Short Communication

Cytochromes of *Rhizobium japonicum* 61A76 Bacteroids from Soybean Nodules

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**ABSTRACT**

Bacteroids of *Rhizobium japonicum* 61A76 were isolated from nodules of field-grown soybean plants by sucrose density gradient fractionation. The major cytochromes, aa₃, b, c, and possibly o were present in the bacteroids throughout the active nitrogen-fixing life of the nodule. This is in contrast with previous reports using other *R. japonicum* strains in which cytochromes aa₃ and o were not found.

The major terminal hemeprotein oxidases of aerobically-grown cells of various species of rhizobia have been reported to be Cyt aa₃ and a (2, 7, 12, 14, 17). Most of these reports also indicated that Cyt aa₃ and aa were not found in bacteroids of the corresponding species. Instead, new CO-binding hemeproteins including Cyt b, Cyt c, and Cyt o were found in bacteroids which were not present in cultured cells (1, 5, 12, 14).

Preliminary experiments in our laboratory with bacteroids from nodules of greenhouse-grown plants inoculated with strain 61A65 indicated that Cyt aa₃ and possibly o were present in bacteroids. To verify this observation, we inoculated field-grown plants with this strain and isolated bacteroids from nodules of various ages. Bacteroids from this *R. japonicum* strain retain Cyt aa₃ and possibly o throughout the active nitrogen-fixing life of the nodule.

**MATERIALS AND METHODS**

*R. japonicum* 61A76 was grown in a yeast extract, gluconate, inorganic salts medium (10). Soybeans (*Glycine max* cv Williams) were field grown during the summer of 1981. The seeds were heavily inoculated with 61A76 by pouring a liquid culture diluted to contain about 10⁶ cells/ml over the seeds in the rows. Uninoculated plots were interspersed with inoculated plots. At appropriate periods, the tap root nodules were harvested, washed, and frozen and kept at −70°C. The field plot had not been used previously for many years, if ever for soybeans. Thus, uninoculated control plants had an average of less than one tap root nodule per plant whereas inoculated plants had an average of 17, 23, and 29 tap root nodules at 29, 43, and 56 d, respectively. Bacteroids were prepared from nodules by grinding with mortar and pestle in the buffer described by Ching et al. (5). The postnuclei supernatant from 15 g nodules was layered on 10 ml of 35% (w/v) sucrose in 50 mM K-phosphate, pH 7.5. After centrifuging for 25 min at 17,000g, the bacteroid pellet was resuspended with 20 ml of the original buffer. Six ml were layered on stepwise gradients prepared in 50 mM phosphate consisting of 2 ml 57% (w/w) sucrose, 16 ml 50% sucrose, 12 ml 45% sucrose. The gradients were centrifuged at 22,000 rpm in a Beckman SW27 rotor for 4.25 h. The bacteroids collecting at the 45 to 50% sucrose interface were harvested and washed with 50 ml phosphate buffer, pH 6.8. This fraction corresponds to the mature bacteroid fraction described by Ching et al. (5). The bacteroids were suspended in phosphate buffer containing 0.1 mM MgCl₂ and 2 µg/ml DNase and ruptured by passage through the French pressure cell. The membrane fraction sedimenting at 183,000g (90 min) was isolated, washed, and used for analysis. Cyt content was determined by spectrophotometry using the difference spectra between ferricyanide oxidized and dithionite reduced or was determined for the CO-reactive hemeproteins using the difference between dithionite + CO minus dithionite spectra. The extinction coefficients used were those used by Appleby (1, 2). L-Malate dehydrogenase was assayed by the method of Ochoa (15). Protein was determined by the Lowry method after solubilization with 0.5 N NaOH for 10 min at 0°C using BSA as the standard.

**RESULTS AND DISCUSSION**

We have been using *R. japonicum* 61A76 for the isolation of mutants defective in terminal oxidase activity (8, 9). We observed that bacteroids of the wild-type strain isolated from greenhouse-grown plants contained levels of Cyt aa₃ similar to those found in cultured cells. This observation was not consistent with reports from other laboratories (2, 12, 14). Inasmuch as greenhouse conditions depart markedly from field conditions, we explored this observation further by inoculating field-grown plants heavily with strain 61A76 during the summer of 1981. The plot used had no recent history of soybean cultivation. Uninoculated plants had only a few nodules and most of them were on lateral roots. Therefore, we used only the earliest formed tap root nodules for these studies. Because an average of less than one nodule per plant was found in this root area of uninoculated plants, there is a high degree of certainty that the nodules contained bacteroids of strain 61A76.

