Applied and Environmental Microbiology, Aug. 1987, p. 1872–1875
0099-2240/87/081872-04$02.00/0
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Vol. 53, No. 8

Ecology of Indigenous Soil Rhizobia: Response of *Bradyrhizobium japonicum* to Readily Available Substrates†

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Received 26 February 1987/Accepted 18 May 1987

Populations of indigenous *Bradyrhizobium japonicum* serocluster 123 and serogroups 110 and 138 were studied after various sugars were added to their soil habitat. Loam soil with approximately 10^4 cells of each group per g of soil were amended every 3 days with 0.1% glucose, sucrose, arabinose, xylose, or galactose. Enumerations of the populations were made every 12 days by immunofluorescence assay. Each *B. japonicum* population in the sugar-treated soils increased by about 1 log during the first 12 days, to a maximum of about 10^6 cells by day 36 or 48, irrespective of the sugar added. Maximum growth rates were similar for each group and occurred during the 12-day incubation period. The most rapid growth was in response to arabinose, with a mean generation time of about 3.0 days. Other mean doubling times were 4.0 days with glucose and galactose treatments, 4.5 days with xylose treatment, and 5.4 days with sucrose amendment. These data provide the first direct evidence that indigenous soil rhizobia can compete successfully with other soil bacteria for readily available substrates in soil in the absence of host legume roots or other rhizospheres. The growth rates in soil of the specific *B. japonicum* populations studied were nearly the same with a given sugar treatment but varied considerably with different sugars. The mean generation times of 3 to 5 days are among the first reported growth rates for heterotrophic bacteria in natural soil.

Information on the biology of rhizobia as indigenous soil bacteria is sparse. Rhizobia, like other indigenous soil bacteria, are limited in distribution, density, and persistence primarily by the nature and amount of carbon and energy available to them. In the vicinity of plant roots, root exudates and root debris are likely substrates since indigenous soil rhizobia are generally capable of colonizing the rhizospheres of both leguminous and nonleguminous plants (10). Suitable substrates must be available in nonrhizosphere soil as well, as populations of indigenous rhizobia can be maintained in fallow soils (10, 12).

There is little specific information on the nature of possible substrates used by rhizobia in soil and rhizospheres or on how well rhizobia can compete with soil bacteria for such substrates. Chowdhury (5) used the most-probable-number (MPN) plant dilution technique to estimate population changes in indigenous *Rhizobium trifolii* and *Rhizobium lupini* after the addition of glucose to the soil. Little or no change in population was detected. Pena-Cabriales and Alexander (11) studied the growth response of four antibiotic-marked mutants of rhizobia in nonsterile soil after the addition of mannitol (0.01, 0.1, 0.5, and 1.0%), alfalfa meal (1.0%), and corn residue (1.0%). Dilution plating of the soil on selective antibiotic media indicated that numbers of each of the rhizobia increased only if 0.5 or 1.0% mannitol was added. Pena-Cabriales and Alexander concluded that the rhizobia were poor competitors in soil unless large amounts of exogenous carbon such as mannitol were added.

The purpose of the present study was to assess the ability of indigenous soil populations of *Bradyrhizobium japonicum* to compete with other soil bacteria over an extended period for readily available sugars. Such information is relevant to the question of whether rhizobia are restricted to a relatively specialized nutrient base in the soil or whether they are competitive for generally available, energy-rich substrates.

MATERIALS AND METHODS

Indigenous populations of *B. japonicum* in soil were enumerated and monitored by immunofluorescence assay after various sugars were added. Experiments were conducted on a composite sample taken from the plow layer of a Waukegan silt loam (fine silty, mixed, mesic Typic Hapludoll) field plot at the University of Minnesota, St. Paul, which had been frequently cropped to soybeans. The portion of the *B. japonicum* indigenous population studied comprised serocluster 123 (16) and serogroups 110 and 138, and serogroup-specific fluorescent antibodies (FAs) were used. These groups were studied previously for rhizosphere response in the same soil under field conditions (10, 13).

Soil treatments. The following reagent-grade sugars were used as soil amendments: sucrose, L-(+)-arabinose, D-(+)-galactose, D-(+)-glucose, and D-(+)-xylose. Sieved soil (2-mm screen) with a 20% moisture content was distributed into four portions, each of which was subsequently subdivided into six 300-g samples to accommodate the five sugar treatments and one control. Each sugar was added to its respective four replicate samples at a concentration of 0.1% and mixed thoroughly. The process was repeated every 3 days for 72 days. Control samples were mixed every 12 days before being sampled.

