Biotypes and Phenotypic Groups of Strains of *Agrobacterium* in South Africa

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ABSTRACT

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Seventy-one South African strains of *Agrobacterium* and 19 reference strains were grouped into three clusters by numerical analysis of 49 phenotypic characters. The major cluster, which corresponds to biotype-2 agrobacteria, contained three distinct groups. A second cluster contained typical biotype-3 as well as strains of *Agrobacterium rubi*. Seven known genetic groups and three additional groups of biotype-1 agrobacteria were distinguished in a third cluster. Diagnostic characters, biotypes, and phenotypic groups of South African strains and reference strains of *Agrobacterium* are discussed.

Additional key words: *Agrobacterium radiobacter, A. rhizogenes, A. tumefaciens,* crown gall.

Agrobacterium spp. in Bergey's Manual (1) are based primarily on phytopathogenic characters. The strains causing crown gall are placed in *Agrobacterium tumefaciens*; those causing hairy root, in *A. rhizogenes*; and those causing cane gall on *Rubus* spp., in *A. rubi*. Nonpathogens are placed in *A. radiobacter*. Other workers recognized three biotypes (12,13) and biotype intermediate strains (2,4,5,22,24) of *Agrobacterium* on the basis of biochemical tests. None of these groups correspond to phytopathogenic characters. Genetic variation among strains of *Agrobacterium* was shown by DNA:DNA hybridizations (6), numerical taxonomic analysis (15), and by numerical analysis of soluble protein patterns (14). Eleven genetic groups, considered as genetic races (7), were identified.

This paper reports the occurrence of different biotypes of *Agrobacterium* in South Africa and the phenotypic grouping of 71 South African and 19 reference strains.

MATERIALS AND METHODS

Bacteria. Strains of *Agrobacterium* were isolated from tumors of infected plant roots and from a soil sample. Tumors were washed in tap water and small pieces of well developed tissue were suspended in a few drops of sterile distilled water for 1 hr at room temperature. The soil sample was diluted tenfold in sterile distilled water and kept for 1 hr at room temperature. The suspensions were streaked on nutrient agar (NA) (Difco Laboratories, Detroit, MI 48232), a selective medium for biotype-1 strains (20), and a selective medium for biotype-2 strains (19). Agar plates were incubated at 28 C for 3 days (NA) or 7 days (selective media). Single colonies were selected and purified by two successive streakings on NA. Cultures that developed small, round, slightly raised, whitish, and translucent colonies on NA were examined for Gram reaction, morphology, production of fluorescent pigment on King's medium B (16), oxidase reaction, and the production of 3-ketolactose. Strains were stored on yeast-mannitol agar (YMA) slants under liquid paraffin at room temperature. Three strains of *Agrobacterium* were supplied by F. G. H. van Zyl, Plant Protection Research Institute, Stellenbosch 7600, South Africa. Nineteen reference strains were also included in the investigation (Table 1).

Pathogenicity tests. Pathogenicity of each strain was determined on 14-day-old tomato seedlings (cultivar Red Khaki) in a greenhouse maintained at ~28 C. Inocula were prepared from 28-hr-old NA slants at 28 C. The suspensions were adjusted to contain about 10^9 colony-forming units (cfu) per milliliter. A drop of bacterial suspension was deposited on a tomato stem and a sterile needle was used to puncture the tomato stem through the drop and thus introduce the bacteria into the plant. Control plants were similarly treated with sterile distilled water. The presence of tumors was assessed visually after 6 wk.

Strains not pathogenic to tomato seedlings were also tested for pathogenicity on broad bean plants as above, and on carrot disks by using the technique of Lippincott and Lippincott (17).

Bacteriological methods. All tests were done at 28 C. Bacterial suspensions were adjusted to ~10^9 cfu/ml of sterile distilled water. Test media were inoculated with one drop of suspension transferred with a sterile loop.

