Changes in the Number, Viability, and Amino-acid-incorporating Activity of *Rhizobium* Bacteroids during Lupin Nodule Development

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WILLIAM D. SUTTON, NEIL M. JEPSEN, AND BRIAN D. SHAW

Plant Physiology Division, Department of Scientific and Industrial Research, Private Bag, Palmerston North, New Zealand

**ABSTRACT**

Between days 10 and 21 after inoculation of *Lupinus angustifolius* seedlings with *Rhizobium* NZP 2257, the average nodule fresh weight increased 3-fold and the number of bacteroids per nodule increased more than 10-fold.

The viability of *Rhizobium* bacteroids, as judged by their ability to form colonies on yeast-extract agar, declined from about 10% on days 10 and 11 after inoculation to about 0.3% on days 14 to 25. Bacteroid viability was highly sensitive to osmolarity.

At optimal pH and K and Mg ion concentrations, the incorporation of $^{14}$C-glycine into isolated bacteroids was also very sensitive to osmolarity, and fell in parallel with bacteroid viability during nodule development.

We suggest that at least two processes contribute to bacteroid nonviability: a reversible change in the cell wall structure occurring between days 10 and 14 after inoculation, and a subsequent irreversible change.

The inability of *Rhizobium* bacteroids isolated from root nodules to divide on nutrient media, reported by Almon (2), has been confirmed (4) but not yet explained. Since nonviable differentiated forms of bacteria are very rare, it has been suggested that there may be some relationship between the inability of bacteroids to divide and the mechanisms whereby the plant controls bacteroid gene expression (9, 11). We are aware of only two sets of observations that may be relevant to this suggestion.

a. In the last 20 years, several groups have suggested that during nodule development, the DNA of *Rhizobium* bacteroids is degraded, reduced in amount, or chemically modified (1, 3, 5, 7). Our own observations have not supported these suggestions: we found that several strains of *Rhizobium* bacteroids had amounts of DNA per cell equivalent to at least one complete bacterial chromosome, and that DNA isolated from purified bacteroids was similar to bacterial DNA in buoyant density, melting temperature, and kinetic complexity (9). Similar findings have recently been reported by Reijnders et al. (8).

b. *Rhizobium* bacteroids have a low ratio of intact ribosomal RNA to DNA (11), suggesting that their capacity for protein synthesis may be restricted.

Neither of these observations has been shown to bear any direct relationship to nonviability, and such questions as whether bacteroids are able to divide within the nodule at a time when they cannot divide on nutrient media, and whether nonviability develops before or after nitrogen-fixing ability seem to have been neglected.

In this paper, we characterize the growth of bacteroid numbers and the development of nonviability in *Rhizobium*-infected lupin nodules, show that one cause of nonviability is the osmotic sensitivity of bacteroids, and explore the use of amino acid incorporation by bacteroids as a biochemical indicator of viability.

**MATERIALS AND METHODS**

**Growth Conditions.** Nodulated lupins (*Lupinus angustifolius* L. cv. Bitter Blue) were grown under controlled environment conditions (10), and *Rhizobium* strain NZP 2257 was cultured as previously described (9).

**Isolation of Bacteroids.** Nodules were detached from the top 2 to 3 cm of the primary roots of at least three plants, rinsed several times with distilled H$_2$O, and ground in a chilled TenBroek glass homogenizer with approximately 10 vol of ice-cold 0.5 mannitol, 50 mm tris-HCl (pH 7.4). The homogenate was filtered through two layers of Miracloth and then centrifuged at 6,000g for 5 min at 4 C. The supernatant was discarded and the bacteroid-containing pellet resuspended in mannitol-tris buffer and either used without further purification or else repelted at 6,000g and resuspended again.

**Viability Measurements.** Nodulated roots were surface-sterilized with 0.1% (w/v) HgCl$_2$ for 5 min, then rinsed three times with sterile distilled H$_2$O, and bacteroids were isolated as above. The bacteroid concentration was measured in a Hawkesley-Thoma counting chamber by dark field microscopy, then 0.1- to 0.2-ml portions of diluted bacteroids in mannitol-tris buffer were spread on Petri dishes containing 20 to 30 ml of yeast extract agar (0.5 g Difco yeast-olate, 0.5 g KH$_2$PO$_4$, 0.2 g MgSO$_4$·7H$_2$O, 0.1 g NaCl/l, adjusted to pH 6.8 with KOH), sealed with Parafilm, incubated at 25 C for 7 to 10 days, and colonies counted. The dilutions were chosen to give 100 to 500 colonies on each of two plates.

