The Taxonomy of the Crown-gall Organism *Agrobacterium tumefaciens* and Its Relationship to Rhizobia and Other Agrobacteria

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SUMMARY

Strains of agrobacteria, rhizobia and other organisms were collected from various sources and tested for their ability to utilize 61 compounds as sole carbon sources, oxidative or fermentative glucose metabolism, hydrolysis of starch, casein and gelatin, anaerobic arginine metabolism and production of 3-ketolactose. The resulting data was used in a numerical analysis and the strains were grouped on the basis of overall similarity.

The resultant groupings confirmed the idea that the genera *Agrobacterium* and *Rhizobium* be combined, and four species of fast-growing rhizobia were recognized: Group I, *Rhizobium radiobacter*; Group II, *Rhizobium meliloti*; Group III, related to *Agrobacterium rhizogenes* and the Biotype 2 strains of Keane, Kerr & New (1970); Group IV, *Rhizobium leguminosarum*. Crown-gall organisms were associated with groups I and III. The four species were defined without reference to pathogenicity or nodulating ability and it appears at present that crown-gall organisms can only be distinguished by pathogenicity testing.

Agrobacterium rubi H36 probably belongs in the genus *Rhizobium* whereas *Agrobacterium gypsophilae* 1948 and *Agrobacterium pseudotsugae* 180 do not.

INTRODUCTION

The crown-gall organism, called *Bacterium tumefaciens* by its discoverers, Smith & Townsend (1907), was placed in the genus *Agrobacterium*, a member of the family Rhizobiaceae, by Conn (1942) with other plant pathogenic and saprophytic species (*Bergey's Manual*, 1957).

Recently, numerical taxonomic studies (Graham, 1964; Moffett & Colwell, 1968) and DNA hybridization experiments (Heberlein, De Ley & Tijtgate, 1967) have demonstrated that, except for its pathogenicity, the crown-gall organism is indistinguishable from the saprophyte *Agrobacterium radiobacter*; both are also closely related to the fast-growing species of root-nodule bacteria of legumes (*Rhizobium leguminosarum*, *R. trifolii*, *R. meliloti*, *R. phaseoli*). The species *A. rhizogenes* and *A. rubi* show affinities with *A. tumefaciens* but *A. pseudotsugae*, *A. gypsophilae* and *A. stellulatum* do not (De Ley, Bernaerts, Rassel & Guilmot, 1966).

Kerr (1969) isolated crown-gall organisms which differed from typical *Agrobacterium tumefaciens*, and Keane *et al.* (1970) proposed that these organisms be designated *A. radiobacter* var. *tumefaciens* Biotype 2, typical crown-gall organisms being designated *A. radiobacter* var. *tumefaciens* Biotype 1. Strains of *A. rhizogenes* showed similar properties to Biotype 2 crown-gall isolates and it was also proposed that *A. rhizogenes* be classified as a variety, *A. radiobacter* var. *rhizogenes* Biotype 2.

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This study was undertaken to re-examine the taxonomy of crown-gall organisms by numerical analysis of nutritional characters, as applied to pseudomonads (Stanier, Palleroni & Doudoroff, 1966; Sands, Schroth & Hildebrand, 1970).

METHODS

Organisms. The organisms studied are shown, with their sources, in Table 1. Tumour-inducing ability was checked by inoculation into tomato plant internodes. Strains of rhizobia and *Agrobacterium rhizogenes* 1855 were maintained on yeast extract, mannitol agar (YMA) (Nutman, 1946) using 0.4% Oxoid yeast extract (Oxoid Limited, London, S.E. 1) in place of yeast water. The agrobacteria (excluding *A. gypsophilae* 1948) were maintained on sucrose, nutrient broth, yeast extract agar (SA) (Lippincott & Lippincott, 1965). Chromobacterium strains 10590 and 10591 were also maintained on this medium. Other organisms were maintained on nutrient agar (NA). All experiments were performed at 25 °C.

