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Viability of Alfalfa Nodule Bacteroids Isolated by Density Gradient Centrifugation

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Abstract

The question of the viability of mature *Rhizobium meliloti* bacteroids has been difficult to resolve unequivocably because extracts of alfalfa root nodules contain a complex mixture of bacterial cells in different stages of development. A non-damaging method based on density gradient centrifugation in Percoll is described for separation of 3 distinct fractions from nodule extracts. An advantage of this method is the retention of nitrogenase activity by isolated intact mature bacteroids indicating minimal loss of coupling between bacteroid respiration and the nitrogenase enzyme system. It was found that the isolated mature bacteroid fraction while containing 90% of the respiration-supported nitrogenase activity of the extracts did not contribute significantly to the number of viable cells present. The viable bacterial cells were not sensitive to gentle sonication while enlarged cellular morphology and induction of loss of cellular integrity by sonication were characteristics of transforming and mature bacteroids.

Nitrogenase activity was associated primarily with the mature bacteroids although the fraction designated as transforming bacteria had 5% of the total nitrogenase activity. Examination of the isolated fractions with phase contrast microscopy revealed that the cells could be classified into two general categories based on size. Cells were either large (6-7 μm in length), pleomorphic, translucent and bacteroid-like in appearance or they were much smaller (1–2 μm in length), uniform and opaque similar to the free-living bacteria in size. Sonication of all three fractions at low power completely destroyed the large type cells and left the smaller, bacteria-like cells intact. When tested for viability, the sonicated and the non-sonicated (control) aliquots from each fraction gave identical results within experimental error. The mature bacteroid, transforming bacteria and the bacterial fractions contained 2%, 27%

and 29% viable cells, respectively. Incubation temperature, osmoticum (mannitol) concentration, carbon source (succinate) and incubation at low oxygen levels after anaerobic extraction has no effect on viability of any of the three fractions. It was concluded that once *R. meliloti* cells had developed to the stage of the larger or y-shaped structures associated with mature bacteroids *in situ*, viability was lost irreversibly.

Keywords: *Rhizobium meliloti*, percoll density gradients, bacteroid fractionation, bacteroid variability, microscopy, nitrogenase

1. Introduction

Previously, determinations of viability have not yielded consistent results when bacteroids and nodule bacteria have been extracted from the nodules of various legumes. The viability of bacterial cells isolated from alfalfa nodules is defined as the ability to reproduce and form colonies on agar plates containing nutrients which support non-diazotrophic growth. Estimates of bacteroid viability have ranged from 90% for cells isolated from soybean nodules (Tsien et al., 1977) to 60–90% for clover nodules (Gresshoff et al., 1977) to 10% for lupin nodules (Sutton et al., 1977). Some factors which influenced bacteroid viability were nodule age and the osmotic environment of the nutrient agar which was used for plating. In addition, a complication affecting the estimation of viability is the fact that crude bacteroid preparations contain a combination of cell types differing in morphology and representing nodule bacteria at various stages of differentiation. Consequently, reports on bacteroidal viability using crude bacteroid preparations may include contributions from cells other than bacteroids as defined here (cf. Gresshoff et al., 1977). In the present work the term "bacteroid" refers specifically to cells which have undergone a process of differentiation culminating in the expression of nitrogenase activity and the associated metabolic support systems.

Separation of crude bacteroid preparations from soybean nodules into 3 different fractions using sucrose density gradients has been reported by Ching et al. (1977). While sucrose gradients have been used to separate crude bacteroid fractions sedimented by centrifugation into the 3 fractions from nodules of soybeans and alfalfa (Paau et al., 1979), self-generating Percoll gradients have not been reported previously for a similar separation of bacterial cells obtained from alfalfa nodules (cf. Reibach et al., 1981). The use of Percoll to form gradients is preferable to sucrose since the colloidal medium avoids any substantial change in osmotic environment that the bacteroids would be subjected to on passage through a sucrose gradient. This study

has utilized 2 successive Percoll density gradient centrifugations to separate 3 fractions of *Rhizobium meliloti* cells from alfalfa nodule extracts. Each fraction was tested for viability, characterized by microscopic examination and assayed for various enzyme activities.

