Theoretical Deoxyribonucleic Acid Homology Between Strains of *Rhizobium japonicum*¹

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Using a theoretical method, the highest possible per cent deoxyribonucleic acid homology between 26 isolates of *Rhizobium japonicum* was determined. The homologies between the isolates and a reference strain ranged from 69.5% to 95%. Least significant difference analysis resulted in three statistically different but overlapping clusters. It was concluded on the basis of homology data that the species is a valid one, although one which shows considerable diversity.

The taxonomy of the genus *Rhizobium* Frank is based primarily upon symbiotic properties, and six species are presently recognized (1). Although some of the species of *Rhizobium* are restricted in their host range, *R. japonicum* can infect a number of legumes including some normally nodulated by other rhizobia. Because there is limited biochemical, nutritional, and physiological information available for *R. japonicum*, the validity of this species has been questioned. Determination of the deoxyribonucleic acid (DNA) base composition of 25 isolates of *R. japonicum* showed that 16 of the isolates differed significantly in base composition [expressed as mean molar percentages of guanine and cytosine (%GC); 4].

Comparisons of the mean base composition of DNA expressed as %GC have proven to be valuable taxonomic tools because of the wide range of variability of the DNA base composition of species of bacteria. The degree of genetic relatedness can then be estimated by establishing the per cent DNA homology between these microorganisms. The DNA homology between two organisms is that fraction of the total genome DNA having GC regions with common sequences. Ordinarily, the per cent DNA homology is determined experimentally by measuring hybridization between DNA from different organisms. However, a theoretical method for determining the highest possible degree of homology was recently reported by De Ley (2). This technique can be used to

calculate the limits of DNA homology between sets of bacteria and depends upon the mean nucleotide composition, the molecular weight, and the distribution of bases in the chromosomal DNA. Homologies determined using this theoretical method compare well with those obtained by hybridization techniques.

Because of the wide range of GC ratios between 25 isolates of *R. japonicum* reported previously, it was questioned whether *R. japonicum* constituted one species (4). The data determined previously, therefore, are included here for the homology determination.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study and their sources are given in Table 1.

Methods. Cultures were grown in 1-liter quantities of yeast extract mannitol medium (4) and incubated at 30 C on a rotary shaker for 4 to 5 days. The cells were harvested by refrigerated centrifugation and washed three times in 0.15 M NaCl containing 0.1 M ethylenediaminetetraacetic acid (EDTA) at pH 8.

DNA isolations were prepared independently at least twice for each strain tested. A 2- to 4-g amount of the harvested cell paste was suspended in 25 ml of saline-EDTA. Two milliliters of sodium lauryl sulfate (25 g/100 ml) were added, and the suspension was shaken vigorously in a water bath shaker at 60 C for 15 to 20 min. The released DNA was purified according to the procedure of Marmur (9). To determine the compositional %GC distribution, the thermal denaturation temperature (T_m) was converted to the %GC by the equation of Marmur and Doty (10):

$$\text{mole \%GC} = (T_m - 69.3)/0.41$$

The thermal denaturation curves were obtained using a sample absorbance recorder (Gilford model 2000)

¹ Paper no. 3501 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N. C.

TABLE 1. DNA base composition and theoretical DNA homology among 26 *Rhizobium japonicum* strains

Strain no.	Denaturation temp (C)	Moles %GC ^e	Highest possible DNA homology with <i>R. japonicum</i> 258 (%)
84 ^a	94.50	61.47	77.2
130 ^a	94.52	61.52	70.6
24 ^a	94.52	61.52	70.6
83 ^a	94.53	61.53	70.9
5563 ^b	94.54	61.57	69.5
508 ^c	94.67	61.89	74.7
6 ^a	94.76	62.08	76.7
31 ^a	94.77	62.13	77.3
W-9 ^b	94.78	62.16	77.7
29 ^a	94.80	62.20	78.2
5633 ^b	94.89	62.41	80.6
120 ^a	94.92	62.50	81.6
40 ^a	94.95	62.56	82.2
g280-1 ^b	94.95	62.56	82.2
46 ^a	94.95	62.56	82.4
62 ^a	94.99	62.65	83.5
125 ^a	95.03	62.76	84.5
122 ^a	95.04	62.78	81.6
10324 ^d	95.09	62.91	85.4
94 ^a	95.15	63.05	87.8
W-18 ^b	95.17	63.09	88.4
85 ^a	95.19	63.13	88.8
44 ^a	95.22	63.21	89.8
505 ^c	95.28	63.37	91.6
g209-8 ^b	95.41	63.67	95.0
258 ^b	95.58	64.09	100.0

^a Obtained from Dean Weber, U. S. Department of Agriculture, Beltsville, Md.

^b Obtained from A. A. Hendrickson, W. R. Grace Co., Princeton, Ill.

^c Obtained from O. N. Allen, Department of Bacteriology, University of Wisconsin, Madison, Wisc.

