Fractionation and Estimation of Particle-Attached and Unattached *Bradyrhizobium japonicum* Strains in Soils

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Rhizobial cells attached or unattached to soil particles were estimated. Nonsterile soils into which antibiotic-resistant mutants of *Bradyrhizobium japonicum* had been introduced were fractionated by a centrifugation technique into two fractions: A, which contained mainly rhizobial cells attached to soil particles, and F, which contained mainly rhizobial cells unattached to them. Rhizobial counts decreased in both fractions during incubation of the soil at 30°C, with a concomitant decrease in the proportion of the count of fraction F to that of fraction A. Sonication of fraction A of the soil incubated for more than 3 weeks caused an increase in the rhizobial count. The ratio of the count of fraction A estimated by the plant infection method to that estimated by the dilution plating method increased after 5 days of soil incubation. More than 90% of the indigenous rhizobia in an agricultural field existed in fraction A. These results suggest that the majority of rhizobial cells are attached to soil particles.

The soil was collected from the 0- to 15-cm horizon of an agricultural field at the University of Osaka Prefecture, Sakai, Japan. The soil was air dried and passed through a 1-mm sieve. The soil texture was silty clay loam. The soil contained 1.67% C and 0.13% N and had a pH of 6.8. Soil sterilization, when needed, was performed by autoclaving subsamples for three periods of 1 h each on three consecutive days.

The antibiotic-resistant strains in the early stationary phase in YEM broth were collected and washed three times with distilled water. The cell suspension was added by a pipette to 10 g (dry weight) of soil with a depth of 2 cm in a 40-ml vial. The moisture content of the soil was adjusted to 30% on a dry weight basis. The vial was capped with aluminum foil and incubated at 30°C in the dark. The loss of water from the soil during the 7 weeks of the incubation was about 15% of the initial amount.

Soil (10 g [dry weight]) containing the introduced rhizobia was suspended in 90 ml of distilled water in a 100-ml Erlenmeyer flask. The soil suspension was shaken by hand for 1 min and then left for 1 min. The upper part of the suspension was collected by decantation and centrifuged for 10 min at 600 × g. The coarse particles which settled down after the shaking procedure and before the centrifugation of the soil suspension were discarded. *B. japonicum* cells in YEM broth were not precipitated by the low-speed centrifugation. The supernatant from the centrifugation was designated as fraction F. A soil particle in this study is defined as an inanimate particle which is precipitated by this low-speed centrifugation. Preliminary observation with a microscope showed that most of the particles in fraction F were smaller than 5 μm. The sediment from the centrifugation was suspended in 100 ml of distilled water. Designated as fraction A, it contained the rhizobial cells which were attached to the soil particles and coprecipitated with them. The sonication (19.5 kHz, 20 W, 1 min) of fraction A was carried out to disperse the cells on the soil particles.

Bacterial counts for each fraction were carried out on YEM agar plates containing the antibiotics. The plates were incubated at 30°C for 10 days before the bacterial counts. Under 1% of the colonies on the plates had no reaction with fluorescent antibodies prepared against the strains (14). The

Materials and Methods

*B. japonicum* USDA 24, USDA 122, USDA 123, USDA 138, and USDA 143 were obtained from the U.S. Department of Agriculture, Beltsville, Md. Mutants occurring spontaneously which were resistant to both 100 μg of nalidixic acid per ml and 100 μg of rifampin per ml or to both 100 μg of erythromycin per ml and 50 μg of tetracycline per ml were obtained from these stock cultures. The parent strains and the mutants were maintained on yeast extract mannitol (YEM) agar medium (15).

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TABLE 1. Viable counts of *B. japonicum* strains in soil fractions A and F

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incubation time (days)</th>
<th>No. of rhizobia/g of dry soil for fraction</th>
<th>F/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA 24 (Nal\textsuperscript{r} Rif\textsuperscript{r})</td>
<td>0</td>
<td>4.0 \times 10\textsuperscript{8}</td>
<td>3.5 \times 10\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>4.2 \times 10\textsuperscript{8}</td>
<td>2.7 \times 10\textsuperscript{8}</td>
</tr>
<tr>
<td>USDA 122 (Ery\textsuperscript{r} Tet\textsuperscript{r})</td>
<td>0</td>
<td>8.0 \times 10\textsuperscript{8}</td>
<td>9.0 \times 10\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.2 \times 10\textsuperscript{9}</td>
<td>3.5 \times 10\textsuperscript{9}</td>
</tr>
<tr>
<td>USDA 123 (Ery\textsuperscript{r} Tet\textsuperscript{r})</td>
<td>0</td>
<td>1.2 \times 10\textsuperscript{8}</td>
<td>2.0 \times 10\textsuperscript{8}</td>
</tr>
<tr>
<td>USDA 138 (Nal\textsuperscript{r} Rif\textsuperscript{r})</td>
<td>0</td>
<td>9.0 \times 10\textsuperscript{7}</td>
<td>1.8 \times 10\textsuperscript{7}</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.6 \times 10\textsuperscript{7}</td>
<td>9.0 \times 10\textsuperscript{7}</td>
</tr>
<tr>
<td>USDA 143 (Nal\textsuperscript{r} Rif\textsuperscript{r})</td>
<td>0</td>
<td>7.2 \times 10\textsuperscript{10}</td>
<td>2.6 \times 10\textsuperscript{10}</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2.5 \times 10\textsuperscript{9}</td>
<td>1.7 \times 10\textsuperscript{9}</td>
</tr>
</tbody>
</table>

