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Adansonian Analysis of the Rhizobiaceae

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SUMMARY

One hundred and ninety-one coded features of 21 strains of the genera *Rhizobium*, 18 of *Agrobacterium*, 11 of *Chromobacterium*, selected strains of *Vibrio cholerae*, *Flavobacterium*, and other representative strains of the families Enterobacteriaceae and Pseudomonadaceae were subjected to Adansonian analysis, with the use of a high-speed computer for the establishment of Similarity (S) and Matching (M) coefficients and for the sorting of the strains into taxonomic clusters. From the frequency of occurrence of features computed for each of the clusters, tables were prepared which provided correlated characteristics suitable for the description of the clusters. Hypothetical median organisms were also computed for each of the clusters and actual strains were selected as neotypes for the new genera, *Rhizobium* and *Phytomyxa*. The genus *Rhizobium* includes the fast-growing rhizobia and the agrobacteria and contains at least four species: *R. meliloti*, *R. leguminosarum*, *R. radiobacter*, *R. rubi*. The genus *Phytomyxa* is reserved for the slow-growing rhizobia which have been placed in the species *R. japonicum*. *Agrobacterium gypsophilae* and *A. pseudotsugae* are removed from both *Rhizobium* and *Phytomyxa* as defined in this study. The lack of significantly high intergeneric relationships amongst the members of the family Rhizobiaceae suggest revaluation of the family structure. From the results obtained here, the genera *Rhizobium* and *Phytomyxa* appear more closely related to the members of the Pseudomonadaceae.

INTRODUCTION

Bacterial taxonomy is at present experiencing a renewal of interest due mainly to developments in information science, molecular biochemistry and molecular genetics. Relationships amongst the soil bacteria as presented in the seven editions of *Bergey's Manual of Determinative Bacteriology* were based primarily on brilliant deductions and the intuitive genius of the pioneer bacteriologists such as Winogradsky, Beijerinck and others.

The soil bacteria received a good deal of attention in the earliest days of bacteriology and the taxonomy of the root nodule bacteria traces to the papers of Schroeter (1886) who separated the root nodule bacteria into two species which were based entirely on root nodule structure. Beijerinck (1888) described the isolation and distinct morphological forms of *Phytomyxa leguminosarum* and *Phytomyxa lupini*, the two species established by Schroeter. The root nodule bacteria have been grouped on characteristic legume root nodulation and subgrouped on the range and type of host plants infected. *Bergey's Manual* (1957) accords six of the subgroups (*Rhizobium*

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leguminosarum Frank, *R. trifolii* Dangeard, *R. phaseoli* Dangeard, *R. meliloti* Dangeard, *R. japonicum* Kirchner, *R. lupini* Schroeter) specific status. This classification, however, has not fully held up to the tests of time and of new methodologies (Lange, 1961; Graham, 1964; De Ley & Rassel, 1965). Furthermore, the generic relationships within the Rhizobiaceae, i.e. intergeneric relationships of *Rhizobium*, *Agrobacterium* and *Chromobacterium*, as well as the intrageneric relationships require some careful reconsideration (Lange, 1961). The DNA homology studies of De Ley, Bernaerts, Rassel & Guilmot (1966*a*) also indicate the need for re-evaluation of the family Rhizobiaceae.

An evaluation of certain diagnostic features currently used in identification and classification of Rhizobium species was presented by Graham & Parker (1964) and an Adansonian analysis of Chromobacterium species was made by Sneath (1957), who provided an extended description for that genus. However, no comparative and complete descriptions of the genera of the Rhizobiaceae are available at the present time.

The study reported here is an Adansonian analysis of the Rhizobiaceae and is intended as an extended survey of the *in vitro* features used in the diagnosis of these bacteria. The relationships amongst the strains representing the genera *Rhizobium*, *Agrobacterium*, *Chromobacterium*, *Pseudomonas*, *Enterobacter*, *Escherichia*, *Vibrio* and *Proteus* have been examined on the basis of all available evidence, i.e. computed similarities, nucleic acid data and enzymic similarities. The frequencies of occurrence of each of the characters tested in the present analysis provide an extended description of the species within the family Rhizobiaceae as well as patterns of characters useful in diagnostic applications.

METHODS

Organisms

A total of 59 cultures was used in the study. The species represented and the source of isolates are listed in Table 1. The Rhizobium and Agrobacterium strains, with the exception of isolates 6466 and 5GLY.FE, were maintained on a modification of the basal medium described by Graham & Parker (1964): (in g./l.) $MgSO_4.7H_2O$, 0·25; $CaSO_4.2H_2O$, 0·03; $Na_2HPO_4.12H_2O$, 1·20; KH_2PO_4, 0·55; NaCl, 0·25; $FeSO_4.7H_2O$, 0·0035; $ZnSO_4.7H_2O$, 0·00016; $CuSO_4.5H_2O$, 0·00008; H_3BO_3, 0·0005; $MnSO_4.4H_2O$, 0·0004; Difco yeast extract, 0·25; Bacto agar, 20·0; mannitol, 10; pH 7·0. This medium (YM) with the addition of 1 % proteose peptone (YMP) was used for the maintenance of the remaining isolates included in the study. Each strain was examined for purity before testing was begun. Furthermore, before inoculation of each test medium, all cultures were streaked out to confirm purity. In addition to the strains listed in Table 1, 26 strains, including the genera *Pseudomonas*, *Serratia*, *Alcaligenes*, *Flavobacterium* and *Xanthomonas*, for which coded data from previous studies were in storage on the computer, were compared by retrieving their data.

Characters and methods of testing

A total of 191 characters were studied and coded for computer analysis. These are listed in the text which follows by character sequence number from the computer analysis (numbers in parentheses). The media for examination of physiological features was prepared by using a salts diluent (Graham & Parker 1964); however, the

Table 1. *Source of isolates included in the computer analysis*

Strain no.	Culture	Source
922*	*Rhizobium* sp.	*Glycine javanica*. M. M. Kennedy, Ayr, Queensland, Australia
942*	*Rhizobium* sp.	*Phaseolus aureus* CB 512 D. O. Norris, Taroom, Queensland, Australia
948*	*Rhizobium* sp.	*Phaseolus aureus* CB 121 D. O. Norris, Australia
952*	*R. japonicum*	*Glycine max* CB 1003 D. O. Norris, Thailand
963*	*Rhizobium* sp.	*Lotononis bainesii* CB 376 D. O. Norris, Australia
965*	*Rhizobium* sp.	*Arachis prostrata* CB 530 D. O. Norris, Australia
988	*Rhizobium* sp.	*Leucaena glauca* NGR 8 ex Trinnick, New Guinea
989	*Rhizobium* sp.	*Clitoria ternatea* CB 930 D. O. Norris, Australia
997*	*Rhizobium* sp.	*Dolichos africanus* CB 756 D. O. Norris, Marandellos, South Rhodesia
998*	*Rhizobium* sp.	*Desmodium intortum* CB 627 D. O. Norris, Ex Bonnier, Congo
WU 425†	*Rhizobium* sp.	*Ornithopus compressus* strain S 3 (M. D. Brocx), Esperance, Western Australia (effective on lupins and Ornithopus)
10317	*Rhizobium* sp.	ATCC
WU 7	*R. lupini*	D 27, Swanbourne, Western Australia (isolated by Dr R. T. Lange) authentic 1960
10318	*R. lupini*	ATCC
SU 216‡	*R. meliloti*	*Medicago sativa* strain 107 S.C.W.-5. Wisconsin, U.S.A. (Parker)
SU 277	*R. meliloti*	*Medicago tribuloides*. M. Tr. Perth, Western Australia (seed from Perth but nodulated plant from Leeton Exp. Farm as 271–279)
SU 298	*R. trifolii*	Isolated from effective plant of Crimson Clover L 17 4A/2
SU 299	*R. leguminosarum*	Vetch and pea culture, New South Wales Department of Agriculture, Australia (originally from U.S.A.)
SU 301	*R. leguminosarum*	Pea B-isolated from effective Field Pea Dunoon, 1952
SU 308	*R. trifolii*	A.G. 14 K. J. Baird, Armidale, New South Wales, Australia
SU 312	*R. phaseoli*	Bean culture, New South Wales Department of Agriculture (1955 Reclassified Nal), Australia
4720	*Agrobacterium tumefaciens*	American Type Culture Collection (ATCC) Washington, D.C.
11156	*A. tumefaciens*	ATCC
11157	*A. tumefaciens*	ATCC
B 6	*A. tumefaciens*	P. K. Chen, Georgetown University, Wash., D.C. (highly virulent strain)
A 6 RI	*A. tumefaciens*	P. K. Chen, Georgetown University, Washington D.C. (virulent strain)
A 66 RI	*A. tumefaciens*	P. K. Chen, Georgetown University, Washington D.C. (attenuated strain)
5 GLY. FE	*A. tumefaciens*	P. K. Chen, Georgetown University, Washington D.C. (avirulent strain)
4718	*A. radiobacter*	ATCC
6466	*A. radiobacter*	ATCC
A. RADIO.	*A. radiobacter*	P. K. Chen, Georgetown University, Wash., D.C.
AR 1001	*A. radiobacter*	P. K. Chen, Georgetown University, Wash., D.C.
AR 1012	*A. radiobacter*	P. K. Chen, Georgetown University, Wash., D.C.
11325	*A. rhizogenes*	ATCC
13333	*A. rhizogenes*	ATCC
13334	*A. rubi*	ATCC
13335	*A. rubi*	ATCC

