Contribution of the Symbiotic Plasmid to the Competitiveness of *Rhizobium leguminosarum*

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Four different symbiotic plasmids from *Rhizobium leguminosarum* were introduced into three different recipient strains that lacked plasmid-linked symbiotic determinants. The twelve synthetic strains so constructed were each tested for competitiveness against a standard reference strain. The recipient strain and the introduced symbiotic plasmid contributed about equally to competitiveness in forming root nodules on pea plants: there was also significant interaction between strain and plasmid, although this was much less important than the main effects. Competitiveness for growth on the legume root surface (the rhizosphere) was attributable entirely to the recipient strain; the introduced plasmid had no significant effect.

INTRODUCTION

Any attempts to improve *Rhizobium* strains for use as inoculants must allow for the fact that indigenous soil rhizobia are often more competitive than an inoculant strain and hence come to occupy the majority of root nodules induced on the legume host (Franc0 & Vincent, 1976; Date & Brockwell, 1978; Jones & Morley, 1981). It is therefore just as important to improve the competitiveness of *Rhizobium* strains as it is to increase the overall rate or efficiency of nitrogen fixation (van Rensburg & Strijdom, 1982; Amarger, 1982).

Recent studies have shown that for fast-growing rhizobia, such as *R. leguminosarum, R. trifolii, R. phaseoli* and *R. meliloti*, many of the determinants for biological nitrogen fixation are located in each strain on a single plasmid (the *sym* plasmid) (Nuti et al., 1979; Hombrecher et al., 1981; Banfalvi et al., 1981; Rosenberg et al., 1981; Hooykaas et al., 1982). Different *sym* plasmids from a single species of *Rhizobium* vary in respect to their symbiotic determinants (Brewin et al., 1983). Moreover, because *sym* plasmids can be transferred by conjugation from strain to strain (Johnston et al., 1978; Brewin et al., 1980a, b, c; Kondorosi et al., 1982), opportunities exist for genetic reassortment in *Rhizobium* by the transfer of entire plasmids between strains (DeJong et al., 1982).

In addition to genes for biological nitrogen fixation, the *sym* plasmids also carry determinants for root hair infection and the induction of legume root nodules: differences in the expression of these determinants on different *sym* plasmids might therefore affect the ability of different *Rhizobium* strains to grow on the surface of legume roots and to initiate root nodules.

In order to examine the contribution of the symbiotic plasmid to the competitiveness of a *Rhizobium* strain, four different symbiotic plasmids were introduced individually into each of three non-nodulating (Nod⁻) recipient strains that were resistant to streptomycin and lacked all the plasmid-borne symbiotic determinants. The competitiveness of each of these 12 Nod⁺ derivatives was then tested against a standard reference strain (726) which was resistant to rifampicin (Johnston & Beringer, 1975). Starting with a mixed inoculum containing equal proportions of streptomycin-resistant and rifampicin-resistant bacteria, competitiveness was evaluated by measuring the relative proportion of streptomycin-resistant bacteria recovered.
from within surface-sterilized nodules, and also from the surface of nodulated roots (the rhizosphere population). In this way the contributions of sym plasmid and genetic background to the overall level of competitiveness could be assessed.

**METHODS**

**Strain construction.** The four symbiotic plasmids are listed in Table 1. All carry transposon Tn5, determining resistance to kanamycin. The three nod- strains used as recipients for these sym plasmids are also listed in Table 1: strains B151 and 8401 entirely lacked their original symbiotic plasmids, whereas 3617 contained pJ1000, a derivative of the sym plasmid pRL101J with a 60 kb deletion that has removed all known symbiotic determinants (Ma et al., 1982). Transfer of each sym plasmid into each recipient was achieved by conjugal matings (Beringer, 1974) from donor strains that were derivatives of R. leguminosarum 300. Lysates of these strains were examined by agarose gel electrophoresis (Hirsch et al., 1980) to confirm that they contained a new plasmid of the expected size.

**Growth of peas.** Pea seeds (var. Wisconsin Perfection) were surface-sterilized in sodium hypochlorite (5%, w/v) for 15 min and allowed to germinate for 7 d on agar containing tryptone yeast-extract (TY) medium. After incubation at 28°C for 2-3 d, cultures were washed off with 5 ml sterile water and the suspensions were adjusted to a uniform optical density at 600 nm by addition of extra water to the denser cultures. Cultures of streptomycin-resistant and rifampicin-resistant bacteria were then mixed in equal proportion, and each mixture was used to inoculate 10 pea plants (0.5 ml samples, 5 x 108 bacteria per plant). In addition, other peas were inoculated with each culture singly at the same concentration, as a control for culture purity.

