Numerical Taxonomic Analysis of *Agrobacterium*

By K. KERSTERS AND J. DE LEY

Laboratory for Microbiology and Microbial Genetics, Faculty of Sciences,
State University, Gent, Belgium

AND P. H. A. SNEATH AND M. SACKIN

M.R.C. Microbial Systematics Unit, Leicester University, Leicester

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SUMMARY

Ninety-two biochemical, physiological and nutritional characters of 70 *Agrobacterium* strains and a few representatives of the genera *Rhizobium*, *Pseudomonas*, *Erwinia*, *Achromobacter*, *Klebsiella* and *Escherichia* were subjected to computer analysis. Sixty-five agrobacteria fell into two distinct clusters. All authentic 3-ketolactose-positive *Agrobacterium tumefaciens* and *A. radiobacter* strains grouped in one rather heterogeneous cluster, whereas the atypical 3-ketolactose-negative tumour-inducing agrobacteria, and the named *A. rhizogenes* strains, grouped in a very tight cluster. *Agrobacterium rubi* and two other 3-ketolactose-negative tumour-inducing strains were not placed in either cluster. Two other strains were misnamed. Diagnostic characters are presented for distinguishing clusters and groups. The present division of the genus into species is based on phytopathogenicity and does not reflect the natural relationships amongst the agrobacteria.

INTRODUCTION

The present taxonomy of the genus *Agrobacterium* is confused. In *Bergey's Manual* (1957) the genus is divided into seven species, on the basis of phytopathogenicity and a few biochemical tests. The major groups are *Agrobacterium tumefaciens*, causing crown-gall, *A. rhizogenes* causing hairy-root and the non-pathogenic *A. radiobacter*, but the species differentiation between *A. tumefaciens* and *A. radiobacter* has been questioned (Graham, 1964; De Ley, Bernaerts, Rassel & Guilmoit, 1966; Heberlein, De Ley & Tijtgaat, 1967; Moffett & Colwell, 1968). The inclusion of *A. rubi* in the *A. tumefaciens*-*A. radiobacter* group has been advocated (Heberlein *et al.* 1967; Keane, Kerr & New, 1970).

The inclusion of the species *Agrobacterium stellulatum*, *A. pseudotsugae* and *A. gypsophilae* in the genus *Agrobacterium* is unjustified (De Ley *et al.* 1966; Heberlein *et al.* 1967; Moffett & Colwell, 1968; Lippincott & Lippincott, 1969; Skyring, Quadling & Rouatt, 1971). The first is probably a marine pseudomonad, the second is an arthrobacter-like micro-organism, and the third is *Erwinia herbicola* (P. H. Graham, unpublished).

Keane *et al.* (1970) observed striking biochemical differences between 3-ketolactose-positive (biotype 1) and 3-ketolactose-negative (biotype 2) *Agrobacterium* strains. Both biotypes did not correlate with current taxonomy. Numerical taxonomic studies (Graham, 1964; 't Mannetje, 1967; Moffett & Colwell, 1968; Skyring *et al.* 1971; White, 1972) and DNA hybridizations (Heberlein *et al.* 1967) indicate a close relationship between agrobacteria and fast growing rhizobia.

In an attempt to clarify the phylogeny and natural relationships amongst the agrobacteria,

we subjected 92 physiological, biochemical and phytopathological properties of 70 cultures of named *Agrobacterium tumefaciens*, *A. radiobacter*, *A. rhizogenes*, *A. rubi* and several unnamed agrobacteria to numerical analysis.

Graham (1964) and Heberlein *et al.* (1967) have suggested profound changes in the nomenclature of *Agrobacterium* and *Rhizobium* and their proposals have been adopted by Moffett & Colwell (1968) and White (1972). This report, however, does not deal with nomenclature, but with phylogeny within the agrobacteria. Strains will simply be referred to by their former species names. A preliminary account of these investigations has been published (De Ley, 1972).

METHODS

Strains. The strains used are listed, with their origin, in Table 1. They were maintained at 4 °C on the following medium: glucose, 2 % (w/v); yeast extract, 1 % (w/v) (Nederlandse Gist- en Spiritusfabriek, Brugge, Belgium); CaCO₃, 2 % (w/v) and agar, 2 % (w/v).

Methods of testing. All tests were performed at 28 °C. Starter cultures were grown at 28 °C for 24 h as described by Moffett & Colwell (1968). Inoculations of all liquid test media were made with 1 or 2 drops of a 100-fold dilution of the starter culture in 0.01 M-phosphate buffer, pH 7.0. Each liquid test medium received 10⁵ to 10⁶ viable organisms. The replica plate technique as described by Stanier, Palleroni & Doudoroff (1966) was not satisfactory in our case, because inocula were too large and nutrients were carried over.

For the study of nutritional and physiological features, the mineral basal medium employed was a modification of the basal medium of Graham & Parker (1964): MgSO₄·7H₂O, 0.25 g; CaCl₂·2H₂O, 0.025 g; Na₂HPO₄·12H₂O, 1.2 g; KH₂PO₄, 0.55 g; NaCl, 0.25 g; FeSO₄·(NH₄)₂SO₄·6H₂O, 4 mg; ZnSO₄·7H₂O, 0.16 mg; CuSO₄·5H₂O, 0.08 mg; H₃BO₃, 0.5 mg and MnSO₄·H₂O, 0.4 mg; distilled water 1 l; pH 7.0.

All nutritional tests were read at 4 days, 1, 2 and 3 weeks.