Ching et al. (5) reported that the bacteroid fraction isolated from 35-d-old soybean nodules could be separated into three fractions by density gradient centrifugation. They suggested that the heaviest fraction corresponded to 'bacteria,' the intermediate fraction corresponded to 'transforming bacteria,' and the lightest fraction corresponded to 'mature bacteroids.' The mature bacteroids were devoid of Cyt aa₃ whereas the bacterial fraction and transforming bacterial fraction contained this Cyt. Ching et al. used a commercial inoculant for their studies so that the rhizobial...
strain is unknown. In order to ascertain whether the Cyt aa₃ which we found to be present in preliminary experiments could be ascribed to the bacterial and transforming bacterial fraction or to the bacteroids, we separated our bacteroid preparation into fractions corresponding to the densities described by Ching et al. A typical gradient of 29-d-old nodule bacteroids is shown in Figure 1. Fraction I contains mitochondria and some bacteroids that are lighter than the major fraction (II). This fraction did not enter the 45% sucrose. Fraction II bands at the 45 to 50% sucrose interface and corresponds to the mature bacteroid fraction. Electron microscopic examination of this fraction demonstrated that it was substantially free of mitochondria. Enzymic assay of fraction II for the marker enzyme for plant mitochondria, malate dehydrogenase (6), showed that this fraction contained less than 2% of the total activity of the nodule homogenate. Fraction III bands at the 50 to 52% sucrose interface and corresponds to the transforming bacterial fraction, and fraction IV bands at the 52 to 57% interface and is the bacterial fraction. Cultured 61A76 cells band at this interface.

Quantitatively, this distribution differs from the results of Ching et al. who used 28 to 35-d-old nodules. Whereas, mature bacteroids represent 50% or less of the total in their results we found 92% in fraction II (Fig. 1). In the 43- and 56-d-old nodules, 99% were mature bacteroids. We used nodules which had been frozen for these studies. During the 1982 growing season, we repeated these studies using freshly harvested nodules. No significant difference in the distribution was observed.

For preparative work, we have used a slightly different gradient to facilitate separation and collection of the mature bacteroid fraction. We did not investigate fractions III or IV inasmuch as they were such a minor part of the total bacteroids. We did not survey nodules younger than 29 d. It is possible that the quantitative distribution may differ in younger nodules, but very little nitrogen is fixed by younger nodules (11, 16).

The hemeprotein concentrations found in bacteroids prepared from various age nodules are shown in Table I. Cyt aa₃ was present in bacteroids throughout their most active nitrogen-fixing period (4, 11, 13, 16), and the level of this Cyt in the 29-d bacteroids was approximately the same as was found in aerobically grown cultured cells of 61A76. The level of this Cyt declined somewhat as the nodules aged. Previous studies have suggested that senescence of tap root nodules begins between 50 and 65 d. Later developing lateral root nodules probably have higher levels of Cyt aa₃, but this has not been determined. Cyt b and c are found in somewhat higher levels than in cultured cells as has been reported for other strains and species of rhizobial bacteroids (1, 2, 12, 14).

We have not included Cyt o in Table I. This Cyt is present in cultured cells and appears to be present in bacteroids in levels similar to those observed in cultured cells. However, the identification in bacteroids is not certain and what appears to be Cyt o in our CO-difference spectra may in fact be P₄₅₀. Further work is needed along these lines to identify Cyt o and P₄₅₀.

Recently, it was reported that R. leguminosarum bacteroids and Rhizobium sp. NGR221 from Parasponia nodules have Cyt aa₃ (3). We have also observed Cyt aa₃ in bacteroids from nodules inoculated with a commercial inoculum. Coupled with our results on R. japonicum 61A76, it is apparent that Cyt aa₃ may be more widely distributed in bacteroids of various species and strains than previously suspected. Experiments with additional strains are in progress. Preliminary results suggest that bacteroids of some strains have Cyt aa₃ while others do not. Thus, it appears that bacteroids may or may not contain Cyt aa₃, and no direct correlation can be ascribed to the development of the bacteroid state and the presence or absence of this Cyt. To study the role of this and other Cyt in nodule respiration, we have isolated a series of Cyt c- and aa₃-deficient mutants. Preliminary studies on the
aerobic physiology of these mutants have been reported (8, 9).

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LITERATURE CITED

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