. Antigens are in the outer wells.

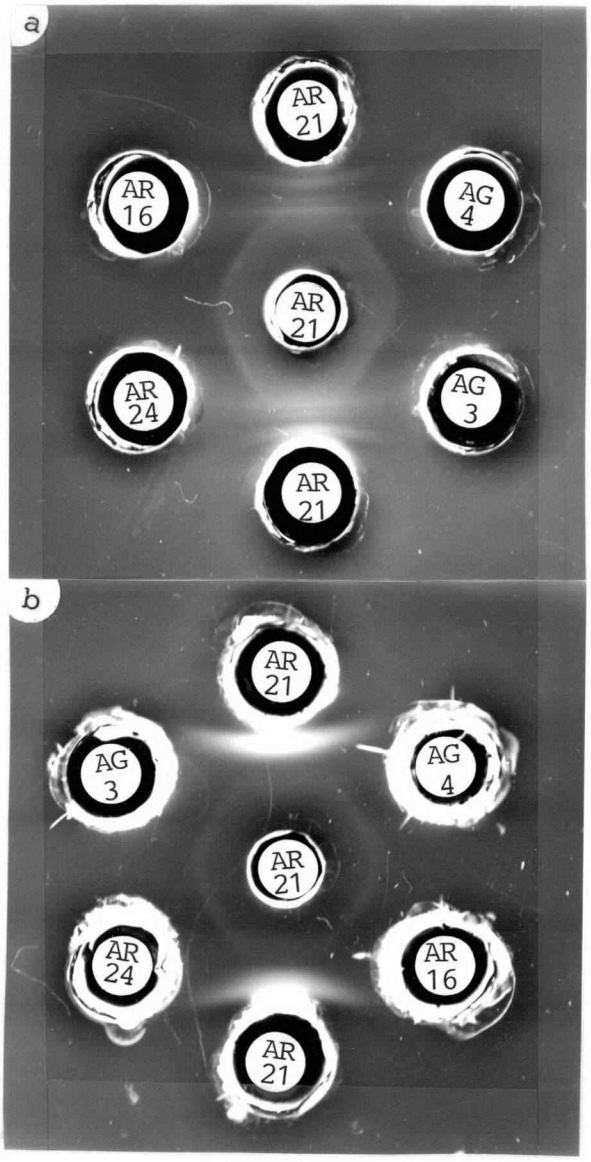
a
AR 21
AR 16
AG 4
AR 21
AR 24
AG 3
AR 21
b
AR 21
AG 3
AG 4
AR 21
AR 24
AR 16
AR 21

Figure 1

Fig. 2 Gel immune diffusion characteristics illustrating the identical antigenic reactions of isolates AR16, AG4 and AV13 against (a) antiserum AR16, (b) antiserum AG4 and (c) antiserum AV13.

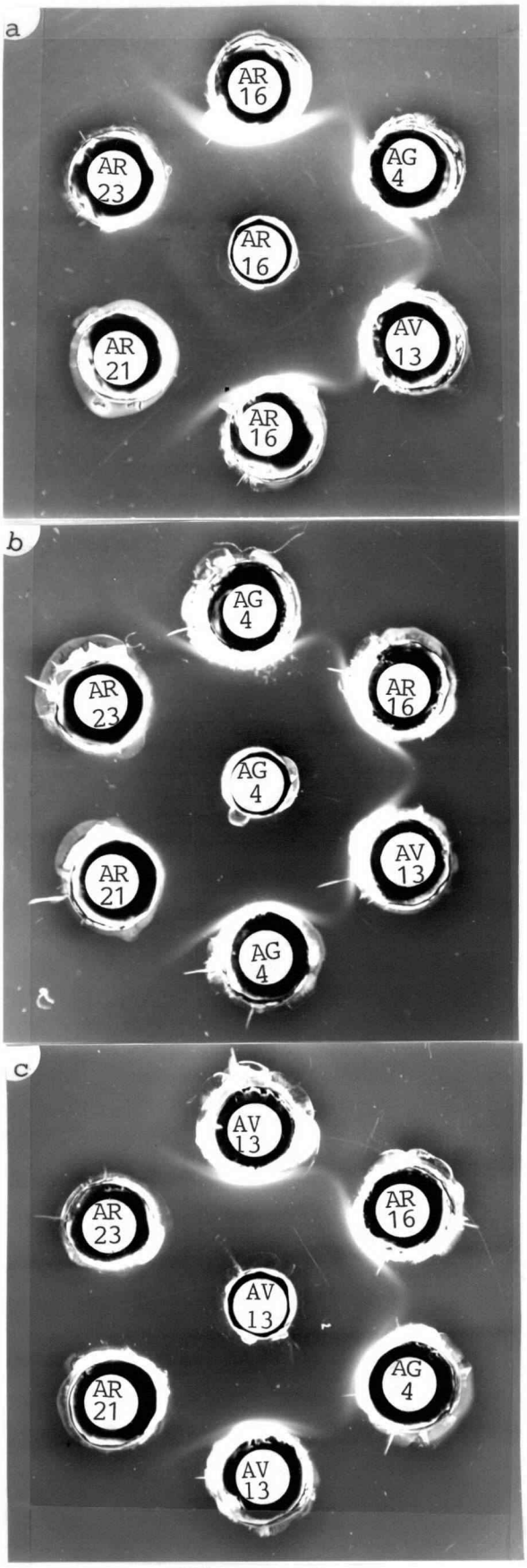
a
AR 16
AR 23
AG 4
AR 16
AR 21
AV 13
AR 16
b
AG 4
AR 23
AR 16
AG 4
AR 21
AV 13
AG 4
c
AV 13
AR 23
AR 16
AV 13
AR 21
AG 4
AV 13