2. Materials and Methods

Plant growth conditions

Alfalfa seedlings (cv. Saranac) were innoculated with *Rhizobium meliloti* (strain Balsac) and grown under controlled environmental conditions as previously described (Miller et al., 1988). After 7 weeks, the nodules were harvested for bacteroid extraction.

Extraction and fractionation of bacteroids

Approximately 8–10 g fresh weight of nodules were homogenized in an extraction medium containing 125 mM KCl; 50 mM disodium succinate; 50 mM sodium N-tris (hydroxymethyl) methyl-2-amino-ethane sulfonate (TES) buffer at pH 7.0 and 0.1% bovine serum albumin (Miller et al., 1988). After removal of the cell debris from the homogenate by centrifugation at 100×g for 10 min, the supernatant fluid was then centrifuged at 1500×g for 5 min. The resulting pellet was resuspended in 1 ml of extraction medium and fractionated into 2 components by layering over 35 ml of 55% Percoll suspended in the extraction medium and centrifuging at 48,000×g for 30 min. After centrifugation, 2 broad, well resolved turbid bands appeared, one occupying the upper half and the other the lower half of the gradient. Both bands were collected with a Pasteur pipette, diluted 1:5 with extraction medium and centrifuged at 5,000×g for 5 min. The pellets were then resuspended in 20 ml of extraction medium and sedimented a second time to ensure the removal of Percoll. The pellet from the upper band was resuspended in extraction medium and is subsequently referred to as the mature bacteroid fraction. The pellet from the lower band was resuspended in 1 ml of extraction medium and layered over 35 ml of 70% Percoll in the extraction medium and centrifuged at 48,000×g for 30 min. The lower band from the 55% Percoll tube separated further into 2 distinct bands after centrifugation with one band located in the upper half and the other located in the bottom half of the tube. Upper and lower bands were collected with a Pasteur pipette and washed free of Percoll as described above. Hereafter, the cells of lower density are referred to as the transforming bacteria and the cells banding at higher density as the bacteria (nodule). Density values along the Percoll

gradient after centrifugation were calibrated with coloured density marker beads (Sigma Chemical Co.). All solutions used in the isolation procedure described were sealed in vials and purged with oxygen-free nitrogen and all subsequent manipulations in the extraction process were carried out in an anaerobic glove chamber.

Enzyme assays

Nitrogenase activity was assayed using the acetylene reduction method (Miller et al., 1988). Aliquots of the bacteroid suspension (prepared anaerobically) were incubated with 10% acetylene and 4% oxygen at 20°C with shaking. After 15 min, a 0.5 ml aliquot was removed and assayed for ethylene. For determination of alanine and β-hydroxybutyric acid dehydrogenase activities, the bacteroid pellets were resuspended in 50 mM TES buffer, pH 7.0 and sonicated for 2, one-minute intervals. Aliquots of each of the sonicated Percoll fractions were then assayed for activity by monitoring the change in absorbance of NADH at 340 nm was described by Reibach et al. (1981).

Sonication

Cells suspended in a tube containing 20 ml of extraction medium were sonicated on ice for two one-minute intervals using a Branson Sonifier (Model S125) tuned to give a meter reading of 5 amp at power setting 2. The stainless steel probe used as 16 cm in length tapering to 3 mm in diameter at the tip.

Viability determinations

Nodules were surface sterilized with 2% sodium hypochlorite (Javex) for 1–2 min followed by at least 10 washes with sterile distilled water. Bacteroids and nodule bacteria were then extracted as usual in a laminar flow cabinet with sterile reagents and materials. The succinate-containing extraction medium was sterilized by passage of a 10-fold concentrated stock solution through a sterile, 0.45 μm millipore filter. Non-autoclavable materials were sterilized by rinsing in 75% ethanol for 10 min followed by 3 rinses in sterile distilled water. Cell counts from the various fractions separated on Percoll were obtained using a Petroff-Hausser bacterial cell counter with a phase contrast microscope equipped with an oil immersion lens. The cells were then diluted to 3 different values (50–300) and plated on yeast extract mannitol agar containing 0.1 g sodium chloride, 0.2 g magnesium chloride, 0.5 g dipotassium hydrogen phosphate, 10 g mannitol, 1 g yeast extract and 15 g of agar per litre.

