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Uniformity of the Microsymbiont Population from Soybean Nodules with Respect to Buoyant Density[1]

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ABSTRACT

The microsymbiont population in soybean root nodules (*Glycine max* L. cv Williams 82 inoculated with *Bradyrhizobium japonicum* 2143) was characterized during symbiotic development to determine the extent of heterogeneity in this population. The microsymbiont population was isolated by centrifugation through a continuous sucrose gradient (44 to 57% weight to weight ratio) and appeared homogeneous at each age examined up to 26 days after planting based on the symmetrical distribution of the population, enzyme activities, poly-β-hydroxybutyrate contents, protein contents, and viabilities. Some differences in viability, protein content, and acetylene reduction activity were observed at later ages. The population migrated to progressively lighter buoyant densities with increasing age until a density equivalent to 48% sucrose was reached. The changing density correlated directly with the increasing poly-β-hydroxybutyrate to protein ratio. The acetylene reduction activity, based on microsymbiont concentration, followed the same developmental pattern as whole nodules. On a protein basis, the decline of acetylene reduction activity was later and reflected the decrease in protein content per cell. These results suggested that the microsymbiont population, which resulted from inoculation of *B. japonicum* 2143 onto Williams 82 cultivar of soybeans, developed as a homogeneous population.

The symbiosis between soybean and *Bradyrhizobium japonicum* involves a complex set of interactions between the plant and the bacteria which permit the bacteria to colonize within the infected host cells. During nodule development, both the plant cells and the bacteria undergo morphological alterations. The development of *B. japonicum* into its symbiotic form(s) cannot be distinguished visually as with other *Rhizobium* species (12, 18). However, numerous biochemical changes have been reported during symbiotic development. These include alterations in enzyme levels, DNA content, cytochrome composition and cell wall structure (3, 9, 13, 15, 18, 20). The most distinctive function of the symbiotic form of *Rhizobium* is the fixation of atmospheric nitrogen which requires the expression of the nitrogenase proteins plus those enzymes necessary to provide low potential electrons and energy in the form of MgATP.

To determine and define microsymbiont subpopulations of *B. japonicum* in the nodule, Ching *et al.* (3) studied several biochemical parameters believed to be indicative of symbiotic development. By using a discontinuous sucrose gradient of 45:50:52:57% sucrose, they separated the microsymbiont population into three forms. The subpopulation at the 45:50% sucrose interface was defined as "mature bacteroids," since it possessed the greatest rates of nitrogenase and hydroxybutyrate dehydrogenase activities and, in addition, had the highest concentrations of Cyt *b*, *c*, and P-450. The population at the 52:57% sucrose interface, which contained the highest levels of Cyt a-a_3, the greatest Cyt *c* oxidase activity, and the lowest nitrogenase activity, was designated undifferentiated bacteria. Those at the 50:52% interface, which possessed intermediate levels of enzyme activities and cytochromes, were designated transforming bacteria. Based on this biochemical evidence, these authors concluded that the discontinuous sucrose density procedure was useful for isolating the various symbiotic forms of *B. japonicum* found within soybean nodules.

Our attempts to isolate distinct subpopulations by these procedures were unsuccessful. The microsymbionts obtained at each density used by Ching *et al.* (3) were indistinguishable from the population defined as mature bacteroids during the period of development from 12 to 34 DAP. Thus, because of the differences between the results reported here and those of Ching *et al.* (3), the term microsymbiont will be used to refer to the *B. japonicum* endophyte of soybean nodules.

The commercial inoculum used by Ching *et al.* (3) contained four different strains of *B. japonicum* (4, 6). Results from a number of laboratories (5, 10, 16) have shown that nodules can be occupied jointly by a mixture of *Rhizobium* species which differ in their effectiveness of nitrogen fixation. Thus, the three different symbiotic forms observed and defined by Ching *et al.* (3) may have resulted from differences in bacteria-host compatibility among the strains (61A101, 118, 124, and 148) in the commercial inoculum, rather than representing differential states of development of the bacterial endophytes. We have reexamined the microsymbiont population present in soybean nodules using a single strain inoculum of *B. japonicum*. The results described here characterize the microsymbiont population of *B. japonicum* 2143 in nodules of Williams 82 cultivar of soybeans.

MATERIALS AND METHODS

Plant Material and Rhizobium Strains. Soybeans (*Glycine max* L. cv Williams 82) were inoculated with *Bradyrhizobium japonicum* 3I1b-143 or 2143 and planted in perlite potting medium in modified Leonard jars containing a nitrogen-free nutrient solution (1). Seeds were surface-sterilized, germinated on water agar, and inoculated with *B. japonicum* for 30 min before planting in autoclaved Leonard Jars. Plants were grown in a Conviron Growth Chamber with a day/night temperature of 28°C (14 h)/25°C (10 h) and watered from the bottom, as needed, with autoclaved nitrogen-free nutrient solution. Light intensity was 350 $\mu E \cdot m^{-2} \cdot s^{-1}$. Nodules were harvested from the crown area of the tap root

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12 to 34 d after seedling planting. *B. japonicum* 3I1b-143 was kindly supplied by Harold Evans, Laboratory for Nitrogen Fixation Research, Oregon State University, Corvallis. Strain 2143 was isolated from 3I1b-143 by its natural resistance to rifampicin and sodium nalidixate at concentrations of 80 μg/ml and 120 μg/ml, respectively. The microsymbionts were reisolated from the nodules and verified as strain 2143 by comparison of their plating efficiency on Vincent's agar medium (21) with or without both rifampicin (80 μg/ml) and sodium nalidixate (120 μg/ml).

