APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1987, p. 2972–2974
0099-2240/87/122972-03$02.00/0

Vol. 53, No. 12

# Variability in Effectiveness of Rhizobia during Culture and in Nodules

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Received 20 April 1987/Accepted 3 September 1987

**The ability of three strains of *Bradyrhizobium* sp. (*Vigna*) to fix dinitrogen in symbiotic association with siratro (*Macropitilium atropurpureum*) was measured after culture in broth and after isolation from nodules. Seven transfers were made between the initial broth culture and the final broth culture. A total of 40 single-colony isolates were obtained from cultures 1 and 7 to test effectiveness. Variation in dinitrogen-fixing effectiveness of the population of one strain did not change on culturing, whereas there was considerable variation in effectiveness of populations of the other two strains. Generally, single-colony isolates from individual nodules had similar levels of effectiveness, but some exceptions occurred. Isolates from different nodules formed by the same *Bradyrhizobium* strain often differed in their effectiveness.**

*Rhizobium* and *Bradyrhizobium* spp. are of scientific and agronomic importance because of their ability to reduce dinitrogen (effectiveness) in root nodules of leguminous plants. During laboratory culture, spontaneous mutation of rhizobia may result in reduced ability to fix dinitrogen. Such mutants have been readily detected during selection for resistance to antibiotics (1, 11, 15). The medium used to culture rhizobia also influences the stability of genes for dinitrogen fixation and promotes selection for such mutants (12, 13). Subculturing *Bradyrhizobium* stock cultures on the typical yeast extract-mannitol-salts medium (17) used in most laboratories may also result in loss of effectiveness (6). Herridge and Roughley (6) tested 17 cultures of *Bradyrhizobium* sp. (*Vigna*) strain CB756 obtained from several stock culture collections. The cultures varied widely in their effectiveness, and some promoted less than half the plant dry-matter production promoted by the most effective cultures. Single-colony isolates from one of the most effective cultures showed wide variability in effectiveness of the subcultures. Nearly half the subcultures were less than half as effective as the most effective subcultures.

Variability in effectiveness of *Bradyrhizobium* sp. (*Vigna*) also is present in the population following inoculation into soil (2). Incubating one strain of *Bradyrhizobium* sp. in moist soil at 45°C for 15 days resulted in a large increase in variability of effectiveness among single-colony isolates. After the incubation period, 37% of the isolates were less than 60% as effective as the parent culture, whereas at the beginning of the incubation period, none of the isolates was so ineffective. Following the incubation period, 8% of the isolates were totally ineffective, but all could nodulate the plant.

Culturing *Bradyrhizobium* sp. for inoculant production is particularly challenging because of a generation time of several hours, the ease with which a faster-growing contaminating bacterium can overtake the culture, and the inherent danger that a large proportion of the population may lose dinitrogen fixation effectiveness. Single-colony isolates of bradyrhizobia belonging to the same strain vary considerably in generation time (15, 16). To reduce the time required for batch production of a culture, a stage-up process is used that provides for a relatively large initial inoculum that amounts to 0.1 to 1% of the final cell yield (3, 4, 10). The large inoculum reduces the lag phase of growth (3), the time required to reach adequate cell yields, and the danger of a few fast-growing organisms overtaking the culture.

We report here on the variability in effectiveness of three strains of *Bradyrhizobium* sp. (*Vigna*) following an initial broth culture and after seven serial transfers in broth. Variability in effectiveness was also demonstrated for isolates cultured from different nodules and between isolates from the same nodule.

Three strains of *Bradyrhizobium* sp. (*Vigna*) were used to determine the genetic stability of the dinitrogen fixation character of these bacteria after culture in broth. These strains were Thai-201 (obtained from Nantakorn Boonkerd, Ministry of Agriculture, Bangkok, Thailand), T-1 (from our collection), and TAL-201 (NifTAL culture collection, Paia, Hawaii). TAL-309 originally came from Sydney University, Sydney, New South Wales, Australia, where it was designated CB756. All strains formed effective nodules on cowpea (*Vigna unguiculata*), peanut (*Arachis hypogaea*), and siratro (*Macroptilium atropurpureum*).

The first experiment evaluated the effectiveness of bradyrhizobia isolated from an initial broth culture of the parent culture and from a culture developed following seven serial subcultures. The measure of effectiveness was the quantity of plant dry matter produced (17). The broth (YMB) and agar (YMA) media containing yeast extract, mannitol, and mineral salts have been described previously (17). The starter culture for broth 1 was from an agar slant of a *Bradyrhizobium* culture that was 10 days old. Cells were scraped from the slant, suspended in YMB, and added to 20 ml of YMB contained in 125-ml Erlenmeyer flasks stoppered with cotton plugs. The starter inoculum provided approximately $5 \times 10^6$ cells per ml of broth, an inoculum anticipated to provide approximately 1% of the final cell yield. Cultures were grown for 5 to 7 days (29°C at 100 rpm), by which time they had reached the early stationary phase of growth. Inoculum (0.2 ml) was removed and used to inoculate broth