Table 1. Characterization of the bacteroid fractions separated by Percoll density gradients

	Mature bacteroids	Transforming bacteria	Bacteria	Free-living bacteria
Relative proportions[a] (% of total cells)	65	24	11	–
Density	1.059	1.090	1.102	1.102
Protein[a] (mg/cell×10^9)	2.17	1.37	0.73	0.30
Nitrogenase[a] (nmoles C_2H_4/hr/mg protein)	155	10	0	0
Alanine Dehydrogenase[a] (η moles/hr/mg protein)	13.7	10.6	1.9	1.2
Hydroxybutyrate Dehydrogenase[a] (η moles/hr/mg protein)	1.86	1.74	0.24	0

[a] Values represent the mean of three separate determinations. All values fell within a standard error range of 10 per cent of the mean values.

Photography

Cells were immobilized on detergent-cleaned, polylysine (800 Kd) coated slides for phase contrast microscopy. Cells were examined with a 100× oil immersion objective on a Nikon phase contrast microscope equipped with a Nikon HFX-II camera. For electron microscopy, portions of the pelleted fractions were fixed with 2% (v/v) glutaraldehyde for 2 hr on ice and 4% (w/v) osmic acid overnight at 2°C. Fixation solutions contained 180 mM KCl and 50 mM TES, pH 7. Following dehydration through an ethanol series and infiltration with propylene oxide and Epon 812 resin, the cured pellets were sectioned and mounted on formvar-coated copper grids. Staining with 5% (w/v) lead citrate-uranyl acetate was carried out for 20 min and the sections were examined and photographed with a Phillips 300 transmission electron microscope.

3. Results

Fractionation of the crude bacteroid preparation by gradient centrifugation on the 55% and 70% Percoll mixtures resulted in 3 distinct bands of cells designated as the mature bacteroids, transforming bacteria and nodule bacteria having mean densities of 1.059, 1.090 and 1.102, respectively (Table 1). All 3 bands were separated completely though very broad in appearance. Density