Nutritional tests. Organisms were tested for their ability to use 61 compounds as sole carbon source, incorporated at a concentration of 0.1% (w/v) in the following medium (modified from Sundman, 1964) (g/900 ml distilled water): NaNO₃, 2.5; KH₂PO₄, 1.0; K₂HPO₄, 2.0; MgSO₄·7H₂O, 0.3; CaCl₂, 0.1; NaCl, 0.1; FeCl₃, 0.01; Oxoid agar no. 1, 10; vitamin solutions A, B and C, 2.5 ml of each. (Vitamin solutions: A—biotin, 20 µg; vitamin B₁₂, 40 µg; distilled water, 100 ml; B—thiamine hydrochloride, 10 mg; nicotinic acid, 10 mg; calcium pantothenate, 10 mg; *p*-amino-benzoic acid, 10 mg; distilled water, 100 ml; C—folic acid, 2.0 mg; 0.001 N-NaOH, 100 ml). The vitamin solutions were autoclaved at 10 lb/in² for 10 min and stored at 4 °C. The medium was dispensed in 90 ml amounts and autoclaved at 15 lb/in² for 15 min. Carbon sources were prepared as 1% solutions and sterilized by filtration through Millipore (Millipore (U.K.) Ltd., Millipore House, Abbey Road, Park Royal, London NW10 7SP) membrane filters (0.22 µm pore size) or by autoclaving; 10 ml of carbon source solution was added to melted basal medium cooled to 50 °C and plates were poured (20 ml per plate). The replica plating technique (Lederberg & Lederberg, 1952) proved unworkable with gummy colonies, consequently plates were inoculated with a zig-zag streak using a wire loop. Minute inocula from young actively growing cultures (2 to 5 days) were used to minimize nutrient carry-over. After 10 days of incubation carbon sources supporting visibly more growth than control plates without added carbon source were scored positive. A few dubious cases showing traces of growth were not scored. All tests were repeated at least twice.

The compounds tested were: glucose, sorbose, xylose, fructose, maltose, lactose, mannose, melezitose, melibiose, cellobiose, trehalose, raffinose, amygdalin, sucrose, galactose, arabinose, inulin, sorbitol, dulcitol, mannitol, erythritol, glycerol, adonitol, rhamnose, aesculin, dextrin, vanillin, mucic acid, sodium malonate, potassium sodium tartrate, sodium gluconate, glucuronic acid, sodium fumarate, sodium formate, tri-sodium citrate, sodium acetate, sodium benzoate, sodium lactate, sodium oxalate, sodium propionate, sodium succinate, sodium malate, sodium hippurate, sodium-*n*-butyrate, urea, biuret, betaine (neutralized), allantoin, ethanol, iso-propanol, ethylene glycol, leucine, lysine, arginine, histidine, cystine, asparagine, sodium glutamate, serine, glycine (D-isomers of sugars except for L-sorbose and L-rhamnose, L-isomers of amino acids).

Glucose utilization. Production of acid from the aerobic or anaerobic breakdown of glucose (Hugh & Leifson, 1953) was examined using the medium of Baird-Parker (1963). Readings were made at intervals up to 21 days. Organisms showing marked acidification of both aerobic and anaerobic tubes were scored positive. Those producing no acid or acid only in the aerobic tube were scored negative.

Table 1. *Strains used and their source*

Species name and strain no.	Source*
<i>Agrobacterium tumefaciens</i>	
B6	J. A. Lippincott
II BNV6	J. A. Lippincott
8150	N.C.I.B.
A6	R. E. Beardsley
A66B	R. E. Beardsley
T37	R. E. Beardsley
TUM6	Ch. Bonnier
TUM7	Ch. Bonnier
TUM8	Ch. Bonnier
TUM15	Ch. Bonnier
TUM17	Ch. Bonnier
1039 ng†	C.C.M.
1041	C.C.M.
1058	C.C.M.
<i>Agrobacterium radiobacter</i> var. <i>tumefaciens</i>	
Biotype 1	
K15	A. Kerr
K19	A. Kerr
K28	A. Kerr
Biotype 2	
K34	A. Kerr
K40	A. Kerr
K41	A. Kerr
<i>Agrobacterium radiobacter</i>	
H29	F. Tanner
M2/1	J. De Ley
L2/2/1	J. De Ley
S1005	Ch. Bonnier
<i>Agrobacterium radiobacter</i> var. <i>radiobacter</i>	
Biotype 1	
K6	A. Kerr
K9	A. Kerr
Biotype 2	
K84	A. Kerr
<i>Agrobacterium rhizogenes</i>	
1855	N.C.P.P.B.
5	N.C.P.P.B.
<i>Agrobacterium rubi</i>	
H36	F. Tanner
<i>Agrobacterium pseudotsugae</i>	
180	N.C.P.P.B.
<i>Agrobacterium gypsophilae</i>	
1948	N.C.P.P.B.
<i>Rhizobium meliloti</i>	
R21	D. C. Jordan
2001	R.C.R.
2003	R.C.R.
2004	R.C.R.
2005	R.C.R.
2006	R.C.R.
2009‡	R.C.R.
<i>Rhizobium phaseoli</i>	
3601	R.C.R.
3602	R.C.R.
3603	R.C.R.