Figure 2

Fig. 3 Gel immune diffusion reactions illustrating the unique characteristics of AR23 and AP17 against (a) antiserum AR23, and (b) antiserum AP17, respectively.

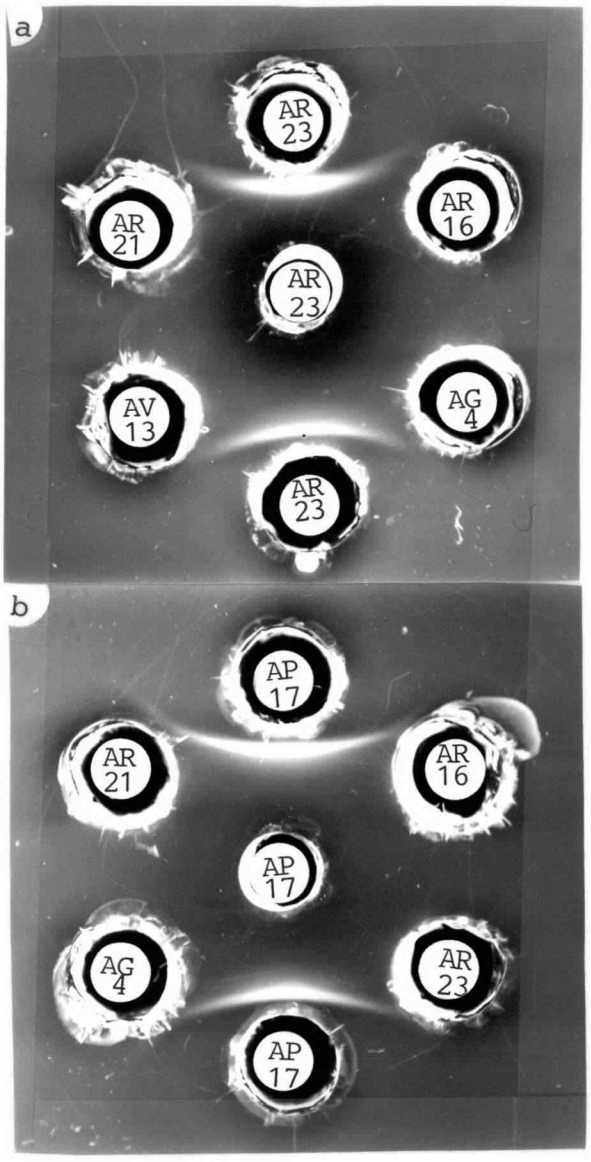
a
AR 23
AR 21
AR 16
AR 23
AV 13
AG 4
AR 23
b
AP 17
AR 21
AR 16
AP 17
AG 4
AR 23
AP 17

Figure 3

Table 2. Nodule occupancy by indigenous serotypes of *Rhizobium leguminosarum* bv. *trifolii* on different species of *Trifolium* sown uninoculated into Abiqua soil.

Trifolium species	Average Number of Nodules Analyzed per Replicate	Serotypes+ AS6	AS16	AR21	AS36	Unknown
		---Nodule Occupancy (%)++				
T. parviflorum	40	4	2	25	8	66
T. patens	36	30	11	37	7	26
T. pratense	38	20	ND*	26	26	28
T. glomeratum	32	6	4	9	9	89

+Nodule occupancy determined by immunofluorescence using fluorescein labeled immunoglobulin conjugates specific to AS6, AS16, AR21, and AS36.

*ND = not determined.

++Total nodule occupancy does not necessarily add up to 100% because some nodules were co-occupied by more than one serotype.

Table 3. Symbiotic effectiveness characteristics of the *Rhizobium leguminosarum* bv. *trifolii* isolates from collections B1, B2, P, and G on *Trifolium subterraneum* in a closed-tube system.

