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DNA Content of Free Living Rhizobia and Bacteroids of Various *Rhizobium*-Legume Associations

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ABSTRACT

The DNA content of bacteroids from 22 different *Rhizobium*-legume associations was compared to that of the corresponding free living *Rhizobium* species using laser flow microfluorometry. In all 18 effective associations, the bacteroids had either similar or higher DNA content than the free living rhizobia. Bacteroid populations isolated from effective clover (*Trifolium repens*) and alfalfa (*Medicago sativa*) nodules had an average DNA content of >1.5-fold higher than free living *R. trifolii* and *R. meliloti*. These populations also contained a significant number of bacteroids with more than 3-fold the DNA content of the free living rhizobia. Populations isolated from effective nodules of winged beans (*Psophocarpus tetragonolobus*), peas (*Pisum sativum*), and mung beans (*Phaseolus aureus*) had an average DNA content of 1.1- to 1.5-fold higher than free living *R.* "cowpeas" and *R. leguminosarum*. Bacteroids from nodules of lupins (*Lupinus angustifolius* and *L. minaretta*), kidney beans (*Phaseolus vulgaris*), and soybeans (*Glycine max*), however, had similar DNA content to the free living forms. Two of the four associations which formed ineffective nodules contained bacteroids with lower DNA content than the free living rhizobia. The other two associations contained bacteroids with slightly higher or similar DNA content to the free living rhizobia. Nodules of the ineffective associations also did not contain leghemoglobin.

The symbiotic association between *Rhizobium* and leguminous plants is of agricultural importance because it reduces atmospheric nitrogen to a biologically useful form. One essential part of establishing this association is the development of a physiological balance between the infecting *Rhizobium* species and the host plants by a successful control in the multiplication of the microsymbionts. *Rhizobium* species in culture have generation times ranging from 3 to 10 h, but if such fast multiplication rates were maintained during nodule development, it would be difficult, if not impossible, for the host to support such a burden and achieve bilateral benefits. Results from Dilworth and Williams (8) show that the multiplication rate of bacteroids in lupin nodules was approximately 6-fold slower than the corresponding cultured *Rhizobium lupini*. The mechanism which controls the multiplication rate of bacteroids is unknown. Such a decrease in multiplication rate may imply changes in nucleic acids since some microorganisms show a positive relationship between growth rate and nucleic acid content (15).

Comparative studies on nucleic acid content between bacteroids and cultured rhizobia, however, have yielded conflicting results. Rautanen and Saubert (21), Schaede (23), and Chizhik (5), using various analyses, reported that bacteroids from soybean, peas, broadbeans, and cowpeas contain less nucleic acid than the free living rhizobia, whereas Bergersen (2), Reijnders *et al.* (22), Bisseling *et al.* (3), and Paau *et al.* (20) reported that bacteroids from a variety of legumes have either similar or higher nucleic acid content than the free living rhizobia. These reports have spanned a period of years and have involved a variety of analytical methods. Because of these differences a comparison of nucleic acid content between cultured *Rhizobium* and symbiotic bacteroids of various *Rhizobium*-legume associations merits a reexamination taking advantage of current analytical procedures. Analytical methods are now available for analyzing the relative nucleic acid content of each individual organism within a population (3, 20). This is especially important for analysis of nodules with heterogeneous bacteroid populations (19). In this communication, we report the use of FMF,[4] a method which can analyze rapidly (500 cells per s) individual bacteroids and bacteria in heterogeneous populations, to compare the DNA content of bacteroids isolated from 22 different *Rhizobium*-legume associations to that of the corresponding cultured rhizobia. The applicability of FMF to the studies of nucleic acid content in bacterial cells has been reported (1, 18). The organisms included in this study represent the seven major species of the genus *Rhizobium* and nine different types of legumes. One of the associations (*R. meliloti* F28-alfalfa) has been studied and reported earlier (20) and was included in this study for comparison purposes and proof of reproducibility of the FMF technique. The term "bacteroid" used in this communication refers to all of the rhizobial cells isolated from nodules regardless of size, shape, and nucleic acid content.