Fractionation of Microsymbiont Populations. Microsymbiont populations were isolated from 5 to 10 g of nodules as described previously (9). Nodules, suspended in MEP[2] (5 mM $MgCl_2$, 1 mM EDTA, 50 mM potassium phosphate buffer, pH 7.3) containing 17% (w/v) sucrose and PVP (1 g nodule: 0.33 g PVP: 10 ml buffer) were disrupted in a Waring blender, and the homogenate was filtered through 4 layers of cheesecloth and centrifuged. The 400*g* to 8000*g* pellet was resuspended in MEP buffer containing 17% (w/v) sucrose and layered onto a 30:42% (w/w) sucrose step gradient (10:15 ml). Gradients were centrifuged in a SW-28 rotor at 72,000*g* in a Beckman L8-55 ultracentrifuge for 35 min at 5°C. The pellet from 10 g of nodules was resuspended in 5 ml of MEP buffer containing 17% (w/v) sucrose and a 2 ml sample was layered onto each 42 to 57% (w/w) continuous sucrose gradient. Gradients were centrifuged for 18 h in a SW-28 rotor at 100,000*g* in a Beckman L8-55 ultracentrifuge at 5°C. Fractions of 1.5 ml were collected with an ISCO model 185 density gradient fractionator, and their refractive index and optical density (A_{630}) determined. For enzyme assays, the fractions were diluted with two volumes of MEP buffer and the cells collected by centrifugation. Cell pellets were resuspended in 6 ml of 20 mM K-phosphate buffer (pH 7.2) containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 1 M glycerol and ruptured with a French pressure cell at 16,000 psi. Cell debris was removed by centrifugation at 30,000*g* for 20 min at 4°C. Aliquots from the supernatant fraction were used for enzyme assays.

Enzyme Assays. Experimental sets included the supernatant fractions, obtained as described above, from at least three ages, with one or two ages in common to a previous experimental set so that data could be normalized if necessary. The mean activity and standard deviation for each fraction were determined from replicate assays, three each at two protein concentrations. Malate dehydrogenase, hydroxybutyrate dehydrogenase, acetoacetyl-CoA thiolase, acetoacetate-succinyl-CoA transferase, isocitrate dehydrogenase, pyruvate dehydrogenase, and fumarase were assayed as described by Karr *et al.* (9).

Protein Assays. The protein content of the cytosol after the centrifugation (30,000*g*) of French press ruptured cells was determined by the method of Lowry *et al.* (11). Total protein content of whole cells was measured after the cells had been digested in 1.0 N NaOH for 50 min at 50°C and the insoluble material had been removed by centrifugation. The supernatant fraction was used for protein measurement. BSA was used as standard.

Plating Efficiency. Viability of strain 2143 was determined from the plating efficiency of the microsymbiont on Vincent's agar medium containing rifampicin and sodium nalidixate. Nodules were surface-sterilized, glassware was autoclaved and dried, and solutions were sterile-filtered before use. Microsymbiont fractions from the sucrose gradient were collected in sterile tubes, and an aliquot was transferred into Vincent's medium or PBS (10 mM K-phosphate and 150 mM sodium chloride, pH 7.0) for further dilution. Thirty to 300 cells (10^9 cells per ml per A_{630}) were plated onto Vincent's agar medium containing rifampicin and sodium nalidixate, 80 and 120 μg/ml, respectively. Refractive index and optical density (A_{630}) of each microsymbiont fraction from the gradient were measured. Viability was expressed as the percentage of CFU per cells plated and was the mean value of six replicate plates, three each at two different dilutions. Experiments were repeated at least twice. The number of cells per ml was determined with a Petroff-Hauser counter. The optical density was proportional to the cell number per ml from 0.08 to 0.8 A_{630}. One unit at 630 nm was equivalent to 1.1×10^9 cells per ml. Values of cell number per ml obtained from 4 d cultured cells were the same (± 10%) as those obtained for the microsymbionts collected at 48% or at 52% (w/w) sucrose at 19 DAP and also agreed with those for 34 DAP microsymbionts at 47 and 50% (w/w) sucrose. No loss of viability was observed between cells plated within 2 h of harvest and those plated after 18 h of centrifugation.

PHB Measurement. PHB analysis was performed either on whole cells or on pellets obtained after centrifugation (30,000*g*) of ruptured cells. The cells or the cell-debris pellets were washed with MEP buffer, digested in Clorox, and the PHB content was measured by the method of Karr *et al.* (8).

