Identification of the Rhizobium Strains in Pea Root Nodules Using Genetic Markers

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SUMMARY

Pea plants were inoculated jointly with pairs of genetically marked strains of *Rhizobium leguminosarum*. Out of 297 nodules examined 56 contained both inoculant strains. The ratios of the strains in the inoculum did not affect the frequencies of mixed nodules. Generally one of the strains consistently occupied the majority of the nodules and in the mixed nodules comprised the majority of bacteria. Transfer of the P-group R factor, RP4, between certain strains of *Rhizobium* within mixed nodules was detected. In some cases the non-parental progeny comprised 10 % of the rhizobia isolated from such nodules.

INTRODUCTION

Studies of competition between *Rhizobium* strains in nodule formation have generally suggested that nodules contain only a single strain (Dunham & Baldwin, 1931; Johnson & Means, 1963; Dudman & Brockwell, 1968), although different nodules on the same plant could contain different strains (Nicol & Thornton, 1941; Hughes & Vincent, 1942; Vincent & Waters, 1953). Other reports have indicated that under some growth conditions up to 10 % of clover and soybean nodules may contain two strains of *Rhizobium* (Vincent, 1954; Skrdleta, 1969, 1970; Pinto, Yao & Vincent, 1974), and Jansen van Rensburg & Strijdom (1971, 1972) have reported that a large proportion of soybean root nodules contained an unidentified fast-growing contaminant as well as *Rhizobium japonicum*.

Most of the above reports involved the use of serological techniques to distinguish *Rhizobium* strains, though Pinto *et al.* (1974) used strains of *R. meliloti* varying naturally in their levels of resistance to streptomycin and kanamycin. This report describes the use of strains of *R. leguminosarum* with genetic markers for auxotrophy and antibiotic resistance for the mixed infection of peas. There are several advantages in the use of such mutants over strains which are distinguished by serological differences. The bacteria isolated from individual nodules can readily be characterized by plating on appropriate selective media; therefore, even if a strain comprised only a small part of the total population within the nodule it could be detected and its frequency determined. The strains can be unambiguously identified and distinguished from contaminants, especially if they carry more than one genetic marker. Furthermore, by plating on media which select against both inoculant strains, recombinant progeny which might have been formed during infection and nodule development could be detected. Also, spontaneous mutation frequencies at the loci under examination can readily be quantified.

Table 1. *Strains of Rhizobium leguminosarum*

Strain	Parent	Additional markers
196	301 (<i>bio</i>)	<i>pur</i>
229	301 (<i>bio</i>)	<i>trp</i> (RP4)*
621	261 (<i>bio</i>)	<i>rif^r-r gly</i>
587	300 (<i>bio lac thi</i>)	(RP4)
726	300 (<i>bio lac thi</i>)	<i>rif^r-r</i>
727	300 (<i>bio lac thi</i>)	<i>str-r phe</i>
728	300 (<i>bio lac thi</i>)	<i>str-r phe</i>
729	300 (<i>bio lac thi</i>)	<i>str-r ura</i>
733	300 (<i>bio lac thi</i>)	<i>str-r liv</i> †

* (RP4) is a P-group R factor conferring resistance to tetracycline, kanamycin/neomycin and carbenicillin (Datta *et al.* 1971) and was transferred to these strains from *Escherichia coli* K12 as described by Beringer (1974).

† *liv* represents growth requirement for leucine, isoleucine and valine.

METHODS

Rhizobium leguminosarum strains are shown in Table 1. The parental strains were field isolates already carrying the markers shown. Further auxotrophic markers were isolated after nitrous acid mutagenesis (Kaudewitz, 1959). Mutations to rifampicin and streptomycin resistance were spontaneous and were isolated by plating on media containing rifampicin or streptomycin at 25 and 500 mg/l respectively.

Genetic markers of strains used in this investigation were designated by standard symbols.

Media TY, SY and VS were prepared as described by Beringer (1974). Where appropriate for the growth of auxotrophic strains, SY was supplemented with amino acids (50 mg/l) and adenine or uracil (10 mg/l).

Inoculation and growth of peas was as described by Beringer (1974). In these experiments, *R. leguminosarum* strains were cultured on TY slants and suspended in distilled water before adding to individual plants in 250 ml conical flasks. About 10^8 bacteria (single or mixed) were added per plant.