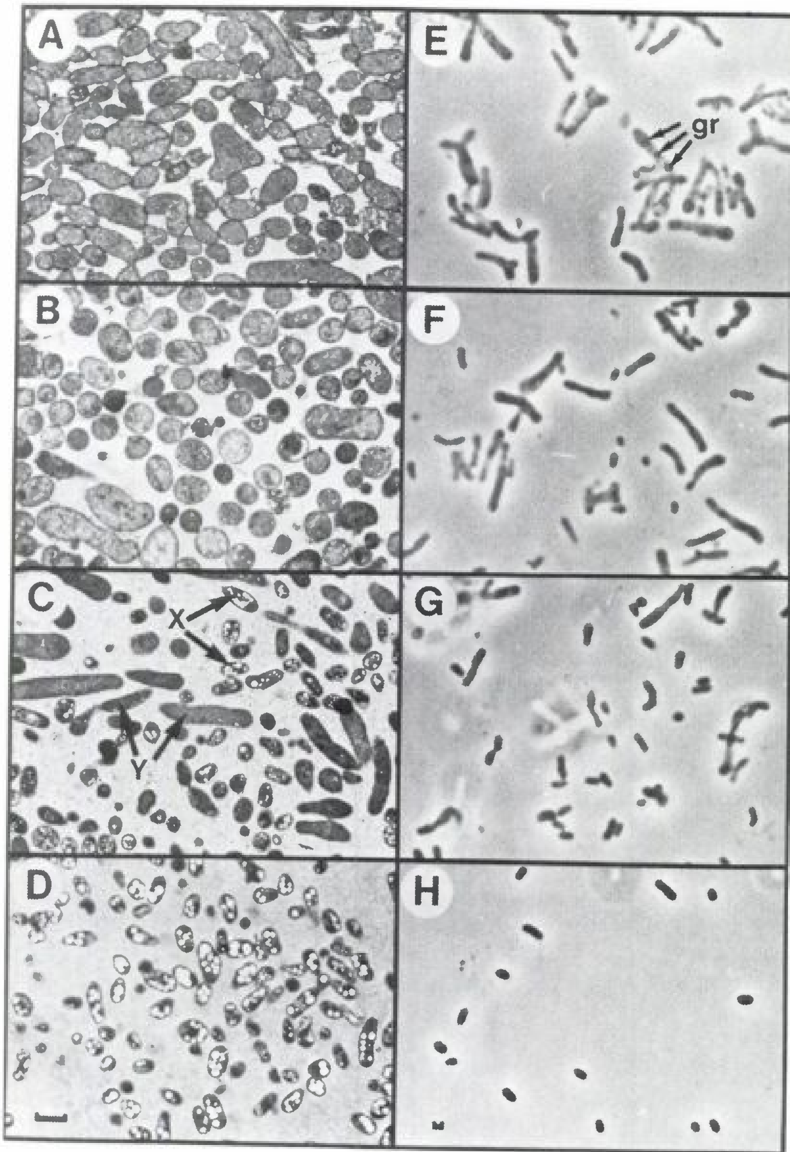

Figure 1. Electron transmission (A–D) and phase contrast (E–H) micrographs of mature bacteroids (A, E); transforming bacteria (B, F); bacteria (C, G); and free-living bacteria (D, H). Cells designated X in (C) contain polyhydroxybutyrate granules. Larger cells (Y) lack these granules. Magnification; bar (⊔) in lower left-hand section in (D) and (H) indicates: 1 μm. The bar in D applies to A–D; bar in H applies to E–H for transmission electron and phase contrast micrographs, respectively. Bacterial and nodule bacterial fractions were prepared as stated under "Methods and Materials".

measurements were based on the density at the center of t he band. Viewed under the electron microscope the bacterial fraction (Fig. 1C) appeared to contain at least two populations. One cell type (X) was smaller than the other and resembled the free-living bacteria in appearance although the white, intracellular granules of polyhydroxybutyrate (PHB) were reduced in size and more numerous than those observed in the free-living bacteria (Fig. 1D). The second cell population were elongated to an average of 6.5 μm and devoid of any well-defined internal structure (Y). The transforming bacterial fraction (Fig. 1B) appeared quite different from the bacterial fraction in that many of the cells were enclosed within a peribacteroidal membrane and they generally appeared to be somewhat larger with a granular interior. The mature bacteroid fraction (Fig. 1A) appeared to be homogenous and slightly larger in size with most cells enclosed in a peribacteroidal membrane. All fractions were free of mitochondria or other recognizable plant cell organelles. When the corresponding unfixed cells were viewed with the phase contrast microscope the cells were surrounded by a halo of light indicating retention of the cellular membranes relatively intact (Hall, 1972). In the mature bacteroid fraction (Fig. 1E) the bacteroids appeared as large translucent structures revealing what appeared as large, darker staining bodies (gr) usually located towards either end of the elongated cells. In both the transforming bacteria (Fig. 1F) and bacterial (Fig. 1G) fractions, the cells could be classified into two distinct populations based primarily on size. Those which were short (approx. 1.5 μm) and opaque resembling the free-living bacteria (Fig. 1H) in appearance and those which were larger (approx. 6.5 μm) and translucent more closely resembling the mature bacteroids in appearance.

When assayed for nitrogenase the mature bacteroid fraction contained 95% of the acetylene reducing activity of the crude bacteroid fraction while the transforming bacteria fraction designated contained only 5% of the total activity and no activity was detectable in the bacterial fraction (Table 1). Alanine dehydrogenase and hydroxybutyric acid dehydrogenase activities are considered to be enzymes associated with bacteroids (Ching et al., 1977; Reibach et al., 1981). In both cases these two enzymes had highest activity in the mature bacteroid fraction with activity being progressively lower in the putative transforming and bacterial fractions (Table 1). Hence, it is probable that the expression of neither nitrogenase proteins nor supporting enzyme systems was maximal in the transforming cells.

