On the DNA Content of the Bacteroids of *Rhizobium japonicum*

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The DNA content of bacteroids from large nodules of soybean plants infected with *Rhizobium japonicum* strain 61-A-101 was found to be 1.20×10^{-14} g per cell. Bacteroids from smaller nodules had slightly less DNA as did the stationary phase, free-living cells; both giving a value of 0.92×10^{-14} g per cell. In comparing these data with those of other workers it was found that there is little evidence to support suggestions that bacteroids possess anything less than a full genetic complement of DNA and that some misinterpretation of older published data has occurred.

Introduction

The often reported low viability (in terms of subsequent ability to form colonies) of mature bacteroids from the nodules of leguminous plants has stimulated a number of workers to investigate the changes in nucleic acid content during nodule development and to compare the content with that of the free-living form of the bacteria.

Some early reports [1, 2] of weakly staining nucleoids in bacteroids prompted studies on the DNA content of these nodules since, as pointed out by Dilworth and Williams [3], a decline in the DNA content of bacteroids would have obvious implications for viability. Results obtained by "gross" methods, in which the DNA content of a large number of bacteroids is estimated by chemical methods and the answer obtained divided by the number of cells present, are said to be at odds with cytofluorometrical methods where the DNA of a number of individual cellular units is estimated [4]. In an effort to resolve the apparent controversy we sought methods by which DNA could be extracted with high efficiency from bacteroids and free-living bacteria and estimated directly by UV absorbance instead of by a colour reaction in a crude extract of whole cells.

Materials and Methods

Seedlings of *Glycine max* var. Caloria were infected with *Rhizobium japonicum* strain 61-A-101 by the method of Werner [5, 8]. Nodules were harvested (nitrogenase activity 6 µmol $C_2H_4 \cdot 9$ g nodule fresh weight$^{-1} \cdot h^{-1}$) 35 d after infection and sorted into size classes by sieving before storing frozen at −75 °C. Approximately 200 mg batches of nodules from a particular size class were processed according to the following scheme:

1. 200 mg nodules were crushed in a cold mortar and then lightly ground after the addition of 2 ml of cold 0.2 M mannitol and the resulting suspension filtered through nylon gauze (ca. 10 µm pores).

2. The pellet obtained by centrifuging 1.2 ml of this suspension for 10 min at 15,000 rpm in a Beckman JA 21 rotor at 5 °C was resuspended in 1.2 ml saline EDTA [6] and 0.3 ml of 25% Na dodecyl sulphate added before the mixture was warmed to 60 °C for 5 min with gentle shaking. Lysis of the bacteroids was completed by forcing the suspension through a French pressure cell (20,000 psi cell pressure).

3. Nucleic acid in 1.2 ml of the lysate was precipitated by the addition of six volumes of the ethanolic perchlorate reagent (EPR) of Wilcockson [7]. This precipitates nucleic acids (and often polysaccharides) but not proteins from crude lysates. The mixture was allowed to stand for at least 3 h before centrifuging at low speed and washing the pellet in cold 70% ethanol. Nucleic acid in the pellet was dissolved by shaking it gently for 1 h with 3 ml of dilute saline sodium citrate (0.1 × SSC) [6]. Insoluble material in the pellet was separated by allowing the suspension to drain through a pasteur pipette which had a small cotton wool plug in the constriction.

4. Nucleic acids in the filtrate were precipitated by the addition of 6 ml of cold ethanol and allowing the mixture to stand overnight. After centrifuging the ethanol was decanted and the pellet dissolved in 0.3 ml of 0.1 × SSC and 0.03 ml of 0.1% RNase (Serva, ribonuclease A, EC 3.1.4.22) in 0.1 M NaCl added

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and incubated at 30 °C for 2 – 3 h before adding six volumes of EPR and allowing to stand for 3 – 4 h at 4 °C.

5. The suspension was centrifuged and the DNA pellet washed in cold 70% ethanol before dissolving in 0.5 ml of 0.1 x SSC and measuring absorbance of appropriate dilutions at 230, 260 and 280 nm in 1 cm pathlength quartz cuvettes in a Zeiss PMQ 2 spectrophotometer. DNA concentrations were estimated using the relationship $E^{0.1\%}_{260\,nm}$ for DNA = 20. For comparison of the DNA content of bacteroids with that of free-living bacteria, *Rhizobium japonicum* 61-A-101 was grown at 28 °C in medium 20 E [8] to a turbidity of 280 Nephelometer units for log phase bacteria and to 1050 units for stationary phase cells. Bacteria were centrifuged to remove medium and washed once with water before resuspending in saline-EDTA and processing as for the bacteroids.