MATERIALS AND METHODS

Rhizobial and Plant Growth. The various *Rhizobium*-legume associations and the source from which the organisms were originally obtained are listed in Tables I and II. Except for *R. japonicum* 705, all cultures were maintained on a yeast-mannitol medium (6, 7). *R. japonicum* 705 was cultured in a medium containing arabinose and glycerol (6, 7). Seeds of the various legumes were surface-sterilized (0.5% sodium hypochlorite) and germinated in Perlite contained in stainless steel pots (20.3-cm diameter) which had been autoclaved overnight. Except for *Psophocarpus tetragonolobus* and *Vicia faba*, the seedlings were inoculated with the indicated rhizobial culture 1 week after germination and the nodules were collected 4 to 6 weeks after inoculation. *P. tetragonolobus* and *V. faba* were inoculated upon cotyledon emergence and the nodules were harvested 8 to 10 weeks after inoculation. Nodules of effective associations were harvested when the nodules were active in acetylene reduction. Nodules of ineffective associations were examined in two different ages and were inactive in acetylene reduction. All plants used in this investigation were grown in a greenhouse under supplemental (15 h per day) light. Cross-contaminations were not observed since uninoculated controls did not nodulate.

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[4] Abbreviation: FMF: flow microfluorometry.

Table I. Relative DNA content of bacteroids isolated from effective nodules as compared to the free living rhizobial strans.

Species	Strain	Source[1]	Host Plant	Category[2]
R. meliloti	F28	1	Medicago sativa (Buffalo)	I
R. trifolii	3D1k5	2	Trifolium repens	I
	TA1	3	" "	I
	CC1303(R3)	4	" "	I
	3D1m5	2	" "	II
R. leguminosarum	3HOq18	2	Pisum sativum	II
	3HOt1	2	" "	II
	SU391	3	" "	II
	3HOt1	2	Phaseolus aureus	II
R. 'cowpea'	NGR156	3	Psophocarpus tetragonolobus	II
	NGR258	3	" "	II
R. lupini	3C3d2	2	Lupinus angustifolius	II
	3C3e1a	2	" "	III
	WU425	3	" "	III
	WU425	3	Lupinus minarette	III
R. phaseoli	CC511	3	Phaseolus vulgaris	III
R. japonicum	705	1	Glycine max (Lee)	III
	705	1	Glycine max (Chippewa)	III

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3 - Dr. R. P. T. Elmes, Dept. Primary Industry, Konedobu, Papua New Guinea (10).
4 - Dr. J. Brockwell, Div. Plant Industry, CSIRO, Canberra City, Australia (4).

[2]Bacteroid populations were categorized according to the relative bacteroid DNA content. See text for further description. All nodules contained leghemoglobin.

Table II. Relative DNA content of bacteroids isolated from ineffective nodules as compared to the free living rhizobial strains.

Species	Strain	Source[1]	Host	Category[2]
R. leguminosarum	3HOq18	2	Phaseolus vulgaris	II
	3HOq18	2	Vicia faba	III
	SU391	3	" "	IV
R. trifolii	CC1316(R16)	4	Trifolium repens	IV

[1]See Table I.

[2]See Table I and text. Nodules did not contain detectable leghemoglobin.

FMF Analyses. Nodule bacteroids were isolated and collected by differential centrifugation and washed as previously described (17). Rhizobial cultures at early stationary phase also were harvested and washed to represent the free living rhizobia. Early stationary phase cultures were chosen for this study because the DNA content of *Rhizobium* species varies to different extents during the earlier phases of growth depending upon the growth rate of the organism (18). The DNA content of rhizobia at early stationary phase growth is low and the least variable and therefore, the most useful for comparative purposes. The microorganisms are readily reculturable (viable) at this growth phase. After washing, the samples were fixed in 64% ethanol overnight at 0 to 4 C and were digested with pancreatic RNase (Sigma, 1 mg/ml, boiled 10 min to inactivate contaminating DNase) at 37 C for 1 h. After RNase digestion, the samples were washed with 50 mM K-phosphate (pH 7.5) and stained with 0.005% ethidium bromide (EB)-1.12% Na-citrate in ice for 60 min. The stained cells were then resuspended in distilled H_2O. After several passages through a 25-gauge syringe needle to eliminate flocculation, the samples were diluted and analyzed. The architecture and operational principles of the FMF were described by Steinkamp *et al.* (24). The fluorescent signals emitted from the bacteria and bacteroids were recorded electronically and displayed on a 256-channel pulse-height analyzer according to relative intensity. In such frequency distributions the channel number is directly proportional to the intensity of the fluorescent signal and the DNA content of the microorganism (1, 18). Cell numbers were automated with a fluorescent counter equipped in the FMF. A minimum of 200×10^3 microorganisms were analyzed in each sample.