Extraction of *B. japonicum* from soil. At 12-day intervals, 10-g samples of soil were placed into milk dilution bottles containing 94 ml of a 10-fold dilution of 1% partially hydrolyzed gelatin in 0.1 M $NH_4H_2PO_4$ (8), 5 drops of Tween 80 (Difco Laboratories, Detroit, Mich.), and 1 drop of antifoam (AF72; General Electric Co., Schenectady, N.Y.). The mixture was placed on a wrist action shaker (Burrell Corp., Pittsburgh, Pa.) and shaken for 30 min. The suspension then

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† Paper no. 15307 in the Scientific Journal Series of the Minnesota Agricultural Experiment Station, St. Paul, MN 55108.

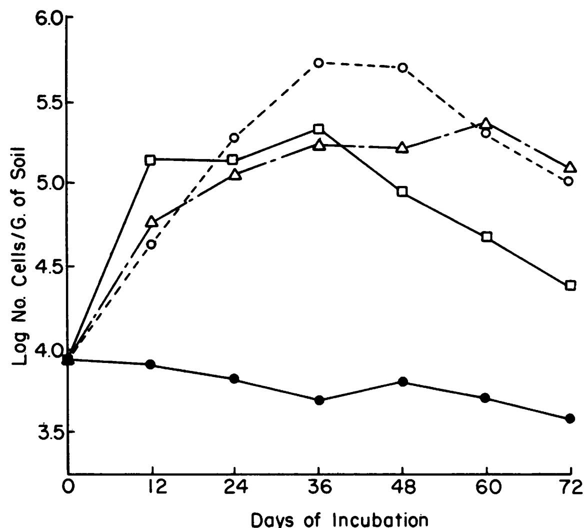

FIG. 1. Averaged growth of indigenous *B. japonicum* groups 110, 123, and 138 in nonsterile Waukegan silt loam, either unamended (●) or amended with arabinose (□), sucrose (○), or xylose (△).

was treated with 1.0 ml of $CaCl_2$ (0.8 g/ml) to promote flocculation of soil particles, shaken again for 1 min, and allowed to stand undisturbed for 30 min before the supernatants were transferred to a large test tube. The *B. japonicum* populations in these supernatants were enumerated.

FA staining and enumeration. Appropriate volumes (usually 1.0 ml) of each supernatant were passed through polycarbonate filters (0.4-μm pore size, 25-mm diameter; Nuclepore Corp., Pleasanton, Calif.) pretreated with irgalan black to reduce autofluorescence (10). Material retained in the filter was treated with gelatin-rhodamine conjugate to control nonspecific fluorescence (3) and then with FAs for group 110, 123, or 138. Preparation of the FAs, procedures for FA staining and microscopic enumeration of fluorescent *B. japonicum* cells, and the features of the fluorescence microscope have been described previously (1, 10, 12, 14, 15). Fifty microscopic fields were counted on each of the four replicate filters, and counts were converted to cells per gram of oven-dried soil.

MPN. Estimates of total *B. japonicum* populations were carried out to supplement some of the FA counts. The MPN plastic pouch procedure of Weaver and Frederick (18) was used with commercial growth pouches (Horticultural Div., Northrup King Co., Minneapolis, Minn.), 10-fold dilutions of soil inocula, and two plants per pouch. Soils for MPN inoculation were dispersed in water by shaking on the wrist-action shaker for 60 min (rather than for 30 min, as in FA analyses). Nodules collected after 6 weeks were crushed, smeared on slides in duplicate, and analyzed for FA to determine nodule occupancy, with 50 nodules per treatment.

Experimental design. The experiment consisted of six treatments replicated four times. Each replication was sampled every 12 days for FA enumerations. Data, expressed in number of cells per gram of soil, were first transformed to logarithms and then subjected to analysis of variance for measurements over time by a complete randomized block design (7). Comparisons of the treatment means were made by the Tukey (Honest Statistical Difference) procedure (17).

RESULTS

For indigenous *B. japonicum* groups 110, 123, and 138, growth responses to the repeated incorporation of various sugars into Waukegan soil were sufficiently similar to justify averaging the data. Cell counts, averaged for the three groups after the treatments with arabinose, sucrose, and xylose are presented in Fig. 1. Initial populations were virtually identical, with about 8×10^3 cells per g by FA count. Populations in the unamended control soils remained essentially stable, with only a slow, progressive decline to about 4×10^3 cells by day 72. In the sugar-amended soils, however, all groups increased their numbers by about 1 log during the first 12 days and peaked at about a 2-log increase by days 36 to 48, irrespective of sugar amendment.

Minor differences in growth responses were observed in the data prior to averaging (some data not shown). For example, the population densities observed for serogroup 110 were highest, and their maxima were reached earliest with the glucose (Fig. 2) and sucrose amendments. The highest population of serocluster 123 was attained with the glucose (Fig. 2) and arabinose treatments, both at day 36. Glucose-amended soils consistently had the highest populations of each group.

