DNA Base Composition, Flagellation and Taxonomy of the Genus *Rhizobium*

By J. DE LEY

*Laboratory for Microbiology, Faculty of Sciences, State University,
Ghent, Belgium*

AND A. RASSEL

*Laboratory for Electron Microscopy, Station for Agricultural Chemistry
and Physics, Ministry of Agriculture, Gembloux, Belgium*

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SUMMARY

There is a correlation between the base composition of pure DNA from 85 strains of *Rhizobium* and their type of flagellation. There are two groups of *Rhizobia*. The peritrichously flagellated organisms, which usually grow fast, have a low % (guanine + cytosine) composition in the range 58.6-68.1 %; these organisms occur in all cross-inoculation groups investigated. From a comparison with literature data it appears that this group is constituted by two subgroups: *Rhizobium leguminosarum* and *R. meliloti*. The subpolarly flagellated, slow-growing strains have a somewhat higher (guanine + cytosine) content, mostly in the range 62.8-65.5 %; these organisms appear to be specialized mainly for the lupin, soybean, cowpea, Lotus, Wistaria and Robinia groups. It is proposed that only one genetic species is involved, to be called *Rhizobium japonicum*. The *rhizobia* would thus contain only three genetic species. The relationship between the high and low (guanine + cytosine) groups is stressed by their closely related % (G + C) values, the near-identity of the average compositional distribution of the DNA molecules and because the peritrichous organisms not infrequently show a subpolar flagellum.

INTRODUCTION

It is well established that the knowledge of the base composition (expressed as molar percentage of guanine + cytosine) of pure DNA will considerably improve and clarify the present bacterial classification.

The present paper is a contribution towards an improved classification of the *rhizobia* along these lines. The present classification (*Bergey's Manual*, 1957) of these bacteria is still based on the cross-inoculation groups proposed by Fred, Baldwin & McCoy (1932). Many authors have expressed dissatisfaction with this system (see Discussion) and one can expect that a future classification will look different. During the course of our work it was noticed that there is a distinct correlation between the average molar (guanine + cytosine) content (% (G + C)) and the type

of flagellation. The conclusion which emerged from it agrees remarkably well with the principle of the proposal recently made by Graham (1964), although in the latter case an entirely different approach was made, namely by Adansonian analysis.

METHODS

Organisms used. All strains with a number of two or three symbols were obtained through the courtesy of Prof. Ch. Bonnier, Chair of Biochemistry and Microbiology, Institut Agronomique de l'Etat, Gembloux, Belgium. All other strains were obtained through the courtesy of Dr L. W. Erdman, U.S.D.A., Beltsville, Md., U.S.A. Upon receipt, and later during the experiments proper, all strains were checked for bacteriological purity.

Growth conditions. Mass cultures were prepared by growing the organisms for 2–3 days at 25° in Roux flasks with 150 ml. of a modified medium of Ashby (1907) containing (in %, w/v) 1 mannitol, 0.02 NaCl, 0.05 K₂HPO₄, 0.02 MgSO₄·7H₂O, 0.01 CaSO₄·2H₂O, 0.01 CaCO₃, 0.1 yeast extract, 2.5 agar. The organisms were harvested; they were washed by repeated suspending in 0.15 M-NaCl, 0.1 M-EDTA buffer pH 8 and centrifuging for 30 min. at 18,000g. Care was taken to remove as much as possible of the polysaccharides.

Preparation and thermal denaturation of pure DNA. The centrifuged cell paste was quickly frozen by immersion of the container in a bath at –80° and lyophilized overnight. It was found that this procedure resulted in an improved cell breakage and release of DNA in the preparation proper. DNA was purified according to the procedure of Marmur (1961). The molecular weights of the samples were determined in the Spinco model E analytical ultracentrifuge and found to be in the range 10–18 × 10⁶. Thermal denaturation was determined by automatic registration as previously described (De Ley & Van Muylem, 1963). T_m , the mid-point of the absorbance–temperature curve, was determined and the average molar (guanine + cytosine) content was calculated with the formula $0.41[\% (G + C)] = T_m - 69.3$ (Marmur & Doty, 1962). We have previously checked this formula by plotting T_m against direct estimations of % (G + C) (De Ley & Schell, 1963) and found it to be quite satisfactory. All results in Table 1 were calculated with this formula. If the calculations of Colwell & Mandel (1964) were followed, the % (G + C) would be 4–4.4 % lower. The standard deviation σ of the compositional distribution around the average % (G + C) was calculated from the same absorbance–temperature curve (Doty, Marmur & Sueoka, 1959).