Table 1 (*cont.*)

Strain no.	Culture	Source
13330	*A. pseudotsugae*	ATCC
13331	*A. pseudotsugae*	ATCC
13329	*A. gypsophilae*	ATCC
12472	*Chromobacterium violaceum*	ATCC
6357	*C. violaceum*	ATCC
12540	*C. violaceum*	ATCC
12541	*C. violaceum*	ATCC
12542	*C. violaceum*	ATCC
553	*C. violaceum*	ATCC
13426	*C. violaceum*	ATCC
6918	*C. viscosum*	ATCC
12473	*C. lividum*	ATCC
6915	*C. amethystinum*	ATCC
16266	*Pseudomonas fluorescens* var. *antirrhinastri*	Pathogenic on antirrhinum seedlings; Queensland, Australia
13430	*P. fluorescens*	ATCC
14216	*P. aeruginosa*	ATCC
14033	*Vibrio cholerae*	ATCC
W 1485	*Escherichia coli* K 12	S. Falkow, Walter Reed Army Institute of Research, Washington D.C.
4115	*Enterobacter aerogenes*	S. Falkow, Walter Reed Army Institute of Research, Washington, D.C.
9247 TR	*Proteus morganii*	S. Falkow, Walter Reed Army Institute of Research, Washington, D.C.
PM-1	*Proteus mirabilis*	S. Falkow, Walter Reed Army Institute of Research, Washington, D.C.
.	*Providence* sp.	S. Falkow, Walter Reed Army Institute of Research, Washington, D.C.

* Strain numbers refer to strains obtained from A. Diatloff, Queensland Department of Primary Industries.

† WU strain numbers refer to strains obtained from Dr C. A. Parker, University of Western Australia.

‡ SU strains received from Dr P. H. Graham, University of Sydney.

media of Simmons and Paton (see below) were made up with distilled water. All tests were read at 1, 2, 7 days and 2, 3, and 4 weeks except for nutritional requirements which were read after 7 days and discarded. Plate inoculations were made by the spot drop (Colwell, 1964) or replica plate technique (Lederberg, 1950). Liquid media were inoculated with 2 drops from a Pasteur pipette of a 3-day culture or, in the case of the slow-growing organisms, 5-day culture.

Morphological characters (1–15) were scored from cultures grown at 25° on agar after 2 days (fast-growing isolates) and 5 days (slow-growing isolates), and examined by phase-contrast microscopy. The characters scored included: rods; curved rods; (oval) spheres; presence of filaments; refractile appearance of organisms; distribution of organisms as predominantly singles, pairs or chains; round or tapered ends; spirals (rods); length 1–3 μ or 3–5 μ; and width 0·6–1·0 μ or 0·2–0·6 μ.

Motility (16) was scored from wet mount preparations examined under phase-contrast microscopy. Examination of broth cultures for motility was done at the same time as the growth characteristics in liquid media were scored (see below).

Flagella stains (17–18) of smears from YM agar slants (Rhizobium species) and

nutrient agar (remaining isolates) were used to distinguish polar from peritrichous flagellar arrangements (Leifson, 1951).

The Gram stain (19–21) used was the Hucker modification (Society of American Bacteriologists, 1957) and cultures were scored as Gram negative, positive, or variable.

Colonial characteristics (22–34) were determined on YM and YMP agar at 3 days and also at 5–10 days for the Rhizobium species. The characters included: small colony size (1–2 mm.); medium colony size (2–5 mm.); convex; rough; opaque; translucent; entire edge, spreading on agar surface; white colony colour; off white colony colour; grey colony colour; butyrous consistency; gum production; mucilaginous colony.

Presence or absence of capsules (35) was determined by using indian ink (Bradshaw, 1964) and *metachromatic granules* (36) with methylene blue (Bradshaw, 1964).

Pigment production (37–40), as a diffusible green pigment, diffusible blue pigment, or visible insoluble pigment, was scored from examination of YM agar, YMP agar, Pseudomonas agar F (Difco), and Pseudomonas agar P (Difco). Fluorescence was determined in a liquid medium (Paton, 1959).

Growth characteristics in liquid media (41–47) included: presence of pellicle or ring; granular or even turbidity; slight, moderate or heavy turbidity with Brown's opacity tubes as reference. Characteristics in liquid media were determined for all isolates in YM broth at 2 and at 5 days, as well as in YMP broth for those isolates which preferentially in a medium with an organic nitrogen source.

Carbohydrate metabolism (48–89). Oxidative and fermentative utilization of carbohydrates was determined by the method of Hugh & Leifson (1953). This medium, without agar and with an inverted inner vial, was also used to detect gas production from carbohydrates. Growth in glucose with and without 10^{-3} M-iodoacetate was determined. The terminal pH value in the glucose fermentation tubes was also measured. Growth and production of acetic acid in ethanol agar was recorded (Shimwell, Carr & Rhodes, 1960). Acid production from carbohydrates (1 %, w/v) and ethanol (5 %, w/v) was determined on agar plates, with the peptone omitted for Rhizobium and Agrobacterium species. The carbohydrates were sterilized by filtration except dulcitol, inulin and dextrin which were steamed for 1 hr on three successive days. The carbohydrates which were tested were: glucose, maltose, lactose, sucrose, galactose, mannitol, adonitol, arabinose, cellobiose, dextrin, dulcitol, fructose, glycerol, inositol, inulin, mannose, melibiose, melezitose, raffinose, rhamnose, salicin, sorbitol, trehalose, xylose, ethanol. Hydrolysis of aesculin and starch, production of dihydroxyacetone from glycerol, and digestion of agar were tested by the methods cited by Colwell (1964). Examination for levan production on YM and YMP agar by the Graham & Parker (1964) modification of the Paton (1960) technique was also done.

The methyl red and Voges-Proskauer tests (90–91) were performed following the techniques described in the *Manual of Microbiological Methods* (1957).

Temperature range of growth (92–102) was determined in YM and YMP broth. Before inoculation for temperature growth tests, the medium was incubated at the given test temperature overnight. Temperatures studied were: 0, 5, 15, 20, 30, 35, 37, 40, 41, 42 and 44°.

NaCl tolerance (103–108) was tested on YM and YMP agar plates (Ionagar, Difco Labs, Chicago, Illinois) adjusted to the required NaCl concentrations: 0, 0·5, 3·0, 5·0, 7·0, 10·0 % (w/v).

The pH range of growth (109–116) was determined in YM and YMP broth adjusted to the required pH values before autoclaving, but in the case of pH 4·0, 4·5, 5·0, 9·0 and 10·0 the media were readjusted and dispensed aseptically after autoclaving. The pH values tested included: 4·0, 4·5, 5·0, 6·0, 7·0, 8·0, 9·0 and 10·0.

The oxidase, cytochrome oxidase, and catalase tests (117–119) were made by the methods cited by Colwell (1964).

The production of phosphatase (120) was detected on YM and YMP agar by the method of Baird-Parker (1963).

Sensitivity to antibiotics and antibacterials (121–133). Sensitivity to the 0/129 pteridine compound was tested following the method of Shewan, Hodgkiss & Liston (1954). Antibiotic sensitivities were determined by using BBL (Baltimore Biological Laboratories, Baltimore, Md.) 'Sensidiscs'. Resistance to concentrations of $HgCl_2$ was determined by incorporation of the compound in agar media. YM and YMP agar was used in all instances where sensitivities to antibiotics and antibacterials were tested. The substances and concentrations (per ml.) used were: penicillin, 10 units; dihydrostreptomycin, 10 μg; chloromycetin, 30 μg; erythromycin, 15 μg.; kanamycin, 30 μg.; aureomycin, 30 μg.; novobiocin, 30 μg.; polymyxin B, 300 units; terramycin, 30 μg.; tetracycline, 30 μg.; $HgCl_2$, 1 μg and 10 μg.

Reduction of nitrate and nitrite, gelatin liquefaction and litmus milk tests (134–143) were made following the technique of the *Manual of Microbiological Methods* (1957) and scored as described by Colwell (1964).

Growth on skim milk agar and casein hydrolysis (144–145) were determined with the skim milk agar medium of Gordon & Mihm (1959).

Production of ammonia from peptone at 14 days and hydrogen sulphide from peptone in lead acetate agar (146–147) was measured following the techniques described in the *Manual of Microbiological Methods* (1957). *Hydrogen sulphide production from cystine and cysteine* (148–149) was determined in micro-tubes with lead acetate paper (Colwell & Quadling, 1962).