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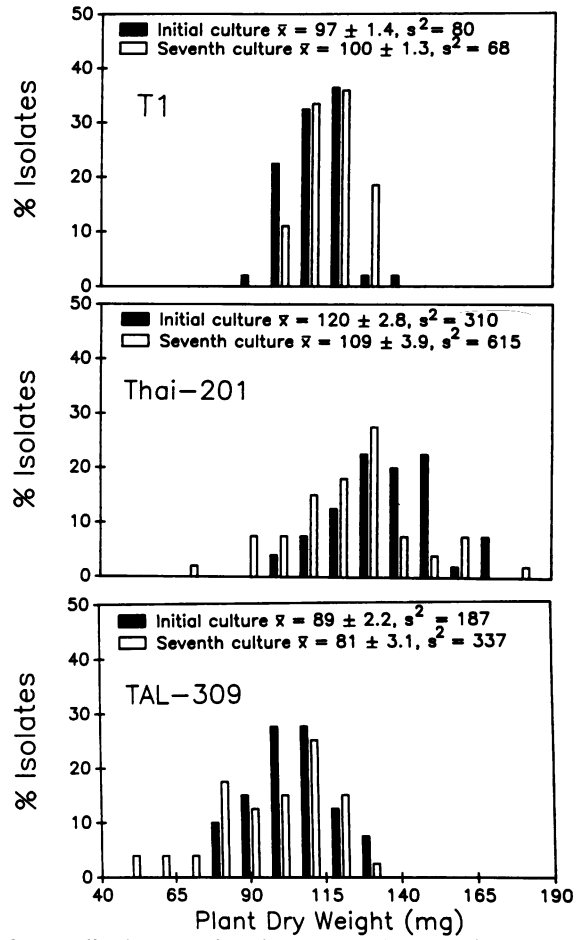


FIG. 1. Effectiveness of 40 single-colony isolates of three strains of bradyrhizobia from initial broth cultures and after seven serial subcultures into broth. Isolates were grouped according to dry-matter production of shoots of siratro.

culture 2. Samples (0.1 ml) were also removed, serially diluted in YMB containing 0.01% (vol/vol) Tween 40 (8), and spread plated on YMA for isolation of single colonies. After 1 week, 40 well-spaced colonies were isolated and subcultured on YMA slants. Broth culture 2 was grown to early stationary phase, and 0.2 ml of culture was used as a starter for broth 3. Culture 2 was then discarded. The same procedure was followed until broth culture 7 had been grown. When broth culture 7 was grown, a sample was removed for isolation of 40 colonies as was done with broth 1. Before plating for isolation, wet mounts of broth cultures were examined by phase microscopy. Cells were observed to be predominately single, with only rare clumps of two or three. Subcultures on slants were allowed to grow for 2 weeks, after which they were stored at 5°C.

To test the effectiveness of bradyrhizobia, siratro plants were grown in cone-tainers filled with sterilized vermiculite (5). Siratro was chosen as the test plant instead of peanut or cowpea because of its small size. Nutrient solution (17) was added to each cone-tainer with an automatic pipette. Solution in excess of that needed to saturate the vermiculite drained from the bottom of the cone-tainer. Plants were grown for 5 weeks under high-intensity fluorescent lights providing 650 microeinsteins/$m^2$ per s at the top of the plants for 14 h/day. The temperature was 31°C during the light period and 21°C during the dark period. Before being planted, seeds were surface sterilized by being soaked for 5 min in a dilute solution of household bleach (0.525 g of sodium hypochlorite per 100 ml of water) and rinsed 10 times in distilled water. Two seeds were planted in each cone-tainer, and seedlings were thinned to one per cone-tainer.

For inoculum preparation, subcultures of the sets of 40 isolates were made on to YMA slants and allowed to grow for 8 days at 28°C. The developed cultures were suspended in sterilized plant nutrient solution at a density of approximately $10^7$ cells per ml. Each isolate was used to inoculate three plants by slowly adding 1 ml of inoculum to each cone-tainer. Several cone-tainers did not receive inoculum; these plants served as uninoculated controls to test for cross-contamination.

Parent culture (Thai-201 and TAL-309) controls were six replicate plants inoculated with $10^7$ cells from a broth culture grown from a stock slant. Plants were grown for 5 weeks, and plant tops were then removed for dry-matter determination. Roots were examined for nodulation.

Two additional investigations were undertaken to determine the effectiveness of isolates from nodules formed on siratro plants inoculated with our stock culture of TAL-309. Five plants were inoculated with approximately $10^6$ cells which had been cultured in YMB, diluted in plant nutrient solution, and grown as previously described. Nodules were collected from all of the plants at 5 weeks after planting, and the nodules were combined. Ten nodules were chosen at random and used to obtain single-colony isolates. The nodules were surface sterilized with $HgCl_2$ (17) and macerated in YMB containing 0.01% (vol/vol) Tween 40. The suspension was streaked on YMA, and five isolated colonies from each nodule were subcultured. In addition, 20 single-colony isolates were obtained from each of two nodules and cultured on YMA slants. Inoculum was prepared and effectiveness tests were conducted with siratro as described above for the previous experiment. There were six replications (cone-tainers) per isolate. Controls were 24 replicate plants inoculated with $10^7$ cells of the parent culture of TAL-309 and 10 uninoculated plants. Uninoculated plants were dispersed among the treatments to check for cross-contamination. Plants were grown for 6 weeks and harvested, and the dry weight of the shoots was measured.