Isolation of bacteria from nodules. A number of well separated and suitably sized nodules were isolated from pea plants about 3 weeks after inoculation. The roots were first washed in sterile distilled water and the excised nodules were surface sterilized by immersion in sodium hypochlorite solution (12 %, w/v, available chlorine) for one minute and then washed thoroughly in distilled water. High levels of chlorine were used for surface sterilization to prevent contamination of the nodule contents by external rhizobia. The effectiveness of this procedure was demonstrated by the absence of *Rhizobium* colonies when surface sterilized nodules were placed directly on TY plates and incubated at 28 °C. Killing of a proportion (or all, in very small nodules) of the rhizobia present within the nodule occurred, but was assumed to be irrelevant to the determination of relative numbers of different phenotypes of *R. leguminosarum* within the nodule.

The sterilized nodules were crushed in 1 ml of 20 % (v/v) glycerol and samples were either plated immediately or were frozen at -20 °C for future use. The presence of rifampicin-resistant, streptomycin-resistant and RP4-carrying strains within the nodule was detected by plating the macerates on TY medium supplemented with rifampicin (25 mg/l), streptomycin (300 mg/l) or kanamycin (20 mg/l), respectively. The presence of auxotrophic strains was detected by plating on SY medium with or without the required metabolite. Non-parental progeny were detected on media selecting against both parental genotypes.

Table 2. *Characterization of rhizobia isolated from nodules excised from peas infected with mixed combinations of strains 196, 229 and 621*

Inoculum strains (A+B)	Input ratio	No. of nodules from which either or both inoculum strains isolated		
		A alone	B alone	A plus B
196+621	1:1	28	1	11
	1:10	3	1	3
	10:1	6	1	8
196+229	1:1	0	9	1
	1:10	3	4	3
	10:1	8	3	7
621+229	1:1	0	22	1
	1:10	0	13	1
	10:1	1	3	0

Acetylene reduction assay of nitrogenase activity. Whole plants were placed in sealed 250 ml conical flasks and acetylene injected to 1 %. Samples (0.5 ml) were taken at intervals and ethylene determined using a Pye Unicam series 104 chromatograph with a 150 cm column (4 mm internal diameter) packed with Poropak R. The carrier gas flow rate was 60 ml/min and the running temperature 60 °C.

RESULTS

Preliminary results showed that, with the exception of strain 733, all *R. leguminosarum* strains described induced the formation of effective nodules as measured by acetylene reduction. The effective strains included 196 and 729, which require purines and uracil respectively. Auxotrophs of both types have been obtained which lost their effectiveness in nitrogen fixation (Scherrer & Dénarié, 1971), though Dénarié, Truchet & Bergeron (1975) have reported that two out of 15 purine- or pyrimidine-requiring mutants were effective. The reason for these exceptions is not clear. The possibility that the auxotrophs were temperature-sensitive was excluded. The mutants were isolated and characterized at 28 °C and were shown to be still auxotrophic on culture media at 22 °C, the temperature at which nodulating ability was determined.

Macerated material from at least 12 pea nodules infected singly with each of the strains was plated on selective media designed to detect both reversion of the auxotrophic markers under study and spontaneous acquisition of resistance to one of the antibiotics used as selective agents. No such mutants were found, suggesting that the occurrence of nodules containing more than one strain of *R. leguminosarum* would not be due to a clone derived from a spontaneous mutation which had occurred during the growth of the bacteria in the course of nodule development.

Mixed infections

Experiments with strains 196, 229 and 621

The strains were mixed together in all possible paired combinations in ratios of 1:1, 10:1 and 1:10. For each mixture three plants were inoculated and usually 15 nodules were excised from each. However, for several combinations this was not possible because large numbers of nodules were closely grouped together and single nodules could not be isolated. The number of nodules yielding data was further reduced because no bacteria could be isolated from many of the smaller nodules. Either bacteria were not present or were killed

Table 3. *Characterization of rhizobia isolated from nodules excised from peas infected with combinations of strains 587, 726, 727, 728 and 729*

Inoculum strains (A+B)	No. nodules from which either or both inoculum strains isolated		
	A alone	B alone	A plus B
587+726	24	1	12
587+727	13	0	0
587+728	6	0	0
587+729	24	1	4
726+727	31	1	0
726+728	17	3	0
726+729	10	4	5

by the severe sterilization procedure. This was not further investigated. Thus the data are biased in favour of isolates from larger nodules.