Anaerobic Isolation and Fractionation of Microsymbionts. All manipulations in preparing microsymbionts were performed in a controlled environmental chamber flushed with purified nitrogen. Anaerobic buffers were prepared immediately before use by repeated cycles of degassing by vacuum and resaturation with nitrogen gas that had been passed over BASF catalyst R3-11. Nodules, 5 to 10 g, were disrupted in MEP buffer containing 17% (w/v) sucrose and PVP as described above. The 400*g* to 8000*g* microsymbiont pellet was used directly in the acetylene reduction assay as described below or resuspended in 5 to 10 ml MEP buffer containing 17% (w/v) sucrose and layered onto a continuous sucrose gradient (14 ml each of 44% and 56% (w/w) sucrose in MEP, overlaid with a 4 ml layer of 44% (w/w) sucrose in MEP buffer and 2 ml of sample). The gradient tubes were centrifuged in a SW-28 rotor at 100,000*g* for 18 h at 5°C in a Beckman L8-55 ultracentrifuge. After puncturing the bottom of the ultracentrifuge tubes, microsymbiont fractions of 1.5 ml were collected into 3-ml disposable, graduated syringes. Between each fraction, 6 to 8 drops were collected to determine the refractive index. The microsymbiont fractions were transferred to screw-capped 30-ml centrifuge tubes, diluted with an equal volume of assay buffer containing 4 mM $MgCl_2$, 4 mM succinate, 0.5 mM EDTA, and 40 mM HEPES buffer, pH 7.4, capped with a serum stopper, and centrifuged at 8000*g* for 30 min at 5°C. The pellet was resuspended in a volume of fresh assay buffer such that the optical density of a 6-fold dilution of the suspension was between 0.2 and 1.2 A_{630}.

Acetylene Reduction Activity. The optimal conditions for the acetylene reduction assay were determined by varying the following parameters: partial pressure of O_2, microsymbiont quantity, succinate concentration, magnesium concentration, and pH. With regard to the partial pressure of O_2 and microsymbiont quantity, it was observed that maximal and reproducible rates of acetylene reduction could be obtained when the ratio (ml of air: A_{630}) was between 0.8 and 1.5 with the microsymbiont quantity between 0.2 and 1.2 A_{630}. The A_{630} refers to the optical density of the cell suspension after a 6-fold dilution. Assay mixtures containing 4 mM $MgCl_2$ and 4 mM succinate in 40 mM HEPES, pH 7.4, microsymbiont quantities between 0.2 and 1.2 A_{630}, and ml of air: A_{630} ratios between 0.8 and 1.5 were optimal for microsymbionts isolated from nodules between 12 and 34 DAP and collected between 45 and 55% (w/w) sucrose (data not shown). Assay mixtures containing 2 ml of the microsymbiont suspension in the assay buffer (40 mM HEPES, pH 7.4, 4 mM $MgCl_2$, 4 mM succinate) were set up in triplicate in nominal 20-ml serum vials under an atmosphere of N_2, one for measuring A_{630} of the diluted sample and two as duplicates for acetylene reduction. Vials were quickly depressurized to one atmosphere

[2] Abbreviations: MEP, magnesium chloride (5 mM), disodium EDTA (1 mM), K-phosphate (20 mM) buffer (pH 7.3); DAP, days after planting; PHB, poly-β-hydroxybutyrate; CFU; colony forming units.

after the addition of the assay mixture, and a volume of air, equal to the A_{630} of the diluted sample, was added. For example, if the suspension, diluted 6-fold, had an A_{630} of 0.55, then 0.55 ml of air was required. The reaction was started immediately by adding 2 ml of acetylene, freshly generated from dithionite-treated water and calcium carbide (17). The vials were agitated at 1400 rpm on an orbital shaker at 25 ± 1°C. Samples of the gas phase, 0.5 ml, were taken every 15 or 20 min throughout a 90 to 120 min period and analyzed by a Varian 940 FID gas chromatograph.

Chemicals. Buffers and chemicals were purchased from Sigma Chemical Company. Polyclar AT (insoluble PVP) was purchased from GAF Corporation, New York. Before use, the PVP was washed with 10% HCl, neutralized, washed with distilled water, and dried. High purity gases (N_2, H_2, O_2) vermiculite, perlite, and sand were obtained from local commercial sources.

RESULTS AND DISCUSSION

Characteristics of Strain 2143. A double-drug-resistant strain of 3I1b-143, designated 2143, was isolated by its natural resistance to rifampicin and nalidixate at concentrations of 80 μg/ml and 120 μg/ml, respectively. The growth rate of strain 2143 in liquid culture and its ability to form colonies on Vincent's agar plates in the absence or presence of these antibiotics was the same as strain 3I1b-143. The latter did not form colonies in the presence of these antibiotics. The temporal profile of acetylene reduction activity obtained from intact nodules formed by inoculation with strain 2143, between 10 and 35 DAP, was indistinguishable from that of the parental strain (data not shown). The microsymbiont population was reisolated from the nodules and verified as strain 2143 by its plating efficiency on Vincent's agar medium in the presence of rifampicin and nalidixate. No microscopic differences were observed in cell size or shape betwen strains 3I1b-143 and 2143.