When the mature bacteroid fraction was sonicated for 2 one-minute periods the bacteroids were all disrupted and the resulting residue appeared as

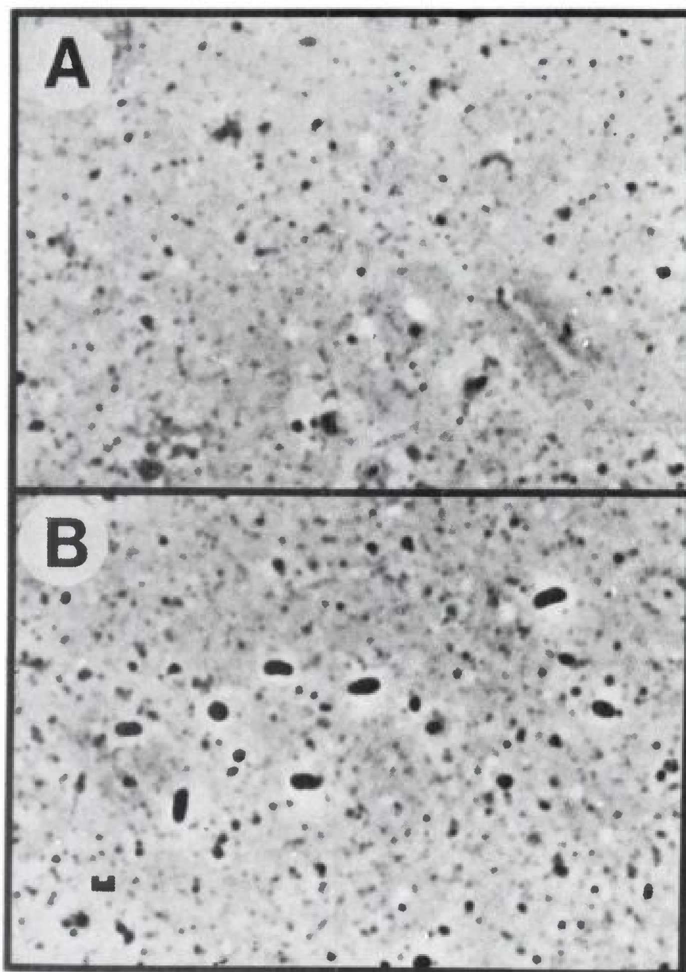

Figure 2. Phase contrast micrographs of sonicated mature bacteroids (A) and sonicated nodule bacteria (B). Magnification; bar (⊔) in lower left-hand section in (B) indicates 1 μm in both A and B.

small particles of debris under the phase contrast microscope (Fig. 2A). When either the transforming bacteria or the bacterial fractions were sonicated in the same way, the elongated, bacteroid-like structures were also disrupted but the small, opaque structures similar to the free-living bacteria remained physically intact (Fig. 2B). Sonicated and non-sonicated (control) cells from the various fractions were counted for cell number and then serially diluted onto agar plates to determine the effect of gentle sonication on cell viability

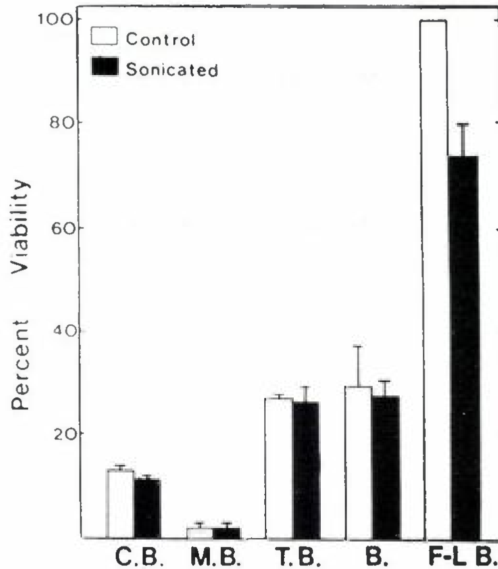

Figure 3. Effect of sonication on bacteroid viability. Crude Bacteroids (CB); Mature Bacteroids (MB); Transforming Bacteria (TB); Bacteria (B); Free-Living Bacteria (FL-B).