Flagella staining. The organisms were grown on agar slants with the modified Ashby medium, containing 1 ml. of distilled water in the bottom (Rhodes, 1958). After several days at room temperature a loopful of the liquid was suspended gently in water; 35 % formalin was added to make a final concentration of 5–10 %. The organisms were centrifuged for 5 min. at 3000 r.p.m. and repeatedly washed by suspending in water and centrifuging at the same speed. A drop of the final suspension (about 10⁷–10⁸ cells per ml.) was applied on a perfectly clean microscope slide and dried (Leifson, 1960). The staining procedure of Rhodes (1958) was followed.

Electron microscopy. The organisms were grown in liquid medium in Kohl flasks and incubated at 20°. Every 48 hr a sample was taken in the following way: 200–

mesh grids covered with a 200 Å thick Formvar film were floated on the surface for 30 min. They were then deposited for 30–120 sec. on a 2% phosphotungstic acid solution, pH 5–7 (Brenner & Horne, 1959; Valentine & Horne, 1962). They were washed for 15 sec. in distilled water and dried in the air in Petri dishes. A Philips EM 200 electron microscope was used. Photographs were taken on Ilford 9B II and enlargements made on Gevarto 8.

RESULTS

The results are compiled in Table 1.

Table 1. *Base composition, expressed as % (G+C), 'melting points' T_m values, standard deviation σ of the compositional distribution about the mean % (G+C) of purified DNA, and type of flagellation of several Rhizobium strains*

Strain no.	Host plant	T_m in °C	% (G+C)	σ	Cross-inoculation group
Peritrichous					
6.2	<i>Lupinus luteus</i>	98.6	59.1	1.18	Lupin
3H0t19	<i>Pisum arvense</i>	98.85	59.7	0.44	Pea
14.1	<i>Vigna sinensis</i>	98.9	59.9	0.12	Cowpea
5.02	<i>Trifolium repens</i>	94.1	60.4	0.0	Clover
316ml	<i>Strophostyles pauciflora</i>	94.15	60.5	0.06	Strophostyles
9.5	<i>Phaseolus vulgaris</i>	94.2	60.6	0.0	Bean
3D1x3	<i>Trifolium dubium</i>	94.2	60.6	0.49	Clover
3E0b5	<i>Lotus americanus</i>	94.2	60.6	0.0	Lotus
5.0	<i>Trifolium pratense</i>	94.4	61.1	0.25	Clover
3F6g2	<i>Caragana arborescens</i>	94.5	61.8	0.75	Caragana
316c10(a)	<i>Phaseolus vulgaris</i>	94.55	61.4	0.06	Bean
3c2nl	<i>Lupinus densiflorus</i>	94.7	61.8	0.25	Lupin
10.0	<i>Arachis hypogea</i>	94.7	61.8	0.0	Cowpea
11.1	<i>Vicia sativa</i>	94.8	62.1	0.38	Pea
9.14	<i>Phaseolus vulgaris</i>	94.9	62.3	0.63	Bean
3D0a30	<i>Medicago sativa</i>	94.9	62.3	0.0	Alfalfa
3F3c1	<i>Wistaria frutescens</i>	95.0	62.5	0.88	Wistaria
1.5	<i>Medicago sativa</i>	95.0	62.5	0.60	Alfalfa
4.1	<i>Pisum sativum</i>	95.0	62.5	0.12	Pea
3F4b7	<i>Robinia pseudoacacia</i>	95.2	63.1	0.50	Robinia
Subpolar					
3i2b1	<i>Erythrina indica</i>	94.6	61.6	0.0	Cowpea
314a8	<i>Pueraria thunbergiana</i>	95.1	62.8	0.87	Cowpea
3.9	<i>Soja hispida</i>	95.2	63.1	0.63	Soybean
3c2k4	<i>Lupinus</i> sp.	95.3	63.3	0.36	Lupin
10.1	<i>Arachis hypogea</i>	95.3	63.3	0.5	Cowpea
316n10	<i>Vigna sinensis</i>	95.3	63.3	0.37	Cowpea
3.15	<i>Soja hispida</i>	95.4	63.5	0.38	Soybean
3c3a1	<i>Ulex europaeus</i>	95.5	63.8	0.48	Cowpea
3.23	<i>Soja hispida</i>	95.5	63.8	0.12	Soybean
3.14	<i>S. hispida</i>	95.5	63.8	0.38	Soybean
311B59	<i>S. hispida</i>	95.6	64.0	0.96	Soybean
3.1	<i>S. hispida</i>	95.6	64.0	0.36	Soybean
3F3d	<i>Wistaria speciosa</i>	95.7	64.3	0.62	Wistaria
3.2	<i>Soja hispida</i>	95.9	64.8	0.0	Soybean
1B0a2	<i>Albizzia julibrissin</i>	96.2	65.5	1.0	Cowpea