Characterization of the Microsymbiont Populations as Related to Buoyant Density. Centrifugation of the *B. japonicum* 2143 microsymbiont population, isolated from nodules of various ages, in continuous sucrose gradients of 42 to 57% (w/w) sucrose produced single, symmetrical distributions (Figs. 1 and 2). The mean buoyant density of the microsymbiont population decreased with nodule age until approximately 25 DAP, after which the buoyant density remained unchanged. Fractions taken at the half-heights at any given age sedimented reproducibly to the same density upon recentrifugation. The profiles of CFU, PHB content, acetylene reduction activity, hydroxybutyrate dehydrogenase activity, and malate dehydrogenase activity were symmetrical and coincident with the A_{630} profile (Figs. 1 and 2). Acetoacetyl-CoA thiolase, acetoacetate-succinyl-CoA transferase, isocitrate dehydrogenase, pyruvate dehydrogenase and fumarase also were coincident with the A_{630} profile (data not shown). Only the microsymbiont population from very young nodules (12-d plants; Fig. 1) showed a slight asymmetry at the high density end of their distribution. A similar asymmetry was observed with early exponential phase, cultured *B. japonicum* cells and probably resulted from the hyperbolic relationship between buoyant density and PHB content (14). Cultured *B. japonicum* in mid- to late-log phase showed a similar accumulation of PHB and buoyant density distribution as the microsymbiont populations obtained from 14 to 27 DAP nodules. Thus, the distribution observed for the microsymbionts appeared typical for a uniform population of *B. japonicum* cells.

PHB and Protein Content Related to Buoyant Density. When considering the entire microsymbiont population at any given age during nodule development, the PHB content on a cellular basis (A_{630}) increased 1.8-fold between 12 and 27 DAP. Differences in the PHB content between the lightest and heaviest fractions of the microsymbionts, at any particular age, with regard

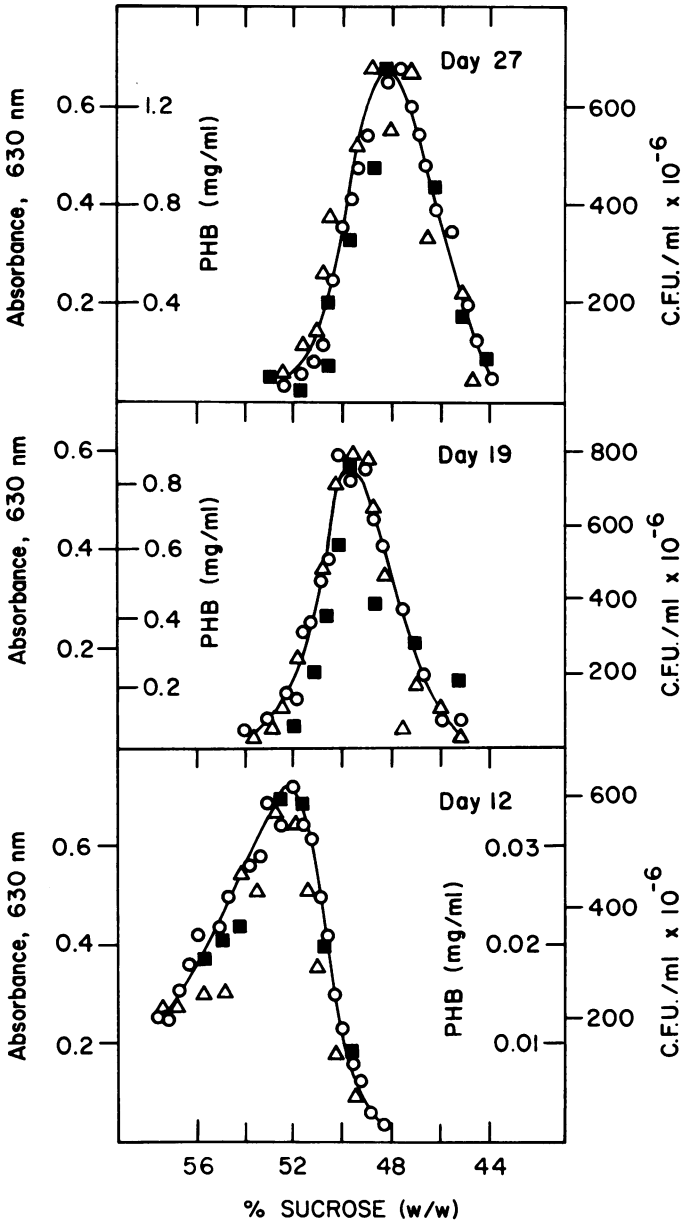

FIG. 1. Distribution of parameters of microsymbionts as a function of plant age and buoyant density. Fractions of the microsymbiont population were collected from continuous 42 to 57% (w/w) sucrose gradients and each fraction was measured for absorbance at 630 nm (○), PHB content (△), and colony forming units (■) as described in "Materials and Methods."