Urease production (150) was tested according to the method of Christensen (1946) with peptone omitted from the media for the Rhizobium and Agrobacterium species.

Indole production (151) was tested by the Kovacs modification cited in the *Manual of Microbiological Methods* (1957).

Lecithinase (152) production was tested on YM and YMP agar plates in which 5 % (v/v) concentrated egg-yolk emulsion (Oxoid, Colab, Inc., Chicago, Illinois) was incorporated.

Lipolytic activity (153–156) was determined by detection of hydrolysis of Tweens 20, 40, 60 and 80, respectively, in YM and YMP agar following the method of Sierra (1957).

The production of 3-ketolactose (157) was determined by the method of Bernaerts & De Ley (1963).

Ability to utilize citrate (158–159) was determined by growth in Koser's citrate (Koser, 1924) modified by the addition of 1·5 % (w/v) Ionagar (Difco Labs., Detroit, Michigan) and inoculation by the replica plate technique. Reaction in Simmons's citrate (Simmons, 1926) was determined on agar slopes.

Oxidation of calcium lactate through acetate to carbonate and the utilization of 0·3 % sodium malonate, 0·1 % sodium acetate, 0·1 % sodium formate as carbon source and 0·1 % $NH_4H_2PO_4$ as nitrogen source were tested (160–164). For the nutritional

tests, a basal salts agar containing 0·00025 % yeast extract and 0·1 % peptone for strains other than the Agrobacterium and Rhizobium species was used. Details of these procedures were as cited by Colwell (1964). Tests for utilization of the carbon or nitrogen source were always done by comparison with a control plate containing only the basal salts medium or basal salts medium containing ·00025 % yeast extract and +0·1 % peptone where incorporated into the test medium.

The utilization of amino acids as carbon and nitrogen source (165–176) was determined from basal salts agar with 0·00025 % yeast extract and 0·1 % of the test amino acid added. The amino acids were filter-sterilized, except for tyrosine and cystine which were steamed for 1 hr on three successive days and used at a final concentration of 0·05 %. Ionagar (Difco Labs., Detroit, Michigan) was used in the nutritional studies and inoculations were by the replica plating technique. Amino acids tested included: L-ornithine monochloride; L-arginine hydrochloride; L-lysine; L-alanine; L-histidine; L-serine; L-phenylalanine; L-proline; L-leucine; L-glutamic acid; L-cystine; L-tyrosine.

Growth, fluorescence, and production of 2-ketogluconate from potassium gluconate in Paton medium (Paton, 1959) were scored (177–179). Growth in Haynes medium and production of 2-ketogluconate from gluconate (Haynes, 1951) was also recorded (180–181).

Arginine dihydrolase and the arginine, ornithine and lysine decarboxylases (182–185) were tested following the methods of Thornley (1960) and Moeller (1955).

Methylene blue reduction (186) was determined by adding 1 % aqueous methylene blue to a 48-hr (fast-growing organisms) or 4-day (slow-growers) YM and YMP broth culture and incubating for 1 hr.

Pectate hydrolysis (187) was detected by the method of Starr (1947).

Ability to produce penicillinase (188) was determined by the technique described by Foley & Perret (1962).

The methods for *production of phenylpyruvic acid and/or melanin from phenylalanine and production of melanin from tyrosine* (189–191) were as cited by Colwell (1964).

The results were scored for computation by recording positive reaction, i.e. growth present or positive test result, as unit features '1', and negative reaction, i.e. no growth or negative test result as '0'. In this study all organisms were tested for all features; however, the programs for analyisng the data were written so that tests not done or not applicable might be scored '3'. The computer then did not include these entries in similarity comparisons.

An IBM 1620 computer, Model II, with 1311 Disk Pack System, was used in the numerical analyses of the data. The programs where the S value was calculated, comparing positive matches, and the M value, comparing positive and negative matches, have been documented for the IBM 1620 Computer User's Library (GTP-2 and GTP-5). The frequency of feature occurrence within the major clusters (IBM Program GTP-3) followed the method of Colwell (1964).

The median organism was obtained by computer by using the program GTP-4 based on the method described by Liston, Wiebe & Colwell (1963).

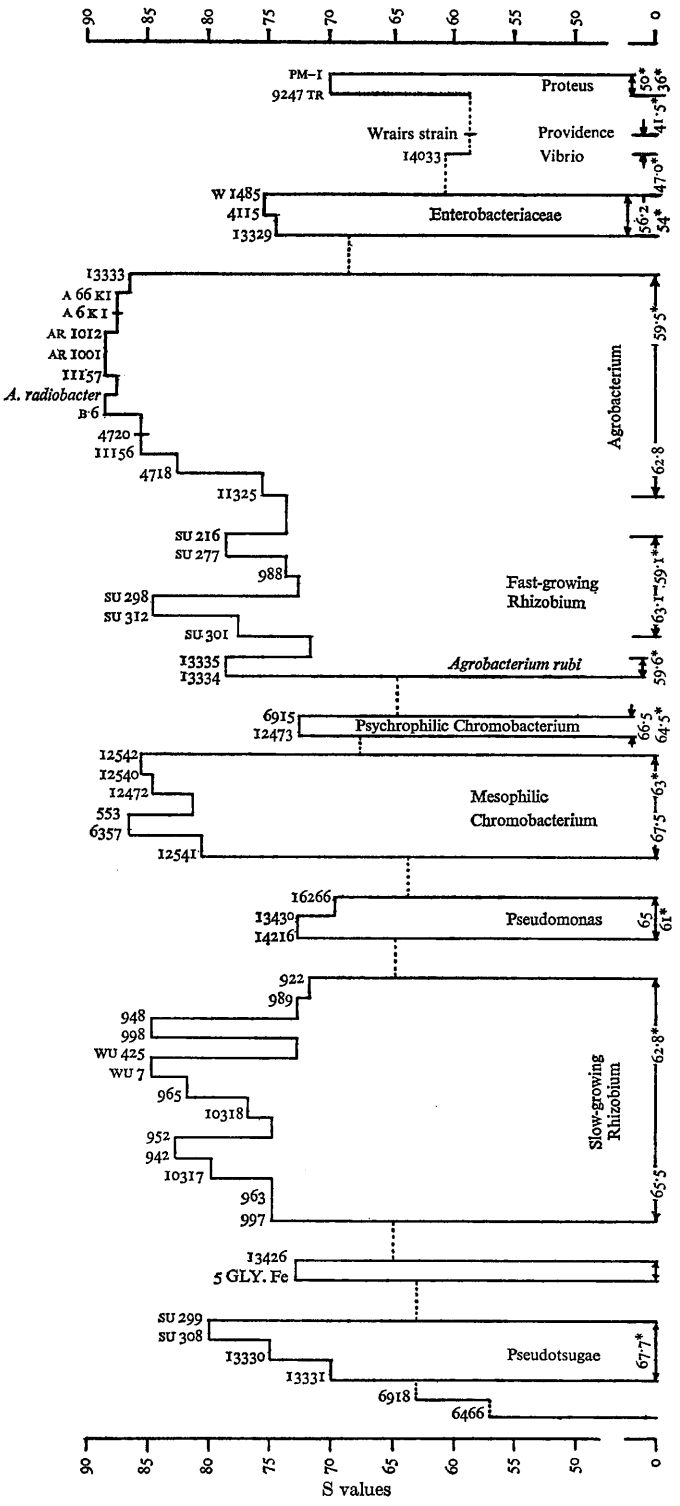

Fig. 1. Sorted clusters from the GTP-2 program output, based on highest linkages.

* Moles % guanine + cytosine DNA base composition

RESULTS

The groupings obtained by using S values from the computer analysis of the taxonomic data for the 59 strains included in the study are presented in Fig. 1. As seen from Fig. 1, at S $\geqslant$ 70, eight clusters were formed. The three major clusters of related strains consisted of: slow-growing Rhizobium strains S $\geqslant$ 72; Agrobacterium species and the fast-growing Rhizobium strains at S $\geqslant$ 72; and the mesophilic Chromobacterium strains S $\geqslant$ 81. The minor clusters were: *Agrobacterium pseudotsugae* and two fast-growing Rhizobium strains at S $\geqslant$ 70; strain 5GLY.FE with *Chromobacterium violaceum* strain 13426 at S = 73; the Pseudomonas species which grouped at S $\geqslant$ 70; psychrophilic Chromobacterium strains grouping at S $\geqslant$ 73; and Proteus species, S $\geqslant$ 70.

The M-value calculation (Silvestri, Turri, Hill & Gilardi, 1962), in which shared negative features are taken into account in computing strain similarities, was also applied to the taxonomic data for the 59 strains. The clusters which formed were found to be essentially the same as those grouped by means of S-value relationships. The main difference, however, was that from M-value computations, strains clustered at what could be interpreted as a much higher degree of similarity, namely the slow-growing Rhizobium strains clustering at M $\geqslant$ 85. Genus or species inter- and intra-relationships were difficult to interpret from inspection of the M-value output. In fact, the strains studied were merged into two major groups at M $\geqslant$ 80. In Fig. 2 the groups sorted by shared M-values of 80% or greater are presented.