Morphological and colonial features. Colonial features and pigmentation were examined after 3, 7 and 14 days of growth on a medium containing: glucose, 1 % (w/v); yeast extract, 1 % (w/v); (NH₄)₂SO₄, 0.1 % (w/v); KH₂PO₄, 0.025 % (w/v) and agar, 2 % (w/v). The type of flagellation was determined by the method of Rhodes (1958) and occasionally by electron microscopy, kindly performed by Dr A. Rassel (Gembloux, Belgium) with the methods described previously (De Ley & Rassel, 1965; De Ley *et al.* 1966).

Organic compounds as sole source of carbon. Growth on organic compounds and acid production from carbohydrates and ethanol were determined on the mineral basal medium supplemented with: NH₄H₂PO₄, 0.05 % (w/v) and yeast extract (Difco), 0.00025 % (w/v). The following carbohydrates were filter-sterilized and tested at 1 % (w/v): L-arabinose, D-arabinose, D-xylose, D-fructose, D-galactose, D-glucose, D-mannose, cellobiose, lactose, maltose, sucrose, dulcitol, glycerol, *meso*-inositol, sorbitol, L-sorbose, sodium-D-gluconate and ethanol. The following organic salts were tested at 0.2 % (w/v): sodium acetate, sodium-D,L-glycerate, sodium-D-lactate, sodium-D,L-mandelate, sodium succinate, sodium benzoate, sodium formate, sodium glyoxylate, sodium oxalate. Bromocresol purple (0.0016 %, w/v) was added to all carbohydrate-containing media. Acid formation was recorded after 4 days, 1, 2 and 3 weeks, and the pH of the culture medium was determined after 3 weeks of growth.

Amino acids as sole source of carbon and nitrogen. Growth on the following filter-sterilized amino acids (0.1 %, w/v) was determined in the mineral basal medium with 0.00025 % (w/v) yeast extract (Difco): L-alanine, L-arginine, L-glutamic acid, L-histidine, L-leucine, L-lysine, L-proline, L-serine, L-threonine, L-ornithine, L-asparagine, L-phenylalanine, L-methionine,

L-tryptophan and L-valine. L-Tyrosine (0.05 %, w/v) was sterilized at 120 °C for 15 min.

Sensitivity to antibiotics. Antibiotic sensitivity discs (Difco) were used on YM agar (Ionagar no. 2, Oxoid): chloromycetin, 30 µg/disc; coly-mycin, 10 µg; erythromycin, 15 µg; kanamycin, 30 µg; penicillin, 10 units; streptomycin, 10 µg; aureomycin, 30 µg; novobiocin, 30 µg; gentamycin, 10 µg; neomycin, 30 µg; terramycin, 30 µg; and tetracycline, 30 µg. A definite inhibition zone around the disc after 2 days of incubation at 28 °C was taken to indicate sensitivity.

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

Reduction of nitrate and nitrite was detected in YM liquid medium with 0.0025 % (w/v) yeast extract (Difco) and either 0.1 % (w/v) KNO₃ or 0.0005 % (w/v) NaNO₂. Nitrite and residual nitrate were detected after 2, 4 and 7 days using the Griess-Ilosvay reagents as described in the *Manual of Microbiological Methods* (1957).

Anaerobic growth in the presence of nitrate was determined on: glucose, 1 % (w/v); yeast extract, 1 % (w/v); KNO₃, 0.5 % (w/v); (NH₄)₂SO₄, 0.1 % (w/v); KH₂PO₄, 0.025 % (w/v) and agar, 2 % (w/v). Slants were inoculated from a 2-day-old YM starter culture and anaerobiosis was obtained with alkaline pyrogallol. Growth at 28 °C was recorded after 7 and 14 days.

Hydrogen sulphide production from cysteine. The liquid YM medium with 0.01 % (w/v) cysteine and lead acetate paper was used.

Hydrolysis of gelatin was determined on YM agar plates supplemented with 0.5 % (w/v) gelatin. After 4 days of growth at 28 °C the plates were flooded with 15 % (w/v) HgCl₂ in 2 N-HCl.

Litmus milk reaction. One drop (about 10⁸ organisms) of a 2-day-old YM starter culture was added to 10 ml litmus milk (Oxoid), mixed well and incubated at 28 °C without further shaking. Peptonization and colour changes were recorded weekly, but only the data after 3 weeks are presented here.

Phytopathogenicity. Infectivity was tested on tomato plants and on carrot discs as described by De Ley *et al.* (1966) and Lippincott & Lippincott (1969), respectively. Tumour and root formation on the discs was recorded after 2 and 3 weeks. A proliferation of the primary xylem observed with some strains was not regarded as tumour tissue.

Growth on liquid sucrose + salts medium, selective medium of Clark and citrate. The media of Lippincott, Webb & Lippincott (1965), Clark (1969) and Koser (1923), respectively, were used. Growth on citrate was also determined on the medium of Simmons (1926), supplemented with 0.0005 % (w/v) yeast extract (Difco).

Absorption of aniline blue was determined by the method of Hendrickson, Baldwin & Riker (1934).

Surface pellicle formation in ferric ammonium citrate and growth on Ca-glycerophosphate agar were determined by the methods of Keane *et al.* (1970).

Coding of features for computation. Most properties were coded as multistate quantitative characters and each multistate character was converted into a series of Z-state unit characters by the additive method of Sokal & Sneath (1963). Strong, weak and no growth, acid production, etc., were coded respectively as 1, 1, 0 and 0, 0. Growth on D,L-glycerate, formate, glyoxylate, L-valine, and H₂S formation were coded as two-state characters. Colonial pigmentation, reaction on litmus milk, infectivity and growth on Ca-glycerophosphate agar were coded as multistate qualitative characters. Each strain was tested for 187 unit characters.