Table 1 (*continued*)

Species name and strain no.	Source*
<i>Rhizobium leguminosarum</i>	
1002	R.C.R.
1003	R.C.R.
<i>Rhizobium trifolii</i>	
2	R.C.R.
33	R.C.R.
36	R.C.R.
204	R.C.R.
0403	R.C.R.
0462	R.C.R.
VANC	J. N. Reeve
Other rhizobia	
3208§	R.C.R.
3404§	R.C.R.
3405§	R.C.R.
3807	R.C.R.
<i>Chromobacterium lividum</i>	
NCTC10590	J. Taylor
NCTC10591	J. Taylor
<i>Xanthomonas campestris</i>	
528	N.C.P.P.B.
<i>Xanthomonas hyacinthi</i>	
599	N.C.P.P.B.
<i>Erwinia carotovora</i>	
312	N.C.P.P.B.
<i>Pseudomonas savastanoi</i>	
1006	N.C.P.P.B.
1506	N.C.P.P.B.
Other soil and root nodule isolates	
RB. 3b	Runner bean nodule isolate
RB. 1b	Runner bean nodule isolate
RED CLOV	Departmental nodule isolate
VETCH	Departmental nodule isolate
E	Soil pseudomonad
Ps. Cogbil	Clinical pseudomonad isolate
Ps. Preen	Clinical pseudomonad isolate
Coral	Isolate from coral

* Investigator or source supplying culture. J. A. Lippincott, Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201, U.S.A.; N.C.I.B., National Collection of Industrial Bacteria, Torry Research Station, 135 Abbey Road, Aberdeen AB9 8DG, Scotland; R. E. Beardsley, Manhattan College, New York, U.S.A.; Ch. Bonnier, Chaire de Microbiologie, Faculté des Sciences Agronomiques de l'État, Gembloux, Belgium; C.C.M., Czechoslovak Collection of Micro-organisms, J. E. Purkyně University, Brno, třída Obránců míru, 10, Czechoslovakia; A. Kerr, Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064; F. Tanner, International Centre for Information on and Distribution of Type Cultures, Institute of Hygiene and Bacteriology, 19 Av. César Roux, Lausanne, Switzerland; J. De Ley, Lab. voor Microbiologie en Microbiele Genetica, Faculteit der Wetenschappen, Rijksuniversiteit, Ghent, Belgium; N.C.P.P.B., National Collection of Plant Pathogenic Bacteria, Plant Pathology Laboratory, Ministry of Agriculture Fisheries and Food, Hatching Green, Harpenden, Herts, England; D. C. Jordan, Department of Microbiology, University of Guelph, Guelph, Ontario, Canada; R.C.R., Rothamsted Collection of Rhizobia, Department of Microbiology, Rothamsted Experimental Station, Harpenden, Herts, England; J. N. Reeve, Department of Microbiology, University of British Columbia, Vancouver 8, Canada; J. Taylor, Salmonella Reference Laboratory, Central Public Health Laboratories, Colindale Avenue, London NW 9, England.

† A clonal variant from the freeze-dried strain received which failed to produce copious amounts of gum on carbohydrate media.

‡ Two clones of this strain, differing in colonial morphology were used in this study. The clones were designated 2009 I and 2009 II but both gave identical results in the work reported here.

