

## *Bradyrhizobium japonicum* Serocluster 123 and Diversity among Member Isolates†

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Diversity was examined within a group of 79 isolates of *Bradyrhizobium japonicum* reactive to fluorescent antibodies (FAs) prepared against *B. japonicum* USDA 123. Analyses were by means of cross-adsorbed FAs, bacteriophage typing, and endonuclease restriction digest patterns. Serogroups 127 and 129 shared antigenic determinants with serogroup 123 but not with each other. Bacteriophage and DNA digest patterns reflected more common features between serogroups 123 and 127 than between 123 and 129. Serogroups 129 and 122 showed FA cross-reactivity. The term serocluster was proposed to reflect interrelationships observed among the serogroups.

Soils of the midwestern United States usually have well-established populations of *Bradyrhizobium japonicum*, the root nodule symbiont of soybean. These indigenous *B. japonicum* populations occupy virtually all soybean nodules to the exclusion of seed inoculant strains introduced at planting with the hope of increasing symbiotic nitrogen fixation. The most competitive of the indigenous *B. japonicum* strains under most midwestern soil conditions are members of serogroup 123, characteristically occupying 60 to 80% of the nodules formed (2, 4, 6, 7). However, the term serogroup designates a group of strains that have antigenic determinants in common but may be diverse with respect to other properties. It is important to determine whether one or a few closely related strains of serogroup 123 predominate in nodules over a relatively large geographical area or whether various different members of the serogroup prove most competitive from place to place as a function of soil environment or soybean cultivar or both.

The question of diversity within serogroup 123 has been addressed in but one paper (3) in which the important observation was made that serogroups 127 and 129 were cross-reactive with serogroup 123. Much more information is needed to define serogroup boundaries and to provide identifying characteristics which individual strains may be studied autecologically.

Data presented in this paper are concerned with the interrelatedness and identifying features of 79 isolates of *B. japonicum*. All of these isolates reacted strongly to fluorescent antibodies (FAs) prepared against strain USDA 123 and hence were designated as belonging to serogroup 123. The term serocluster is proposed to reflect the interrelationships observed and to describe better this important segment of *B. japonicum* indigenous to soil.

### MATERIALS AND METHODS

**Cultures.** The primary reference cultures of *B. japonicum* used for antibody production were USDA 123, USDA 127, and USDA 129, all obtained from the *Rhizobium* culture collection maintained by the U.S. Department of Agriculture at Beltsville, Md., and Webster 48, a Minnesota soil isolate apparently serologically identical to USDA 127. An addi-

tional 75 isolates were obtained from sources indicated in Table 1. All cultures were maintained on yeast extract-mannitol agar slants (11).

**FA preparation.** Young female New Zealand White rabbits were used to produce antibodies against somatic antigens of *B. japonicum* USDA 123, USDA 129, and USDA 127 or Webster 48. Antigen preparation, injection, and bleeding were as previously described (10), and globulin separation, conjugation with fluorescein isothiocyanate, and titration of the FAs followed the procedures of Belser and Schmidt (1). Adsorption of FAs to remove cross-reactivity was done by the protocols of Robert and Schmidt (9) and usually required two exposures to the adsorbing antigens with 1 to 2 h of incubation followed by a third exposure and overnight incubation.

**Bacteriophage isolation and typing.** Soil samples were collected from around the roots of senescent soybean plants from experimental fields at the University of Minnesota, St. Paul. About 10 g of soil was extracted in 95 ml of H<sub>2</sub>O on a wrist-action shaker for 60 min. After centrifugation at 2,000 rpm for 10 min, the supernatant was filtered successively through filters with pore sizes of 1.2, 0.45, and 0.2 μm. The soil filtrate was plated on yeast extract-mannitol plates against two isolates FA typed as 123, three typed as 127, and three as 129; plaque formation was observed during 14 days. Pure bacteriophages were obtained by successive reisola-

TABLE 1. Source and number of isolates reactive to unadsorbed *B. japonicum* USDA 123 FA, included in the study of serocluster 123

| Geographical origin                                       | Source       | No. of isolates |
|---|--------------|-----------------|
| United States and People's Republic of China <sup>a</sup> | H. Keyser    | 11              |
| Wisconsin, Dane County <sup>b</sup>                       | B. Kammicker | 9               |
| Iowa, Plymouth County                                     | This study   | 2               |
| Minnesota, University field plots (St. Paul)              | This study   | 25              |
| Minnesota, Sherburne County                               | This study   | 12              |
| Minnesota, Isanti County                                  | This study   | 8               |
| Minnesota, Waseca County                                  | This study   | 6               |
| Minnesota, Houston County                                 | This study   | 2               |

<sup>a</sup> Keyser et al. (5).