Computation. The simple matching coefficient (S_{SM}) (Sokal & Michener, 1958) was computed. To reduce dissimilarities due to differences in growth rate the pattern difference

Table 1. *Strains used and their origin*

The origin of the strains is given only for the agrobacteria. For the other organisms it is not relevant.

Strain no.	Species name as received	Received from*	Isolated from, in,
Predominantly 3-ketolactose-positive <i>Agrobacterium</i> strains, assigned to cluster 1			
B6 group			
B/1	<i>A. tumefaciens</i>	D. Zutra	Bitter almond root stock, Israel, 1961
E/1	<i>A. tumefaciens</i>	D. Zutra	Myrobalan plum root stock, Israel, 1961
TRI	<i>A. radiobacter</i>	ICPB	U.S.A., 1924
B2A	<i>A. tumefaciens</i>	G. Heberlein	Subclone from strain B2 (T. Stonier, personal comm.)
TT2	<i>A. tumefaciens</i>	ICPB	Synonym of B2: from apple seedling crown-gall, Shenandoah, Iowa, U.S.A., 1932-1934
3/1	<i>A. tumefaciens</i>	D. Zutra	Apple, Israel, 1962
RV3	<i>A. tumefaciens</i>	CIP	No information available
B6	<i>A. tumefaciens</i>	CIP	Apple seedling crown-gall, Shenandoah, Iowa, U.S.A., 1934
B6-806	<i>A. tumefaciens</i>	G. Heberlein	Phage-sensitive isolate from u.v.-irradiated B6
TR6	<i>A. radiobacter</i>	ICPB	No information available
TT11	<i>A. tumefaciens</i>	ICPB	Gall on incense cedar, U.S.A., 1916
Kerr 14	<i>Agrobacterium</i> sp.	A. Kerr	Soil around almond tree, South Australia, 1967
0322	<i>Agrobacterium</i> sp.	A. C. Hayward	Gall on peach stock, South Australia, 1967
S1005	<i>A. radiobacter</i>	C. Bonnier	Presumably in U.S.A.
1641	<i>A. tumefaciens</i>	NCPPB	<i>Flacourtia ramontchi</i> , U.K.
4452	<i>A. tumefaciens</i>	ATCC	Raspberry (presumably in U.S.A.)
4718	<i>A. radiobacter</i>	ATCC	Soil, U.S.A., 1927
11156	<i>A. tumefaciens</i>	ATCC	Smooth colony variant from B6, 1942
F	<i>A. tumefaciens</i>	Delft	Unknown
F/1 group			
F/1	<i>A. tumefaciens</i>	D. Zutra	<i>Dahlia</i> , Israel, 1961
925	<i>A. tumefaciens</i>	NCPPB	<i>Dahlia</i> sp., South Africa
NIAS 1 group			
A-2-1-1	<i>A. tumefaciens</i>	NIAS	Cherry, Saitama, Japan, 1956
I	<i>A. tumefaciens</i>	NIAS	<i>Sedum alboroseum</i> , Japan, 1958
0362 group			
0362	<i>Agrobacterium</i> sp.	A. C. Hayward	John Innes potting soil, Australia
0363	<i>Agrobacterium</i> sp.	A. C. Hayward	John Innes potting soil, South Australia, 1968
TT111 group			
Mushin 3	<i>A. tumefaciens</i>	R. Mushin	Apple, Wisconsin, U.S.A., 1935
Mushin 5	<i>A. tumefaciens</i>	R. Mushin	Unknown
Mushin 6	<i>A. tumefaciens</i>	R. Mushin	Gall on hop plant, Victoria, Australia, 1960
TT8	<i>A. tumefaciens</i>	ICPB	No information available
E.III.9.6.1	<i>A. tumefaciens</i>	H. Veldstra	Unknown
Mac 37	<i>A. tumefaciens</i>	J. E. Tanner	Unknown host plant, Canada
TT111	<i>A. tumefaciens</i>	ICPB	U.S.A., no further information available
143	<i>A. tumefaciens</i>	ATCC	Unknown host plant, probably U.S.A.
396	<i>A. tumefaciens</i>	NCPPB	<i>Dahlia</i> , presumably in Germany
398	<i>A. tumefaciens</i>	NCPPB	Host plant unknown, presumably in Germany
1001	<i>A. tumefaciens</i>	NCPPB	<i>Vitis vinifera</i> , Roumania
AB1558	Presumed <i>Agrobacterium</i>	H. Lautrop	Probable contamination of human blood culture, Denmark
1674	<i>A. tumefaciens</i>	NCPPB	<i>Beta vulgaris</i> , U.K.
4720	<i>A. tumefaciens</i>	ATCC	Presumably subclone of T5, itself from black raspberry gall, Iowa, U.S.A., 1926
8149	<i>A. radiobacter</i>	NCIB	Soil, the Netherlands or Germany, 1904
ATB	<i>A. tumefaciens</i>	M. Rubio-Huertos	<i>Vitis vinifera</i> crown-gall, Benidorm (Alicante), Spain, 1961
ATV	<i>A. tumefaciens</i>	M. Rubio-Huertos	<i>Vitis vinifera</i> crown-gall, Province of Toledo, Spain, 1961

Table 1 (cont.)