(Fig. 3). In all 3 fractions, the conditions under which sonication was carried out had no effect on the viability of cells compared to the non-sonicated controls. The mature bacteroids had the lowest number of viable cells of all 3 fractions with only 2% of the counted cells forming colonies. The transforming bacteria and bacterial fractions contained viable cells corresponding to 27% and 29% respectively, of the total cell count. With the crude bacteroid preparation, sonication again had no effect on viability as compared to the non-sonicated cells (12% of cells viable). Sonication of the free-living bacteria grown to late log phase in liquid culture killed approximately 30% of the viable cells in contrast to the undifferentiated nodule bacteria which appeared to be resistant to gentle sonication.

In order to determine whether other factors such as incubation temperature, osmoticum concentration, carbon source and the level of oxygen present during the bacteroid extraction and incubation of the agar plates had any influence on cell viability, experiments were carried out in which the impact of these various parameters (Table 2) was examined. No colony growth on agar plates could be detected at 15°C after 7 days. If the plates were then transferred to 25°C, colony growth appeared the next day with no observ-

Table 2. Effect of various parameters on the viability of bacteroid cells plated on yeast extract mannitol agar plates

Treatment variable	Mature bacteroids	Transforming bacteria	Bacteria
	– – – – – – percent viable cells – – – – –		
Control	2±1	27±2	29±8
Temperature (15°C)	1±1	25±5	31±7
Osmoticum (mannitol 0.4 M)	1±1	27±6	28±7
Carbon source (succinate 50 mM)	2±2	28±6	32±6
Oxygen (0.5%, 1%, 2%)	2±1	28±4	30±7

Mean values of 5 determinations are given and standard deviation from the mean indicated.

able difference in viability between these plates and controls. If the mannitol in the agar was increased to 0.4 M to provide an isotonic medium for the bacteroids, no effect on viability in any of the 3 fractions could be detected. Finally, bacteroids were extracted and plated anaerobically and then incubated under atmospheres of 0.5%, 1% and 2% oxygen for a period of 3 days. No colony growth could be detected after this period of exposure to low oxygen levels. Growth only began after atmospheric levels of oxygen were restored. However, no differences in the viability of the fractions could be detected following this treatment. Mature bacteroids were not able to grow under any of the sets of experimental conditions described above.

4. Discussion

Self-generating Percoll gradients have been widely used to achieve separation of various plant cellular organelles differing in density such as mitochondria, chloroplasts and vacuoles (Jackson and Moore, 1979; Mills and Joy, 1980). A major advantage in the use of Percoll density gradients is that the Percoll colloid does not contribute significantly to osmolarity thus ensuring that isotonicity can be maintained throughout the gradient. This is in contrast to sucrose gradients where the components being separated experience large differences in osmotic strength as they sediment through the gradient. Single stage, isopycnic centrifugation in Percoll has been used previously in the purification of bacteroids from soybean nodules (Reibach et al., 1981) but

resulted in only a single band which contained a mixture of all bacteria from the nodule. Previously, Ching et al. (1977) and Paau et al. (1979) separated bacteroid preparations into mature bacteroids, transforming bacteria and bacteria using sucrose gradients. In the present report mature bacteroids were separated from 2 other fractions which co-migrated during an initial separation on 55% Percoll. The separation of the transforming bacteria and bacterial fraction was then achieved by using a 70% Percoll mixture. The entire procedure was accomplished easily and quickly taking 2 hr from the time that nodules were homogenized.