^d Obtained from American Type Culture Collection, Rockville, Md.

^e Mean molar percentage of guanine and cytosine.

attached to a monochrometer (Beckman model DU). When these distributions are obtained for each of two taxa, they can be used to assess the maximal possible amount of DNA homology between the taxa as measured by the amount of overlapping of the two distributions. This is essentially the method of De Ley (2) and is adapted here to determine the degree of relatedness between strains of *R. japonicum*.

The T_m and the mole mean %GC (% $\overline{GC}$) for the 26 strains of *R. japonicum* are reported in Table 1. The % $\overline{GC}$ is calculated from the above equation using T_m for the value of T . Because the DNA of the different strains is of equal molecular weight, the area under the compositional curves is the same. The amount of overlap was determined by using the error integral, because the thermal denaturation curve can be considered as the error function of the %GC distribution. Use of this function for comparing two strains requires

the standard deviation of the strains sampled and the difference between the % $\overline{GC}$ of the two strains. The standard deviation is a measure of the range of the %GC compositional distribution of the species.

Calculation of the species standard deviation is complicated by the asymmetry of the %GC distribution, which is most likely associated with the existence of an excessive number of adenine-thymine-rich regions (2). However, the distribution appears to be approximately normal, and an approximate standard deviation, based on normal distribution theory, can be calculated. De Ley found (2), in addition, that each half of the distribution below and above T_m is almost perfectly normal, although each has a separate standard deviation, σ_1 and σ_r . In the study, these observations also appeared correct, with the left or lower side of the distribution always being broadest, thus necessitating that the standard deviations for the two halves

be calculated separately and averaged to obtain a reasonable approximation to the species standard deviation under the assumption that the underlying distribution is normal.

In this experiment, two replicates of each of the 26 strains were measured. To determine the standard deviation for each half of the distribution, the %GC's around %GC were calculated for each replicate. Sums of squares for each half of the distribution were obtained and adjusted for the variation in replicates and strains. The resulting error sum of squares was used to determine the standard deviation, giving a standard deviation of $\sigma_1 = 5.49$ and $\sigma_r = 2.85$. The average standard deviation is 4.17, and the corrected average standard deviation, $\sigma_c = \sigma_u - .75$, is 3.42 for the strains studied. To calculate the per cent homology, the complement error integral was evaluated over the range of zero to ΔGC (when ΔGC is the %GC for strain 258 minus the %GC for the strain under consideration) and multiplied by 100. De Ley (2) gave the details of this procedure.

RESULTS AND DISCUSSION

Strain 258 was chosen as the reference strain because its isolated DNA had the highest T_m of any of the strains. The degrees of homology between this reference strain and the other 25 strains are summarized in Table 1. The homologies ranged from 69.5 to 95.0%. These homology data were plotted against T_m or %GC as shown in Fig. 1. Least significant difference analysis of the homology percentages resulted in three statistically significant but overlapping clusters. However, the degree of homology between some of the strains (24, 130, 83, 5563) and strain 258 is only about 70%. Since Heberlein, De Ley, and Tijtgat (8) concluded that authentic strains of the same species

should display at least 70% DNA homology based on their method of analyzing base composition data, we can say this species shows rather broad genetic diversity. Despite the questionable diversity of this species, *R. japonicum* strains exhibit closer affinity to each other than is shown between *R. japonicum* and other members of the family *Rhizobiaceae*. Thus, if one accepts the limit of 70% homology within a species, this species appears to be a valid one based on DNA homology data although it exhibits just about the limits of diversity. These conclusions are supported by nutritional, serological, and physiological studies being conducted in our laboratory (5, 6). In all of these studies, considerable diversity is observed, and yet the organisms appear more closely related to other isolates of *R. japonicum* than to other rhizobia.

Recently, some investigations on numerical taxonomy of the genus *Rhizobium* have suggested that *R. japonicum* and other slow growing species are so different from the other rhizobia as to warrant a separate genus for *R. japonicum* and related species (7, 11). One of these studies included six isolates of *R. japonicum* (7), whereas the other included only one representative of this species (11). DNA base homology studies of the taxonomy of the genus *Rhizobium* (3, 8) also included only a limited number of *R. japonicum* strains. In view of the genetic diversity of this species as found in the present study, a large sampling of representative strains of *R. japonicum* must be included in any valid taxonomic studies of this species or in establishing the status of the genus *Rhizobium*.

ACKNOWLEDGMENTS

This study was supported by grant GB-4378X from the National Science Foundation, Public Health Service research grant AI-107247 from the National Institute of Allergy and Infectious Diseases, and by U.S. Department of Agriculture, CSRS grant 916-15-04.

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FIG. 1. Relatedness within the species *Rhizobium japonicum* on the basis of the highest possible theoretical DNA homology between 26 strains relative to *R. japonicum* strain 258 (DNA homology = 100%).

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