Strain no.	Species name as received	Received from*	Isolated from, in,
II Chr	<i>A. tumefaciens</i>	D. Knösel	<i>Chrysanthemum frutescens</i> tumour, Province of Brandenburg, Germany, 1927
M2/1 group			
M2/1	<i>A. radiobacter</i>	M. Bernaerts	Ditch water, near Mechelen, Belgium, 1952
L2/2/1	<i>A. radiobacter</i>	M. Bernaerts	Ditch water, near Mechelen, Belgium, 1952
TT9 group			
TT9	<i>A. tumefaciens</i>	ICPB	Probably synonymous with B37
B37	<i>A. tumefaciens</i>	NRRL	Hop?, U.S.A.
T37	<i>A. tumefaciens</i>	G. Heberlein	Walnut gall, California, U.S.A., 1926
Border cases			
Gembloux A	<i>A. tumefaciens</i>	C. Bonnier	Prunus galls, Gembloux, Belgium
IIBV7	<i>A. tumefaciens</i>	G. Heberlein	Derived from Stapp's strain IIB, itself isolated from <i>Chrysanthemum frutescens</i> , Germany, 1927
417	<i>A. tumefaciens</i>	IPO	Apple, The Netherlands, about 1959
3-Ketolactose-negative <i>Agrobacterium</i> strains, assigned to cluster 2			
PPI I	<i>A. tumefaciens</i>	I. Popova	<i>Vitis vinifera</i> , near Sofia, South Bulgaria, 1959
SI	<i>A. tumefaciens</i>	NIAS	Rose, Japan, 1962
PPI6	<i>A. tumefaciens</i>	I. Popova	<i>Vitis vinifera</i> , near Sofia, South Bulgaria, 1959
TR7	<i>A. rhizogenes</i>	ICPB	Hairy root on apple, U.S.A.
Kerr 38	<i>Agrobacterium</i> sp.	A. Kerr	Plum gall, South Australia
Kerr 39	<i>Agrobacterium</i> sp.	A. Kerr	Plum gall, South Australia
PPI49b	<i>A. tumefaciens</i>	I. Popova	<i>Prunus cerasifera</i> , near Targovishte, North Bulgaria, 1959
TR101	<i>A. rhizogenes</i>	ICPB	No information available
TR107	<i>A. rhizogenes</i>	ICPB	No information available
TT133	<i>A. tumefaciens</i>	ICPB	Almond tumour, Israel, 1961
223	<i>A. tumefaciens</i>	NCPBP	Unknown
0321	<i>Agrobacterium</i> sp.	A. C. Hayward	Gall on peach, South Australia, 1967
1649	<i>A. tumefaciens</i>	NCPBP	<i>Rosa</i> sp., South Africa
1651	<i>A. tumefaciens</i>	NCPBP	<i>Prunus persica</i> , South Africa
3-Ketolactose-negative <i>Agrobacterium</i> strains, which could not be assigned to either cluster			
TR2	<i>A. rubi</i>	ICPB	Probably from black raspberry gall, U.S.A.
1650	<i>A. tumefaciens</i>	NCPBP	<i>Rosa</i> sp., South Africa
1771	<i>A. tumefaciens</i>	NCPBP	<i>Vitis vinifera</i> , Iran
Doubtful cases and reference strains			
M39	<i>A. tumefaciens</i>	P. Manigault	Received as a mutant of B6; thought to be a contaminant (van der Plaats <i>et al.</i> 1969)
1772	<i>A. tumefaciens</i>	NCPBP	<i>Vitis vinifera</i> , Iran
ABI196	<i>Achromobacter</i> sp.	H. Lautrop	
ABI293	<i>Achromobacter</i> sp.	H. Lautrop	
8129	<i>Achromobacter hartlebii</i>	NCIB	
PA	<i>Erwinia lathyri</i>	Graham	
B	<i>Escherichia coli</i>		
<i>Klebsiella rubiacearum</i>	<i>Klebsiella rubiacearum</i>	Silver	
518	<i>Pseudomonas aureofaciens</i>	CCEB	
316C10A	<i>Rhizobium leguminosarum</i>	USDA	
I.5	<i>R. meliloti</i>	Gembloux, Belgium	

* ATCC: American Type Culture Collection; CCEB: Culture Collection of Entomogenous Bacteria (Prague, Czechoslovakia); CIP: Collection de l'Institut Pasteur (Paris, France); ICPB: International Collection of Phytopathogenic Bacteria (Davis, California, U.S.A.); IPO: Instituut voor Plantenziektenkundig Onderzoek (Wageningen, The Netherlands); NCIB: National Collection of Industrial Bacteria (Torry Research Station, Aberdeen, Scotland); NCPBP: National Collection of Plant Pathogenic Bacteria (Harpenden, Hertfordshire); NIAS: National Institute of Agricultural Science (Tokyo, Japan); NRRL: Northern Utilization Research and Development Division (Department of Agriculture, Peoria, Illinois, U.S.A.); USDA: United States Department of Agriculture (Beltsville, Maryland, U.S.A.).

coefficient (D_p) was also calculated (Sneath, 1968). Both coefficients were clustered by both single linkage (Sneath, 1957) and unweighted average linkage (Sokal & Michener, 1958). All calculations were carried out on an ICL 4130 computer at Leicester University.