Single strain inoculants of the three strains formed similar numbers of nodules. Table 2 shows that strain 621, when used in the presence of either of the other two strains, competed badly in nodule formation. Out of 62 nodules examined from inoculant combinations of strains 621 and 196, only three contained 621 alone, although 22 nodules contained a mixture of 196 and 621. When 621 and 229 were used for the mixed inoculum only one nodule out of 39 contained 621 alone. A further two nodules contained both strains. Strains 229 and 196 appeared closer in their nodulating abilities: the number of nodules occupied by each singly and the number from which both strains were isolated were about the same.

There was no pronounced effect of the ratio of the strains in the inoculum. The strain comprising the greater part of the inoculum did not necessarily form the majority of the nodules. Such a lack of effect of the composition of the inoculum has been noted several times (e.g. Vincent & Waters, 1953; Johnson, Means & Weber, 1965; Robinson, 1969; Skrdleta & Karimová, 1969). The reason for this lack of correlation is not known, though Pinto *et al.* (1974) have found that the relative proportions of strains of rhizobia in the rhizosphere can differ greatly from the proportions in the inoculum. Such differential fitness of bacterial strains in the rhizosphere might overcome any experimental manipulation of the inoculum ratios.

Experiments with strains 587, 726, 727, 728 and 729

In these experiments equal numbers of bacteria from each strain were present in the inocula. Samples (0.1 ml) from the nodule macerates were plated on appropriate selective media and the identity of the strains occupying the nodules determined qualitatively (Table 3).

It is striking that for all combinations tested, one of the strains in each mixture formed most of the nodules examined. For example strain 727, although able to form effective nodules when used as the sole inoculant, formed only one out of 45 nodules excised from plants which had been inoculated with this strain in conjunction with 587 or 726. If competitiveness can be equated with the number of nodules occupied, then strain 587 was the most competitive, followed by strain 726. Both were superior to the streptomycin-resistant strains.

An attempt was made to determine the relative competitiveness of the strains when cultured together in liquid medium. Starting inoculants, paired as in Table 3, were prepared to give an initial population for each strain of 10^6 /ml in VS medium. These cultures were

Table 4. Numbers of viable bacteria of the different strains isolated from mixed nodules

Inoculum strains (A + B)	Nodule code	No. bacteria/nodule			Frequency of non-parentals/ R ⁻ parent
		Parental		Non-parental	
		A	B		
587 + 726	1	8.4 × 10 ³	3.3 × 10 ³	0	—
	2	1.3 × 10 ⁵	7.6 × 10 ⁴	6.4 × 10 ³	8.4 × 10 ⁻²
	3	2.5 × 10 ⁵	3.5 × 10 ⁴	3.4 × 10 ³	9.7 × 10 ⁻³
	4	1.2 × 10 ⁴	4.1 × 10 ²	0	—
	5	5.9 × 10 ⁵	2.0 × 10 ²	0	—
	6	3.8 × 10 ⁵	1.6 × 10 ³	0	—
	7	3.4 × 10 ⁴	2.9 × 10 ⁴	0	—
	8	5.6 × 10 ³	4.0 × 10 ²	0	—
	9	2.6 × 10 ⁴	2.2 × 10 ³	2.0 × 10 ²	9.1 × 10 ⁻²
	10	1.1 × 10 ⁶	4.1 × 10 ²	0	—
	11	1.2 × 10 ⁵	1.3 × 10 ³	3.1 × 10 ²	2.5 × 10 ⁻¹
	12	2.1 × 10 ⁴	3.1 × 10 ³	0	—
587 + 729	13	8.6 × 10 ⁴	1.0 × 10 ³	0	—
	14	5.1 × 10 ⁶	1.1 × 10 ⁶	0	—
	15	5.1 × 10 ³	3.0 × 10 ³	0	—
	16	1.0 × 10 ⁴	4.0 × 10 ²	0	—
726 + 729	17	8.1 × 10 ⁴	4.3 × 10 ²	0	—
	18	1.6 × 10 ³	1.2 × 10 ⁶	0	—
	19	1.9 × 10 ⁴	4.7 × 10 ²	0	—
	20	2.7 × 10 ³	2.7 × 10 ⁵	0	—
	21	1.0 × 10 ³	6.8 × 10 ³	0	—

shaken on an orbital shaker at 28 °C, and samples were taken at intervals, diluted and plated on appropriate selective media. In these conditions the growth rates of both strains in all the combinations were essentially similar. The conditions of competition in such an experiment are of course quite different from those in the rhizosphere and within the nodule. The fact that there were large selective differences between the strains for nodulation suggests that either competition in the rhizosphere is different, or that the plant exerts a selection on the infection pattern.

