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# Viability of *Rhizobium* Bacteroids

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Bacteroids prepared from nodules of soybean and bean were tested for viability. Contrary to the prevailing view that bacteroids are nonviable, it was found that bacteroids averaged 90% viability, irrespective of *Rhizobium* strain, nodule age, or nodule environment.

*Rhizobium* bacteroids—the vast majority of the rhizobia occurring in mature, functional legume root nodules—are widely accepted to be incapable of cell division (2, 5, 6, 8). Support for the view that bacteroids are nonviable appears to stem from two papers. In the earlier of these, Almon (1) reported in 1933 that single bacteroids isolated by microtechniques from legume nodules reproduced rarely, if at all, in microslide culture systems. In 1968 Bergersen (3) investigated viability of washed soybean bacteroids by using direct microscopic counts and plate counts. He concluded that bacteroid viability was less than 0.02% as compared with 80 to 90% for cells in culture.

In preliminary experiments by fluorescent-antibody (FA) enumeration, we observed that populations of *Rhizobium japonicum* increased substantially in field soils during nodule senescence at the end of the soybean growing season and were led to a reexamination of the question of bacteroid viability. We now report that, contrary to the prevailing view, a large majority of bacteroids are viable.

Most experiments involved soybean (cultivar Chippewa) and strains USDA 31, 123, and 138 of the soybean symbiont *R. japonicum*. Late-log (6-day) shaken cultures grown in yeast extract-mannitol medium (4) were used either for plant inoculation or as the control in viability experiments. One experiment involved the bean cultivar Seafarer and *R. phaseoli* isolated from a Minnesota soil. Plants were grown in pots containing a local field plot soil partially sterilized by flowing steam for 1.5 h and inoculated with $2 \times 10^8$ cells/g just before planting the surface-sterilized seeds. In some cases plants were grown in the absence of fixed nitrogen in sand in modified Leonard jar assemblies (7) rather than in soil. Plants were grown in a plant growth chamber (Conviron, Controlled Environments, Pembria, N.D.) with a 16-h daylight cycle and day and night temperatures of 25 and 16°C, respectively.

Thirty nodules of uniform size were picked from five plants at each sampling time and then surface sterilized (7), washed 10 times with sterile water, and ground in 10 ml of sterile phosphate buffer (50 mM; pH 7.0) or in 10 ml of half-strength rhizobia growth medium with a manual glass tissue grinder and Teflon pestle. Coarse particles were removed by centrifugation at $500 \times g$ for 10 min. The supernatant fraction constituted a "crude bacteroid" preparation. Portions of the crude bacteroid preparations were centrifuged at $3,000 \times g$ for 20 min, and the pelleted cells were suspended in 10 ml of buffer or medium to comprise a "washed cell" preparation.

Bacteroids and controls were enumerated by direct microscopic count of cells in 20 squares of 0.0025 $mm^2$ each in a Petroff-Hausser (P-H) counting chamber and by membrane filter-FA procedures (11) by using a Zeiss Universal microscope equipped both for epifluorescence and phase-contrast. Cells stained by strain-specific FA were determined on six to nine filters at 3 or more dilutions for each preparation, counting 10 to 60 fields per filter. In addition to enumeration, the FA was used (i) to confirm that the strain of bacteroid in the nodule preparation was identical to the strain of rhizobia used as plant inoculum, (ii) to insure that all cells in a bacteroid preparation were positive to the appropriate FA, and (iii) to confirm that the plate colonies originating from bacteroid seeding were *R. japonicum* of the appropriate strain.

Viable counts were made from serially diluted samples, surface plated on the yeast extract-mannitol medium solidified with 1.5% Noble agar (Difco).

Triplicate plates for each of three dilutions were inoculated. Plates were incubated at 28°C until colonies could be seen clearly—about 7 days for culture control and 9 to 14 days for bacteroids.

Table 1 shows a typical experiment illustrating the viability of both crude and washed bac-

TABLE 1. *Typical experiment on the determination of bacteroid viability*[a]

| Prepn | Plate count ($\times 10^9$) | FA count[b] ($\times 10^9$) | % Viable[c] by FA | P-H count[b] ($\times 10^9$) | % Viable[c] by P-H |
|---|---|---|---|---|---|
| Crude bacteroid | 2.5 ± 1.1[d] | 2.6 ± 0.4[d] | 99 | 3.2 | 79 |
| Washed bacteroid | 5.5 ± 0.4 | 5.1 ± 1.2 | 107 | 6.6 | 83 |
| Control[e] | 1.8 ± 0.5 | 1.3 ± 0.2 | 138 | 2.0 | 88 |

[a] Bacteroids isolated from 3-week-old soybean plant root nodules inoculated with *R. japonicum* USDA 31.
[b] Membrane filter FA count. Direct count with a P-H counting chamber.
[c] Calculated from plate count/FA count or plate count/direct P-H count.
[d] Mean ± standard deviation from six to nine counts.
[e] Free-living cells of *R. japonicum* USDA 31 from a 6-day culture.