Flagellation. The strains from the Beltsville collection were selected because their type of flagellation is known (Leifson & Erdman, 1958). Several of them were

rechecked and the type of flagellation was confirmed. The strains of the Gembloux collection were studied both by electron microscopy and by ordinary staining. The latter procedure yields less reliable results because the flagella are easily detached. This appears to happen during the drying of the drop containing the live bacteria. The use of formalin improves the results. Electron microscopy is preferable.

There are two types of strains: peritrichously (Fig. 1) and subpolarly (Fig. 2) flagellated. This confirms previous observations (Hansen, 1919; Löhnis & Hansen, 1921; Shunk, 1921; Leifson & Erdman, 1958). Several peritrichously flagellated strains, such as 5.62 from *Trifolium repens* (Fig. 3), 5.0 from *Trifolium dubium* and 11.1 from *Vicia sativa*, are surrounded by fimbriae. We are not aware that these structures have previously been observed in rhizobia.

The peritrichous organisms bear 2–5 flagella, dispersed all over the cell. Quite often one of these flagella is subpolarly implanted. There appears to be a difference between the subpolar and the peritrichous flagella of the same cell since the latter are very easily detached. In several strains only careful inspection reveals the peritrichous individuals amongst an excess of apparently subpolarly flagellated cells, which frequently still have short stumps of broken-off peritrichous flagella. This phenomenon may give rise to erroneous conclusions, e.g. such as the one by Krassilnikov (1959) that all rhizobia have polar flagella. It is also possible that peritrichous strains indeed contain a number of subpolar individuals, a situation which exists, for example, in several acetic acid bacteria (Asai, Iizuka & Komagata, 1964).

DNA base composition. All strains have a T_m in the narrow range 93.6°–96.2° and a % (G + C) between 59.1 and 65.5. The peritrichous organisms have the lowest % (G + C), ranging from 59.1 to 63.1; nearly all the subpolarly flagellated strains are in the upper range from 62.8 to 65.5 (except strain 312B1, which has 61.6%). Both groups are not sharply separated; there is overlapping in the range 61.6–63.1 % (G + C). The compositional distribution σ of the DNA molecules for both groups is nearly identical: these values range from 0 to 1.2 with an average of 0.3 for the peritrichous strains and of 0.46 for the subpolar ones.

DISCUSSION

The classification within the genus *Rhizobium*. In the first generally accepted system Fred *et al.* (1932) proposed 16 cross-inoculation groups. Six of them were elevated to species level: *R. meliloti* (alfalfa), *R. trifolii* (clover), *R. leguminosarum* (pea), *R. phaseoli* (bean), *R. lupini* (lupin), and *R. japonicum* (soybean); the other groups did not receive species status. This is the origin of the species differentiation in *Bergey's Manual* (1957) and in Prévot's *Traité* (1961). Krassilnikov (1959) added another four species to them. Later the number of cross-inoculation groups was increased to 22 (Allen & Allen, 1947). However, innumerable exceptions show that a better system of classification is required (Wilson, 1939, 1944; Johnson & Allen, 1952; Bonnier, 1958; Lange, 1961; Manil, 1963; Graham, 1964).