Relative numbers of each strain within nodules with dual infections. The nodule macerates from plants inoculated with strains 587, and 726 to 729 were stored at -20 °C. The macerates from the mixed nodules were melted, diluted, and plated on the appropriate selective media and the resultant colonies counted. In addition, a portion from each nodule was plated on media which selected against both parent strains so that only non-parental progeny could grow. Table 4 shows that there was a considerable variation both in the total number of bacteria within the nodules and in the proportions of each strain, even when the nodules were formed from the same inoculum of mixed strains. For example, in nodule 18 strain 729 was the most numerous whereas in nodule 17 it was strain 726. However, with one exception (nodule 3), in all the nodules containing strains 587 and 726 the former was in the majority. Strain 587 may thus be considered a superior competitor to 726 on the dual bases of forming more nodules containing only one strain than 726 and also of being the majority strain in those nodules containing both strains.

Transfer of genetic markers within the nodule. Table 4 shows that four of the nodules containing both strains 726 and 587 yielded progeny which were able to grow on TY medium supplemented jointly with rifampicin and kanamycin. Each of these colonies was replicated on to TY medium supplemented with tetracycline at 10 mg/l. All colonies were

resistant to this second antibiotic, thus demonstrating that these colonies carried the R factor RP4. The possibility that these non-parentals were in fact clones of strain 587 which had spontaneously acquired resistance to rifampicin cannot be ruled out, but is very unlikely. Nodules 2, 3, 9 and 11 were derived from four different plants. Therefore, the non-parental classes in these nodules could not have been members of the same mutant clone which established itself during growth of the bacteria in the rhizosphere or within the plant. Large numbers of rifampicin-resistant mutants were not present in the original inoculum, as it was examined and the frequency of rifampicin-resistant mutants shown to be 3.2×10^{-8} . The low spontaneous mutation rate of rifampicin resistance (2×10^{-8} to 5×10^{-8}) observed for a number of *R. leguminosarum* strains means that the chance of non-parentals arising as four independent mutations is very low. It is unlikely that rifampicin-resistant derivatives of strain 587 were more competitive in nodule formation, as no rifampicin-resistant bacteria were isolated from 19 nodules (representing 1.6×10^7 bacteria) excised from plants inoculated with strain 587 alone.

Beringer (1974) showed that RP4 could be transferred in culture between strains of *Rhizobium* at a maximum frequency of 1.4×10^{-3} /recipient. This figure is considerably exceeded in the plasmid frequencies shown in Table 4. Of course these figures do not represent the real frequencies of RP4 transfer between strains 587 and 726 since there may have been differential selection pressure on the genotypes after transfer. If the R⁺ rifampicin-resistant progeny were at a selective advantage compared with either strain 587 or 726, then the calculated frequency of transfer would be over-estimated.

The results do not tell where the transfer of the R factor occurred. However, the fact that the non-parental progeny were detected only in nodules containing both 587 and 726 suggests that the transfer of RP4 occurred within the nodule itself. This finding also provided further evidence against spontaneous mutation to rifampicin resistance by strain 587, since there is no *a priori* reason to suppose that such mutations would arise preferentially in nodules containing both strains 587 and 726.

Two of the nodules containing non-parental progeny (nodules 2 and 3) contained relatively high numbers of both parental strains. However, nodules 9 and 11 did not have such a high population. In fact, nodule 7 had greater numbers of both parents than nodule 9 but no non-parentals were isolated from the former. Optimal levels of RP4 transfer between *R. leguminosarum* in liquid medium are lower than those observed within nodule tissue. This implies that a more intimate packing of bacteria within the nodule may favour more successful pair formation for gene transfer. Recent crosses between *R. leguminosarum* strains impinging on to Millipore membranes, resulting in a frequency of transfer of RP4 of up to 10 %, are compatible with this observation.