RESULTS

The bacteria selected for this study consisted of 70 *Agrobacterium* strains of widely divergent origin (Table 1). Six strains of the following genera were included for comparison: *Erwinia*, *Escherichia*, *Klebsiella*, *Pseudomonas* and *Rhizobium*. Three supposed *Achromobacter* strains (AB1196, AB1293 and NCIB8129) were also investigated, because previous numerical analysis and DNA hybridization studies suggested a distant relationship with *Agrobacterium* (J. M. Shewan and J. De Ley, unpublished).

The results are represented in a dendrogram (Fig. 1). Sixty-five of the 70 agrobacteria examined grouped in one of the two major clusters, formed at the 80 % similarity level. Five strains, two of them being misnamed (M39 and NCPPB1772), did not fall into any of the clusters.

All authentic agrobacteria (strains M39 and NCPPB1772 are not regarded as such) were Gram-negative rods, 1.5 to 3 μ m long, peritrichously flagellated; grew on L-ornithine and L-asparagine; were sensitive to 30 μ g aureomycin, 10 μ g gentamycin, 30 μ g neomycin, 30 μ g novobiocin, 30 μ g terramycin, 30 μ g tetracycline; reduced nitrite; produced H_2S from cysteine and possessed catalase; failed to grow on sodium benzoate, sodium formate, sodium glyoxylate, sodium oxalate, L-phenylalanine, L-methionine, L-tryptophan and L-valine; did not produce acid from glycerol, *meso*-inositol, maltose and sodium-D-gluconate; did not hydrolyse gelatin. The DNA base composition varied from 58 % to 62 % GC.

Cluster 1. Predominantly 3-ketolactose-positive strains

This is a complex cluster, consisting of seven groups (subclusters), tentatively designated by representative strain numbers (Fig. 1, Table 2). The similarity within the groups of cluster 1 is at least 90 %.

Cluster 1 consists of named *Agrobacterium tumefaciens* and *A. radiobacter* strains, some unspecified *Agrobacterium* strains but no *A. rhizogenes* strains. The following properties differentiate them from cluster 2 (Table 2): colonies on glucose-yeast extract medium were beige and usually mucous; all strains were 3-ketolactose-positive (except strains 0362 and 0363); produced characteristic brownish coloured curd and serum zones in litmus milk, grew on D-lactate, produced a brown surface pellicle on ferric ammonium citrate, grew abundantly on most carbohydrates with weak acid production and failed to grow on L-tyrosine. The 'biotype 1' of Keane *et al.* (1970), and Group I of White (1972) almost certainly fall in our cluster 1.

Certain characters can be used to differentiate the groups (Table 2). Although very similar, they are retained as groups because it is known from DNA hybridization (De Ley, 1972) that the genomes are at least 80 % homologous within each group, and about 50 % between groups (except the NIAS I group).

B6 and TT111 Groups. Both large groups could be distinguished from each other by the ability of the former to grow aerobically on L-sorbose and anaerobically on a glucose-yeast extract medium containing nitrate. It should be emphasized that the B6 and TT111 groups are genetically only 50 % homologous with each other and with the other groups (De Ley, 1972).

Fig. 1. Dendrogram of S_{SM} similarity coefficients, grouped by the unweighted average-linkage-cluster analysis, showing the relationships amongst various *Agrobacterium* strains and some reference strains. ●, Crown gall former; ~, root former; ◐, doubtfully pathogenic; ○, non-pathogenic.

F/1 Group. This group consists of two pathogenic strains (F/1, 925); they formed a black pigment on Ca-glycerophosphate agar and failed to grow on dulcitol.

NIAS 1 Group. Both Japanese strains (NIAS 1, NIAS A-2-1-1) were pathogenic and could be differentiated from the B6 group by their inability to grow on sodium acetate, ethanol and L-sorbose.

0362 Group. Both 3-ketolactose-negative Australian strains (0362, 0363) could be differentiated from all other agrobacteria by their ability to grow on Koser's citrate.

M2/1 Group. Both Belgian isolates (L2/2/1, M2/1) were non-pathogenic and grew on acetate and ethanol but not on dulcitol.

TT9 Group. This group consists of three pathogenic strains. They grew anaerobically on the glucose-yeast extract medium containing nitrate and were sensitive to coly-mycin, whereas strains of the B6 group were generally resistant.

Cluster 2. 3-Ketolactose-negative strains

Fourteen strains joined above the 85 % S-level to form a very homogeneous cluster (Fig. 1) containing all named authentic *Agrobacterium rhizogenes* strains, some atypical crown-gall producers, and some unspecified *Agrobacterium* strains. Colonies on glucose-yeast extract medium were white and mucous. All strains were 3-ketolactose-negative, produced a characteristic pink-coloured curd zone in litmus milk, turned the modified Simmons citrate medium blue, grew weakly on most carbohydrates with pronounced acid production from the following sugars: D-xylose, D-galactose, D-glucose and D-mannose (pH values of the culture media after 3 weeks of growth were between 4.0 and 5.0). They failed to grow on D-lactate, did not form a brown surface pellicle on ferric ammonium citrate and grew weakly on L-tyrosine (Table 2). Our cluster 2 has almost identical biochemical and nutritional properties as Group III of White (1972) and 'biotype 2' of Keane *et al.* (1970). The latter authors describe a few more tests, that distinguish biotype 1 from 2. The tests for oxidase, growth on glycerophosphate agar and sodium selenite agar were not successful for separating our strains.