Fig. 2. Cluster output from GTP-2 showing groups forming at M = 80% (M = 'Matching coefficient' of Silvestri *et al.* 1962).

Group 1: (16266) *P. fluorescens* var. *antirrhinastri*+(13430) *P. fluorescens*+(14216) *P. aeruginosa*+(922) *Rhizobium* sp.+(988) *Rhizobium* sp.+(948) *Rhizobium* sp.+(998) *Rhizobium* sp.+(WU 425) *Rhizobium* sp.+(WU 7) *R. lupini*+(965) *Rhizobium* sp.+(10318) *R. lupini*+(952) *R. japonicum*+(942) *Rhizobium* sp.+(10317) *Rhizobium* sp.+(963) *Rhizobium* sp.+(997) *Rhizobium* sp.+(13426) *C. violaceum*+(5 Gly. Fe) *A. tumefaciens*+SU 299 *R. leguminosarum*+(SU 308) *R. trifolii*+(13330) *A. pseudotsugae*+(13331) *A. pseudotsugae*+(6466) *A. radiobacter.*

Group 2: (W 1485) *E. coli*+(4115) *E. aerogenes*+(13329) *A. gypsophilae*+(4718) *A. tumefaciens*+(11156) *A. tumefaciens*+(AR 1012) *A. radiobacter*+*A. radiobacter*+(11157) *A. tumefaciens*+(AR 1001) *A. radiobacter*+(B 6) *A. tumefaciens*+(A 6 KI) *A. tumefaciens*+(A 66 KI) *A. tumefaciens*+(4720) *A. tumefaciens*+(13333) *A. rhizogenes*+(11325) *A. rhizogenes*+(SU 277) *R. meliloti*+(SU 216) *R. meliloti*+(988) *Rhizobium* sp.+(SU 301) *R. leguminosarum*+(SU 312) *R. phaseoli*+(SU 298) *R. trifolii*+(13335) *A. rubi*+(13334) *A. rubi.*

Group 3: (6915) *C. amethystinum*+(12473) *C. lividum*+(P 66) *X. phaseoli*+(12542) *C. violaceum*+(12540) *C. violaceum*+(12472) *C. violaceum*+(533) *C. violaceum*+(6375) *C. violaceum*+(12541) *C. violaceum.*

Group 4: (PM-1) *P. mirabilis*+(9247 TR) *P. morgani.*

Group 5: (17) *Pseudomonas*+*P. viscosa*+(P 51) *P. atrofaciens*+(P 8) *P. atrofaciens*+(P 9) *P. coronafaciens*+(P 28) *P. lachrymans*+(P 26) *P. pisi*+(P 11) *P. pisi*+(P 33) *P. phaseolicola*+(P 3) *P. phaseolicola*+(P 10) *P. glycinea.*

Group 6: (4358) *F. arborescens*+(8315) *F. flavescens*+(958) *F. suaveolens*+(338) *P. maltophila*+(16) *A. radiobacter.*

Group 7: (BS) *Alc. faecalis*+(14 L) *Pseudomonas*+*Achr. iophagus.*

Group 8: (SM 10) *S. marcescens*+(SMI) *S. marcescens*+(SM 21) *S. marcescens.*

The S value relationship ($S = NS/N_S + N_D$, where N_S = number of similar matches and N_D = number of dissimilar matches, i.e. features positive for one strain but not the other and vice versa) were used for subsequent analyses of the taxonomic data. In Fig. 3 the formation of the groups can be followed. Eleven groups formed at

Fig. 3. Cluster output from GTP-2 showing groups formed at selected levels of similarity based on S-value calculations (Sokal & Sneath, 1963).

Clustering at S = 80.

Group 1: (948) *Rhizobium* sp.+(998) *Rhizobium* sp.

Group 2: (WU 425) *Rhizobium* sp.+(WU 7) *R. lupini.*

Group 3: (952) *R. japonicum*+(942) *Rhizobium* sp.+(10317) *Rhizobium* sp.

Group 4: (SU 308) *R. trifolii*+ (SU 299) *R. leguminosarum.*

Group 5: (SU 312) *R. phaseoli*+(SU 298) *R. trifolii.*

Group 6: (4718) *A. radiobacter*+(11156) *A. tumefaciens*+(4720) *A. tumefaciens*+(B 6) *A. tumefaciens*+*A. radiobacter*+(11157) *A. tumefaciens*+(AR 1001) *A. radiobacter*+(AR 1012) *A. radiobacter*+(A 6 RI) *A. tumefaciens*+(A 66 RI) *A. tumefaciens*+(13333) *A. rhizogenes.*

Group 7: (12542) *C. violaceum*+(12540) *C. violaceum*+(12472) *C. violaceum*+(553) *C. violaceum*+(6357) *C. violaceum*+(12541) *C. violaceum.*

Group 8: (P 51) *P. atrofaciens*+(P 8) *P. atrofaciens*+(P 9) *P. coronafaciens.*

Group 9: (P 11) *P. pisi*+(P 26) *P. pisi*+(*P* 28) *P. lachrymans.*

Group 10: (609) *Alc. faecalis*+(14 L) *Pseudomonas.*

Group 11: (SM 10) *S. marcescens*+(SMI) *S. marcescens*+(SM 21) *S. marcescens.*

Clustering at S = 75.

Group 1: (948) *Rhizobium* sp.+(998) *Rhizobium* sp.

Group 2: (WU 425) *Rhizobium* sp.+(WU 7) *R. lupini*+(965) *Rhizobium* sp.+(10317) *Rhizobium* sp.+(10318) *R. lupini*+(952) *R. japonicum*+(942) *Rhizobium* sp.+(963) *Rhizobium* sp.+(997) *Rhizobium* sp.

Group 3: (SU 216) *R. meliloti*+(SU 277) *R. meliloti.*

Group 4: (SU 301) *R. leguminosarum*+(SU 312) *R. phaseoli*+(SU 298) *R. trifolii.*

Group 5: (13330) *A. pseudotsugae*+(SU 308) *R. trifolii*+(SU 299) *R. leguminosarum.*

Group 6: (11325) *A. rhizogenes*+(4718) *A. radiobacter*+(11156) *A. tumefaciens*+(4720) *A. tumefaciens*+(B 6) *A. tumefaciens*+*A. radiobacter*+(11157) *A. tumefaciens*+(AR 1001) *A. radiobacter*+(AR 1012) *A. radiobacter*+(A 6 RI) *A. tumefaciens*+(A 66 RI) *A. tumefaciens*+(13333) *A. rhizogenes.*

Group 7: (13334) *A. rubi*+(13335) *A. rubi.*

Group 8: (12542) *C. violaceum*+(12540) *C. violaceum*+(12472) *C. violaceum*+(553) *C. violaceum*+(6357) *C. violaceum*+(12541) *C. violaceum.*

Group 9: (W 1485) *E. coli*+(4115) *E. aerogenes*+(13329) *A. gypsophilae.*

Group 10: (P 53) *P. phaseolicola*+(P ii) *P. pisi*+(P 26) *P. pisi*+(P 28) *P. lachrymans*+(P 8) *P. atrofaciens*+(P 9) *P. coronafaciens*+(P 3) *P. phaseolicola.*

Group 11: (609) *Alc. faecalis*+(14 L) *Pseudomonas.*

Group 12: *P. viscosa*+(17) *Pseudomonas.*

Group 13: (SM 10) *S. marcescens*+(SMI) *S. marcescens*+(SM 21) *S. marcescens.*

Clustering at S = 70.

Group 1: (922) *Rhizobium* sp.+(989) *Rhizobium* sp+(948) *Rhizobium* sp.+(998) *Rhizobium* sp.+(WU 425) *Rhizobium* sp.+(WU 7) *R. lupini*+(965) *Rhizobium* sp.+(10318) *R. lupini* +(952) *R. japonicum*+(942) *Rhizobium* sp.+(10317) *Rhizobium* sp.+(963) *Rhizobium* sp.+(997) *Rhizobium* sp.