Effectiveness and Leghemoglobin Determinations. Nodule effectiveness was determined by the ability of nodules to reduce acetylene (13). The rate of acetylene reduction was determined with a Perkin-Elmer 900 gas chromatograph. The reaction tubes contained 65% helium, 25% oxygen, and 10% acetylene. Nodules (approximately 0.5 g) that failed to reduce detectable quantities of acetylene after 1-h incubation at 23 C were considered ineffective. Leghemoglobin content was determined spectrophotometrically using the nodule cytosol (19). The cytosol was centrifuged at 20,000*g* for 20 min and 2.5 ml of the supernatant was added to 0.5 ml of 5 N KOH containing 0.1 g Na-dithionite. The increase in A_{555} was used as a measure of leghemoglobin content.

RESULTS

Our studies initially included 25 possible *Rhizobium*-legume associations. Three of the inoculations, *R. leguminosarum* SU391 and 3HOq18 to *P. aureus* and *R.* "cowpea" to *Sesbania drummondii,* failed to form nodules and therefore were not analyzed. Eighteen of the 22 associations which formed nodules were effective in acetylene reduction and contained leghemoglobin (Table I). The remaining four associations formed nodules but the nodules did not have detectable acetylene-reducing activity or leghemoglobin (Table II).

Bacteroids isolated from nodules of the effective *Rhizobium*-legume associations had an average DNA content similar to or higher than the DNA content of free living organisms. The distribution of bacteroids according to their DNA content (Figs. 1–3) showed that all of the effective nodules contained a popula-

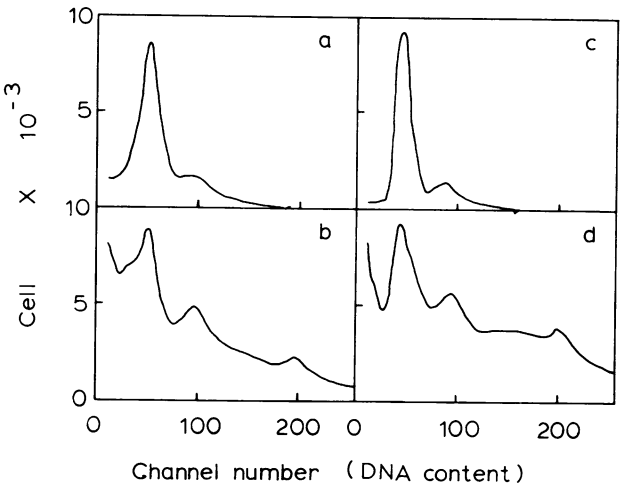

FIG. 1. Distribution of free living *R. trifolii* CC1303 and 3Dlk5 and respective bacteroids from *T. repens* according to DNA content. Channel number is directly proportional to DNA content of microorganism. a: *R. trifolii* CC1303; b: bacteroids of CC1303 from *T. repens;* c: *R. trifolii* 3Dlk5; d: bacteroids of 3dlk5 from *T. repens.* Distributions in b and d typify category I bacteroid populations.

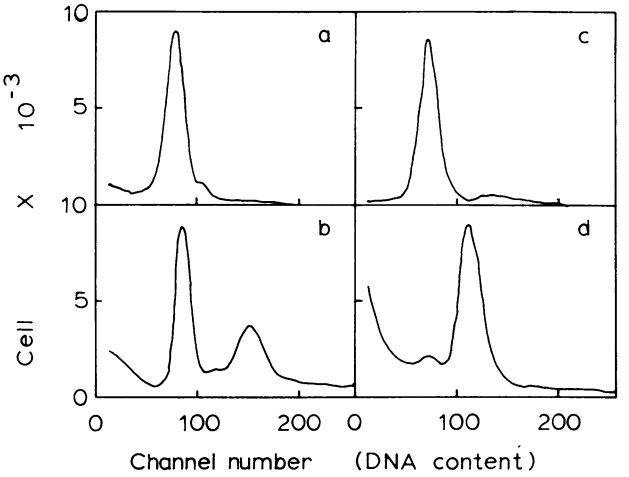

FIG. 2. Distributions of free living *R. leguminosarum* SU391 and 3HOq18 and respective bacteroids from *P. sativum* and *P. vulgaris* according to DNA content. a: *R. leguminosarum* SU391; b: bacteroids of SU391 from *P. sativum;* c: *R. leguminosarum* 3HOq18; d: bacteroids of 3HOq18 from *P. vulgaris.* Distributions in b and d typify category II bacteroid populations.

tion of bacteroids with identical DNA content to the early stationary phase rhizobia. In several of the associations a significant portion of the bacteroid populations had a DNA content that was more than 2-fold that of cultured rhizobia. Based upon FMF analysis of bacteroid DNA content, the bacteroid populations from different effective associations were divided into three general categories (Table I).