to buoyant density were within ± 15% of the mean. However, during nodule development, PHB content on a protein basis increased 10-fold and analysis by linear regression (r^2 = 0.92) correlated the increasing PHB/protein ratio with decreasing buoyant density (Fig. 3). The large increase in PHB/protein ratio resulted from the combined effect of PHB accumulation and protein loss. The loss of protein from cells (μg protein/A_{630}) was apparent, although small, in the lighter microsymbiont fractions by 19 DAP. The greatest decreases in protein content (3-fold) occurred at later ages (Fig. 4). Whereas a definite correlation between PHB content per mg of protein as a function of buoyant density was obtained, this was not true for the specific activities of malate dehydrogenase and hydroxybutyrate dehydrogenase (Fig. 5) and other enzyme activities measured (data not shown).

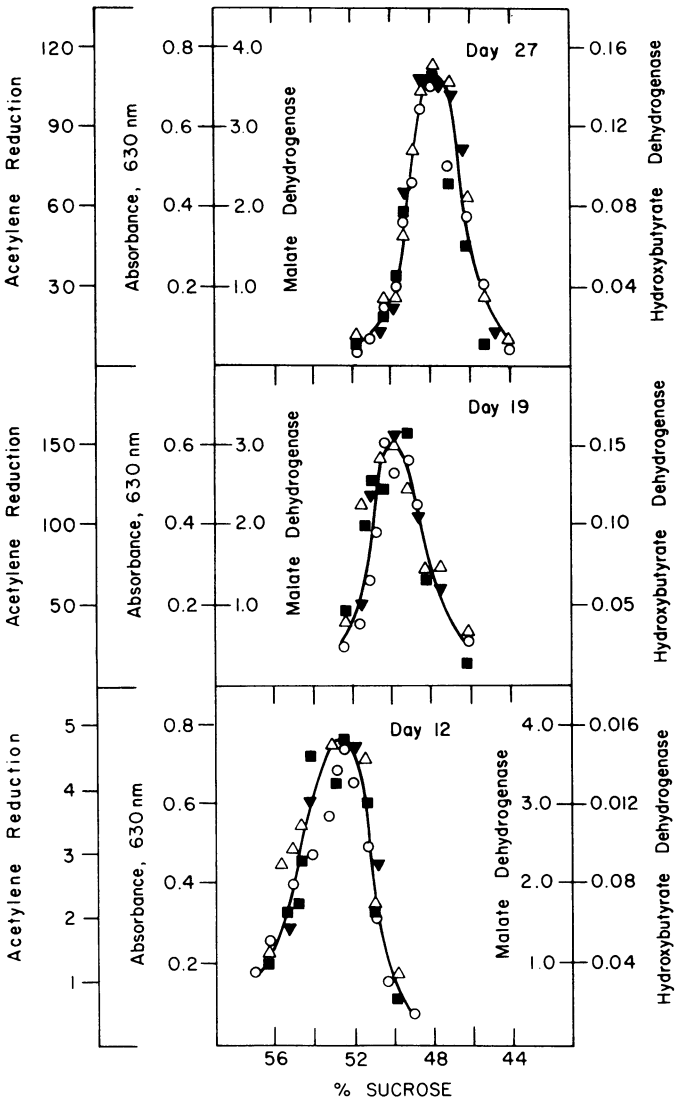

FIG. 2. Distribution of parameters of microsymbionts as a function of plant age and buoyant density. Fractions of the microsymbiont population were collected from continuous 42 to 57% (w/w) sucrose density gradients, and each fraction was measured for absorbance at 630 nm (○), malate dehydrogenase (△) (μmol/min · ml), hydroxybutyrate dehydrogenase (■) (μmol/min · ml) and acetylene reduction (▼) (nmol/h·ml) as described in "Materials and Methods."

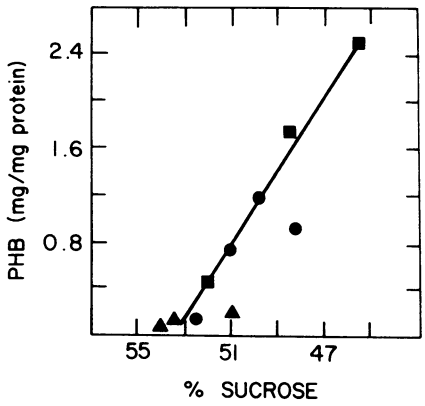

FIG. 3. PHB content on a protein basis as a function of buoyant density. The microsymbiont population was isolated from soybean root nodules and centrifuged into continuous 42 to 57% (w/w) sucrose gradients as described in "Materials and Methods." Fractions of microsymbionts were collected from sucrose gradients containing the population from 12 (▲), 19 (●), or 27 (■) DAP nodules.