Some decline in population densities was noted after day 48 or 60 for each population and with every sugar treatment except xylose. Declines were most marked in the glucose-treated soil (Fig. 2) and with arabinose treatment (data not shown). Lesser decreases in the population by day 60 were observed in the galactose-treated (Fig. 3) and sucrose-amended (data not shown) soils.

Indigenous *B. japonicum* of populations other than groups 110, 123, and 138 also increased following the addition of readily available energy to the soil. The total indigenous *B. japonicum* populations, as estimated by the MPN method, was compared with the total identified populations of groups 110, 123, and 138 at days 36 and 60 (Table 1). MPN counts of the total indigenous population were about 2 log units greater than counts of the untreated control at both sampling times, with an increase rather than a decline at day 60. This count

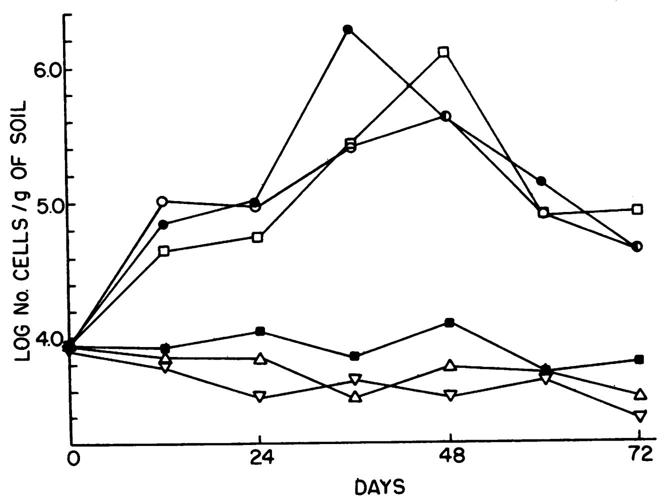

FIG. 2. Growth of indigenous *B. japonicum* in glucose-amended or unamended nonsterile Waukegan silt loam soil. Each point is the average of four replications. Amended soil: □, serogroup 110; ●, serocluster 123; ○, serogroup 138. Unamended soil: △, serogroup 110; ■, serocluster 123; ▽, serogroup 138.

is in contrast to the total FA counts, which showed a 10-fold decrease from days 36 to 60 in both the arabinose- and glucose-treated soils.

The abrupt drop in FA counts was more likely due to the failure to release FA-detectable cells from the soil by the usual extraction procedure than to a selective decline in the three serologically identifiable groups. An indication of this is the consistent proportion (about 33%) of the total MPN enumerated by FA at day 36 for xylose and at day 60 for the control. The repeated additions of glucose and arabinose resulted in a highly developed crumb structure in those soils by day 60, whereas the granulation brought about by galactose and sucrose was less obvious, and that brought about by xylose was barely noticeable. Cementation of most of the soil particles into small granules probably decreased sharply the efficiency of the dispersion step used to release bacteria from the soil prior to FA enumeration.

DISCUSSION

The data presented in this paper constitute the first direct evidence that indigenous soil rhizobia can benefit from the presence in soil of readily available substrates in the absence of a legume or any other plant root. Despite the ready availability to the soil community of simple carbon sources used in this study and the enhanced general microbial activity fueled by repeated small additions of these substrates, *B. japonicum* was nevertheless competitive for substrate and remained so throughout the experiment. This competitiveness was indicated by consistent population increases of up to 2 logs for each of the *B. japonicum* groups monitored and for each of the sugar amendments.

For the three *B. japonicum* groups, growth responses to any given sugar amendment were generally similar, but group 123 responded somewhat better than did groups 110 and 138 in arabinose-treated soil, and group 110 responded somewhat more poorly than the others did with arabinose and galactose treatments. The treatments with glucose and sucrose elicited the best growth responses in each of the groups, but differences usually did not exceed 0.5 log. A

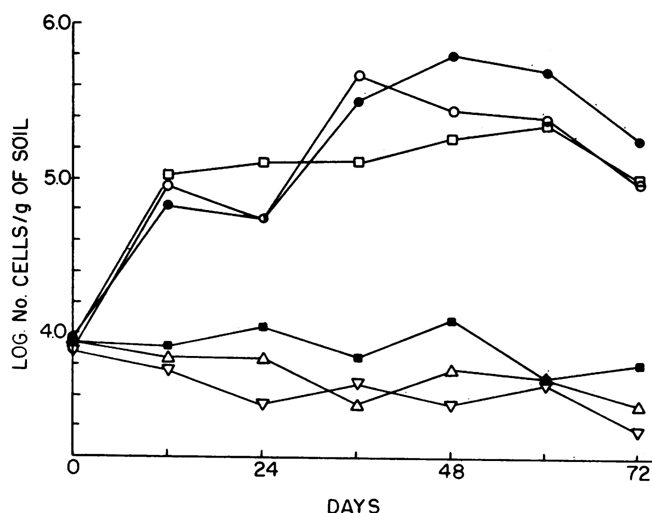

FIG. 3. Growth of indigenous *B. japonicum* in galactose-amended or unamended nonsterile Waukegan silt loam soil. Each point is the average of four replications. Amended soil: □, serogroup 110; ●, serocluster 123; ○, serogroup 138. Unamended soil: △, serogroup 110; ■, serocluster 123; ▽, serogroup 138.

