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Characterization of Indigenous Isolates of *Rhizobium leguminosarum* bv *trifolii* in terms of Plasmid Profiles

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ABSTRACT. *Twenty Rhizobium leguminosarum bv trifolii isolates were obtained from white clover (Trifolium repens) nodules from a single site. Plasmid profiles of the isolates were analyzed according to the modified Eckhardt (1978) method. Three to five plasmids of different sizes were identified in each isolate. Eleven isolates had unique profile patterns. However, 90% of the population shared at least two plasmids of similar size. The isolates sharing the same profile were considered as one strain. Altogether fourteen strains were identified in the studied population.*

With the exception of two plants, plasmid profiles of rhizobia nodulating the same clover plant were not identical. Similar profiles were observed between rhizobia nodulating different plants. This suggested that there is no selection of plants by a particular strain of Rhizobium leguminosarum bv trifolii. It was concluded that many strains could be identified by comparing the plasmid profiles, even in a small population of Rhizobium leguminosarum bv trifolii isolated from a single site.

INTRODUCTION

The symbiotic nitrogen fixing soil bacteria of the genus *Rhizobium* play an important role in agriculture, because of their ability to fix atmospheric nitrogen. Except for *Parasponia* of the Ulmaceae family (Trinick, 1979), all known rhizobia-induced nodules are confined to the Leguminosae family.

It has been established that rhizobia carry one or more plasmids, the extra-chromosomal circular DNA. The size and the number of plasmids vary within and between species of rhizobia. The plasmids account for more than

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25% of the total DNA in some strains or even as much as 50% in some cases (Beynon *et al.*, 1980). Two to ten plasmids, size ranging from 50-600 kb, were reported in *R. leguminosarum* bv *trifolii* (Thurman *et al.*, 1985; Harrison *et al.*, 1989a).

It is now well documented that in the majority of *Rhizobium* species, genes responsible for symbiotic nitrogen fixation are carried on large plasmids, namely, the Sym plasmids. However, there are cryptic plasmids of which functions are not fully understood.

Studies of the identification of rhizobia have established that soil rhizobia have a phenotypically and genotypically diverse clonal structure. Plasmid profile analysis has been widely used in order to study the genetic variation in *Rhizobium*. Plasmid profiles give a good measure of isolate heterogeneity, since a unique profile can reasonably be taken to represent a single strain.

In this study, an attempt was made to identify the variation between isolates in a small natural population of *R. leguminosarum* bv *trifolii* by comparing their plasmid profiles.

MATERIALS AND METHODS

Isolation of rhizobia from nodules

Twenty white clover (*Trifolium repens*) plants located about 1m apart were obtained from a clover pasture field (Frongoch, Aberystwyth, U.K.) which had no prior history of being inoculated with *R. leguminosarum* bv *trifolii*. Each plant was given a number. To find out whether there is any preference of the strains to a particular location of the root zone, the rhizobia were isolated from nodules obtained from different depths of the root system of the selected plants .

The *Rhizobium* isolates were designated by the number of the plant they obtained along with a letter denoting the position of the nodule on the plant root system. The rhizobia isolated from a nodule taken from the top, second, third and fourth quarter of the root system were denoted as a, b, c and d, respectively. In the plants 18 and 19, the position of the nodules on the root system was not recorded properly.

Nodules were detached from the plant root system by cutting the root 2 mm on either side of the nodule; and were surface sterilized by keeping them in 95% (v/v) ethanol for 1 min and in 20% (v/v) sodium hypochlorite for 3 min. The nodules were washed 5 times in sterile distilled water allowing 10 min for each wash.

After sterilization, the nodules were crushed and streaked on to TYC (Tryptone Yeast Calcium Chloride) plates (Beringer, 1974). Following growth at 27°C, the cultures were streaked into single colonies to ensure purity.

Plasmid profile analysis

Twenty *Rhizobium* isolates were analyzed for their plasmid content by agarose gel electrophoresis, according to the Eckhardt (1978) method modified by Rosenberg *et al.*, (1982).

RESULTS AND DISCUSSION

Isolation of rhizobia from nodules

Though twenty plants were selected, the isolation of rhizobia was not successful for some of them. The plants numbered 6, 8, 11 and 12 were devoid of nodules. Plants numbered 10, 14 and 15 had only pale coloured ineffective nodules, hence, rhizobial isolation was not carried out. Following isolation, no rhizobial growth was observed from the nodules obtained from the plants numbered 3, 7, 9 and 17. This could be due to destruction of rhizobia by high stringent surface sterilization.

Therefore, the isolates used in this study were 1a, 1b, 1c, 2a, 2b, 4a, 4b, 4c, 4d, 5a, 5b, 13a, 13c, 16a, 16b, 16c, 18, 19, 20a and 20b.

Plasmid profile analysis

Plasmid profiles of the twenty isolates are given in the Figure 1. Three to five plasmids of different sizes were identified per isolate. Isolates 1a, 1c, 2a, 2b, 18, 20a and 20b carried 3 plasmids, whereas, all other isolates tested, except isolate 13c, had 4 plasmids each. Only isolate 13c carried 5 plasmids. One plasmid band could represent more than one plasmid of the

same size. A thick band above average intensity could represent co-migrating plasmids. Alternatively, less intense bands would suggest plasmids that exist in a reduced copy number. However, occurrence of co-migrating plasmids were thought to be infrequent (Schunmann, 1990). Therefore, throughout the study, one plasmid band was considered as representing a single plasmid.