A new system of classification. Graham (1964) proposed, after Adansonian analysis, that there would be only three species of rhizobia: the fast-growing *Rhizobium meliloti* and *R. leguminosarum* (a pool of *R. leguminosarum*, *R. trifolii* and *R. phaseoli* from *Bergey's Manual*) and the slow-growing *Phytomyxa japonicum* (a

renamed pool of *R. lupini*, *R. japonicum* and organisms from the cowpea group). Our results likewise advocate a renewal in the classification of the rhizobia and they are in agreement with the basic aspects of Graham's proposal. The following facts are new: (1) there is a distinct correlation between the DNA base composition and the type of flagellation: the peritrichous organisms have a low $\%(G+C)$ and the subpolarly flagellated ones have a high $\%(G+C)$; (2) within each group the $\%(G+C)$ range is very narrow, indicating that the organisms are genetically closely related; (3) the $\%(G+C)$ of both groups are close together and even overlap, an argument in favour of their close genetic relatedness.

The basis for the new classification may be summarized as follows:

(a) *The fast-growing, peritrichous, low (G+C) group.* This group coincides with *Rhizobium leguminosarum* and *R. meliloti* of Graham (1964). This finer distinction between species cannot be detected by the method of DNA base composition, because all strains have $\%(G+C)$ which are very close together. *R. leguminosarum* has a $\%(G+C)$ from 59.1 to 63.1. Both strains of *R. meliloti* are in the vicinity of 62.4%. Organisms of the leguminosarum type have been isolated from almost all host plants and they thus belong to almost all cross-inoculation groups. Our results on the peritrichous flagellation of the alfalfa, clover, pea and bean groups are in perfect agreement with previous reports by Hansen (1919), Löhnis & Hansen (1921), Shunk (1921), Müller & Stapp (1925) and Leifson & Erdman (1958). Several of the other smaller groups are likewise peritrichously flagellated (Leifson & Erdman, 1958; Leifson, 1960). Table 1 shows this also to be the case for bacteria isolated from the Lotus, Strophostyles, Robinia, Caragana and Wistaria groups.

(b) *The slow-growing, subpolarly flagellated, high (G+C) group.* This group coincides with *Phytomyxa japonicum* from Graham (1964). Their base composition is in the range 62.8–65.5, except for an aberrant strain 312B1 which has 61.6%. Organisms of this type have been mainly isolated from plants of the lupin, soybean and cowpea groups; a few subpolar strains from *Wistaria*, *Lotus* and *Robinia* are known. Both the results from the present paper and from Graham (1964) favour the view that only one genetic group is involved. Whether it is a species of *Rhizobium*, to be called *R. japonicum*, or a separate genus, to be called *Phytomyxa japonicum*, can only be decided unbiasedly after DNA-hybridization experiments.

(c) *Arguments for the close genetic relatedness of both groups.* (i) Their $\%(G+C)$ are very near and even overlap. The compositional distribution σ of their DNA molecules is small and of the same order.

(ii) Similarities in morphology and root-nodule-forming properties.

(iii) Frequently peritrichous strains bear a more firmly attached subpolar flagellum.

(iv) The Adansonian similarity is of the order of 80% (Graham, 1964).

(v) R. Balassa (Balassa, 1963) demonstrated exchange of genetic material by transformation between strains of the alfalfa, lupin and soybean groups.

(vi) Plants of the lupin, soya, cowpea and some of the minor groups are a host to both peritrichous and subpolar strains. This is exemplified in the present paper by strains 6.2, 3c2n1 and 3c2k4 from *Lupinus*, 10.0 and 10.1 from *Arachis*, 14.1 and 316n10 from *Vigna*, 3F3cl and 3F3d from *Wistaria*. Similar situations have been encountered by Wilson (1917), Fred & Davenport (1918), Löhnis & Hansen (1921), Shunk (1921), Müller & Stapp (1925) and Leifson & Erdman (1958). The peritrichous

organisms can grow in a wide variety of leguminous plants, whereas the subpolar ones are much more narrowly specialized and limited to plants of the lupin, soybean, cowpea and some of the minor groups (*Lotus*, *Wistaria*, *Robinia*).

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EXPLANATION OF PLATE

Fig. 1. Electron micrograph of a peritrichously flagellated cell of a *Rhizobium*, strain 10.0 from *Arachis hypogea* (22,200 $\times$).

Fig. 2. Electron micrograph of a subpolarly flagellated cell of a *Rhizobium*, strain 3.15 from *Glycine hispida* (39,500 $\times$).

Fig. 8. Fimbriated cells from a *Rhizobium*, strain 5.62 from *Trifolium repens*. Fragments of flagella (A) may be seen (30,000 $\times$).