Results

The first line of Table I shows that these bacteria have in the bacteroid form a similar amount of DNA to stationary phase cells. We were able to show that at least for the larger nodules the bacteroids had some one third more DNA per cell than the stationary phase cells. The data are corrected for a recovery of DNA of 70% as found when ^{14}C-labelled *E. coli* K 12 DNA was added after suspension of the bacteroids in saline-EDTA. About 90% of the added radioactivity could be recovered after the first EPR precipitation in the filtrate after separation of polysaccharide. The enzymatic digestion of the RNA is preceded by an ethanol precipitation and followed by another EPR precipitation and resulted in a further loss of 20% of the radioactivity. Addition of radioactive *E. coli* RNA indicated that the overall procedure leads to the preparation of DNA free from contaminating RNA. The dissolved EPR precipitates either before or after RNase treatment normally had a ratio of absorbance at 260 nm to 280 nm in excess of 1.8 and 260/230 of more than 2. On occasions when ratios less than this were found a brief centrifugation of the material increased the ratios suggesting that contaminating suspended material rather than dissolved protein or polysaccharide was the cause. The material separated by filtration after the first EPR precipitation stage was, after overnight hydrolysis in 6 N HCl, only very weakly ninhydrin positive and probably consists almost entirely of polysaccharide material.

Discussion

Bisseling *et al.* [4] using a cytofluorometrical method found 0.91×10^{-14} g per cell for stationary phase *R. japonicum* strain WA 107. Although our own value for stationary phase cells of *R. japonicum*

Table I. DNA content of bacteroids and free-living cells of *Rhizobium*.

Rhizobium species	strain	DNA content per cell (fg [a])				
		free-living bacteria		bacteroids		
		log	stationary	early	mature	Reference
R. japonicum	61-A-101	18.6	9.2		12 [b]	this work
R. japonicum	WA 107		9.1		19.3	Bisseling *et al.* [4]
R. "cowpea"	CB 756		7.1		7.1	Bisseling *et al.* [4]
R. "cowpea"	32 H 1	13 [c]			2.8	Bergersen [13]
R. lupini	D 25			30	11	Dilworth and Williams [3]
R. lupini	GK 82		7.5		13.7	Bisseling *et al.* [4]
R. "lotus"	NZP 2257	(7.5	7.1) [d]		8.3	Sutton [11]
R. "lotus"	NZP 2037	(13.2	8.8) [d]		3.8	Sutton [11]
R. "lotus"	NZP 2037		10.7		31.8	Bisseling *et al.* [4]
R. leguminosarum	PRE	14	12		38	Reijinders *et al.* [12]
R. leguminosarum	PRE		7.8		44.4	Bisseling *et al.* [4]

[a] 1 fg = 1×10^{-15} g.
[b] Value given is for bacteroids, from 3 – 4 mm nodules; 2 – 3 mm nodules gave 9.5 fg per bacteroid and nodules less than 2 mm 9.2 fg per bacteroid.
[c] Chemostat grown bacteria not necessarily growing at maximum rate.
[d] Values are for cells from shaken (left) and not shaken (right) broth cultures and are not necessarily log and stationary phase cultures.

strain 61-A-101 is very similar (0.92×10^{-14} g) we found only about a 30% increase in the bacteroids of mature nodules whereas Bisseling *et al.* recorded a doubling to 1.93×10^{-14} g per bacteroid. It is possible that our method, which averages the DNA content of mature bacteroids and developing bacteroids within the nodule, gives a lower value for DNA content than the individual cell cytofluorometrical method because in the latter method selection for mature bacteroids in the estimation may occur.

On the other hand Paau and Cowles [9] found that the DNA content of *R. japonicum* bacteroids was similar to that of the free-living cells and also used a cytofluorometrical method.

At least part of the "controversy" Bisseling *et al.* [4] found between the gross average methods and the cytofluorometrical method for the determination of DNA content rests not so much in any weakness in the methods used but in the interpretation of some of the results obtained by the original workers and by subsequent authors.Thus Bisseling *et al.* [4] record that both Bergersen [10] and Dilworth and Williams [3] found less DNA per cell after bacteroid formation. Bergersen [10] did not look at DNA but only at total nucleic acid in *Rhizobium japonicum* bacteroids. Dilworth and Williams [3] did indeed record a fall in the DNA content per bacteroid during the development of *R. lupini* strain D 25 in the nodules of lupin and commented on the significance this could have for the viability of bacteroids. They did not, however, say that the mature bacteroids had less DNA than stationary phase cells. They did not measure DNA of free-living cells, stationary or growing. Sutton [11] stressed that even the lowest DNA content he found in bacteroids was still enough to represent one whole *E. coli* sized genome and that the kinetic complexity of the bacteriod DNA was unchanged from that of the free-living cells.