§ 'Slow-growing' strains.

Hydrolysis of starch, caesin and gelatin. YMA or NA was modified by the addition of either (i) an autoclaved 10 % solution of soluble starch to give a final concentration of 1 % or (ii) an autoclaved 10 % solution of Oxoid skim milk to give a final concentration of 2 %, (iii) a 10 % solution of gelatin, sterilized by steaming on three consecutive days, to give a final concentration of 0.4 %.

Plates were inoculated with streaks of organisms and incubated for up to 14 days. Casein hydrolysis was recorded by observing a clearing around the growth, starch and gelatin hydrolysis were observed by the application of Lugol's iodine and Frazier's reagent respectively (Cowan & Steel, 1965).

Alkali from arginine anaerobically. Ability to utilize arginine under anaerobic conditions with the release of alkali was examined in the medium of Møller (1955) using 0.001 % bromothymol blue as the only indicator. Test medium contained 1 % L-arginine and control medium contained none. Development of a blue coloration in the arginine medium, but not the control medium, within 14 days was scored positive.

3-Ketolactose. Ability to produce 3-ketolactose from lactose was examined by the method of Bernaerts & De Ley (1963).

Numerical methods. All tests were scored qualitatively as positive, negative, or in a few cases unavailable or inapplicable. The data was punched on cards and analysed on the KDF 9 computer of the University of Birmingham using a computer program which:

(i) calculated and printed similarity coefficients between pairs of organisms. Two types of coefficients were calculated: simple matching (or S.M.) similarity coefficients (Sneath, 1962) in which both shared negative and positive results are regarded as similarities and simple matching coefficients ignoring negative matches or positive only (P.O.) similarity coefficients (Sneath, 1957);

(ii) clustered the similarity coefficients by the pair-group average linkage method of Sokal & Michener (1958).

The data from the 3-ketolactose test was excluded from the numerical analysis.

RESULTS

The results of the numerical analysis in dendrograms 1 and 2 (Fig. 1, 2) show that the majority of fast-growing rhizobia and agrobacteria can be separated into four groups, defined by *a posteriori* weighting of certain characters, and completely unrelated to pathogenicity.

The major difference between the clustering of the S.M. coefficients and the P.O. coefficients is the unexpectedly wide separation of group IV in dendrogram 2 from the other rhizobia and agrobacteria. This can be explained by the general inactivity of strains in this group; for example, *Agrobacterium tumefaciens* B6 gave 42/66 positive results whereas *Rhizobium trifolii* 2 gave 34/66. Since not all of the 34 positives were also positive for B6, the number of shared positive matches was low.

The major groups formed are as follows:

Group I: 3-ketolactose +, erythritol -, lysine +, ornithine +, tartrate -, mucic acid -.

Strains not included in this group did not produce 3-ketolactose from lactose. Both tumourigenic and non-tumourigenic strains are present within the group but are not resolvable.

Group II: 3-ketolactose -, erythritol + (except *Agrobacterium rubi* H36), lysine +, ornithine +, tartrate -, mucic acid -.

This group is made up essentially of strains of *Rhizobium meliloti* but one strain of *R. phaseoli*, 3602, and *A. rubi* H36 are also included.

Group III: 3-ketolactose —, erythritol +, lysine +, ornithine +, tartrate +, mucic acid +.

This group contains all the Biotype 2 strains of Keane *et al.* (1970) and as in Group I the pathogens are indistinguishable from the non-pathogen (K84). *Rhizobium* strain 3807 is also a member of this group as is *Agrobacterium rhizogenes* 1855.

Group IV: 3-ketolactose —, erythritol +, lysine —, ornithine —, tartrate and mucic acid variable.

All named strains of *Rhizobium leguminosarum*, *R. trifolii* and *R. phaseoli*, except strains 0462 and 3602, together with unnamed nodule isolates RB.1b and RB.3b are included in this group. It is not possible to resolve the three named species of rhizobium within the group.