\(a\) Each value is the mean of five replicates. Values followed by the same letter are not significantly different at the 5% level as determined by Student’s *t* test.

soil contained (per gram [dry weight]) \(10^2\) to \(10^3\) indigenous bacteria which formed colonies on YEM agar containing the antibiotics.

The rhizobial population in each fraction was also estimated by the plant infection method (4). A 2-day-old soybean seedling (*Glycine max* cv. Tamahomare), aseptically germinated on vermiculite in a test tube (2 by 20 cm), was inoculated with 1 ml of a 10-fold dilution series of each fraction. Five plants were inoculated with each dilution. The plants were grown for 3 weeks after inoculation as described elsewhere (19) and then were examined for the presence of nodules. The most probable number of rhizobia in each fraction was calculated from the proportion of plants forming nodules at each dilution level.

**RESULTS**

All of the strains used in this study occurred in both fractions A and F, and all except strains USDA 122 (Ery\textsuperscript{r} Tet\textsuperscript{r}) and USDA 138 (Nal\textsuperscript{r} Rif\textsuperscript{r}) at day 0 had higher counts in fraction A than in fraction F (Table 1). The existence of the rhizobia in both fractions was confirmed in 14 other soils obtained from different regions of the Osaka Prefecture (data not shown). *B. japonicum* USDA 138 (Nal\textsuperscript{r} Rif\textsuperscript{r}) was used for the following experiments. The rhizobial counts of fractions A (count A) and F (count F) decreased during 7 weeks of soil incubation (Fig. 1). The rate of decline in count F was larger than that in count A, so that the ratio of count F to count A (F/A ratio) decreased with incubation of the soil. Rhizobial cells in a number of soils which differed from one another in inoculum size and incubation time were estimated (Fig. 2). The F/A ratio of a soil depended upon the rhizobial population size, not the incubation time, of the soil. The soil in which the sum of count A and count F was under \(10^6\) cells per g of dry soil revealed nearly 100 times as much count A as count F. The F/A ratio was above 0.1 in the soil with the sum of the counts at \(10^7\) or above. The result implied that the rhizobia saturated soil-adsorbing surfaces, with an increase in fraction A.

About 5% of the rhizobia of fraction A were counted in the supernatant from the low-speed recentrifugation of fraction A. Observation with a microscope revealed the existence of unattached bacteria in fraction A. These suggested detachment of a part of the rhizobia from soil particles. Sonication techniques have previously been used to detach bacteria from soil particles (20, 21). Sonication of fraction A of the soil incubated for more than 3 weeks caused an increase in the rhizobial count (Fig. 3). This suggested that with increasing incubation there is an increasing chance that a colony arises from more than one cell on a soil particle.

The assumption that a colony on the YEM agar plate of fraction A originates from plural cells may be supported by a comparison of rhizobial numbers estimated by two different methods: the dilution plate method and the plant

**FIG. 1.** Comparison of counts of *B. japonicum* USDA 138 (Nal\textsuperscript{r} Rif\textsuperscript{r}) in fractions A (○) and F (●). Δ, F/A ratios. Cells (2 \(\times\) 10\textsuperscript{8}) were added to 10 g (dry weight) of soil. Each point is the mean of five replicates. Bacterial counts at the same incubation time, except at 7 days, were significantly different at the 5% level.

**FIG. 2.** Relationship between total count and F/A ratio. F + A, total count for the two fractions. The populations of *B. japonicum* USDA 138 (Nal\textsuperscript{r} Rif\textsuperscript{r}) in the soil fractions with different inoculum sizes (10\textsuperscript{6} to 10\textsuperscript{9} cells per g of dry soil) were estimated at different incubation times (0 to 49 days).
infection method. The more rhizobia an inoculum contains means that there is a higher probability of nodule formation. Therefore, the ratio of the estimate by the plant infection method to that by the dilution plate method (P/D ratio) may depend upon the number of rhizobia per CFU. B. japonicum USDA 138 (Nal\(^+\) Rif\(^+\)) (10\(^5\) cells per g of dry soil) was introduced into sterilized soil. The soil was fractioned as above after incubation at 30°C. The rhizobial cells in the two fractions were estimated simultaneously by the two methods. Both rhizobial counts of the two fractions, when estimated by the dilution plate method, increased to 10\(^8\) cells per g of dry soil within 1 week of the incubation and remained at this population level in the following incubation period. The P/D ratio in fraction F was constant during the incubation period (Fig. 4). The P/D ratio in fraction A, on the other hand, increased after 5 days of incubation.