Reijinders *et al.* [12] using a gross average method and DNA estimation by a colour reaction found a three fold increase in the DNA content per cell (to 3.8×10^{-14} g per bacteroid) compared with the DNA content of free-living stationary phase cells. Surprisingly they found almost no difference between the DNA content of stationary phase and log phase cells of *R. leguminosarum* strain PRE. We found that *R. japonicum* 61-A-101 in log phase had almost twice the DNA content of stationary phase cells. Also with *R. leguminosarum* Bisseling *et al.* [4] using the cytofluorometrical method found rather less DNA in the stationary phase cells of the same strain (0.78×10^{-14} g per cell) and about six times that amount in bacteroids.

From Table I which includes a summary of most of the published data for the DNA content of rhizobia it can be seen that the range for the DNA content of stationary phase cells of different *Rhizobium* strains found by a number of workers is quite narrow ($0.71 - 1.2 \times 10^{-14}$ g per cell). There is, in fact, no example of a work which demonstrates unequivocally that mature bacteroids have less DNA than a stationary phase free-living bacterium. As already mentioned, Dilworth and Williams [3] reported a decline during bacteroid maturation but did not compare their values with stationary phase cells. The 1.10×10^{-14} g per cell found by these authors is not very different from the 1.37×10^{-14} g per cell found by Bisseling *et al.* [4] who found that the bacteroids of their strain of *R. lupini* had almost twice the DNA content of stationary phase cells. There are, however, two pieces of data at variance with the others in the Table. Sutton [11] with the *Lotus* nodulating strain NZP 2037 found a value of only 0.38×10^{-14} g per bacteroid representing a large fall relative to his values for shaken or unshaken broth cultures. No direct comparison with stationary phase cells was made and furthermore Bisseling *et al.* [4] using the same strain found a value eight times higher for the bacteroids and this was some three times higher than the DNA content of stationary phase cells of the same strain as measured using the cytofluorometrical method. The other result is that of Bergersen [13] who also reported a very low value for the bacteroids of the cowpea nodulating strain 32 H 1, 0.28×10^{-14} g per cell. Again no direct comparison was made with stationary phase cells but the value was some five times lower than that of the cells in a nitrogen-fixing chemostat culture of the same strain. It is unlikely that such a culture of this relatively slow-growing bacterium would have more than two full chromosomes per cell and thus Bergersen's report seems to suggest that bacteroids of 32 H 1 have less than one full genome. However, Bisseling *et al.* [4] used the cowpea strain CB 756 and in a direct comparison with stationary phase cells found the same DNA content as in the bacteroids, 0.72×10^{-14} g per cell. Thus with two exceptions the data from different workers suggest that bacteroids have either a similar or a greater DNA content than that of stationary phase cells of the same strain.

The extent of the increase in DNA content in the bacteroids from that of the free-living stationary phase cells seem to depend on the state of "differentiation" of the bacteroid. Thus those rhizobial strains which have bacteroids appearing not very different from free-living bacteria under the light microscope have the same or perhaps up to two times the DNA content of the free-living stationary phase cells. The morphologically differentiated bacteroids of the faster growing strains show a DNA content several times greater than that of the free-living cells. Bisseling *et al.* [4] and Van den Bos *et al.* [14] demonstrate conclusively with *Rhizobium leguminosarum* that the DNA content per unit was higher in the Y-shaped bacteroids than in rod forms and higher again in multiply branched bacteroids. We support the suggestion of Bisseling *et al.* [4] that the higher DNA content of bacteroids relative to stationary phase free-living bacteria is probably a phenomenon related to the formation of filaments. It is well known that many bacteria (*e. g.* the *E. coli* B family) will under a variety of constraints, UV light, toxic chemicals etc., form long filaments through a failure of normal septum formation. It is interesting to note that also *R. japonicum* will produce swollen and even branched forms when grown in the presence of high concentrations of the organic acids, malonate and succinate [15, 16]. These acids although not themselves good carbon sources for the growth and nitrogen fixation by free-living *R. japonicum* appear to stimulate the utilization of other carbon sources [16] and occur in the soybean plant especially in the nodules [17]. Some recent experiments with up to 100% plating efficiency of bacteroids of *Rhizobium trifolii* [18] in osmotically protective media (0.2 M mannitol) show that the morphological differentation of the bacteroids is reversible. They also suggest a full genetic complement DNA in the bacteroids.

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