Group 2: (13334) *A. rubi*+(13335) *A. rubi*+(SU 301) *R. leguminosarum*+(SU 312) *R. phaseoli*+(SU 298) *R. trifolii*+(998) *Rhizobium* sp.+(SU 277) *R. meliloti*+(SJ 216) *R. meliloti*+(11325) *A. rhizogenes*+(4718) *A. radiobacter*+(11156) *A. tumefaciens*+(4720) *A. tumefaciens*+(B 6) *A. tumefaciens*+*A. radiobacter*+(11157) *A. tumefaciens*+(AR 1001) *A. radiobacter*+(AR 1012) *A. radiobacter*+(A 6 RI) *A. tumefaciens*+(A 6 RI) *A. tumefaciens*+(13333) *A. rhizogenes.*

Group 3: (13331) *A. pseudotsugae*+(13330) *A. pseudotsugae* +(SU 308) *R. trifolii*+(SU 299) *R. leguminosarum.*

Group 4: (12473) *C. lividum*+(6915) *C. amethystinum.*

Group 5: (P 60) *X. phaseoli*+(12542) *C. violaceum*+(12540) *C. violaceum*+(12472) *C. violaceum*+(6357) *C. violaceum*+(553) *C. violaceum*+(12541) *C. violaceum.*

Group 6: (16266) *P. fluorescens* var. *antirrhinastri*+(13430) *P. fluorescens*+(14216) *P. aeruginosa.*

Group 7: (SM 10) *S. marcescens*+(SMI) *S. marcescens*+(SM 21) *S. marcescens*+(13329) *A. gypsophilae*+(4115) *E. aerogenes*+(W 1485) *E. coli.*

Group 8: (PM-1) *P. mirabilis*+(9247 TR) *P. morgani.*

Group 9 (13426) *C. violaceum*+(5 GLY. FE) *A. tumefaciens.*

Group 10: (P 10) *P. glycinea*+(P 33) *P. phaseolicola*+(P 11) *P. pisi*+ (P 26) *P. pisi*+(P 28) *P. lachrymans*+(P 8) *P. atrofaciens*+(P 51) *P. atrofaciens*+(P 9) *P. coronafaciens*+(P 3) *P. phaseolicola.*

Group 11: (609) *Alc. faecalis*+(14 L) *Pseudomonas.*

Group 12: *P. viscosa*+(17) *Pseudomonas.*

S ⩾ 80, 13 groups at S ⩾ 75, and 12 groups at S ⩾ 70. Figures 2 and 3 also include the data for other reference strains retrieved from the computer data library. As can be seen from Figs. 1 and 3, separation of the Rhizobia and Agrobacteria from Chromobacterium, Pseudomonas, Serratia, Enterobacter, Escherichia, and Proteus species was at S-value levels of < 70%.

From examination of Fig. 3 certain results may be noted. The slow-growing Rhizobia and the fast-growing Rhizobia/Agrobacteria clusters are distinct, forming from pairs at S ⩾ 80 into the two clusters at S ⩾ 70. The strains *Agrobacterium pseudotsugae* 13330 and 13331, *Rhizobium trifolii* SU 308 and *R. leguminosarum* SU 299 formed a separate and distinct cluster, unrelated to the two major groups. DNA base-composition data available for these strains (see Fig. 1) indicated a significantly different overall guanine+cytosine DNA base composition for these strains when compared with strains of the two major groups. Other DNA base composition data for these groups also correlated well with the sorting of the strains obtained from the computer analysis.

Agrobacterium gypsophilae 13329 shared high relationships with the strains of *Escherichia coli*, *Enterobacter aerogenes* and *Serratia marcescens* (S = 70, group 7; Fig. 3). An electrophoretic study of the isozymes and protein patterns of *A. gypsophilae* and other agrobacteria currently underway in our laboratories has provided confirmatory evidence for removing *A. gypsophilae* from the agrobacteria (P. K. Chen, personal communication).

Proteus strains (S = 70, group 8) did not cluster with other strains representing the Enterobacteriaceae (S = 70, group 7). Soil isolates labelled by other investigators as Pseudomonas species (S = 70, group 10) did not cluster with *Pseudomonas fluorescens* and *P. aeruginosa* (S = 70, group 6). These strains and the strains of groups 11 and 12 will be considered in a separate publication.

The Chromobacterium strains formed two major clusters corresponding to the mesophilic (*C. violaceum*) and psychrophilic (*C. lividum*) groups described by Sneath (1960). These two groups joined at S = 68%. The single strain of *C. viscosum* which was tested did not cluster with the Chromobacterium groups but rather joined at a low level (S = 63%) to the Rhizobium and *A. pseudotsugae* strains. Also, as noted above, *C. violaceum* 13426 joined only with *A. tumefaciens* 5 GLY. FE and neither of these strains shared significantly high similarities with any of the other isolates tested.

Thus, intra-group relationships for the Rhizobiaceae, and inter-group relationships for the family with the Pseudomonadaceae and Enterobacteriaceae can be observed from Fig. 1. Relationships with Serratia, Flavobacterium, Alcaligenes and Xanthomonas strains tested are given in Figs. 2 and 3.

The characters shared by all strains of the species and genera comprising the family Rhizobiaceae examined in the present study are listed in Tables 2 and 3. Thus, all the strains tested as Gram-negative rods, 1–3 μ length × 0·6–1·0 μ width, round-ended, motile, and occurring mainly as single or paired cells. Colonies on YM and YMP agar were 1–2 mm diameter, convex, opaque, and entire. Growth within the temperature range 20–30°, pH 6–8, and NaCl concentration of 0–0·5% was noted. All members of the Rhizobiaceae studied were catalase-positive. None of the strains produced a capsule detectible by the method employed. None grew in YM or YMP broth with NaCl added to final concentrations of 5% or greater. None produced gas from carbohydrates, acetic acid from ethanol, dihydroxyacetone from glycerol, or a urease detectible within 48 hr. The strains were all methyl-red negative and Voges–Proskauer

negative. None of the strains demonstrated starch or pectate hydrolysis, agar digestion, or production of phenylpyruvic acid from phenylalanine. All strains were negative for hydrogen sulphide production in lead acetate agar but positive tests were recorded when other methods were employed for hydrogen sulphide production (see Table 4). The characters which were variable for the groups are listed in Table 4.

Table 2. *Positive characters, i.e. feature frequency of 1·00, for the major groups defined by the computer analysis: (1) slow-growing Rhizobium; (2) Agrobacterium/fast-growing Rhizobium; and (3) Chromobacterium.*

Character no.	Character	Character no.	Character
1	Straight rods	19	Gram-negative
6	Predominant arrangement as single cells	24, 26, 28	Convex, opaque colonies with entire edge
9	Round ends	95, 96	Growth within temperature range 20–30°
12	1–3 μ length	103, 104	Growth at NaCl concentrations 0–0·5%
14	0·6–1·0 μ width	112–114	Growth within pH range 6·0–8·0
16	Motile	119	Catalase-positive

Table 3. *Negative characters, i.e. feature frequency of 0, for the major groups defined by the computer analysis: (1) slow-growing Rhizobium; (2) Agrobacterium/fast-growing Rhizobium; and (3) Chromobacterium*

Character no.	Character	Character no.	Character
2	Curved rods	88	Agar digestion
3	(Oval) spheres	90	Methyl-red reaction
4	Filaments	91	Voges–Proskauer reaction
5	Refractile cells	108	Growth in 10% NaCl
10	Tapered end	147	H_2S produced from peptone in lead acetate agar
11	Spiral (rods)	150	Urease production (48 hr)
35	Capsule	151	Indole production
41	Pellicle formation in YM broth	157	3-ketolactose production
50	Glucose, gas production	184	Ornithine decarboxylase
53	Acetic acid production from ethanol	185	Lysine decarboxylase
86	Starch hydrolysis	187	Pectate hydrolysis
87	Dihydroxyacetone production from glycerol	189	Phenylpyruvic acid production from phenylalanine

The slow-growing Rhizobium species cluster demonstrated, in addition to the characters listed in Tables 2 and 3, the following features: polar flagella; mucilaginous colonies; metachromatic granules; a slight turbidity in YM broth; little or no detectible utilization of carbohydrates; production of levan; growth in the temperature range 15–37°; tolerance of NaCl in concentrations up to 3·0%; growth in the pH range 4–8; oxidase positive; no sensitivity to the 0/129 pteridine compound or to polymyxin B and marked sensitivity to $HgCl_2$ at 10 μg. concentration; production of an alkaline reaction in litmus milk and a detectible penicillinase. Of the characters for which the strains tested uniformly negative, the following may be noted: phosphatase; nitrite reduction; gelatin liquefaction; litmus milk peptonization; casein hydrolysis;

Table 4. *Characters with the variable frequency of occurrence amongst the major groups and subgroups defined by the computer analysis*

Characters with frequency of occurrence 0–0·25, scored (−), or 0·75–1, scored (+), were selected and tabulated. The sequence of characters as listed is as coded for the computer. For details of methods for performing tests, scoring, etc., see Methods. ± = Variable occurrence within the set of strains tested. Actual frequency of occurrence is given in parentheses.