<sup>b</sup> Unpublished data.

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TABLE 2. Specificity of FAs used to characterize isolates of *B. japonicum* serocluster 123

| FA               | FA dilution | Relative fluorescence of strains <sup>a</sup> : |      |      |     |
|------------------|-------------|---|------|------|-----|
|                  |             | 123   | 127  | 129  | 122 |
| 123 (unadsorbed) | 1:32        | 4+  | 3+   | 4+   | —   |
| 127 (unadsorbed) | 1:32        | 2-3+  | 4+   | —    | —   |
| 129 (unadsorbed) | 1:32        | 4+  | —    | 3-4+ | 4+  |
| 122 (unadsorbed) | 1:32        | —   | —    | 3-4+ | 4+  |
| 123 <sup>b</sup> | 1:16        | 3+  | —    | —    | —   |
| 127 <sup>c</sup> | 1:16        | —   | 2-3+ | —    | —   |
| 129 <sup>d</sup> | 1:8         | —   | —    | 3-4+ | 4+  |

<sup>a</sup> Relative fluorescence on increasing scale: — (none) to 4+ (bright).

<sup>b</sup> Adsorbed with strain 127 and 129 cells.

<sup>c</sup> Adsorbed with strain 123 cells.

<sup>d</sup> Adsorbed with strain 123 cells.

tions from individual plaques. The titer of phage stocks varied from  $10^5$  to  $10^9$  PFU/ml. Phages were stored over chloroform.

Strains of *B. japonicum* tested for bacteriophage sensitivity were grown to the stationary phase in yeast extract-mannitol broth. Approximately  $10^8$  bacteria were seeded per plate of the yeast extract-mannitol medium with a soft-agar overlay. High-titered phage stocks (between  $10^5$  and  $10^9$  PFU/ml) were then spotted on the plates in drops of 2 to 3  $\mu$ l. Plates were observed for lysis after incubation for 8 days. A slight clearing was judged to be due to nonspecific phage enzymes and was not rated positive for confluent lysis.

**Restriction endonuclease analysis.** Cultures used for isolation of DNA were grown to the stationary phase with shaking in yeast extract-maltose medium (8). The cells (1.5 ml) were centrifuged and washed first in TE medium (10 mM Tris, 1 mM EDTA, pH 8.0) with 0.1 M NaCl, and then in TE and 25% sucrose and were finally suspended in a 200- $\mu$ l mixture at pH 8.0 containing 0.005 mM Tris, 12.5% sucrose, 500  $\mu$ g of lysozyme per ml, and 0.04 M EDTA. After a 10-min incubation at room temperature, 250  $\mu$ l of a mixture containing 0.8% sodium lauryl sulfate and 200  $\mu$ g of pronase per ml (10 U/mg) was incubated at 37°C for 1 h. The sample was extracted by adding an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1, vol/vol/vol) and mixing for 2 min at room temperature. The layers were separated by low-speed centrifugation, and the top layer was reextracted with the phenol-chloroform-isoamyl alcohol mixture. Phenol was removed by extraction with ether. The DNA was precipitated overnight at -20°C in the presence of 2 volumes of ethanol and 0.04 M sodium acetate. DNA was pelleted by centrifugation, washed in 70% ethanol, and resuspended in TE to a concentration of 100  $\mu$ g/ml. DNA (1  $\mu$ g) was digested with 30 units of the restriction enzyme *Eco*RI (Promega) for 1 h at 37°C. The digestion buffer contained Tris hydrochloride (0.05 M), MgCl<sub>2</sub> (0.1 M), and NaCl (0.085 M), pH 7.5. The entire reaction mixture was loaded on a 0.7% agarose gel (14 by 20 cm) at 2 V/cm for 18 h. The gel was stained in ethidium bromide at 0.5  $\mu$ g/ml and visualized with UV light.

## RESULTS

All isolates used in this study reacted strongly (2 to 4+) with FA prepared against strain USDA 123. Strains USDA 127 and 129 representing two other serogroups also were reactive with USDA 123 FA (Table 2). USDA 123 was made specific for 123 by adsorption with USDA 127 and USDA 129 cells (Table 2). Similarly, FA prepared against USDA 127 (and Webster 48), originally cross-reactive with 123 but

not 129, was USDA 127 specific after adsorption with USDA 123 cells. Unadsorbed USDA 129 FA also stained USDA 123 but did not react with USDA 127. Cross-reactivity to USDA 123 was removed completely by adsorption with USDA 123 cells. Clearly, both USDA 127 and USDA 129 share antigens with USDA 123 but do not share non-USDA 123 antigens.