Time of separation is also an important factor if respiration-coupled nitrogenase activity is to be retained since the coupling between respiration and nitrogenase is extremely sensitive to structural or chemical damage to the bacteroid membranes (Miller et al., 1988). While the resolution of the various components was good, the bands obtained were rather broad. Such broad banding was also observed with bacteroids isolated from lupin nodules by Sutton and Mahoney (1977) who used continuous sucrose gradients suggesting that within each distinct fraction there is a wide range of cell densities. High nitrogenase activity detected in the mature bacteroids combined with the finding that the cells from all three fractions exhibited a halo of light around the cells when viewed with the phase contrast microscope (Hall, 1972) indicated that the cells sustained little peribacteroidal membrane damage during purification. The increased number of smaller granules of polyhydroxybutyrate in the bacterial (nodule) fraction and the absence of such granules in isolated mature bacteroids and transforming bacteria in whole nodules (Paau et al., 1978) suggests that the large granules of PHB in the free-living bacteria may be utilized as an energy source for development during bacterial differentiation. This hypothesis is supported by the finding that the activity of hydroxybutyrate dehydrogenase increased by about 5-fold in the transition from the nodule bacteria to the mature bacteroids. In contrast, it is of interest that in the soybean-*B. japonicum* symbiosis the situation is reversed whereby the mature bacteroids contain large granules of PHB while the free-living bacteria do not (Ching et al., 1977).

Concurrent with the increase in both hydroxybutyrate dehydrogenase and nitrogenase activity in mature bacteroids was an increase in alanine dehydrogenase activity. This enzyme has been previously detected in the bacteroidal fraction (Reibach et al., 1981; Dunna and Klucas, 1973). Since the observed specific activity of this enzyme was at a level comparable to the acetylene--reducing activity of bacteroid nitrogenase, this ammonia utilizing system

(pyruvate + NH_3 = alanine) may account for the assimilation of some of the ammonia produced inside the bacteroids by the action of nitrogenase as has been previously suggested for soybean bacteroids (Dunn and Klucas, 1973).

Viability measurements of the cells isolated in each of the Percoll fractions indicated that 27% and 29% of the transforming bacteria and bacterial fractions, respectively, were able to regenerate colonies on agar plates while the mature bacteroids had a very low viability of only 2% of the enumerated cells. Given the percentage of viable cells in each fraction (Table 1) and calculating back to the crude bacteroid preparation, the percent viability of the crude bacteroid preparation should have been 11%. This compares favourably with an actual value of 12% viable cells in the crude bacteroid preparation as determined before exposure to Percoll or separation. The results indicate that the manipulations and reagents involved in the Percoll purification had no discernable effect on the viability of the bacteroids or undifferentiated cells.

In contrast to the report of Sutton et al. (1977) raising the concentration of mannitol in the agar to a level which was iso-osmotic with respect to the bacteroids did not enhance bacteroid viability. When cells which are accustomed to growth under a microaerophilic environment are suddenly exposed to levels of atmospheric oxygen they may lose their ability to grow and divide. Thus, bacteroid cells which are present in an environment wherein the oxygen concentration is regulated by leghemoglobin may have lost their defense mechanisms against oxygen radicals (Morris, 1979; Elstner, 1982). However, when bacteroids were extracted anaerobically and maintained under very low oxygen levels (i.e. 0.5 to 2%), no increase in viability was detected, suggesting that oxygen concentration was not a factor in determining bacteroid viability on nitrogen rich media.

Sonication at low power selectively destroyed only the large translucent bacteroid-like cells. This finding correlates with the increased osmotic sensitivity of bacteroids (Sutton and Paterson, 1979) and supports the hypothesis that *R. meliloti* bacteroids have undergone extensive modification of the cell wall during differentiation (Miller and Tremblay, 1983). Since viability is unaffected by sonication, the results strongly suggest that once the bacteroids have enlarged and developed active nitrogenase support systems they no longer retain the ability to regenerate viable cells on growth medium. This conclusion is supported by the studies of Sutton and Paterson (1983) and Yanzhen et al. (1984) who, after examining a wide range of legume nodules, concluded that loss of bacteroid viability was associated with a marked increase in cell size. The proliferation of bacteria within the infection thread

and the development of mature bacteroids within the peribacteroidal membrane is a complex process involving many biochemical and physical modifications. Which of these many changes are irreversible and lead to loss of viability is not known. The use of successive Percoll density gradients to isolate and study distinct fractions in which cells representing progressive stages of bacteroid development are concentrated should prove useful in further characterizing the process of differentiation.

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REFERENCES

Ching, T.M., Hedtke, S., and Newcomb, W. 1977. Isolation of bacteria, transforming bacteria, and bacteroids from soybean nodules. *Plant Physiol.* **60:** 771–774.