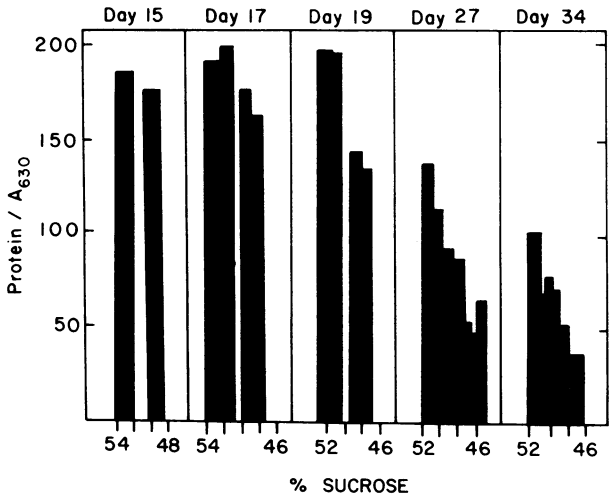

FIG. 4. The protein content of microsymbiont cells as a function of DAP and buoyant density. Units are μg protein from whole cells per *A* at 630 nm. Fractions of the microsymbiont population were collected from continuous 42 to 57% (w/w) sucrose gradients after their isolation from plants at the indicated ages as described in "Materials and Methods." The value for each fraction is the mean of triplicates (SD ± 5%) and typical of other experiments at these ages.

Enzyme Specific Activity as Related to Buoyant Density. Patterns of enzyme specific activities might be informative in relation to microsymbiont development if a particular metabolic process was associated with a specific subpopulation. Malate dehydrogenase and hydroxybutyrate dehydrogenase were chosen for this type of analysis because of their different developmental profiles (9). Malate dehydrogenase activity remained unchanged during nodule development, whereas hydroxybutyrate dehydrogenase increased at least 3-fold over the same time period (9). Ching *et al.* (3) had reported that the latter activity was 2.5 times greater in the mature bacteroid fraction than in the bacterial fraction. Malate dehydrogenase, on the other hand, might be elevated in the mature bacteroid because organic acids, particularly malate, succinate, and fumarate, caused the greatest increase in acetylene reduction rates when added to isolated suspensions of soybean microsymbionts (2).

As shown in Figure 5, two different aspects of microsymbiont development with regard to enzyme specific activity were apparent. First, considering the entire population, the development patterns of hydroxybutyrate dehydrogenase and malate dehydrogenase were similar to those we had reported previously (9); hydroxybutyrate dehydrogenase increased with age, whereas malate dehydrogenase remained constant. Second, no trends in the specific activities of malate dehydrogenase or hydroxybutyrate dehydrogenase within the microsymbiont population, at any given age, were observed as a function of buoyant density. The variation between the specific activity of each fraction was within the standard deviation of the populations' average activity (SD ± 7 to 10% at 18 and 19 DAP and within 20% at 12 DAP). At 27 DAP, the variation in activity was less than 10% among fractions between 47 and 50% (w/w) sucrose, which contained over 85% of the population. The overall pattern at 27 DAP reflected the change in protein content (Fig. 4). The same pattern of uniformity in the specific activities of fumarase, isocitrate de-

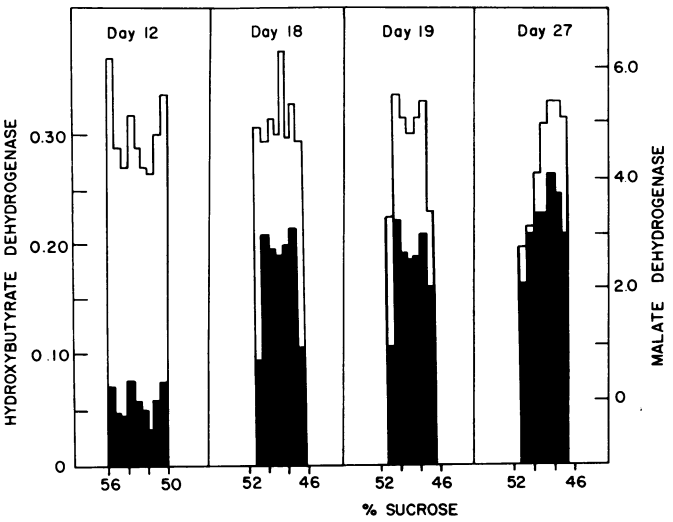

FIG. 5. Specific activity of malate dehydrogenase and hydroxybutyrate dehydrogenase within the microsymbiont population as a function of buoyant density and plant age. Fractions of the microsymbiont population were collected from continuous 42 to 57% (w/w) sucrose density gradients, and the specific activity (μmol/min/mg protein) of malate dehydrogenase (open bars) and hydroxybutyrate dehydrogenase (solid bars) was determined for each fraction as described in "Materials and Methods." Values are the mean of six assays (SD < 10%) except at 12 DAP (SD ± 20%) and typical of other experiments at these ages.