					Agrobacterium/fast-growing *Rhisobium*			
			Chromobacterium					
Character no.	Character	Slow-growing *Rhizobium*	*C. violaceum*	*C. lividum**	*A. radiobacter tumefaciens rhizogenes*	*R. meliloti**	*R. leguminosarum trifolii phaseoli*	*A. rubi**
	Morphological and cultural:							
17	Polar flagella	+	+	+	−	−	−	−
18	Peritrichous flagella	−	+	+	+	+	+	+
22	Colony size 1–2 mm dia.	+	+	+	+(0·75)	+	(±0·66)	+
34	Mucilaginous colonies	+(0·92)	−	−	+	+	+	+
36	Metachromatic granules	+	+(0·83)	−	−(0·17)	±(0·50)	−	+
45	Slight turbidity in YM broth	+	+	±(0·50)	−	±(0·50)	±(0·33)	±(0·50)
46	Moderate turbidity in YM broth	−	−	±(0·50)	+(0·92)	+	±(0·66)	±(0·50)
	Carbohydrates:							
48	Glucose (Hugh & Leifson, 1953) oxidative, Acid	−(0·08)	+	+	+(0·92)	+	+	±(0·50)
49	Glucose (Hugh & Leifson, 1953) fermentative, Acid	−	+	−	+	+	+	+
51	Glucose+iodoacetate, growth	+(0·85)	+	±(0·50)	+(0·92)	+	±(0·66)	±(0·50)
52	Glucose+iodoacetate, acid	−	±(0·50)	−	(+0·75)	+	±(0·33)	−
55	Maltose, acid	−(0·08)	+	±(0·50)	+	+	+	+
57	Lactose, acid	−	−	−	+(0·92)	+	+	+
59	Sucrose, acid	(0·15)	±(0·33)	+	+(0·83)	+	+	+
61	Galactose, acid	±(0·54)	−(0·17)	+	+	+	+	+
63	Mannitol, acid	−	−	−	±(0·58)	±(0·50)	+	+
65	Terminal pH in glucose fermentation							
	pH 6·5–7·0	+(0·92)	N.T.†	N.T.	+(0·82)	±(0·50)	−	+
	pH 5·0–6·0	−	N.T.	N.T.	−(0·09)	−	±(0·33)	−
66	Adonitol, acid	−	−	−	+	+	+	+
67	Arabinose, acid	±(0·30)	−	+	+	+	+	+
68	Cellobiose, acid	−	−	+	+	+	+	±(0·50)
69	Dextrin, acid	−	±(0·50)	−	−	−	−	−
70	Dulcitol, acid	−	−	−	+	±(0·50)	+	−
71	Fructose, acid	−(0·15)	+(0·83)	±(0·50)	+(0·75)	−	+	+
72	Glycerol, acid	−	−(0·17)	+	+	±(0·50)	+	+
73	Inositol, acid	−	−	+	+(0·92)	−	+	±(0·50)
74	Inulin, acid	−	−	−	−(0·25)	±(0·50)	+	−
75	Mannose, acid	−(0·15)	±(0·66)	+	+	+	+	+
76	Melibiose, acid	−	−	−	+(0·75)	+	+	±(0·50)
77	Melezitose, acid	−	−	−	+(0·92)	+	−	±(0·50)
78	Raffinose, acid	−	−	−	−(0·17)	−	+	−
79	Rhamnose, acid	−(0·23)	±(0·50)	−	+	+	+	±(0·50)
80	Salicin, acid	−(0·08)	±(0·33)	−	+	+	±(0·33)	+

Table 4. (*Continued*)

Character no.	Character	Slow-growing *Rhizobium*	*Chromobacterium*		*Agrobacterium*/fast-growing *Rhizobium*			
			C. violaceum	*C. lividum**	*A. radiobacter tumefaciens rhizogenes*	*R. meliloti**	*R. leguminosarum trifolii phaseoli*	*A. rubi**
81	Sorbitol, acid	−	−(0·17)	+	+(0·75)	+	+	±(0·50)
82	Trehalose, acid	−	+(0·83)	−	+	+	+	+
83	Xylose, acid	±(0·38)	−	±(0·50)	+(0·92)	+	+	±(0·50)
84	5% Ethanol, acid	−(0·08)	−(0·17)	−	±(0·33)	−	−	−
85	Aesculin hydrolyzed	±(0·61)	−	+	+	±(0·50)	±(0·33)	+
89	Levan produced	+(0·77)	−	±(0·50)	+	+	+	−
	Physiological:							
93	Growth at 5°	−	−	+	−	−	−	±(0·50)
94	Growth at 15°	+(0·77)	+	+	+	+	+	+
97	Growth at 35°	+	+	+	+	+	±(0·33)	+
98	Growth at 37°	+(0·85)	+	−	+(0·92)	+	−	+
101	Growth at 42°	−	±(0·66)	−	−(0·08)	±(0·50)	−	−
105	Growth in 3·0% NaCl	+	−	−	+	+	+	+
106	Growth in 5·0% NaCl	−	−	−	±(0·67)	−	−	−
109	Growth at pH 4·0	+	−	−	±(0·58)	−	±(0·33)	+
110	Growth at pH 4·5	+	+	+	+	±(0·50)	+	+
116	Growth at pH 10·0	±(0·30)	+	±(0·50)	+	+	−	+
117	(Kovacs) oxidase	+	+	±(0·50)	±(0·83)	+	±(0·66)	+
120	Phosphatase	−	+	+	±(0·50)	−	−	±(0·50)
	Antibacterials:							
121	Sensitivity to o/129 pteridine compound	−	−	−	−	−	−	±(0·50)
	Sensitive to:							
122	Penicillin, 10 units	−(0·15)	−	±(0·50)	−(0·17)	−	±(0·66)	+
123	Dihydrostreptomycin, 10μg.	±(0·61)	+(0·83)	+	−	±(0·50)	−	+
124	Chloromycetin, 30 μg.	−(0·23)	+(0·83)	+	+(0·92)	+	+	±(0·50)
125	Erythromycin, 15 μg.	−(0·23)	+(0·83)	+	±(0·33)	−	+	+
126	Kanamycin, 30 μg.	±(0·69)	+	+	+	+	+	+
127	Aureomycin, 30 μg.	+(0·77)	+(0·83)	+	+	+	±(0·66)	+
128	Novobiocin, 30 μg	±(0·69)	+	+	+(0·83)	+	±(0·66)	±(0·50)
129	Polymyxin B, 300 units	−	+	+	±(0·50)	+	+	+
130	Terramycin, 30 μg	±(0·46)	+	+	+	+	+	±(0·50)
131	Tetracycline, 30 μg.	+(0·85)	+	+	+	+	+	+
132	$HgCl_2$, 1 μg.	+(0·92)	−	−	−(0·08)	−	−	−
133	$HgCl_2$, 10 μg.	+	+	±(0·50)	+	+	+	+
	Biochemical tests:							
134	Nitrate reduced to nitrite	+(0·85)	+	+	+	−	+	+
135	Nitrite reduced	−	±(0·50)	±(0·50)	±(0·58)	−	−	−
136	Gelatin liquefied	−	+	±(0·50)	−	−	−	−
137	Litmus milk, peptonized	−	+	−	+	−	+	+
140	Litmus milk, alkaline	+	−	+	+(0·92)	+	+	+
145	Casein hydrolysed	−	+	−	−	−	−	−
146	Ammonia produced in peptone water	+	+	+	±(0·42)	±(0·50)	−	+
148	Hydrogen sulphide produced from cystine	±(0·46)	+	+	+	+	±(0·66)	+
149	Hydrogen sulphide produced from cysteine	±(0·69)	+	+	+	+	+	+

Table 4. (*Continued*)

Character no.	Character	Slow-growing *Rhizobium*	*Chromobacterium*		*Agrobacterium*/fast-growing *Rhizobium*			
			C. violaceum	*C. lividum**	*A. radiobacter tumefaciens rhizogenes*	*R. meliloti**	*R. leguminosarum trifolii phaseoli*	*A. rubi**
150	Urease (1 week)	±(0·69)	±(0·50)	±(0·50)	+	+	+	+
152	Lecithinase	−	+	±(0·50)	−	−	−	−
153	Lipase (Tweens 20, 40)	+(0·85)	+	+	−	−	−	+
154	Lipase (Tween 60)	+(0·85)	+	±(0·50)	−	−	−	−
155	Lipase (Tween 80)	−	+	±(0·50)	−	−	−	−
158	Growth in Koser's citrate	−(0·23)	+	+	±(0·66)	±(0·50)	−	±(0·50)
159	Simmons citrate	−	+(0·83)	±(0·50)	−	−	−	−
160	Oxidation of calcium lactate through acetate to carbonate	−	+	+	+	+	±(0·66)	+
	Nutrition:							
161	Utilization of 0·3% sodium malonate as C source	+(0·77)	−	+	−(0·25)	+	±(0·66)	+
162	Utilization of 0·1% sodium acetate as C source	+	−	±(0·50)	+	+	+	+
163	Utilization of 0·1% sodium formate as C source	+(0·92)	±(0·33)	±(0·50)	+	+	±(0·66)	+
164	Utilization of $NH_4H_2PO_4$ as N source	−	−(0·17)	+	+(0·92)	+	±(0·33)	±(0·50)
	Utilization as C and N source:							
165	Ornithine monochloride	±(0·69)	+	−	+	+	±(0·66)	−
166	Arginine hydrochloride	±(0·31)	±(0·67)	+	+	+	±(0·66)	−
167	L-lysine	±(0·69)	+	+	+(0·83)	+	−	−
168	L-alanine	±(0·69)	+	+	+	+	+	−
169	L-histidine	−(0·23)	+	+	+	+	±(0·66)	+
170	L-serine	±(0·38)	+(0·83)	+	+(0·75)	+	+(0·66)	−
171	L-phenylalanine	+(0·77)	+	−	−(0·08)	+	±(0·33)	−
172	L-proline	+(0.77)	+(0·83)	+	+	+	±(0·66)	−
173	L-leucine	±(0·69)	−	+	−	±(0·50)	−	−
174	L-glutamic acid	±(0·54)	+	±(0·50)	±(0·42)	−	+	−
175	L-cystine	±(0·54)	−	−	−(0·08)	±(0·50)	−	−
176	L-tyrosine	+(0·77)	+	+	−(0·17)	±(0·50)	+	−
	Other:							
179	Production of 2-ketogluconate from potassium gluconate	−	−	−	−(0·08)	−	−	−
182	Arginine dihydrolase	−	±(0·33)	−	−	−	−	−
183	Arginine decarboxylase	−	+	−	−(0·08)	−	−	−
186	Methylene-blue reduction	−	−(0·17)	+	+(0·75)	−	−	−
188	Penicillinase	+	±(0·33)	+	+	+	±(0·66)	+
190	Melanin produced from phenylalanine	−	+(0·83)	−	−	−	−	−
191	Melanin produced from tyrosine	−	±(0·50)	+	+(0·83)	−	−	−