TABLE 2. *Percent viability of Rhizobium bacteroids*

| Prepn | USDA 31[a] | USDA 138[a] | Field isolate[b] | USDA 123[c] | *R. phaseoli*[d] |
|---|---|---|---|---|---|
| Bacteroid | 91.7 ± 20.6[e] | 90.5 ± 34.5[e] | 97 | | 51 |
| Control[f] | 83.2 ± 34.5[g] | 97.5 ± 35.8[g] | | 110.5 | |

[a] Bacteroids from nodules of soybean plants grown in partially sterilized soil or nitrogen-free sand in growth chamber. Plants inoculated with *R. japonicum* USDA 31 or USDA 138, as indicated.
[b] Bacteroids from soybean field at the end of growth season. About 50% of isolated bacteroids were derived from strain USDA 123. Percent viable obtained from plate count/direct count.
[c] *R. japonicum* USDA 123, free-living culture.
[d] Bacteroids from nodules of 6-week-old plants of navy bean cultivar Seafarer grown in a sterile, nitrogen-free system. Plants were inoculated with a strain of *R. phaseoli* isolated from Minnesota soil. Viability obtained from plate count/direct count.
[e] Mean ± standard deviation from four experiments, each including four values determined from plate count/FA count and plate count/direct count for both crude and washed bacteroid preparations.
[f] Free-living cells from 6-day culture.
[g] Mean ± standard deviation from three independent experiments including total of six values.

teroids of *R. japonicum* strain USDA 31. Virtually all of the rhizobia taken from the nodules of this 3-week-old soybean plant were viable, as indicated by the ability to reproduce on the plate culture medium. No significant differences on viability were seen between crude and washed bacteroid preparations. The data reflect the large experimental error that resulted in considerable variation from experiment to experiment. The P-H direct count tends to overestimate the cell number, whereas the FA direct count gives much better accuracy but underestimates to some extent due to burial of some cells in the cellulose matrix of the filter. When data derived from all four experiments with strain 31 were pooled, the viability of the bacteroids was 86.8 ± 24.1% based on P-H direct count and 96.9 ± 16.6% based on FA direct count. The comparable figures for the vegetative cell controls were 87.3 ± 35.2% and 99.1 ± 31.0%.

Data for all experiments with *R. japonicum* bacteroids are included in Fig. 1. The values plotted are the means from determinations on both crude and washed bacteroid preparations and on viability calculated from both P-H and FA data. Included also are data on the percent viability of vegetative control cells of each strain. Bacteroid viability was high irrespective of soybean symbiont strain, the age of the plant, and

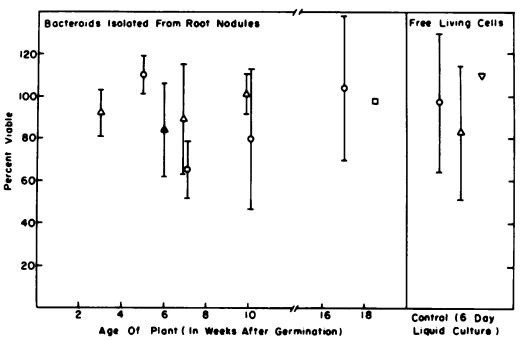


FIG. 1. *Percent viability of R. japonicum bacteroids obtained from root nodules of soybean plants at various developmental stages and of free-living cell controls. Mean ± standard deviations of each age group were calculated from four determinations as described in the text. Symbols: △, ○, and ▽, strains USDA 31, 138, and 123, respectively; □, bacteroids prepared from nodules of mature, field-grown soybeans and comprised of approximately 50% strain USDA 123; ▲, bacteroids from plants grown in nitrogen-free system inoculated with strain USDA 31.*

the root nodule circumstances. Also evident is large experimental error inherent in the determinations; this error is of the same magnitude be it bacteroids or vegetative controls.

Table 2 summarizes the viability determina-

tions on *R. japonicum* bacteroids and controls and includes a single experiment with *R. phaseoli* bacteroids. No significant differences in percent viability can be noted among the different strains of soybean bacteroids. The viability of the *R. japonicum* bacteroid is undoubtedly high, and that of the *R. phaseoli* bacteroid may be equally high, given the poor precision of one enumeration. In any event, probably a large majority of the bacteroids prepared and counted in these experiments proved to be viable.

The great discrepancy between our data and those of Bergersen (3) is not easily explained. Bergersen used soybeans and a similar approach, except that FA enumeration was not available, but did not give a detailed account of the experiments. Negative results derived from the experiments of Almon (1) are easier to explain since single cells of many bacterial species are difficult to grow under conditions of micromanipulation and microslide growth chamber conditions. The finding that bacteroids are viable is consistent with the results of several recent investigations in which no major differences were found in buoyant density, base sequence, and molecular weight between deoxyribonucleic acid isolated from bacteroids and from free-living cells (9, 10, 12).

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