The effectiveness of 40 single-colony isolates from broth culture varied considerably for the three strains (Fig. 1). Isolates from subcultures 1 and 7 of T-1 were the least variable. The statistical measure of variability, the F test for the ratio of variances (14), indicated no significant difference (at the $P = 0.05$ level) between the two cultures. However, the mean effectiveness for culture 7 (F test, $P = 0.05$) was higher than for culture 1. Even though the difference was slight (3%), it indicated that the population was shifting to higher effectiveness with development of new generations. Populations of the other two strains were considerably more variable with respect to effectiveness than was T-1 (Fig. 1), and continued subculturing significantly increased variability and reduced the mean effectiveness of the populations by approximately 9%.

Parent culture controls of T-1 and TAL-309 produced plants with dry-matter contents in shoots of 101 (±4.5) and 111 (±6.5) mg, respectively. The quantity of dry matter produced by plants treated with the parent cultures was not significantly different from the mean of the population treated with T-1 (Fig. 1), but was significantly higher for plants treated with TAL-309 (Fig. 1). Strain TAL-309 (CB756) has a history of being a variable strain (6). These

TABLE 1. Ability of 20 single-colony isolates of *Bradyrhizobium* sp. (*Vigna*) strain TAL-309 from two siratro nodules to stimulate dry-matter production in shoots of siratro grown in a nitrogen-deficient medium

| Isolate | Dry wt (mg/plant)[a] | |
|---|---|---|
| | Nodule 1[b] | Nodule 2[c] |
| 1 | 193 | 273 |
| 2 | 181 | 259 |
| 3 | 177 | 253 |
| 4 | 171 | 250 |
| 5 | 155 | 245 |
| 6 | 155 | 244 |
| 7 | 154 | 244 |
| 8 | 153 | 242 |
| 9 | 150 | 239 |
| 10 | 148 | 237 |
| 11 | 148 | 232 |
| 12 | 143 | 232 |
| 13 | 143 | 228 |
| 14 | 135 | 227 |
| 15 | 128 | 225 |
| 16 | 125 | 222 |
| 17 | 118 | 220 |
| 18 | 114 | 215 |
| 19 | 112 | 193 |
| 20 | 111 | 185 |

[a] Each value is the mean of results for six plants.
[b] Least significant difference, 45; coefficient of variation, 28. Average dry weight was 145 mg. per plant.
[c] Least significant difference, not significant, coefficient of variation, 24. Average dry weight was 233 mg. per plant.

results confirm those of Boonkerd and Weaver (2), who found that plants treated with the parent culture tend to yield the same amount of or slightly more dry matter than a mean of the amount produced by plants treated with individual isolates.

The mean effectiveness of five isolates of TAL-309 from each of 10 nodules ranged from 122 to 241 mg of dry matter produced per plant. Isolates from half the nodules were considerably less effective than the most effective isolates, indicating that both highly effective and relatively ineffective bradyrhizobia were forming nodules on the plant (data not shown). Single-colony isolates from individual nodules were generally of similar effectiveness. However, there was one instance with strain TAL-309 of a statistically significant difference (at the $P = 0.05$ level) between isolates from a single nodule (data not shown).

To provide a better probability of detecting differences in the population of TAL-309 within individual nodules, 20 single-colony isolates were obtained from each of two nodules. Differences in effectiveness among the 20 isolates were apparent (Table 1), and there was a large difference in the mean effectiveness of isolates from the two nodules.

It is possible that the differences in effectiveness within nodules was due to mixed infection. However, given the low probability of having mixed infections at our inoculum density (9), it seems unlikely that this occurred. Also, when mixed infections occur, one strain is usually dominant (7). With the high degree of variability expressed by 40 isolates from the parent culture in the first experiment, it seems more probable that as a population developed in a nodule, some variability in effectiveness developed.

The results of our investigation indicate that populations of bradyrhizobia in cultures and in nodules may be highly variable with respect to the ability to promote dinitrogen fixation. It does not appear that continuous subculturing of bradyrhizobia results in large shifts in effectiveness. It may be convenient to use cells from a broth subculture to serve as a starter for another culture, and our results indicate that this may not be detrimental to production of a quality inoculant. Individual strains should be examined for stability of effectiveness before stability in serial subcultures is assumed.

This work was supported by United States Department of Agriculture-United States Agency for International Development BNF Limiting Factors Program grant 84-CRSR-2-2521 and Texas Agricultural Experiment Station project H-3701.

We thank Lisa Lucia and Linda Adair for technical assistance.

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