**Glycine Incorporation Experiments.** Bacteroid suspensions were diluted to 5 × 10$^6$/ml and 5-ml portions were shaken at 25 C and 250 rpm in 25-ml conical flasks with 0.016 μCi/ml of U-$^{14}$C-glycine (10 Ci/mol, The Radiochemical Centre, Amersham, England). Samples (0.7-1.0 ml) taken at intervals were diluted into 10 ml of ice-cold mannitol-tris buffer, centrifuged 5 min at 6,000g, resuspended in 5% (w/v) trichloroacetic acid, loaded onto 22-mm diameter glass fiber discs (Whatman GF/A), rinsed twice with 5% (w/v) trichloroacetic acid and twice with 1:1 (v/v) ethanol-ether, dried, and counted in a toluene-based scintillation fluid at 65% efficiency.

Quantitative comparisons between different incubation treatments (see Figs. 4 and 5) were based on the 2-hr time points. Other assessments in the results section were based on two to five time points for each treatment.
RESULTS

Nodule Development. In lupins grown under our standard conditions, nodules were first visible as small swellings on the primary root 9 days after inoculation. Between days 10 and 15, the acetylene reduction activity/mg fresh weight of nodule tissue increased more than 1,000-fold (10).

Between days 10 and 21 after inoculation, the nodules steadily increased in size (Fig. 1). In addition, between days 11 and 13, there was a 3-fold increase in the concentration of bacteroids per unit of nodule tissue, fresh weight. The combined effect is shown in Figure 1 b: a 10-fold increase in the number of bacteroids per nodule between days 10 and 19, with the multiplication rate rapidly falling off after day 12.

It was concluded that at least some nodule bacteroids are capable of dividing in situ for the first 15 to 19 days of nodule development. The doubling time of *Rhizobium* NZP 2257 in broth cultures at 25°C was 8 to 12 hr (results not shown). By assuming a similar doubling time of 8 to 12 hr for bacteroids within nodules and fitting the growth curve (Fig. 1 b) to a biphasic semilogarithmic plot, we calculated that in day 10 to day 12 nodules, 25 to 45% of the total population of bacteroids were dividing, whereas in day 13 to 19 day nodules, only 2 to 4% of the bacteroids were dividing.

Viability Measurements. When bacteroids isolated from nodules of various ages were cultured on yeast extract agar, the proportions capable of cell division and subsequent colony formation (Fig. 2) were much lower than calculated above. However, very different results were obtained when different concentrations of mannitol were added to the culture medium (Fig. 3). Adding 0.3 M mannitol increased the number of colonies formed more than 10-fold, and similar effects were obtained with 0.3 M sucrose (results not shown).

The effect of mannitol was probably not caused by a general stimulation of carbon metabolism, because when broth-cultured *Rhizobium* bacteria were plated out on the same range of media, the largest colonies were obtained on 0.1 M mannitol, a concentration that had little effect on bacteroid colony formation. Mannitol at higher concentrations reduced the size of bacterial colonies, and also slightly reduced the numbers (Fig. 3).

When bacteroid colony-forming ability on 0.3 M mannitol medium was followed as a function of nodule development, it was found to drop gradually from about 50% on days 11 to 12 to about 2% on day 25 (Fig. 2). Concomitant with this, the time taken for most colonies to reach a countable size increased from 5 days to about 10 days. The variation in colony size also increased. These results suggest that many bacteroids from older nodules go through a long lag phase before commencing cell division in vitro.

The ability of isolated bacteroids to form colonies when plated out was not affected by incubating crude nodule homogenates for 30 min at 0°C, but was reduced about 2-fold after 30 min at
25 C. Since our routine procedure for preparing bacteroids was carried out at 0 to 5 C and took less than 30 min, it seems unlikely that the results in Figure 2 could have been caused by bacteroids being exposed to toxic substances or degradative enzymes during isolation.

Glycine Incorporation. Bacteroid suspensions incorporated 14C-labeled glycine into acid-insoluble material at a constant rate for 2 to 6 hr. No lag was detected when samples were taken as early as 5 min after the addition of labeled glycine. The optimum pH was 6.5. With either MES-KOH or KCl, the optimum K ion concentration was about 30 mM. The optimum Mg ion concentration was zero, even 0.1 mM Mg2+ being slightly inhibitory (6%). EDTA at 1 mM was also inhibitory (64%). Similar optimum values of pH, K+, and Mg2+ were obtained with rinsed, broth-cultured cells of Rhizobium NZP 2257.