The surface (0 to 15 cm deep) soil of the agricultural field not inoculated with B. japonicum was sampled just after the soybean harvest in October. The soil was fractionated by the same method as above, and indigenous rhizobia in fractions A and F were estimated by the plant infection method (Table 2). The rhizobial estimate in fraction A was about 10 times as large as that in fraction F. Sonication of fraction A caused an increase in the rhizobial estimate.

**DISCUSSION**

Although we used a somewhat imprecise manual shaking procedure, the reproducibility of the fractionation method in this study was sufficiently high. However, some problems may arise when we infer the location of rhizobial cells in situ from the results obtained here. (i) It is not known how well cell location in the soil suspension relates to that in situ. Fraction F may contain the cells which are dislodged from large particles by the shaking procedure, in addition to the cells which are free and not associated with soil particles in situ. (ii) Soil particles are defined in this study as the particles which have not settled down after the soil suspension has been allowed to stand for 1 min and has been precipitated by low-speed centrifugation. Fraction F seems to contain small clay particles which are not precipitated by centrifugation. It is not known how many rhizobia are associated with these small clay particles. In addition, there may be cells on large particles or in soil aggregates which are settled and discarded during the fractionation.

Microscopy studies have revealed colonies of soil bacteria on particles (10). Nioh and Furusaka have tried to fractionate soil bacteria into attached and unattached populations by a centrifugation technique (17). The previous investigations on the survival of rhizobia introduced into soils have not fully been taken into consideration the rhizobial attachment to the soil particles. We demonstrated in this study that B. japonicum cells introduced into soil were attached to the soil particles in the suspension. The degree of attachment, which was expressed by the F/A ratio, varied with incubation of the soil.

Each colony appearing on an agar plate does not necessarily originate from one cell. The data in the present study make it clear that a lot of cell clumping occurs and that the extent of this varies between the two fractions and with incubation time. Cell attachment to the soil particles may cause the clumping. As it is not known how many cells make up a CFU, the population size of rhizobial cells cannot be determined exactly by the dilution plate method.

Attachment of a rhizobial cell to a soil particle may affect

**FIG. 4.** Comparison of rhizobial numbers estimated by the plant infection and dilution plate methods. P/D represents the ratio of the rhizobial counts estimated by the plant infection and dilution plate methods. ●, fraction A; ○, fraction F.

**TABLE 2.** Populations of indigenous rhizobia in fractions A and F of an agricultural field soil as determined by the plant infection method

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>No. of rhizobia/g of dry soil for fraction*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.9 × 10(^3)</td>
<td>7.9 × 10(^2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2.2 × 10(^5))b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.4 × 10(^4)</td>
<td>1.3 × 10(^3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(7.0 × 10(^5))b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Each value is the mean of three replicates. Differences among the means are significant at the 5% level as determined by Student's t test.

*b Values in parentheses represent the estimates of fraction A sonicated before the estimation.

![FIG. 3. Effect of sonication on rhizobial count of fraction A. The relative count represents the ratio of the count after the sonication of fraction A to the count before the sonication. Cells (2 × 10\(^5\)) of B. japonicum USDA 138 (Nal\(^+\) Rif\(^+\)) were added to 10 g (dry weight) of soil.](image)
its survival in the soil. The findings in some previous studies suggested that protozoa are able to prey on the rhizobial cells introduced into soils \((9, 12)\). Protozoa seem to play a role in regulating the rhizobial population. The rhizobial count of fraction \(A\), which contains mainly particle-attached cells, did not decrease as rapidly as that of fraction \(F\) (Fig. 1). Danso and Alexander demonstrated that the existence of physical barriers in the form of glass microbeads depresses the predation by protozoa \((8)\). Adsorption on soil particles may influence the availability of bacterial cells as prey.

The effect of solid surfaces on bacterial metabolism has been examined with several species of bacteria \((23)\). Attachment on solid surfaces stimulated the glucose uptake by \textit{Pseudomonas} sp. \((11)\). Hattori et al. demonstrated the enhancement of the activity of \textit{Escherichia coli} by its adsorption to ion exchange resin \((13)\). Microbial metabolic activity can be related to the degree of bacterial adsorption. A change in the metabolic activity of a rhizobial cell would affect its survival.

The attachment of introduced rhizobia to a soil particle may influence their behavior, such as their movement in soils and competition with indigenous rhizobia for infection. Rhizobial cells unattached to soil particles may be able to move much more easily than the attached cells. The increase in the \(P/D\) ratio shown in Fig. 4 may indicate an increase in the nodulation ability of rhizobia in fraction \(A\), as well as an increase in the number of rhizobia in a CFU. We have previously reported, however, that in a strain population, the proportion of the rhizobial cells able to nodulate soybeans decreased with time after the introduction of rhizobia to soils \((18)\). Nodulation ability and the competitiveness of rhizobial cells in fractions \(A\) and \(F\) are now under study.

**LITERATURE CITED**