Dunn, S.D. and Klucas, R.V. 1973. Studies on possible routes of ammonium assimilation in soybean root nodule bacteroids. *Can. J. Microbiol.* **19:** 1493–1499.

Elstner, E.F. 1982. Oxygen activation and oxygen toxicity. In: *Ann. Rev. Plant Physiol.* Vol. 33. W.R. Briggs, P.B. Green and R.L. Jones, eds. Palo Alto, CA, pp. 73–96.

Gresshoff, P.M., Skotnicki, M.L., Eadie, J.F., and Rolfe, B.G. 1977. Viability of *Rhizobium trifolii* bacteroids from clover root nodules. *Plant Sci. Lett.* **10:** 299–304.

Hall, D.O. 1972. Nomenclature for isolated chloroplasts. *Nature, New Biol.* **235:** 125–126.

Jackson, C. and Moore, A.L. 1979. Isolation of intact higher-plant mitochondria. In: *Plant Organelles,* Methodological Surveys (B) Biochemistry, Vol. 9. E. Reid, ed. Ellis Horwood, Ltd., Chichester, West Sussex, pp. 1–12.

Miller, R.W. and Tremblay, P.A. 1983. Cytoplasmic membrane of *Rhizobium meliloti* bacteroids. I. Alterations in lipid composition, physical properties and respiratory proteins. *Can. J. Biochem., Cell Biol.* **61:** 1334–1340.

Miller, R.W., McRae, D.G., Al-Jobore, A., and Berndt, W.B. 1988. Respiration supported nitrogenase activity of isolated *Rhizobium meliloti* bacteroids. *J. Cell. Biochemistry* **38:** 35–49.

Mills, R.W. and Joy, K.W. 1980. A rapid method for isolation of purified, physiologically active chloroplasts, used to study the intracellular distribution of amino acids in pea leaves. *Planta* **148:** 75–83.

Morris, J.G. 1979. Nature of oxygen toxicity in anaerobic microorganisms. In: *Strategies of Microbial Life in Extreme Environments.* M. Shilo, ed. Verlag Chemie, pp. 149–162.

Paau, A.S., Cowles, J.R., and Raveed, D. 1978. Development of bacteroids in alfalfa (Medicago sativa) nodules. *Plant Physiol.* **62:** 526–530.

Paau, A.S., Oro, J., and Cowles, J.R. 1979. Flow-microfluorometric analysis of bacteroids fractionated from alfalfa and soybean nodules by buoyant density sedimentation. *Plant Sci. Lett.* **15:** 63–68.

Reibach, P.H., Mask, P.L., and Streeter, J.G. 1981. A rapid one-step method for the isolation of bacteroids from root nodules of soybean plants, utilizing self-generating Percoll gradients. *Can. J. Microbiol.* **27:** 491–495.

Sutton, W.D., Jepsen, N.M., and Shaw, B.D. 1977. Changes in the number, viability, and amino-acid-incorporating activity of *Rhizobium* bacteroids during lupin nodule development. *Plant Physiol.* **59:** 741–744.

Sutton, W.D. and Mahoney, P. 1977. Preparation and fractionation of *Rhizobium* bacteroids by zone sedimentation through sucrose gradients. *Plant Physiol.* **60:** 800–802.

Sutton, W.D. and Paterson, A.D. 1979. The detergent sensitivity of *Rhizobium* bacteroids and bacteria. *Plant Sci. Lett.* **16:** 377–385.

Sutton, W.D. and Paterson, A.D. 1983. Further evidence for a plant host effect on *Rhizobium* bacteroid viability. *Plant Sci. Lett.* **30:** 33–41.

Tsien, H.C., Cain, P.S., and Schmidt, E.L. 1977. Viability of *Rhizobium* bacteroids. *Appl. Environ. Microbiol.* **34:** 854–856.

Yanzhen, C., Junchu, Z., and Huakui, C. 1984. Differentiation and viability of nodule bacteria in host cells. *Scientia Sinica* **27:** 593–600.