Other groups

The three strains of slow-growing rhizobia examined cluster together with *Rhizobium trifolii* 0462. It is of interest that 0462, a pink pigmented S₂P₁₁ phage mutant of *R. trifolii* 0403, grew slowly and utilized butyrate, as did the three slow-growing strains, but no strain in groups I–IV. *Agrobacterium gypsophylae* 1948 is most similar to *Erwinia carotovora* 312 and *A. pseudotsugae* 180 to the chromobacterial strains 10590 and 10591. The pseudomonads and xanthomonads do not form a distinct group.

DISCUSSION

Previous detailed numerical analyses of agrobacteria either failed to include *Agrobacterium rhizogenes* (Graham, 1964) or failed to detect the differences between *A. rhizogenes* and *A. tumefaciens* (Moffett & Colwell, 1968). However, the results presented here clearly indicate that *A. rhizogenes* (as represented by strain 1855) and the Biotype 2 strains of *A. tumefaciens* cluster separately from *A. radiobacter*, and *A. tumefaciens*. *Agrobacterium rhizogenes* 5 is a member of the tumefaciens/radiobacter cluster, and indeed De Ley *et al.* (1966) and Lippincott & Lippincott (1969) report that this strain is a typical crown-gall-forming organism.

Nomenclature of the groups

There is an obviously close relationship between the organisms in groups I–IV and this is most evident in dendrogram 1, where all the groups form a single cluster at over 75 %. The separation of group IV, when P.O. coefficients are analysed has been explained. On the basis of these results taken in conjunction with genetic evidence (Klein & Klein, 1953; Kern, 1965*a, b*, 1969) phage work (Roslycky, Allen & McCoy, 1960, 1962) serological cross-reactions (Graham, 1963, 1971; Vincent & Humphrey, 1970; White, 1971) and DNA hybridization (Heberlein *et al.* 1967) this author agrees with the proposal, first made by Graham (1964), that *Agrobacterium* and *Rhizobium* should be combined into a single genus *Rhizobium* and it is suggested that groups I–IV should be taken to represent four species-groups within this genus.

Group I: Rhizobium radiobacter. A species containing both saprophytic and tumour-inducing organisms. Graham (1964) defined varieties within this species on the basis of pathogenicity, and at present varieties can only be distinguished by pathogenicity-testing. As observed by De Ley (1968), ability to form 3-ketolactose seems to be characteristic of this species and Keane *et al.* (1970) isolated 3-ketolactose-positive strains capable of producing hairy root disease.

Group II: Rhizobium meliloti. The true position of *Agrobacterium rubi* H36 is dubious since

Fig. 1. Dendrogram I produced as a result of clustering s.m. similarity coefficients by the pair-group average linkage method (Sokal & Michener, 1958). Organisms are designated by strain numbers (see Table 1).

Fig. 2. Dendrogram 2 produced as a result of clustering P.O. similarity coefficients by the pair-group average linkage method (Sokal & Michener, 1958). Organisms are designated by strain numbers (see Table 1).

Table 2. *Proposed nomenclature for pathogenic strains of Rhizobium*

Disease	Proposed designation (species group I*)	Old designations
Non-pathogenic	<i>Rhizobium radiobacter</i> state, saprophytic	<i>Agrobacterium radiobacter</i> <i>A. radiobacter</i> var. <i>radiobacter</i> <i>Rhizobium radiobacter</i> var. <i>radiobacter</i> <i>Agrobacterium radiobacter</i> var. <i>radiobacter</i> Biotype 1
Crown gall	<i>Rhizobium radiobacter</i> state, tumourigenic	<i>Agrobacterium tumefaciens</i> <i>A. radiobacter</i> var. <i>tumefaciens</i> <i>Rhizobium radiobacter</i> var. <i>tumefaciens</i> <i>Agrobacterium radiobacter</i> var. <i>tumefaciens</i> Biotype 1
Hairy root	<i>Rhizobium radiobacter</i> state, rhizogenic	<i>A. radiobacter</i> var. <i>rhizogenes</i> Biotype 1

* Strains of species Group III capable of producing the same disease would be designated by the same 'state' epithets.

it failed to cluster with this group when morphological and physiological characters were analysed (White, 1971). Moffett & Colwell (1968) examined two strains designated *A. rubi* and their results showed that the two strains differed in several respects. The taxonomy of strains named *A. rubi* still needs clarification.