The ungrouped strains

Of the five strains which did not cluster with any of the above agrobacteria (Fig. 1), two are probably misnamed. Strain NCPPB 1772, isolated from *Vitis vinifera* in Iran, is yellow, polarly flagellated and non-pathogenic. Strain M39, a supposed u.v.-induced mutant of *Agrobacterium tumefaciens* CIP51 (which is a str^r mutant of *A. tumefaciens* B6) is non-pathogenic, phenotypically and genotypically aberrant, and perhaps related to *Alcaligenes denitrificans* (van der Plaats, Vernie & De Ley, 1969).

The three remaining 3-ketolactose-negative, crown-gall forming *Agrobacterium* strains (NCPPB 1650, NCPPB 1771 and *Agrobacterium rubi* ICPBTR2) are clearly separate from each other and from both major clusters. Some of their features are listed in Table 2.

The 'vigour' (proportion of positive character states; Sneath, 1968) for strains TR2 and NCPPB 1650 is considerably lower (0.2 and 0.3 respectively) than for clusters 1 and 2 (0.54 to 0.65 and 0.42 to 0.48 respectively). A lack of appropriate growth factors in the media is probably the major reason for the large number of negative nutritional tests of both strains. Starr (1946) and Keane *et al.* (1970) found that *Agrobacterium rubi* requires L-glutamate and cofactors, present in yeast extract.

Strain NCPPB 1771, isolated from *Vitis vinifera* in Iran, is aberrant in several nutritional and biochemical tests (Table 2). The genomes of these three strains hybridize to an extent of less than 20 % with each other and with all other agrobacteria (De Ley, 1972).

Table 2. *Diagnostic characters for identification of clusters and major groups of Agrobacterium. For experimental conditions see Methods*

Cluster	...	I										2	TR2	1650	1771
Group	...	B6	F/I	NIAS I	O362	TT111	M2/I	TT9							
No. of strains tested		19	2	2	2	18	2	3							
Colonies white		—	—	—	—	—	—	—							
Colonies beige		+	+	+	+	+	+	+							
Colonies light-yellow		—	—	—	—	—	—	—							
3-Ketolactose formation		+	+	+	—	+	+	+							
Litmus milk reaction, colour of curd zone after 3 weeks		brown	brown	brown	brown	brown	brown	brown							
Anaerobic growth on glucose-yeast extract medium containing 0.5 % (w/v) nitrate		+	+	+	d	—	—	+							
Brown surface pellicle on ferric ammonium citrate		+	+	+	+	d	+	+							
Black pigment on Ca-glycerophosphate		—	+	—	—	d	—	—							
Sensitivity to coly-mycin		—	d	—	—	d	—	+							
Growth on:															
Sodium acetate		+	+	—	+	+	+	+							
Simmons citrate + yeast extract		—	—	—	++	—	—	—							
Koser's citrate		—	—	—	+	—	—	—							
Sodium-D-lactate		+	+	+	+	+	+	+							
D-Arabinose, L-arabinose, D-galactose		++	++	++	++	++	++	++							
D-Xylose, D-glucose, D-mannose		+	+	+	+	+	+	+							
Cellobiose		++	++	++	++	++	++	++							
Sucrose		++	++	++	++	++	++	++							
L-Sorbose		++	—	—	—	—	—	d							
Dulcitol		++	—	+	+	+	+	+							
Sorbitol		++	++	++	++	++	++	++							
Ethanol		+	+	—	+	+/+	+	+							
L-Tyrosine		—	—	—	—	—	—	—							
Acid formation from D-galactose, D-glucose, D-mannose, D-xylose		+	+	+	+	+	+	+							

+ or ++, 90 to 100 % of the strains yield a moderate (+) or strong (++) positive reaction; d, 11 to 89 % of the strains yield a positive reaction; —, 0 to 10 % of the strains yield a positive reaction.

In addition, this phenotypic analysis indicates that the *Achromobacter* strains AB1196, AB1293 and NCIB8129, and all reference strains, are clearly separate from the agrobacteria.

Comparison of data using the simple matching coefficient, pattern difference coefficient and different cluster analysis techniques

There was very good agreement between the clusters formed by single linkage and unweighted average linkage of the simple matching and pattern difference coefficients. In all analyses the same 51 and 14 strains grouped in clusters 1 and 2 respectively, whereas strains NCPPB 1650, NCPPB 1771 and *Agrobacterium rubi* ICPB TR2 always occupied a separate position. The groups in the average linkage clustering of S_{SM} were the same as in the average linkage clustering of D_p . Therefore the differences between the groups and clusters are not simply accounted for by differences in growth rate or vigour.

Identification of clusters and major groups

Table 2 should aid the identification of clusters and major groups. Strains from clusters 1 and 2 are separated by 20 out of 90 characters.

DISCUSSION

The two major clusters 1 and 2 (Fig. 1), correspond rather well with biotypes 1 and 2, respectively, of Keane *et al.* (1970). Although fewer strains and less features were used, the recent results of White (1972), are essentially similar to ours.

Cluster 1 contains 3-ketolactose-positive tumour-inducing and non-pathogenic agrobacteria. It seems very likely that biotype 1 of Keane *et al.* (1970) and Group I of White (1972) are identical with our cluster 1. The seven groups of cluster 1 are genetically different (De Ley, 1972) but phenotypically very similar. They can be differentiated from each other only by less than 4 characters out of 92 checked. An extensive study of many other agrobacteria (unpublished) showed that, at the present time, groups F/1, 0362 and M2/1 consist of two strains only.