Antibodies were raised against strain USDA 122 when it was learned that USDA 129 FA cross-reacted with strain USDA 122. This cross-reactivity (as shown in Table 2), however, did not extend to USDA 123 and USDA 127. Strains of serogroup 122 that may have occurred among our isolates were eliminated by virtue of their nonreactivity with USDA 123 FA.

All isolates were grouped according to the serogroup-specific FAs and were typed for bacteriophage sensitivity against 12 phages freshly isolated from soil. Results are summarized in Table 3. The bacteriophages were restricted to hosts among serogroups 123, 127, and 129 and were unreactive with all other bacteria tested except for strains USDA 110 and USDA 62 of *B. japonicum* (Table 3, footnote a). Serogroups 123 and 127 constituted a phage susceptibility group separate from serogroup 129. Sensitivity to phage J was shared by most isolates of serogroups 123 and 127. Phages I, V, and W were most likely to be diagnostic of serogroup 123 strains, infecting more than twice as many serogroup 123 as 127 strains. Phage N appeared to be the best indicator of a serogroup 127 isolate.

Strains of serogroup 129 were much restricted in phage sensitivity as compared with other serocluster isolates. Phages J and K infected virtually all strains of serogroup 129. Sensitivity to these phages and resistance to nearly all others of the phage bank were a good indicator of a strain 129 isolate. Phage J was the most promiscuous, infecting not only serocluster 123 but also a strain of serogroup 110 and a strain of serogroup 62.

DNA restriction endonuclease digests yielded patterns reflecting differences among serogroups 123, 127, and 129 (Fig. 1). Fragments of DNA distributed quite similarly in lanes 1 to 7 with possible differences between serogroup 123 (lanes 1 to 3) and serogroup 127 (lanes 4 to 7) near the top of the gel. Lanes 8 to 11 are made up of serogroup 129 isolates.

TABLE 3. Distribution of bacteriophage susceptibility among 79 *B. japonicum* isolates belonging to three serogroups of serocluster 123<sup>a</sup>

| Bacteriophage | No. of serogroup 123 (n = 30) positive (%) | No. of serogroup 127 (n = 19) positive (%) | No. of serogroup 129 (n = 30) positive (%) |
|---------------|--|--|--|
| A             | 4 (13)                                     | 8 (42)                                     | 0 (0)                                      |
| I             | 20 (67)                                    | 6 (32)                                     | 0 (0)                                      |
| J             | 21 (70)                                    | 14 (74)                                    | 2 (7)                                      |
| M             | 12 (40)                                    | 10 (53)                                    | 0 (0)                                      |
| N             | 4 (13)                                     | 10 (53)                                    | 0 (0)                                      |
| P             | 7 (23)                                     | 6 (32)                                     | 0 (0)                                      |
| V             | 23 (76)                                    | 6 (32)                                     | 0 (0)                                      |
| Z             | 15 (50)                                    | 2 (11)                                     | 0 (0)                                      |
| K             | 10 (33)                                    | 6 (32)                                     | 27 (90)                                    |
| L             | 7 (23)                                     | 4 (21)                                     | 0 (0)                                      |
| D             | 13 (43)                                    | 5 (26)                                     | 0 (0)                                      |
| W             | 15 (50)                                    | 5 (26)                                     | 0 (0)                                      |

<sup>a</sup> The following isolates outside of serocluster 123 were also typed: *B. japonicum* USDA 110, 62, 135, 24, 117, 71, 38, 31, and 138 and Nitragin 61A93; nine fast-growing strains of *R. japonicum*; one *R. phaseoli*; one *Agrobacterium tumefaciens*. Strain USDA 110 reacted with phages J and K, and strain USDA 62 was sensitive to phages I, J, V, and D. All other isolates were negative to all phages.