In general, the nodules obtained after mixed inoculation with strains 587 plus 829 contained fewer 'viable' bacteria than did those obtained after infection with 587 plus 726. However, nodule 14 had a dense population of both genotypes yet no non-parentals were isolated. Whether their absence was due to sampling or whether there was a genuinely lower transfer of RP4 from strain 687 to 729 than to 726 is unknown. Possibly the introduction of RP4 into the streptomycin-resistant strain 729 renders it less competitive than the parents, leading to selection against the non-parental progeny.

The absence of any non-parental progeny in the nodules containing strain 726 plus 729 was not surprising since Beringer (1974) found no evidence for recombination between chromosomal markers in conjugation experiments with *R. leguminosarum*; although not rigorously tested, the *rif-r* and *str-r* markers of strains 726 and 729 respectively were presumed to be chromosomal.

Experiments with the non-infective strain 733

Strain 733 requires leucine, isoleucine and valine (*liv*) for growth and is streptomycin resistant. It was found to be non-infective. The possibility that an effective strain could act as a helper for 733 in infection was investigated.

Peas were inoculated with equal numbers of strain 733 plus 587 or 733 plus 726. The nodules from these peas were excised, surface sterilized, macerated, and spread on selective plates of TY medium supplemented with streptomycin, rifampicin or kanamycin to detect strains 733, 726 and 587, respectively. Of 48 nodules examined (23 representing 733+726, and 25 representing 733+587), none yielded any streptomycin-resistant progeny though the other strain was always isolated. This shows that the defect in 733 responsible for its lack of infection was not remedied by the presence of either of the two infective strains. It is not known where the symbiosis with strain 733 breaks down or the causes of this. Nor do we know whether the *liv*⁻ mutation alone is responsible since the effectiveness of *liv*⁺ revertants has not been tested.

DISCUSSION

The results demonstrate the usefulness of genetically marked strains of *Rhizobium* in studies of competition between strains in nodule formation. As Skrdleta (1970) showed for soybean and Pinto *et al.* (1974) for *Medicago* and clover, a substantial minority of pea root nodules may contain more than one strain of *Rhizobium*. In some cases the frequency of one of the strains was less than 0.1 % of the total number of viable bacteria isolated from the nodule, yet this strain was readily detected. Using standard serological techniques, such low frequencies might well remain undetected and the nodule be classified as singly infected. However, the fluorescent antibody technique of van der Merwe, Strijdom & Jansen van Rensburg (1972) has the potential to detect low frequencies of serologically-distinct bacteria within the nodule.

Electron micrographs of young root nodules of *Medicago sativa* (Jordan, Grinyer & Coulter, 1963) showed clearly that several bacteria could be released from the tip of an infection thread. The data obtained from the mixed infection experiments do not indicate whether there is a mixture of bacterial strains within a single infection thread or whether two separate threads, each containing a single genotype, are involved. In any event, it is apparent that if nodules are initiated by the progeny of a single bacterium there is no exclusion mechanism against subsequent invasion by other bacteria.

A large number of different mutations in *Rhizobium*, for example to bacteriophage, anti-metabolite or antibiotic resistance and to certain metabolic requirements, are associated with a loss of infectivity or effectiveness (Dénarié *et al.* 1975). In no case is there an explanation of the means by which such mutants lose their symbiotic proficiency. The existence of mixed nodules may allow some of the ineffective mutants to be classified, in future, by complementation tests. In the present study, failure of the non-infective strain 733 to be 'rescued' by 726 or 587 could be due to a structural defect in 733 rather than a metabolic defect, which might have been expected to be simply repairable. It remains to be seen whether mixed infection of legumes by certain combinations of two different ineffective strains results in the formation of mixed nodules able to fix nitrogen. If so, this could provide a useful tool for the classification of such mutants and for indirect assessment of their defects. For example, complementation in terms of cross-feeding of some diffusible product from one strain to another within the nodule is more conceivable than the interaction of altered structural components.

Although the nodules on a pea may contain two strains of free-living bacteria, it should be noted that it is not possible to deduce from a study of this type whether the bacteroids within the nodule can also comprise more than one strain, since bacteroids cannot be cultured and thus genotypes cannot be determined by plating procedures.

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