The 3-ketolactose test (Bernaerts & De Ley, 1963) is of paramount importance for quick identification of cluster 1. It is a well-established test in practical phytopathology and bacterial identification. However, Moffett & Colwell (1968) reported that none of their agrobacteria were 3-ketolactose-positive, whereas Lippincott & Lippincott (1969) and we found unequivocally positive 3-ketolactose reactions for their strains 4718, 4720, 11156, B6, 11157, 13333 and 5GLYFE. In our experience with several hundreds of different strains this test has always been perfectly reproducible when performed as described by Bernaerts & De Ley (1963).

Cluster 2 contains most of the 3-ketolactose-negative tumour-inducing bacteria and all real *Agrobacterium rhizogenes* strains. Their genomes are about 97 % similar and they hybridize at 20 % or less with the strains of cluster 1. Cluster 2 contains the 3-ketolactose-negative *A. tumefaciens* strains P2, AG1, AG6 and all root-forming *A. rhizogenes* studied by Lippincott & Lippincott (1969) (J. De Ley and K. Kersters, unpublished). Our results reinforce those of Keane *et al.* (1970) and Lippincott & Lippincott (1969), that both types of strains are phenotypically indistinguishable, except for their phytopathological behaviour. The biochemical properties of the 3-ketolactose-negative tumour-inducing agrobacteria of our cluster 2 correspond with the definition of *A. rhizogenes* by Riker *et al.* (1930). According to Keane *et al.* (1970) two named *A. rhizogenes* strains (TR104 and TR108) belonged in their biotype 1 (roughly our cluster 1). However, strain TR104 (ATCC 13333, NCPPB 5) is probably misnamed: it is a normal 3-ketolactose-positive, crown-gall producing strain, which fails

to induce root-formation (Lippincott & Lippincott, 1969; K. Kersters, unpublished). We suspect that the rhizogenic properties of strain TR108 should be rechecked.

The ungrouped 3-ketolactose-negative tumour-inducing strains *Agrobacterium rubi* ICPBTR2, *A. tumefaciens* NCPPB1650 and 1771 may form nuclei around which future *Agrobacterium* isolates may cluster. This is certainly so for TR2, because acrylamide gel electrophoresis and DNA:DNA hybridizations indicate that it forms a separate, distinct cluster with *A. rubi* ATCC13335 and *A. tumefaciens* EU6. Keane *et al.* (1970) included the *A. rubi* strains in their biotype 2, but according to our present data they form a very small separate cluster. Strains NCPPB1650 and 1771 are genetically and phenotypically unique in more than 250 *agrobacteria* investigated (unpublished).

The groupings obtained with numerical analysis agree on the whole excellently with the results from DNA:DNA hybridizations, DNA hybrid stability (De Ley, Tijtgaat, De Smedt & Michiels, 1973) and protein electrophoresis (J. De Ley & K. Kersters, unpublished). In some cases these methods complement each other. For example, strains NIAS1 and NIASA-2-1-1 form phenotypically a small group (Fig. 1, Table 2), but genotypically they belong in the B6 group. Strains CIPRV3, Zutra 3/1, and NCPPB1641 are phenotypically indistinguishable from the B6 group, whereas genotypically they form a distinct group at 50 % DNA-homology with all other groups. DNA hybridization and electrophoretic protein profiles indicate that strain Mushin 6 is a member of the small TT9 group. Strains IPO417, IIBV7 and Gembloux A appear to be separate cases in cluster 1, but DNA hybridization shows them to be members of the TTIII group.

The non-pathogenic *Agrobacterium radiobacter* strains are scattered over four groups of cluster 1 (Fig. 1). They are indistinguishable from the 3-ketolactose-positive *A. tumefaciens* strains, except for phytopathogenicity. In contradiction to some previous claims (Bergey's *Manual*, 1957), the following features appeared to have no diagnostic value: congo red and aniline blue absorption (Riker *et al.* 1930; Hendrickson *et al.* 1934), brown pigment production on calcium glycerophosphate agar (Hendrickson *et al.* 1934) and the ability to utilize nitrate in nitrate glycerol phenol-red medium (Sagen, Riker & Baldwin, 1934). Huisingh & Durbin (1967), Lippincott & Lippincott (1969), and Keane *et al.* (1970) likewise could not detect any physiological or biochemical difference between *A. tumefaciens* and *A. radiobacter*. A similar situation exists for the 3-ketolactose-negative *A. tumefaciens* and *A. rhizogenes* strains, which differ only in their phytopathological behaviour.

The three main species names in the genus *Agrobacterium* refer mainly to their state of phytopathogenicity. In the present approach tumour formation is just one of 92 features and it appears to be found in all groups and both clusters. A taxon separation on the sole criterion of pathogenicity is very valuable for the practical phytopathologist, but a species separation on the same grounds is not justified biologically. The current classification of the genus *Agrobacterium* into species according to pathogenicity does not reflect the natural relationships amongst these bacteria.