Colony characters, morphology, and pigmentation. These were examined on glucose-yeast extract agar described by Kersters et al (15). Results were recorded after 3, 7, and 14 days.

Production of 3-ketolactose. The method of Bernaerts and De Ley (3) was used.

Litmus milk reaction. Acid or alkali production was recorded after 2 and 3 wk of growth in 10 ml of litmus milk (Difco).

Growth in the presence of 2% sodium chloride. Growth in nutrient broth (Difco) containing 2% NaCl was recorded daily for a period of 7 days.

Growth at different incubation temperatures. Growth on NA slants in water baths at 30, 35, and 37 C was recorded after 7 days.

Acid production from erythritol and melezitose. Media described by Kerr and Panagopoulos (13) were used. Acid production was recorded after 14 days.

Alkali production from malonate and tartrate. The media of Kerr and Panagopoulos (13) were used. Alkali production was recorded after 7 days.

Growth on selective media. Selective agar media of Schroth et al (20) and of New and Kerr (19) were used. Growth was recorded after 14 days.

Growth on media containing dulcitol, L-sorbose, ethanol, or sorbitol. The basal medium of Kersters et al (15), with slight modifications, was used. It contained 0.25 g $MgSO_4 \cdot 7H_2O$, 25 mg $CaCl_2 \cdot 2H_2O$, 1.2 g $NaH_2PO_4 \cdot 2H_2O$, 0.55 g KH_2PO_4, 0.25 g NaCl, 4 mg $FeSO_4 \cdot 7H_2O$, 0.16 mg $ZnSO_4 \cdot 7H_2O$, 0.08 mg $CuSO_4 \cdot 5H_2O$, 0.5 mg H_3BO_3, 0.4 mg $MnSO_4 \cdot H_2O$, 0.5 g $NH_4H_2PO_4$, 2.5 mg yeast extract (Difco), 16 mg bromocresol purple, 18 g agar (Biolab

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TABLE 1. Origin of the bacterial strains used in a numerical taxonomic study of biotypes and phenotypic groups of *Agrobacterium* in South Africa

Cluster	Group	Host and strains[a]	Locality and date
		Prunus persica	
1	1	A1SA, A5SA, A6SA, C5SA	South Africa, Franschhoek, 1978
		39ASA, 39BSA, 39CSA, 39FSA, 39GSA, 39HSA, 39ISA, 39KSA, 39MSA, 39NSA, 39OSA	South Africa, Vereeniging, 1978
		41ASA, 41BSA, 41CSA, 41DSA	South Africa, Krugersdorp, 1978
		27[b]	South Australia
		Prunus avium	
		Z6SA, Z8SA, Z12SA, Z13SA, 46SA, 47SA, 48SA, 49SA, 51SA, 53SA	South Africa, Ficksburg, 1978
		62SA	South Africa, Grabouw, 1979
		Vitis vinifera	
		Z36SA, Z38SA	South Africa, Douglas, 1979
		W2SA, W3SA	South Africa, Paarl, 1979
		Prunus salicina	
		72SA, 73SA, 74SA	South Africa, Grabouw, 1979
		KERR38[c]	South Australia
		Unknown	
		ICPB TR101[c]	Unknown
		Soil	
		84[b]	South Australia
		Prunus persica	
	2	D1SA, D2SA, D3SA, D4SA, D5SA, D6SA, D7SA, D8SA, D9SA, D10SA	South Africa, Ceres, 1978
		Prunus salicina	
	3	69SA, 70SA, 71SA, 75SA	South Africa, Grabouw, 1979
		Rosa spp.	
2	4	NCPPB 1650[c]	South Africa, 1960
		Rubus spp.	
	5	ICPB TR2[c]	U.S.A.
		Vitis vinifera	
	6	NCPPB 1771[c]	Iran, 1965
	7	2160SA, 2164SA	South Africa, Villiersdorp
		2221SA	South Africa, Upington
		Soil	
3	8	0362[c]	Australia
		Unknown	
	9	CIP RV3[c]	No information available
		Chrysanthemum spp.	
	10	K2SA	South Africa, Pretoria, 1978
		Prunus persica	
		24[b]	South Australia
		Dahlia spp.	
	11	F/1[c]	Israel, 1961
		NCPPB 925[c]	South Africa, 1959
		Chrysanthemum spp.	
	12	K21SA	South Africa, Pretoria, 1978
		Ditch water	
	13	M2/1[c]	Belgium, 1952
		Unknown	
	14	ICPB TT111[c]	U.S.A.
		ATCC 143[c]	Probably U.S.A.
	15	ICPB TT9[c]	Probably = B37 (see Kersters et al [15])
		Juglans spp.	
		T37[c]	U.S.A., California, 1926
		Chrysanthemum spp.	
		K22SA	South Africa, Pretoria, 1978
	16	K12SA, K14SA, K16SA, K17SA, K27SA, K28SA, K30SA, 45ASA, 45BSA, 45CSA, 45DSA	South Africa, Pretoria, 1978
		Soil	
		56SA, 57SA, 59SA	South Africa, Ficksburg, 1978
		Pyrus malus	
	17	CIP B6[c]	U.S.A., 1934
		Rubus spp.	
		ATCC 4452[c]	Probably U.S.A.