No differences in bacteroid glycine incorporation resulted from the replacement of 0.3 mM mannitol with either 0.3 mM sucrose or 0.3 mM glucose, or from supplementing 0.3 mM mannitol with either 10 mM succinate or 10 mM malate. At a constant cell density of 5 × 10^6/ml, there was no effect of shaking rate (0-250 rpm) or flask volume (25-50 ml). There were no consistent differences in bacteroid glycine incorporation when nodules were homogenized in media of different pH (6.5-7.4), mannitol concentration (0.3-0.5 mM), carbohydrate composition (mannitol, sorbitol, or sucrose), or reducing power (0-5 mM 2-mercaptoethanol).

When bacteroids were incubated with 2 mM K glutamate, glycine incorporation was reduced by about 50%, but a variety of other nitrogenous compounds (potassium nitrate, ammonium sulfate, l-asparagine, and l-glutamine, all at 2 mM and adjusted to pH 6.5) had no effect. When bacteroids were supplied with an equimolar mixture of 20 l-amino-acids (at concentrations of each ranging from 1.6-8 mM in different experiments), they incorporated no more 14C-glycine than bacteroids supplied with the same concentration of glycine alone.

A major difference between bacteroids and broth-cultured Rhizobium was in their response to mannitol. Even 0.1 mM mannitol had an inhibitory effect on glycine incorporation into the broth-cultured bacteria, yet bacteroids in the absence of mannitol were virtually inactive (Fig. 4). The optimum mannitol concentration for isolated bacteroids increased from about 0.25 mM on days 10 to 11 to about 0.4 mM on days 17 to 19, but 0.3 mM was near optimal for all ages (Fig. 4).

Correlation between Glycine Uptake and Colony-forming Ability. The pronounced sensitivity to mannitol concentration shown by both bacteroid viability (Fig. 3) and bacteroid glycine incorporation (Fig. 4) suggested that there might be some relationship between the two phenomena. We therefore character-

Fig. 4. Glycine incorporation by bacteroids and bacteria as a function of mannitol concentration. All media contained 50 mM MES-KOH (pH 6.5) (K+ = 35 mM). The greatest 2-hr incorporation observed for each sample was taken as 100%: a: (●): 10-day bacteroids, peak 2-hr incorporation 9,790 counts/min·ml; (○): 19-day bacteroids, peak 2-hr incorporation 5,321 counts/min·ml; b: (△): broth-cultured Rhizobium NZP 2257, peak 2-hr incorporation 3,220 counts/min·ml.

Fig. 5. Glycine-incorporating activity of bacteroids in different mannitol concentrations as a function of time after inoculation; (•): 0.1 mM mannitol, single measurements; (○): 0.2 mM mannitol, single measurements; (▲): 0.3 mM mannitol, each point represents the mean (± SE) of five to 10 independent bacteroid preparations; (-----): colony-forming ability of bacteroids on media containing zero or 0.055 mM mannitol, data taken from Figure 2.

ized the changes in bacteroid glycine incorporation as a function of nodule age.

The glycine-incorporating activity of bacteroids in a medium containing 0.1 mM mannitol declined over the same period of time, and to about the same extent, as colony-forming ability on yeast extract agar (Fig. 5). Glycine incorporation in a medium containing 0.2 mM mannitol fell about 20-fold between days 11 and 21. Glycine incorporation in 0.3 mM mannitol fell about 2-fold over the same period (Fig. 5), a result confirmed with many independent preparations of bacteroids.

DISCUSSION

Although bacteroid numbers in the nodule increased for at least 15 days after inoculation (Fig. 1), the ability of bacteroids to form colonies on nutrient media of low osmolarity declined rapidly after day 11, apparently because of their increasing osmotic sensitivity (Figs. 2, 3, and 4). This is not likely to be a simple adaptation by bacteroids to the high osmolarity of nodule cytoplasm, because growth of Rhizobium NZP 2257 for up to 10 days on 0.3 mM mannitol medium had no effect on subsequent colony-forming ability (results not shown). A more likely explanation for the osmotic sensitivity of bacteroids would appear to be a change in the structure of the cell wall, perhaps resulting from digestion by plant enzymes. Differences between the cell walls of bacteroids and broth-cultured bacteria have already been reported (6).

Although many apparently nonviable bacteroids from day 12 to day 14 nodules were able to form colonies when cultured on a medium containing 0.3 mM mannitol, this ability was lost as the nodules grew older (Fig. 2). We would like to propose two possible explanations. The first is that there may be a further and irreversible change in the structure of the bacteroid cell walls. The second is that the initial change in bacteroid cell wall structure may result in greater permeability of the wall as well as greater osmotic sensitivity, and that as a consequence plant substances enter the bacteroids and cause irreversible damage.

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