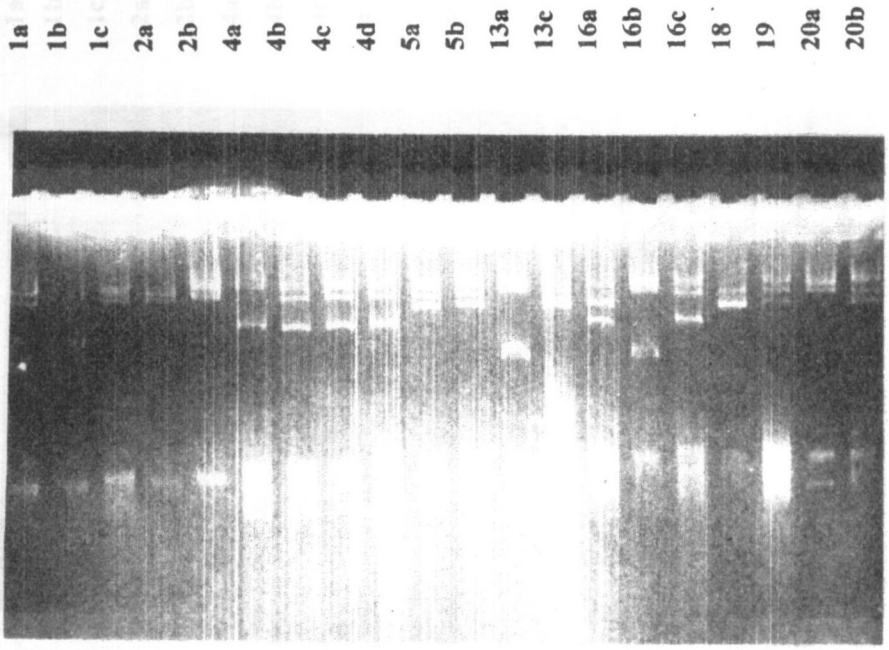

Figure 1. **Plasmid profiles of the isolates.** (The numbers represent the name of the Isolate)

Plasmids migrate through the gel according to their size. The plasmids which move further in the gel are smaller than the ones located closer to the wells of the gel.

The total number of plasmids of the isolates studied did not vary significantly, ranging only from 3 to 5. In some isolates, very faint bands could be seen in the lower area of the gel. It was difficult to suggest whether these were small intact plasmids, or broken plasmids or chromosomal fragments. In this study, those were not considered as intact plasmids. Future investigation would, however, be of interest.

Eleven isolates (55% of the population) had four plasmids of different sizes, whereas, 8 isolates (40% of the population) had 3 plasmids. Only one isolate namely, 13c representing 5% of the population had 5 different sizes of plasmids. This might not be a true representation of the population as a whole, since the sample size was small. However, the isolates tested were assumed fairly representative of the nodule population of *R. leguminosarum* bv *trifolii*.

It was observed in this study that some rhizobia with different plasmid profiles nodulate a single plant. Moreover, similar profile patterns could be observed between rhizobia nodulating different plants. Isolate distribution was considered random among the white clover plants. These results agree with those of Glynn *et al.*, (1985) who found no uniformity in the isolates of *R. leguminosarum* bv *trifolii* identified by plasmid profiles taken from single clover plants. Similar results were observed in the same biovar (Harrison *et al.*, 1988) from single clover plants, and in *R. leguminosarum* bv *viciae* (Young *et al.*, 1987) from single pea plants, using electrophoretic types (ET) as the basis of identity.

From the twenty isolates, only a few isolates shared the same plasmid profile pattern; but, 90% shared at least 2 plasmids of similar size. Plasmid profiles of 11 were not comparable to any of the plasmid profiles of the other isolates. Isolates having an identical plasmid profile were considered as one strain. For instance, isolates 2a, 2b and 1b had similar profiles and they were considered to be of the same strain. However, they nodulated 2 different plants (1 and 2). Isolates 5a and 5b which nodulated the same plant were considered as one strain. Isolates 4b, 4c, 4d and 16c showed similar profiles (same strain); whereas, 4a exhibited a totally different profile pattern which showed no close relationship to that of the other isolates. Nevertheless, 4a, 4b, 4c and 4d were isolated from the same plant, but from different root depths. There was no evidence of preference to a certain depth of the root zone by any of the strains. One strain could occupy the nodules located at any depth of the root system.

All 20 isolates, except 4a and 16a shared at least 2 similar-sized plasmids implying little variation in the size distribution of the plasmids within the population. However, among the 3 biovars of *R. leguminosarum* isolated from the same soil, a wide diversity had been observed in the size and number of plasmids (Laguerre *et al.*, 1993).

This study of plasmid profiles revealed certain degree of similarity among isolates. It is possible that the chromosomal backgrounds of these isolates are different, and the plasmids could be of the same size purely by coincidence, but it is unlikely. However, it is difficult to explain plasmid profile data to a greater extent. Predicting the exact degree of relatedness between isolates through an examination of their plasmid content is impossible, as it is primarily based on the number of plasmids per isolate and their molecular weight. Isolates containing the same number of plasmids may not be more related than isolates having a different number of plasmids due to the occurrence of one conjugational event. However, similar profiles show a good indication of being the same strain.

CONCLUSIONS

There were three groups of isolates which showed similar profile patterns. One consisted of isolates 1b, 2a and 2b. Isolates 4b, 4c, 4d and 16a belonged to the second group, whereas, isolates 5a and 5b were included in the third group. Isolates within one group could be considered as one strain in terms of plasmid profiles. All isolates, except 4a and 16a, shared at least 2 plasmids of similar size.

No uniformity in the plasmid profiles was observed in the isolates taken from a single clover plant. Moreover, one strain did not dominate in the nodules of a particular root zone depth of the plants.

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