Group III. As with group I, crown-gall-forming organisms, the Biotype 2 strains of Keane *et al.* (1970), also occur in this group, but Group III is clearly separated from Group I by the *Rhizobium meliloti* cluster. Keane *et al.* (1970) demonstrated the close similarity between *Agrobacterium rhizogenes* and the Biotype 2 crown-gall isolates but failed to demonstrate the clear difference between typical crown-gall isolates and the Biotype 2 strains which is apparent here. Strain 1855 gives typical reactions of the species *A. rhizogenes* (White, 1971) and it may be that all group III strains should be incorporated into this species. However, since the type culture of *A. rhizogenes* was not included in this work it is suggested that no new epithet is applied until further studies have been made on organisms related to those in group III.

Group IV: Rhizobium leguminosarum. In line with the findings of Graham (1964) strains of *R. leguminosarum*, *R. trifolii* and *R. phaseoli* are included in this single enlarged species.

The position of the other agrobacteria

As remarked, *Agrobacterium rubi* is probably closely related to the groups described above but the exact relationship needs further elucidation. As regards the species *A. gypsophilae* it was suggested (De Ley, 1968) on the basis of DNA base composition that the organism is probably related to *Erwinia* and the results presented here show that *A. gypsophilae* 1948 and *E. carotovora* 312 might well be considered as members of the same genus. There is no evidence to support the inclusion of *A. gypsophilae* in the genus *Xanthomonas* (Prévot, 1961). De Ley (1968) suggested that *A. gypsophilae* be re-named *E. herbicola* since both have yellow pigmentation. Both strains 1948 and 312 strongly ferment glucose.

The results also confirm the suggestions of others that *Agrobacterium pseudotsugae* 180 is not closely related to the other agrobacteria. The apparent relationship with the chromobacteria demonstrated here is of interest since the DNA base composition of strain 180 is within the chromobacterial range (De Ley *et al.* 1966). However, a close phenetic relation-

Table 3. *Possible diagnostic characters for separating the four species of fast-growing Rhizobium recognized in this work*

Test	<i>Rhizobium radiobacter</i>	Group III	<i>Rhizobium meliloti</i>	<i>Rhizobium leguminosarum</i>
3-Ketolactose formation	+	o	o	o
Litmus milk brown in 14 days	+	o	o	o
Ethanol*	+	o	+	o
Erythritol*	o	+	+	+
Ornithine*	+	+	+	o
Lysine*	+	+	+	o
Tartrate*	o	+	o	o
Mucic acid*	o	+	o	±
Melezitose*	+	o	+	o
Growth on 2 % NaCl	+	o	+	o
Growth at 37 °C	+	o	+	o
Growth at 40 °C	+	o	+	o
Malonate*	o	±	+	o
Propionate*	+	o	+	o
Butyrate*	o	o	o	o

* Utilization of.

+

± More than half strains positive.

+

o Few or no positive strains.

ship was not apparent in an analysis of other properties (White, 1971). The position of this organism must remain undecided.

The slow-growing rhizobia

Our results confirm that there is less similarity between the fast and slow-growing rhizobia than between the fast growing rhizobia and the agrobacteria. However, no suggestion can be made as to whether the slow growers should be placed in a new genus.

Designation of pathogenic rhizobia

Some members of both groups I and III are capable of producing crown-gall tumours and it would appear that group III strains can transfer the property to group I strains *in vivo* (Kerr, 1971). It thus appears that the property of pathogenicity has little or no taxonomic value and it is suggested that the varietal epithets suggested by Graham (1964) be reduced to the infra-subspecific rank 'state'. To do so removes the requirement to designate crown-gall organisms in different species groups by different varietal epithets.

Thus strains in groups I and III may be found in one of at least three states (Table 2) and there is some evidence (MacGregor & Alexander, 1971) that root nodulating rhizobia may also have a transitory tumourigenic state.

Identification

Using this scheme, crown-gall organisms could belong to one of at least two species. Table 3 gives a list of properties taken from the nutritional results analysed here and also from the results of White (1971) which should aid the identification of the four species of *Rhizobium* related to the tumour-inducing and root-proliferating pathogens.

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