**Recovery of rhizobia.** The proportions of rifampicin-resistant and streptomycin-resistant bacteria were ascertained as follows for the initial inoculum, the rhizosphere population and the root nodule bacteria. Each mixture used for inoculation was diluted to 10^-6 and samples (0.1 ml) of this dilution were then plated on five TY plates containing streptomycin (200 μg ml⁻¹) and five TY plates containing rifampicin (20 μg ml⁻¹). Rhizosphere bacteria were recovered from five nodulated roots for each treatment, harvested 21 d after inoculation: each root system was washed by vortexing with 10 ml saline solution (0.85%, w/v, NaCl), diluted to 10^-4 and 0.1 ml samples were plated on TY plates containing streptomycin or rifampicin. In order to analyse nodule occupancy 21 d after inoculation, whole root systems from each of 10 plants were surface-sterilized by being shaken for 45 s with sodium hypochlorite (5%, w/v, available chlorine) rinsed twice with sterile water, and transferred to a sterile glass plate: individual nodules (20 per plant) were stabbed with a sterile toothpick, and the contents were transferred to a master-grid on TY agar. Each stab on the master-grid therefore represented the contents of a single nodule. After incubation for 2 d at 28°C, this was replica-plated to selective plates containing streptomycin or rifampicin. A proportion of the nodules (20-50%) yielded no ex-nodule bacteria because of the intensity of the surface sterilization treatment.

Sample clones from the inoculant cultures and from ex-nodule bacteria were checked for antibiotic resistance markers and plasmid content on agarose gels.

### Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Rhizobium strains</th>
<th>Derivation</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>726</td>
<td>R. leguminosarum 300 rif</td>
<td>Johnston &amp; Beringer (1975)</td>
</tr>
<tr>
<td>3617</td>
<td>R. leguminosarum 300 str nod-6007 (phe' trp' revertant of strain 6007)</td>
<td>Brewin et al. (1980a)</td>
</tr>
<tr>
<td>8401</td>
<td>R. phaseoli 8002 str, cured of pRL2J1</td>
<td>Lamb et al. (1982)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>R. leguminosarum sym plasmids</th>
<th>Size (kb)</th>
<th>Strain of origin of sym determinants</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJ1008</td>
<td>300</td>
<td>128C53</td>
<td>Brewin et al. (1982)</td>
</tr>
<tr>
<td>pJB5J1</td>
<td>200</td>
<td>248</td>
<td>Johnston et al. (1978)</td>
</tr>
<tr>
<td>pJ1019</td>
<td>240</td>
<td>300</td>
<td>Brewin et al. (1982)</td>
</tr>
<tr>
<td>pJ1016*</td>
<td>250</td>
<td>TOM</td>
<td>Brewin et al. (1980b)</td>
</tr>
</tbody>
</table>

* pJ1016 (pRL5J1::Tn5) was obtained after random transposon mutagenesis of a strain containing pRL5J1 following the procedure of Beringer et al. (1978) and Johnston et al. (1978). This plasmid, but none of the other three, confers the ability to nodulate pea cv. Afghanistan (Brewin et al., 1980b).
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Table 2. Percentage of each synthetic strain (streptomycin-resistant) present in mixtures with the standard strain 726 (rifampicin-resistant) in the initial inoculum, in the final root surface population and in the contents of individual nodules

<table>
<thead>
<tr>
<th>Synthetic strain</th>
<th>Initial inoculum</th>
<th>Final rhizosphere</th>
<th>Final nodules*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Background</td>
<td>Mean</td>
<td>S.E.M.</td>
</tr>
<tr>
<td>B151</td>
<td>pIJ1008</td>
<td>54.9</td>
<td>3.7</td>
</tr>
<tr>
<td>B151</td>
<td>pJB5</td>
<td>37.4</td>
<td>2.2</td>
</tr>
<tr>
<td>B151</td>
<td>pIJ1019</td>
<td>52.7</td>
<td>3.7</td>
</tr>
<tr>
<td>B151</td>
<td>pIJ1016</td>
<td>52.9</td>
<td>1.8</td>
</tr>
<tr>
<td>3617</td>
<td>pIJ1008</td>
<td>49.8</td>
<td>1.2</td>
</tr>
<tr>
<td>3617</td>
<td>pJB5</td>
<td>56.1</td>
<td>2.2</td>
</tr>
<tr>
<td>3617</td>
<td>pIJ1019</td>
<td>48.2</td>
<td>3.0</td>
</tr>
<tr>
<td>3617</td>
<td>pIJ1016</td>
<td>47.4</td>
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<tr>
<td>8401</td>
<td>pJB5</td>
<td>59.3</td>
<td>2.2</td>
</tr>
<tr>
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<td>pIJ1019</td>
<td>56.6</td>
<td>2.6</td>
</tr>
<tr>
<td>8401</td>
<td>pIJ1016</td>
<td>59.4</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* The minority of mixed nodules (containing both Str-r and Rif-r bacteria) have not been included in this analysis.
† n, number of replicates.