Table I. *Viability of Microsymbionts of Various Ages as Function of Buoyant Density*

Buoyant Density	Percent Viability[a] at Nodule Age (d):				
	12	14	19	27	34
% sucrose, w/w					
55.0	63				
54.0	70	52			
53.1	80	50	85		
52.2	78	—[b]	95	88	84
51.3	66	48	95	84	81
50.0	72	44	105	78	66
49.1		38	100	68	48
48.2		42	90	62	39
47.3			80	51	33
46.5				40	24

[a] Values are the mean of six determinations, three each at two different dilutions, SD ± 15%. Experiments at each age repeated at least twice. [b] — = sample lost.

hydrogenase and pyruvate dehydrogenase was observed (data not shown). When all experiments were considered, no obvious patterns indicative of a subpopulation were observed.

Viability of Microsymbionts. Since overall changes in the metabolic processes or the integrity of the microsymbiont cell might be reflected by its plating efficiency, viability of the microsymbiont was measured during nodule development. Subjecting the microsymbiont population to centrifugation in continuous sucrose gradients did not affect plating efficiencies. At 12 DAP, viability increased from 27% to 75% after fractionation. This probably reflected the removal of extraneous plant material from the relatively small microsymbiont mass in the 8000*g* pellet. Through the period of maximum acetylene reduction activity, viabilities of all the fractions were similiar and approached 100% (Table I). These values were in agreement with those reported by others (7, 19). However, by 27 DAP, viability in the fractions at lighter buoyant densities had decreased by 50% as compared to the heavier densities. At 47 DAP the maximum viability was about 90 ± 12% and only 6 ± 2% in the lighter fractions. This trend toward lower viability in the lighter fractions of microsymbionts from older nodules was similar to those observed in protein content and acetylene reduction activity (Figs. 4 and 6). These differences occurred during the period when whole nodule acetylene reduction activity was declining (9).

Acetylene Reduction of Microsymbionts. Two aspects of the development of acetylene reduction of the microsymbiont population were examined. The first was the temporal development of acetylene reduction activity of the entire microsymbiont population (Fig. 6). We had previously reported that, under our plant growth conditions, the maximum acetylene reduction activity of nodulated root segments appeared around 21 DAP, and the increase in activity between 14 and 21 DAP was generally 3-fold (8, 9). The subsequent decrease was about 50% by 28 DAP (8, 9). A similar developmental pattern in acetylene reduction activity, when expressed as microsymbiont concentration (A_{630}), was maintained in the isolated microsymbionts (Fig. 6). The acetylene reduction activity increased 5- to 6-fold between 12 and 20 DAP (3 ± 0.5 to 18 ± 2 nmol C_2H_4/h · A_{630}), and by 27 DAP the activity had diminished to 33% (6 ± 2 nmol C_2H_4/h · A_{630}) of the maximum activity (Fig. 6). With respect to the temporal development of activity of the microsymbionts at a given buoyant density, the same pattern was observed. For example, the activity of microsymbionts collected at 49% (w/w) sucrose increased 5- to 8-fold between 12 and 20 DAP and decreased at older DAP. The same pattern occurred in the microsymbionts collected at heavier densities (Fig. 6).

The acetylene reduction activity, on a protein basis, of the isolated microsymbionts increased in all microsymbiont fractions and reached maximum activity at 27 DAP. The shift in maximum activity to older microsymbionts reflected the 50% decrease in cellular protein content observed between 19 and 27 DAP (Fig. 4). Thus, the temporal changes in activity, whether expressed on a cellular or protein basis, were a common property shared throughout the microsymbiont population.

The similarity of the developmental patterns of acetylene reduction activities of (a) nodulated root segments, (b) the microsymbionts obtained from nodule homogenates (8000*g* pellet), and (c) the microsymbionts obtained after centrifugation through continuous sucrose gradients, indicated that the experimental manipulations did not result in substantial losses of nitrogen fixation capacity. Accurate estimation of actual recoveries of acetylene reduction activity of the microsymbionts during the various steps in the isolation procedures was difficult due to the variable amount of contaminating plant material in the nodule homogenate. The extraneous plant material affected both the A_{630} and protein measurements.

The second aspect considered was the relationship, at a given age, between the acetylene reduction activity of the microsymbionts and their buoyant density. Based on microsymbiont concentration (A_{630}), the acetylene reduction activity appeared unrelated to the buoyant density of the microsymbionts (Fig. 6). Differences in activities, at a given age, between fractions collected at various buoyant densities (% sucrose) were less than 20%. The activity of each fraction was within 7% of the populations' mean activity. Likewise, when based on protein, activity differences between fractions, at a given age, were within 25% of the population's mean activity, except at 17 DAP (Fig. 6). The apparent differences at 19 and 34 DAP did not seem indicative of subpopulations with different acetylene reducing activities, because, at 19 DAP, the fraction of lowest activity contained less than 10% of the population and therefore was not