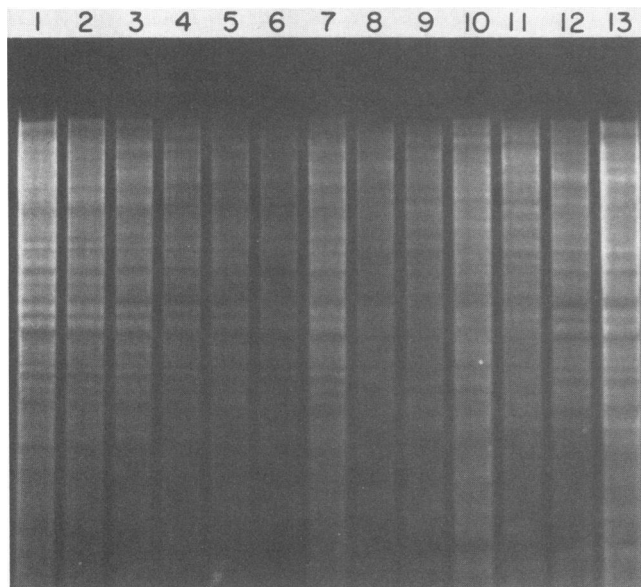


FIG. 1. Gel patterns of *Eco*RI restriction endonuclease digest of DNAs from members of *B. japonicum* serocluster 123. Lanes 1 to 3, Serogroup 123 (strains USDA 123, St. Paul 42, and Lambertson 1, respectively); lanes 4 to 7, serogroup 127 (strains USDA 127, Webster 48, Waseca 1, and Rosemount 2, respectively); lanes 8 to 11, serogroup 129 (strains USDA 129, KS6, Becker 5, and Becker 4, respectively); lane 12, PRC50 (serogroup 123); lane 13, PRC83 (serogroup 127).

Agreement within this group is good, and the pattern differs from those of 123 and 127 in the upper third of the gel. Serogroup 129 isolate Becker 5 (lane 10) shows somewhat distinctive multiple bands near the top of the middle third of the gel. Isolate PRC50 (lane 12) was serotyped as 123, and PRC83 (lane 13) was serotyped as 127. Bands in these lanes appear to agree well with lanes 1 to 7, but based on its rather poorly distinguished uppermost bands, PRC83 may differ from other isolates.

#### DISCUSSION

Evidence of interrelationships based on serology, bacteriophage sensitivity, and DNA restriction digest analyses led us to group isolates of serogroups 123, 127, and 129 together as serocluster 123, presented diagrammatically in Fig. 2. The likelihood is that serocluster 123 is not a discrete grouping but rather is linked to other serogroup clusters. An indication of this is the position of serogroup 122 relative to serocluster 123. Serogroup 122 shares antigens with serogroup 129 which in turn has antigens in common with 123. Serogroups 122 and 123, however, have no antigens in common, but serocluster 123 may be linked to another serocluster by way of serogroup 122. Similarly, there may be linkage of serocluster 123 in still another direction by way of serogroup 127. Although we did not encounter strains that have antigens in common with serogroup 127 but not with serogroup 123, neither did we look for that relationship.

One indication that serocluster 123 is perhaps more open-ended on the serogroup 129 side than on the 127 side is the evidence of greater similarity between 123 and 127 than between 123 and 129. Bacteriophage sensitivity patterns (Table 3) as well as DNA digest patterns (Fig. 1) point to this likelihood. We found that these same patterns, at the level of the individual isolate, can be sufficiently distinctive to dif-

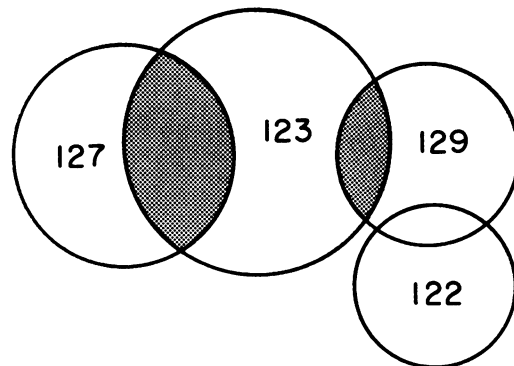


FIG. 2. Diagrammatic representation of interactions among serogroups 123, 127, and 129 to make up serocluster 123. Shaded areas represent common antigens.

ferentiate among even closely similar strains since very few individual patterns were repeated.

The point is sometimes raised that serological properties of rhizobia may not be extrapolated to a correlation with other important properties, notably symbiotic nitrogen fixation. While this may be true, there is no good evidence on the point since comparisons have never been made under natural soil and plant growth conditions. On the other hand the close agreements observed in this study with respect to serological, bacteriophage, and DNA properties suggest that serological features indeed may reflect biological expressions of significance to the rhizobium-legume symbiosis. A case in point is the well-known competitive success of the group we refer to as serocluster 123. It will be important to learn how strains within serocluster 123 compare in relative competitiveness as indigenous soil populations and how refined an indicator antigenic determinants may be of competitiveness. The possibility exists that a monoclonal antibody directed against a determinant that correlates strongly with competitiveness may be the tool of choice to select or engineer the superior *Bradyrhizobium* species tailored as an inoculant for a given soil-climate-cultivar complex.

#### ACKNOWLEDGMENTS

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