Table 3. Analyses of variance for percentage of streptomycin-resistant strain (after angular transformation)

The angular transformation is given by arcsin Jpercent/100 (in radians). DF, degrees of freedom; MS, mean square value.

<table>
<thead>
<tr>
<th>Source</th>
<th>Inoculum</th>
<th>Rhizosphere</th>
<th>Nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>MS</td>
<td>DF</td>
</tr>
<tr>
<td>Plasmid</td>
<td>3</td>
<td>0.0014</td>
<td>3</td>
</tr>
<tr>
<td>Strain</td>
<td>2</td>
<td>0.0308***</td>
<td>2</td>
</tr>
<tr>
<td>Interaction</td>
<td>6</td>
<td>0.0229***</td>
<td>6</td>
</tr>
<tr>
<td>Error</td>
<td>47</td>
<td>0.0028</td>
<td>48</td>
</tr>
</tbody>
</table>

*** Highly significant effects (P < 0.0005).

RESULTS

Table 2 shows the percentage of each synthetic strain (streptomycin-resistant) in the inoculant mixtures, and after competition with the standard strain, 726 (rifampicin-resistant). An analysis of variance of these data is in Table 3. Although an attempt was made to adjust the proportions of each strain to a 50:50 ratio in the inocula, this was not entirely successful: there was still significant variation amongst the inocula (Table 2). It is possible that these variations reflected differences between strains with respect to the relationship between optical density and the number of viable bacteria in cell suspension culture. However, variations in ratios between the inocula were small compared to those after competition (see the smaller mean square values in Table 3). This point was confirmed by an analysis (not reported) in which final values were corrected for differences in inoculum value: this did not alter the conclusions.

Competitiveness in the rhizosphere was attributable entirely to the host strain: the nature of the sym plasmid had no significant effect (Tables 2 and 3). Derivatives of 8401 were the most competitive, and derivatives of B151 the least. Differences in strain and in plasmid contributed about equally to the variation in nodulation competitiveness as judged by the similar mean square values in Table 3. There was also significant interaction between strain and plasmid, although this was much less important than the main effects (Table 3).
In some cases the frequency of Rif-r in nodules was extremely low, and the variance in frequency was also low as a consequence. This meant that the variances were not homogeneous overall, and hence the assumptions for analysis of variance were not strictly met in the nodule analysis. However, the large magnitude of the observed effects ensures that the conclusions are not seriously affected by this.

**DISCUSSION**

In this experiment, we have tried to analyse two different components of competitiveness, namely the ability of one strain to grow faster than another on the surface of legume roots, i.e. rhizosphere competitiveness, and the ability of one strain to induce more root nodules than another, i.e. nodulation competitiveness. We believe that these two aspects of competitiveness can be analysed independently because the pea seedlings used were already 14 d old at the time of inoculation and would have initiated infection threads leading to nodule development very shortly after the application of inoculum. The proportions of bacteria in the rhizosphere were analysed after growth for a further period of 21 d on the surface of pea roots.

The results show that both the *sym* plasmid and the background genotype can exert a major influence on competition for nodulation. The background genotype is determined by the chromosome and by any resident plasmids other than the *sym* plasmid. This distribution of effects between the *sym* plasmid and elsewhere was also observed in the effects of plasmid content on nitrogen fixation efficiency (DeJong et al., 1981, 1982). It is also consistent with the observation that, for randomly induced symbiotically-defective mutations, about half were complemented by wild-type *sym* plasmid determinants (Brewin et al., 1980a; Forrai et al., 1983).

Competitiveness for survival and growth in the rhizosphere was due entirely to *Rhizobium* strain and not to the introduced plasmid. Strain 8401, which in this study proved to be the most competitive both for nodule initiation and rhizosphere growth, is a cured strain derived from *R. phaseoli* and not *R. leguminosarum* (although the introduction of a *sym* plasmid from *R. leguminosarum* converted 8401 into a strain that was indistinguishable from *R. leguminosarum* itself).

This study was concerned with the influence of components of the *Rhizobium* genotype on the competition with one particular reference strain (726). The effects of host plant genotype on this interaction were not examined. There is evidence that host genotype may influence the outcome of competition for nodulation between strains of *Rhizobium* (May & Bohlool, 1983), so it is possible that our results would have differed, in detail at least, if a different pea variety had been chosen as host.

In conclusion, it is reasonable to expect that conjugal transfer of *sym* plasmids between *Rhizobium* strains could result in new plasmid/strain combinations with enhanced competitiveness as well as improved performance in symbiosis (DeJong et al., 1981, 1982). Moreover, plasmid and strain effects appeared to be largely independent in this study; if this finding proves to be general, each component could perhaps be selected separately for improved performance, without the need to evaluate each plasmid in every background.

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