The first category (I) represented populations containing bacteroids with an average DNA content >1.5-fold of that of the free living forms. A significant portion of the bacteroids of these populations contained more than 3-fold the DNA content of free living rhizobia. Bacteroids containing as much as 6-fold more DNA than cultured rhizobia were also present in this category. Category I was represented by bacteroids isolated from alfalfa and clover nodules infected with *R. meliloti* and *R. trifolii,* respectively. The distributions of two of the *R. trifolii* strains and the respective category I bacteroids from nodules of *Trifolium repens* are shown in Figure 1.

The second category (II) represented populations which contained bacteroids with a slightly higher (1.1- to 1.5-fold) average DNA content than the free living forms. These populations contained some bacteroids with 2- to 2.5-fold higher DNA content than cultured rhizobia. Bacteroids with 3-fold or higher DNA content than cultured rhizobia were not present in significant number. This category was represented by bacteroids isolated from nodules of peas, mung beans, and winged beans infected with *R. leguminosarum* or *R.* "cowpea." The distributions of bacteroids representing category II are shown in Figure 2.

The third category (III) represented populations which contained bacteroids with an average DNA content similar to cultured rhizobia. The distribution of bacteroids from these nodules also was similar to that of free living rhizobia cultures (Fig. 3). Category III was represented by bacteroids isolated from nodules of lupines, kidney beans, and soybeans infected with *R. lupini, R. phaseoli,* and *R. japonicum,* respectively. Some of the associations in this category contained cells with lower DNA content than cultured rhizobia (Fig. 3b, channels 10–40). Only a few if any of these cells were observed when the nodules were active in acetylene reduction. Perhaps they originated from the senescent part of the nodules and represented deteriorating bacteroids (19).

The average DNA content of bacteroids isolated from the four ineffective associations varied from a slightly increased (category II) to a lower content than the cultured rhizobia (Table II). The bacteroid populations with lower DNA content than cultured rhizobia were observed exclusively in ineffective nodules and were referred to as category IV in Table II. The ineffective nodules also did not contain detectable levels of leghemoglobin. The distribution of category IV bacteroids from two ineffective associations is shown in Figure 4.

DISCUSSION

The 22 *Rhizobium*-legume associations analyzed in this study revealed four different types of bacteroid populations with respect to changes in DNA content upon nodulation. Three types of bacteroid populations (categories I–III) were observed in the effective associations while the fourth category (category IV) was seen only in ineffective nodules. *Rhizobium* spp. which form bacteroid populations representative of categories I to III seem to be grouped along the general division lines suggested by earlier workers (9, 11, 12, 14, 16) for the genus *Rhizobium.* Certainly a sharp distinction relative to DNA changes in bacteroids was observed between category I represented by *R. meliloti* and *R.*

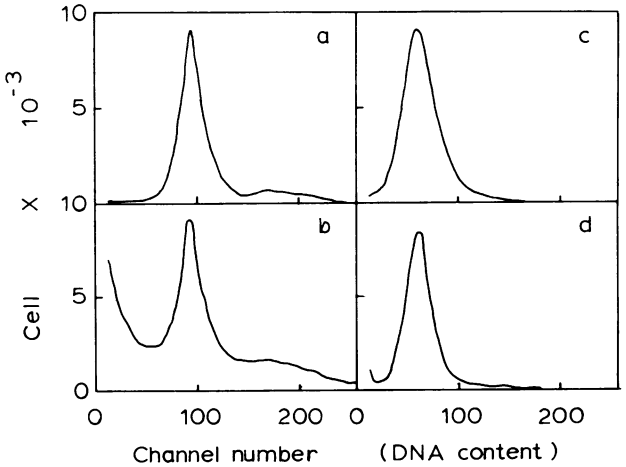

FIG. 3. Distributions of free living *R. lupini* WU425 and *R. phaseoli* CC511 and the respective bacteroids from *L. minarette* and *P. vulgaris* according to DNA content. a: *R. lupini* WU425; b: bacteroids of WU425 from *L. minarette;* c: *R. phaseoli* CC511; d: bacteroids of CC511 from *P. vulgaris.* Distributions in b and d typify category III bacteroid populations.