± = Variable occurrence with the set of strains tested. Actual frequency of occurrence is given in parentheses.
* Only two strains tested.
† N.T. = Not tested.

lecithinase; utilization of $NH_4H_2PO_4$ as nitrogen source; production of 2-keto-gluconate from gluconate; arginine decarboxylase; and methylene-blue reduction.

The Chromobacterium strains formed two clusters corresponding to the mesophilic (*C. violaceum*) and psychrophilic (*C. lividum*) strains described by Sneath (1960). These two groups joined at S = 68 %. There were only a few characters in each group which varied from the descriptions provided by Sneath (1960). The data are presented in Table 4. The differences may be cited as follows. Mesophilic strain ATCC 12541 produced acid from sorbitol. Chromobacterium strain no. 12540 did not produce acid from trehalose. Failure to produce acid from salicin, lactose and inulin, by the psychrophilic group agreed with Leifson's results (Sneath, 1960). However, the two strains in this group did not produce acid from mannitol. Growth was not detected in either

Table 5. *S value of Agrobacterium strains with the hypothetical median organism*

Strains		S value	No. of tests*
11157	*A. tumefaciens*	94·4	107
A. RADIO	*A. radiobacter*	92·9	112
AR 1001	*A. radiobacter*	92·9	112
B6	*A. tumefaciens*	90·1	111
A6RI	*A. tumefaciens*	87·7	114
4720	*A. tumefaciens*	87·4	111
A66RI	*A. tumefaciens*	86·2	116
AR 1012	*A. radiobacter*	85·2	115
11156	*A. tumefaciens*	84·2	114
4718	*A. radiobacter*	83·9	112
13333	*A. rhizogenes*	82·2	118
11325	*A. rhizogenes*	75·4	118

* Number of tests (n_s+n_d) on which S value ($S = n_s/n_s+n_d$) is based. Total number of coded tests was 191.

group with 3 % NaCl but all strains grew in the range pH 4·5–10·0 except strain no. 6915 which did not grow at pH 10·0. Pigment description was excluded from the tables because although all strains characteristically produced a dark blue non-diffusible pigment, this character was not stable, as non-pigmented or palely pigmented colonies frequently were present on agar plate streaking of a pure culture. De Ley (1964) made similar observations concerning the pigmentation of Chromobacterium.

Neither Chromobacterium cluster produced acid from adonitol, dulcitol, melibiose, melezitose or raffinose. Both groups possessed lipase (Tween 20, 40) and were sensitive to $HgCl_2$, 10 μg. The two groups differed in several respects. The mesophiles were fermentative in glucose metabolism, were not sensitive to dihydrostreptomycin 10 μg., peptonized milk, hydrolysed casein, did not grow in a minimal medium with malonate or acetate as carbon source, and tested positive for arginine decarboxylase. The mesophiles utilized ornithine and phenylalanine as a nitrogen and carbon source while the psychrophiles utilized leucine. These features may be worth further analysis to determine their usefulness in diagnostic applications, since they represent metabolic functions most probably governed by gene complexes, and would therefore, as a combination, or plexus of features, provide a determinative key.

The Agrobacterium/fast-growing Rhizobium cluster, although divisible into four subgroups consisting of *A. radiobacter*+*A. tumefaciens*+*A. rhizogenes*, *R. meliloti*,

R. leguminosarum+*R. trifolii*+*R. phaseoli*, and *A. rubi* (Fig. 1), have many features in common. Several differ from those scored for the slow-growing Rhizobium and the Chromobacterium clusters. In contrast to the slow growers, the Agrobacterium/fast-growing Rhizobium cluster was peritrichously flagellated, slightly fermentative in glucose medium (decrease in Ph in fermentation tube) and produced acid in a number of the carbohydrates tested. The strains also were sensitive to Polymyxin B and lipase negative. These and other characters useful in distinguishing the subgroups of this cluster are listed in Table 4.

Table 6. *S value of slow-growing Rhizobium strains with the hypothetical median organism*

	Strain	S value	No. of tests*
965	*Rhizobium* sp.	84·2	76
998	*Rhizobium* sp.	82·3	79
952	*R. japonicum*	82·3	79
10318	*R. lupini*	81·8	77
942	*Rhizobium* sp.	80·5	82
997	*Rhizobium* sp.	80·5	82
WU7	*R. lupini*	79·7	74
948	*Rhizobium* sp.	78·3	83
10317	*Rhizobium* sp.	77·8	81
WU425	*Rhizobium* sp.	75·6	78
963	*Rhizobium* sp.	73·2	82
989	*Rhizobium* sp.	73·2	82
922	*Rhizobium* sp.	71·3	80

* Number of tests (n_s+n_d) on which the S value ($S = n_s/n_s+n_d$) is based. Total number of coded tests was 191.

Table 7. *S value of Chromobacterium violaceum strains with the hypothetical median organism*

	Strain	S value	No. of tests*
553	*C. violaceum*	90·0	100
12472	*C. violaceum*	88·3	103
12542	*C. violaceum*	87·6	105
12541	*C. violaceum*	86·8	106
6357	*C. violaceum*	85·6	104
12540	*C. violaceum*	82·2	107
13426	*C. violaceum*	55·9	118

* Number of tests (n_s+n_d) on which the S value, $S = n_s(n_s+n_d)$, is based. Total number of tests employed was 191.

Hypothetical median organisms (Liston *et al.* 1963) were calculated for the Agrobacterium, slow-growing Rhizobium, and *Chromobacterium violaceum* groups. A measure of homogeneity of the clusters can be obtained from the tabulation of strain S-values of a cluster with the hypothetical median organism for that cluster. The Agrobacterium strains each share S-values > 75% with the hypothetical median organism (Table 5). Thus a suitable representative or neotype strain for this group, for example, would be *A. tumefaciens* 11157 (S = 94%).

Tables 6 and 7 contain S-value calculations of each strain with the respective hypothetical median strains for the other two groups which were analysed. *Chromobacterium*

violaceum 13426, not a member of the *C. violaceum* cluster from the initial data results (Fig. 1), yielded a very low S-value (S = 56%) with the hypothetical median organism for the major *C. violaceum* cluster, reaffirming its lack of membership in the group.

Fig. 4. Inter-S-values computed from the hypothetical median organisms for the Agrobacterium, *Chromobacterium violaceum*, slow-growing Rhizobium and the *A. pseudotsugae* clusters.

		1	2	3	4
Slow-growing Rhizobium	1	100	.	.	.
Agrobacterium	2	45	100	.	.
A. pseudotsugae	3	51	60	100	.
Chromobacterium violaceum	4	46	48	53	100

Fig. 5. Inter-S-values for the median organisms of the Rhizobiaceae clusters and for other representative strains included in the study.