TABLE 1. *B. japonicum* population size measured by immunofluorescence assay after repeated sugar additions to nonsterile Waukegan soil

Addition	No. of cells (10^5)/g at:			
	Day 36		Day 60	
	Total[a]	Fluorescent (%)[b]	Total	Fluorescent (%)
Arabinose	33.0	10.0 (30)	49.0	1.5 (3)
Glucose	70.0	24.0 (34)	95.0	2.5 (3)
Xylose	17.0	5.4 (32)	2.4	8.4 (35)
Control	0.4	0.2 (36)	0.5	0.2 (29)

[a] Estimated by the MPN method.
[b] Total of groups 110, 123, and 138.

similar uniformity in the population dynamics of the same three *B. japonicum* groups was observed in the rhizosphere studies of Moawad et al. (10).

The growth patterns of the three groups were generally similar in response to glucose, galactose, xylose, and arabinose. The most rapid population increases occurred during days 0 to 12, followed by a slower rate of growth between day 12 and the day of peak population, usually at day 36 or 48. Soil that was amended with sucrose resulted in a different pattern, in which the initial growth rates of the three *B. japonicum* populations were again nearly the same but slower than those observed with the other sugars. This initial growth rate with sucrose, however, in contrast to rates with the other sugars, was maintained to day 24 and even to day 36 with serogroup 110.

The data permit an approximation of the average maximum growth rate of *B. japonicum* for each group as a function of sugar amendment during the early period of incubation (0 to 12 days). The most rapid growth was in response to arabinose, with a mean generation time of about 3.0 days. This rate was followed by the mean generation times in response to glucose and galactose (about 4.0 days), xylose (4.5 days), and sucrose (5.4 days). *B. japonicum* is characterized as a slow-growing rhizobium with a generation time of 6 to 12 h in pure culture and favorable media, but estimates of the growth rates in nature of this bacterium or any other specific soil bacteria are virtually nonexistent. The 10- to 15-fold decreases in the pure-culture growth rates in soil that we observed are reasonable in view of the size and diversity of the soil microbial community, whose populations are competing for readily available substrates. According to Brock (4), the generation time of *Escherichia coli* slows from 20 to 30 min in pure culture to 12 h in the intestinal tract, and *Leucothrix mucor*, a marine bacterium, has a generation time of 2 h in culture compared with 11 h in nature. Belser and Schmidt (2) estimated the growth rate of the chemosynthetic autotrophic nitrifier *Nitrobacter winogradskyi* at 14 h in pure culture and 140 h in actively nitrifying soil.

Although each of the three *B. japonicum* populations increased from 50- to 100-fold in all sugar-amended soils, it is not known whether they responded to the primary substrate or to by-products of more successful competitors for the primary substrate. Whether *B. japonicum* "sits at the first table" or at a second or later table is of little ecological significance. What is important is that the presence in soil of high-energy, readily available substrates promotes active *B. japonicum* growth which can be sustained when the species is competing with other soil biota. That a sugar substrate in soil may be modified before use by *B. japonicum* is sug-

gested by the data for the disaccharide sucrose. Sucrose reportedly is not metabolized by *B. japonicum* in pure culture (6, 9). However, not only did *B. japonicum* respond to sucrose, but sucrose amendment was the only treatment after which all three groups maintained initial growth rates well beyond day 12 (Fig. 1). The reduced but prolonged growth rate associated with sucrose is consistent with the hypothesis that sucrose may become available to *B. japonicum* only after it is split into glucose and fructose by other members of the soil community.

The concept of rhizobia as specialized bacteria dependent for their survival on the legume root nodule niche clearly does not hold for *B. japonicum*. Instead, this rhizobium appears to be as well adapted to the soil at large as it is to the rhizosphere (10, 12) and competes favorably in both environments for readily available substrates. Consequently, once introduced into a suitable soil, *B. japonicum* populations will integrate into the indigenous soil communities and equilibrate at population densities commensurate with the general level of microbial activity of the soil. It is likely that the naturalization of rhizobia is less successful and more dependent on nodulation success in soils, where microenvironments are marginal with respect to the tolerance of rhizobia for pH, temperature, and other environmental parameters.

ACKNOWLEDGMENT

This work was supported by grant 84-CRGR-1-1403 from the U.S. Department of Agriculture Competitive Research Grants Office.

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