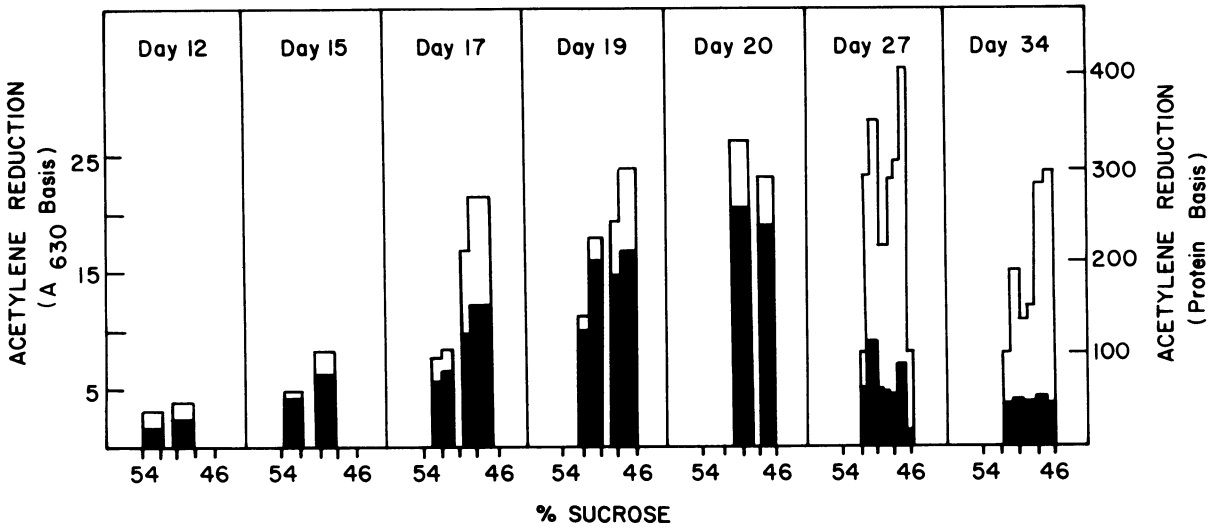

FIG. 6. Distribution of the acetylene reduction activity of microsymbionts as a function of plant age and buoyant density. The microsymbiont population was isolated from nodules of plants at the indicated ages and centrifuged into continuous 44 to 56% (w/w) sucrose gradients. Fractions were collected from the gradients and resuspended in 4 mM $MgCl_2$, 4 mM succinate, 40 mM HEPES (pH 7.4) and assayed for acetylene reduction activity as described in "Materials and Methods." The acetylene reduction activity of the microsymbionts at each buoyant density was calculated on a protein (open bars; nmol/h · mg protein), and on a A_{630} basis (closed bars; nmol/h · A_{630}). Values are the average of duplicate assays, two each at air:A_{630} ratios of 0.8, 1.0 and 1.5, (SD ± 5 to 15%). A_{630} refers to the optical density of the assay suspension diluted 6-fold as described in "Materials and Methods." The data represent measurements collected from a series of experiments in which microsymbionts from nodules at three different ages were examined. Experiments were conducted such that at least one age was in common with previous experimental sets. Each bar represents the following percentage of the total population: 12, 15, and 20 DAP, 30%; and 17 and 19 DAP, 10 to 20%. The region between 48 and 54% (w/w) sucrose at 12 and 15 DAP, and between 46 and 52% (w/w) sucrose at 19, 20, 27, and 34 DAP, contained 90 to 95% of the total microsymbiont population.

considered meaningful. At 34 DAP, the 3-fold difference between the fractions of lightest buoyant densities and those of the heaviest resulted from the 3-fold difference in protein content of the microsymbiont (Fig. 4). At 17 DAP, acetylene reducing activity doubled on microsymbiont concentration basis (A_{630}) and tripled on a protein basis. This difference was greater than the combined variation (SD ± 25%) of the specific activity between light and heavy fractions and could not be explained by experimental variables, since similar quantities of microsymbionts had been collected in each fraction and assays contained equivalent concentrations of cells or protein.

Ching *et al.* (3) reported an approximately 7-fold difference in acetylene reduction activity between the fractions they defined as mature bacteroids and bacteria. We observed only about a 3-fold difference, and this difference was related to the gradually changing protein concentration rather than an individual subpopulation of definable buoyant density. Because of the differences in the experimental parameters, plant cultivars and *Rhizobium* inoculum used, direct comparison cannot be made between our results and Ching *et al.* (3). The disparity between our studies and those of Ching *et al.* suggest that the characteristics of the microsymbiont population may depend on the nature of inoculum, strain and cultivar used.

SUMMARY

The evidence presented here did not support the presence of discrete subpopulations of microsymbionts with distinctly different properties during symbiotic development. Symbiotic development of the microsymbiont population in nodules formed on Williams 82 soybeans after inoculation of *B. japonicum* 3I1b-143 or its derivative, 2143, proceeded as a single, uniform population.

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