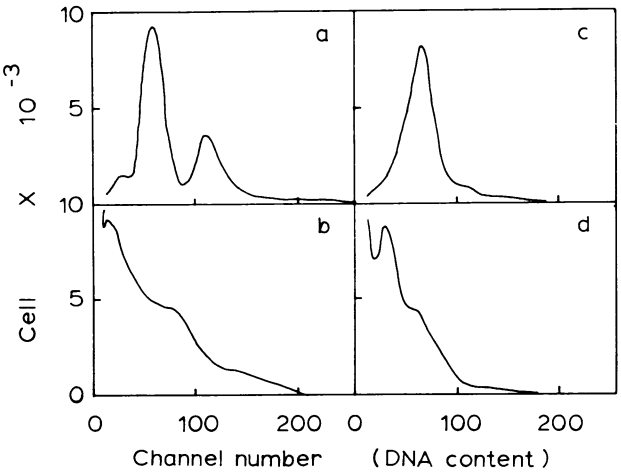

FIG. 4. Distributions of free living *R. trifolii* CC1316 and *R. leguminosarum* SU391 and the respective bacteroids from *T. repens* and *V. faba* according to DNA content. a: *R. trifolii* CC1316; b: bacteroids of CC1316 from *T. repens;* c: *R. leguminosarum* SU391; d: bacteroids of SU391 from *V. faba.* Distributions in b and d typify category IV bacteroid populations.

trifolii, and category III represented by *R. japonicum* and *R. lupini* (Table I).

We have examined bacteroid populations from alfalfa (category I) and soybean (category III) nodules at different stages of nodule development to determine if they maintained their respective distribution categories. Bacteroid populations from soybean nodules maintained the category II distribution for up to 12 weeks. Bacteroid populations from alfalfa nodules maintained the category I distribution for up to 8 weeks. In older alfalfa nodules, however, the number of bacteroids with high DNA content slightly decreased. In 10- to 12-week-old alfalfa nodules a small number of bacteroids with slightly lower DNA content than cultured rhizobia also were observed. These bacteroids probably represent senescent bacteroids in the process of deterioration (19).

Bisseling *et al.* (3) recently suggested that the host plant could influence the change in DNA content of the infecting rhizobia. There is some evidence in this investigation to support the suggestion. Bacteroids isolated from nodules of *P. sativum, P. vulgaris,* and *V. faba* all infected with *R. leguminosarum* 3HOq18 had different DNA content (Tables I and II). Bacteroids isolated from *V. faba* nodules had DNA content similar to the cultured rhizobia while those isolated from *P. sativum* and *P. vulgaris* contained more DNA than the free living rhizobia.

The ineffective associations between *R. trifolii* CC1316 (R16) *T. repens;* and *R. leguminosarum* SU391 and *V. faba* demonstrated incompatability among the host and the *Rhizobium* species because the microorganisms failed to develop into bacteroids with DNA content comparable to the effective bacteroids of *R. trifolii* and *R. phaseoli.* The decrease in DNA content probably signifies premature deterioration of the rhizobia in the nodules (Fig. 4 and Table II). The ineffective associations between *R. leguminosarum* 3HOq18 and *P. vulgaris* and *V. faba* may represent another type of incompatibility since bacteroids of strain 3HOq18 from these two legumes had DNA content comparable to effective bacteroids in other legumes. The ineffectiveness perhaps stemmed from the incapability of these bacteroids to induce leghemoglobin formation in nodules of *P. vulgaris* and *V. faba.* Since the two ineffective associations in category IV (*R. trifolii* CC1316 *versus T. repens,* and *R. leguminosarum* SU391 *versus V. faba* also did not contain leghemoglobin, it is tempting to speculate that the induction of leghemoglobin in nodules comes after development of the infecting rhizobia into bacteroids, at least with respect to DNA content.

Since significant qualities of bacteroids with DNA content lower than the free living rhizobia were observed only in ineffective nodules and not in any of the effective nodules during stages of active acetylene reduction, we conclude that the development or transformation of free living rhizobia into functional acetylene-reducing bacteroids does not involve a reduction of DNA content in the microsymbiont. The maintenance of at least one genome equivalent of DNA in the bacteroids seems to be essential for the successful establishment of effective symbiosis in *Rhizobium*-legume associations. The relative increase in DNA content in categories I and II bacteroid populations during the development phase associated with active acetylene reduction may be related to gene amplification or simply a change in the rate of division of the microorganisms. Additional investigation is necessary to determine this point since our present data cannot discriminate the two possibilities.

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