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REFERENCES

- Bergey's Manual of Determinative Bacteriology* (1957), 7th edn. Edited by R. S. Breed, E. G. D. Murray and N. R. Smith. Baltimore: The Williams and Wilkins Co.
- BERNAERTS, M. J. & DE LEY, J. (1963). A biochemical test for crown gall bacteria. *Nature, London* **197**, 406-407.
- CLARK, A. G. (1969). A selective medium for the isolation of *Agrobacterium* species. *Journal of Applied Bacteriology* **32**, 348-351.
- DE LEY, J. (1972). *Agrobacterium*: intrageneric relationships and evolution. In *Proceedings of the Third International Conference on Plant Pathogenic Bacteria*, pp. 251-259. Edited by H. P. Maas Geesteranus. Wageningen (the Netherlands): Centre for Agricultural Publishing and Documentation.
- DE LEY, J., BERNAERTS, M., RASSEL, A. & GUILMOT, J. (1966). Approach to an improved taxonomy of the genus *Agrobacterium*. *Journal of General Microbiology* **43**, 7-17.
- DE LEY, J. & RASSEL, A. (1965). DNA Base composition, flagellation and taxonomy of the genus *Rhizobium*. *Journal of General Microbiology* **41**, 85-91.
- DE LEY, J., TIJTGAT, R., DE SMEDT, J. & MICHIELS, M. (1973). Thermal stability of DNA:DNA hybrids within the genus *Agrobacterium*. *Journal of General Microbiology* **78**, 241-252.
- GRAHAM, P. H. (1964). The application of computer techniques to the taxonomy of the root-nodule bacteria of legumes. *Journal of General Microbiology* **35**, 511-517.
- GRAHAM, P. H. & PARKER, C. A. (1964). Diagnostic features in the characterisation of the root-nodule bacteria of legumes. *Plant and Soil* **20**, 383-396.
- HEBERLEIN, G. T., DE LEY, J. & TIJTGAT, R. (1967). Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium* and *Chromobacterium*. *Journal of Bacteriology* **94**, 116-124.
- HENDRICKSON, A. A., BALDWIN, I. L. & RIKER, A. J. (1934). Studies on certain physiological characters of *Phytomonas tumefaciens*, *Phytomonas rhizogenes* and *Bacillus radiobacter*. Part II. *Journal of Bacteriology* **28**, 597-618.
- HUISINGH, D. & DURBIN, R. D. (1967). Physical and physiological methods for differentiating among *Agrobacterium rhizogenes*, *A. tumefaciens* and *A. radiobacter*. *Phytopathology* **57**, 922-923.
- KEANE, P. J., KERR, A. & NEW, P. B. (1970). Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. *Australian Journal of Biological Sciences* **23**, 585-595.
- KOSER, S. A. (1923). Utilization of the salts of organic acids by the colon-aerogenes group. *Journal of Bacteriology* **8**, 493-520.
- LIPPINCOTT, J. A. & LIPPINCOTT, B. B. (1969). Tumour-initiating ability and nutrition in the genus *Agrobacterium*. *Journal of General Microbiology* **59**, 57-75.
- LIPPINCOTT, J. A., WEBB, J. H. & LIPPINCOTT, B. B. (1965). Auxotrophic mutation and infectivity of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **90**, 1155-1156.
- Manual of Microbiological Methods* (1957). Society of American Bacteriologists. New York: McGraw-Hill Book Co.
- 'T MANNETJE, L. (1967). A re-examination of the taxonomy of the genus *Rhizobium* and related genera using numerical analysis. *Antonie van Leeuwenhoek* **33**, 477-491.
- MOFFETT, M. L. & COLWELL, R. R. (1968). Adansonian analysis of the *Rhizobiaceae*. *Journal of General Microbiology* **51**, 245-266.
- VAN DER PLAAT, J. B., VERNIE, L. N. & DE LEY, J. (1969). Suspected mutant of *Agrobacterium tumefaciens* with an altered DNA. *Mutation Research* **7**, 466-468.
- RHODES, M. E. (1958). The cytology of *Pseudomonas* species as revealed by a silver-plating staining method. *Journal of General Microbiology* **18**, 639-648.
- RIKER, A. J., BANFIELD, W. M., WRIGHT, W. H., KEITT, G. W. & SAGEN, H. E. (1930). Studies on infectious hairy root of nursery apple trees. *Journal of Agricultural Research* **41**, 507-540.
- SAGEN, H. E., RIKER, A. J. & BALDWIN, I. L. (1934). Studies on certain physiological characters of *Phytomonas tumefaciens*, *Phytomonas rhizogenes* and *Bacillus radiobacter*. Part I. *Journal of Bacteriology* **28**, 571-595.
- SIMMONS, J. S. (1926). A culture medium for differentiating organisms of the typhoid-colon aerogenes groups and for isolation of certain fungi. *Journal of Infectious Disease* **39**, 209-214.
- SKYRING, G. W., QUADLING, C. & ROUATT, J. W. (1971). Soil bacteria: principal component analysis of physiological descriptions of some named cultures of *Agrobacterium*, *Arthrobacter* and *Rhizobium*. *Canadian Journal of Microbiology* **17**, 1299-1311.

- SNEATH, P. H. A. (1957). The application of computers to taxonomy. *Journal of General Microbiology* **17**, 201-226.
- SNEATH, P. H. A. (1968). Vigour and pattern in taxonomy. *Journal of General Microbiology* **54**, 1-11.
- SOKAL, R. R. & MICHENER, C. D. (1958). A statistical method for evaluating systematic relationships. *Kansas University Science Bulletin* **38**, 1409-1438.
- SOKAL, R. R. & SNEATH, P. H. A. (1963). *Principles of Numerical Taxonomy*. San Francisco: W. H. Freeman.
- STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966). The aerobic pseudomonads: a taxonomic study. *Journal of General Microbiology* **43**, 159-271.
- STARR, M. P. (1946). The nutrition of phytopathogenic bacteria. II. The genus *Agrobacterium*. *Journal of Bacteriology* **52**, 187-194.
- WHITE, L. O. (1972). The taxonomy of the crown-gall organism *Agrobacterium tumefaciens* and its relationship to rhizobia and other agrobacteria. *Journal of General Microbiology* **72**, 565-574.