Collection	Host Species	No. of Isolates Tested	Effectiveness Categories		
			E1	E2	E3
B1	*T. pratense*	5	1	1	3
B2	*T. pratense*	6	4	0	2
P	*T. parviflorum*	3	1	0	2
	T. glomeratum	1	1	0	0
	T. patens	1	1	0	0
G	*T. glomeratum*	31	4	13	14

E_1 = Isolates producing shoot dry weights not significantly different from the uninoculated plants according to Tukey's W-procedure at P = 0.05.

E_2 = Isolates producing shoot dry weights significantly greater than uninoculated plants and less than nitrate supplemented plants according to Tukey's W-procedure at P = 0.05.

E_3 = Isolates producing shoot dry weights not significantly different from nitrate supplemented plants according to Tukey's W-procedure at P = 0.05.

Table 4. Gel immune diffusion characteristics of representative isolates from B1, B2, G, and P collections when challenged with antisera representing six serotypes of Rhizobium leguminosarum bv. trifolii.

Collection	No. of isolates tested	Antisera AP17	AS36	AR21	AS27	AS6	AS16
		---No. of isolates showing antigenic reaction with the antisera					
G	14	0	0	12[a]	0	0	0
B1	4	0	0	0	0	0	0
B2	6	0	0	6[a]	0	0	0
P	4	0	0	0	0	0	0

[a]Reaction of non-identity with AR 21.

Table 5. Symbiotic effectiveness characteristics of the five isolates of Rhizobium leguminosarum bv. trifolii selected for antisera production in an open-tube system.

Collection	Isolate	Shoot Dry Weight (g)[+]
B2	AR16	0.27^{b++}
P	AP17	0.27^{b}
P	AV13	0.30^{b}
G	AG4	0.31^{b}
B1	AR23	0.49^{a}
	$LSD_{0.05}$	0.08

[+]Mean shoot dry weight of four replicates

[++]Shoot dry weights not followed by the same letter are significantly different according to Tukey's W-procedure at P = 0.05.

Table 6. Gel immune diffusion characteristics of the isolates from collections B1, B2, G and P when challenged with antisera AP17, AR23, AG4, AR16, AV13 and AR21.

Collection	Host species	No. of isolates	Antisera					
			AP17	AR23	AR21	AG4	AR16	AV13
			Precipitin reactions					
G	T. glomeratum	1	I[a]	-	-	-	-	-
		1	-	I	-	-	-	-
		2	-	-	NI[b]	-[c]	-	-
		9	-	-	NI	I	I	I
		3	-	-	-	-	-	-
B1	T. pratense	4	-	I	-	-	-	-
		1	-	-	-	-	-	-
B2		5	-	-	NI	I	I	I
		1	-	-	-	-	-	-
P.	T. parviflorum,	2	-	-	-	I	I	I
	T. patens	1	-	-	-	-	-	-
	T. glomeratum	1	-	-	NI	-	-	-

a: precipitin line is identical to the homologous interaction
b: precipitin line is non-identical to the homologous interaction
c: No precipitin lines are visible.

Table 7. Nodule occupancy by indigenous serotypes of *Rhizobium leguminosarum* bv. *trifolii* on four *Trifolium* species sown uninoculated into Abiqua soil.

Host species	Average No. of isolates analyzed/ replicate	Serotypes (% nodule occupancy)[a]									LSD[c] 0.05
		AG4	AR23	AS6	AS27	AS16	AR21	AP17	AS36	Unknowns	
T. repens	10	48.2*[b]	14.9	6.7	12.6	13.0	13.0	0.9	1.0	12.6	18.9
T. patens	8	40.9	21.5	11.0	11.0	21.5	11.0	16.6	1.1	15.6	N.D.
T. pratense	10	41.3*	23.9	7.0	6.7	20.9	15.1	0.9	0.9	14.9	15.8
T. parviflorum	9	38.0*	17.0	1.0	6.9	23.0*	9.8	1.0	9.6	24.0*	17.9

[a]Percentages represent arcsin $\sqrt{\%}$ transformed values of the mean of three replicates. Only two replicates were analyzed for *T. patens*.

[b]Within a *Trifolium* species percentage values followed by an asterisk are not significantly lower ($P = 0.05$) than the occupancy of serotype AG 4.

[c]Analysis of variance was carried out on the transformed data. LSD values are represented as transformed values.

Table 8. Nodule occupancy of indigenous serotypes of *Rhizobium leguminosarum* bv. *trifolii* on four *Trifolium* species exposed to an aqueous suspension (1:10) of Abiqua soil.

Source	No. of isolates analyzed	Un-known	Serotypes (nodule occupancy) AR23	AS6	AP17	AS27	AG4	AS16	AR21	AS36
T. patens	9	2	1	3	-	-	-	1	1	1
T. repens	8	1	1	-	-	-	1	1	4	-
T. parviflorum	10	0	1	2	-	-	1	3	1	2
T. pratense	10	0	2	1	-	-	4	3	-	-

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