Rhizobium sp. 965	100										
R. trifolii SU308	48	100									
A. tumefaciens 11157	45	54	100								
Chromobacterium violaceum 553	43	53	46	100							
Pseudomonas fluorescens 13430	51	51	49	55	100						
P. fluorescens 16266 var. *antirrhinastri*	46	58	55	52	70	100					
P. aeruginosa 14216	42	54	53	57	73	68	100				
Escherichia coli W1485	42	46	57	52	49	52	55	100			
Enterobacter aerogenes 4115	35	51	62	52	53	62	64	76	100		
Proteus mirabilis PM-I	41	46	50	54	45	49	51	53	57	100	
P. morgani 9247TR	42	45	50	53	46	49	49	55	52	70	100
	965	SU308	11157	553	13430	16266	14216	W1485	4115	PM-I	9247TR

By using the computed hypothetical median organisms for the four groups Agrobacterium, Chromobacterium (mesophilic), slow-growing Rhizobium and *A. pseudotsugae*, an S-value table of inter-group relationships was obtained (see Fig. 4). The Agrobacterium and the *A. pseudotsugae* groups shared S-value relationship of 60% but the other values for the groups were much lower. An ordering of the groups on the basis of hypothetical median organism similarities would thus be as given in Fig. 4.

An S-value table for actual median organisms (selected on the basis of highest computed relationship with the hypothetical median strain for the given group) was computed for 11 of the species from the analysis (Fig. 5). The intra-S-value for the three Pseudomonas strains was *c.* 70% and for *Escherichia coli* W1485 and *Enterobacter aerogenes*, 4115, 76%, S-value levels which, from earlier studies (Colwell & Liston, 1961), would be considered at least a generic level of similarity. The two Proteus species, showed a similar S-value, 70%. The order in which the strains are listed in Fig. 5 represents a final arrangement based on the computed similarities amongst the strains as given in the triangular matrix.

DISCUSSION

The relation of the Rhizobia to other bacteria, the relationships amongst the crown gall organisms, the root nodule organisms, *Agrobacterium radiobacter*, and the hairy-root lesion organism (*A. rhizogenes*), and the division of the root nodule bacteria into two broad groups were problems facing the early students of soil bacteriology and remain to the present as points of debate amongst the workers in the field. As early as 1903 the Rhizobia were divided into two groups on the basis of cultural characteristics, including carbohydrate reactions, gelatin reaction, etc. (Fred, Baldwin & McCoy, 1932). Löhnis & Hansen (1921) established both the so-called fast-growing Rhizobium group, characterized as producing rapid profuse slimy growth, appreciable acid formation and a 'serum zone' in milk, and the slow-growing group which exhibited slower, less profuse growth and an alkaline reaction in carbohydrate media and no change in milk. As a result of the work of Fred *et al.* (1932), 6 species were established for the nodule bacteria and the species were placed into the new genus *Rhizobium* (*Bergey's Manual*, 1957).

Lange (1961) clearly demonstrated that the cross-inoculation group separations of the rhizobia for species designation was not, in practice, satisfactory. Distinctly different host groupings were apparent from the data collected during his study of nodule bacteria associated with the indigenous legumes of south-western Australia. As pointed out by Lange, the economic importance attached to agronomic legumes and the resulting disproportionate attention to these has resulted in a skew classification of the nodule bacteria.

Lange's suggestion that an Adansonian analysis of the root nodule bacteria should be done was taken up by Graham (1964). Graham's analysis of strains of the genera *Rhizobium*, *Agrobacterium*, *Chromobacterium*, *Beijerinckia* and *Bacillus* provided some valuable new data. From the results of his analysis, Graham concluded that *Chromobacterium*, *Bacillus* and *Beijerinckia* showed little affinity with the nodule bacteria studied. The results of the analysis reported in the present work provide strong support for the separation of *Chromobacterium* from the other genera of the Rhizobiaceae. In fact, there seems little reason to retain the family as presently composed. Of the several genera included in the present analysis, the highest relationship of the *Chromobacterium violaceum* was to *Pseudomonas aeruginosa* (57% S; see Fig. 5). The root nodule bacteria might better be placed in the family Pseudomonadaceae, a conclusion also arrived at by De Ley *et al.* (1966*b*) from DNA homology studies. Furthermore, the *C. violaceum* and *C. lividum* inter-species relationships were so low as to suggest treatment of these clusters as different genera.

Graham also pointed out that the genus *Rhizobium* required major revision and suggested, from his results, that *R. trifolii*, *R. leguminosarum* and *R. phaseoli* be consolidated into a single species, that *Agrobacterium* and *A. tumefaciens* be included as *R. radiobacter* in the genus *Rhizobium* and that the genus *Phytomyxa* be applied to the strains of slow-growing root-nodule bacteria. We find all of these suggestions most useful after examination of the results of our analyses. From Fig. 1 it is obvious that the major separation of the Rhizobium species is into the slow growers and the fast growers as originally described by Löhnis & Hansen (1921). Generic level of separation, at the least, is clearly indicated. Since Buchanan (1926) concluded that the two names, Phytomyxa and Rhizobium, were available for

the bacteria of leguminous plants, it seems logical for historical reasons to retain the fast-growers within the genus *Rhizobium* and include the slow-growers in the new genus *Phytomyxa*. The intra-generic relationships of the fast-growers and the Agrobacterium strains are not as clear-cut as Graham (1964) would imply. It is clear, however, that the results reported here support the consolidation of the remaining Rhizobium into two or more species. Our analysis would support retention of *R. meliloti* (strains SU 277 and SU 216, Fig. 1) and *R. leguminosarum* (SU 301, SU 312, SU 298, Fig. 1). However, besides *Rhizobium radiobacter*, into which we would place *A. tumefaciens*, *A. rhizogenes* and *A. radiobacter*, we would also propose the retention of species *Rhizobium rubi*, into which strains presently designated *A. rubi*, 13334 and 13335, should be placed.

Agrobacterium gypsophilae strain 13329 should not be included in the Rhizobiaceae; that is, the results of the computer study indicate that it may more logically be placed in the family Enterobacteriaceae (see Fig. 1).

The *Agrobacterium pseudotsugae* strains included in the present study would be not considered members of the species *R. radiobacter*, as were the other Agrobacterium species. *Agrobacterium pseudotsugae* appears to warrant a separate generic designation. De Ley, Park, Tijtgat & Van Ermengem, 1966*b*) also questioned the relationship of *A. pseudotsugae* to the other agrobacteria on the basis of DNA base composition.

Genera, other than those of the Rhizobiaceae, which were studied provide some interesting information. Members of the Enterobacteriaceae formed two clusters linked via the *Vibrio cholerae* type strain 14033 (Hugh, 1965) and the four isolates of *Serratia marcescens*, data for which were included in the computations as they were available from an earlier study (Colwell & Mandel, 1965). The genus *Vibrio* might better be placed in the family Enterobacteriaceae. Strains of the genus *Vibrio* are being studied in our laboratory in order to resolve this point.

The clustering together of the two strains 5GLY.FE, a non-virulent *Agrobacterium tumefaciens* strain and *C. violaceum* 13426, and the rather nebulous position of *A. radiobacter* strain 6466 and *Chromobacterium viscosum* 6918, were not readily explicable. In the case of *A. radiobacter* 6466 and *A. tumefaciens* 5GLY.FE, misidentification by the persons isolating these strains may be the explanation for the anomalous results. Sneath (1960) also concluded that *C. viscosum* does not rightly belong in the genus *Chromobacterium*. The following facts should be made clear. In our hands, *C. violaceum* 13426 did not produce pigment under any of the conditions tested and *C. viscosum* produced a purple water-soluble pigment. None of the other Chromobacterium strains tested in this analysis produced a water-soluble pigment, the pigment being in every case restricted to intracellular location.

From the data in Table 7 it appears that *C. violaceum* 12472, the proposed neotype strain (Sneath, 1960) was a suitable choice (S = 88% with the hypothetical median organism of the mesophilic cluster of the genus *Chromobacterium*). Other neotype strains, as indicated from Tables 5 and 6, would be 11157 for *Rhizobium radiobacter* and 965 for *Phytomyxa japonicum*.

Family relationships, as determined from Figs. 4 and 5 would result in the elimination of only *Phytomyxa* (represented by Rhizobium 965) and *Proteus*, if an inter-S level of 50–60% were selected as the demarcation. The lack of over-all similarity of Proteus species one to the other, as well as of the genus to the rest of the genera of the Enterobacteriaceae, was noted by Krieg & Lockhart (1966). Perhaps, more

logically, it would be better simply to order the genera, as was done in Fig. 1, according to inter-group S-values, since the over-all DNA base compositions correlated extremely well with the intra- and inter-group arrangements thus obtained.

The feature frequency of occurrence for *Rhizobium radiobacter* and *R. meliloti*, *R. leguminosarum* and *R. rubi* for *Phytomyxa japonicum* and for *Chromobacterium violaceum* and *C. lividum* (Table 4) permit establishment of characteristics useful in forming descriptions for each of these species, independent as in the case of the Rhizobium and Phytomyxa species, of plant inoculation studies. These characteristics thus provide reference markers, or base-points, for purposes of comparison with new data obtained in future or may be employed in a determinative key for identifying the species of the genera listed.

It is important to emphasize that bacterial taxonomy is in a dynamic situation, a fluid state with new information constantly becoming available. As more knowledge concerning the biochemical pathways and genetic control mechanisms operating in these organisms is accumulated, it is to be expected that the descriptions and limits of the taxa proposed here will be appropriately altered.

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