[a]ATCC = American Type Culture Collection; CIP = Collection de l'Institut Pasteur (Paris, France); ICPB = International Collection of Phytopathogenic Bacteria (Davis, California); NCPPB = National Collection of Plant Pathogenic Bacteria (Hertfordshire, England).
[b]Supplied by A. Kerr, Adelaide, South Australia.
[c]Supplied by K. Kersters, Gent, Belgium.

Laboratories, Pretoria 0001, South Africa) and 1 L distilled water. Dulcitol, L-sorbose, ethanol, and sorbitol were filter sterilized and added separately to the basal medium to yield final concentrations of 1%. Cultures were inoculated on agar plates and results were recorded after 7 and 14 days on the dulcitol, L-sorbose, and sorbitol media and after 7 days on the ethanol medium.

Anaerobic growth in the presence of nitrate. The medium of Kersters et al (15) containing 10 g glucose, 10 g yeast extract (Difco), 5 g KNO_3, 1 g $(NH_4)_2SO_4$, 0.25 g KH_2PO_4, 20 g agar (Biolab Laboratories), and 1 L of distilled water was used. Cultures were inoculated on agar plates. Anaerobiosis was maintained with disposable hydrogen plus carbon dioxide generator envelopes (Becton, Dickinson and Co., Cockeysville, MD 21030) in anaerobic jars. Growth was recorded after 4, 7, and 12 days.

Growth on Stonier's medium. Growth on agar plates containing Stonier's (23) medium, supplemented with 200 μg/L biotin, was determined after 2, 7, and 14 days.

Computer-assisted numerical analysis of phenotypic properties. All tests were recorded as positive or negative. Good, moderate, poor, and no growth were coded as +++, ++−, +−−, and −−−, respectively. The no comparison code was used where no growth occurred in cases for comparison of colonial pigmentation. The resemblance of each strain to every other strain was calculated by using the similarity coefficient (S_{sm}) of Sokal and Michener (21). Strains were clustered by the unweighted-pair group method. Redundant characters and pathogenicity were not used for calculating coefficients of association. All calculations were made on a Univac 1100 computer.

RESULTS

General features and pathogenicity. The strains of *Agrobacterium* that were studied are listed in Table 1. The 71 South African strains from 11 localities were from five different plant host species and one soil sample. Agrobacteria could be isolated successfully from the soil sample only on the selective medium for biotype-1. All strains were Gram-negative, oxidase positive, non-spore-forming rods, that produced no fluorescent pigments.

Pathogenicity of the strains on tomato seedlings is indicated in Fig. 1. Sixty-eight South African and 14 reference strains caused tumors on tomato stems. Strains not pathogenic on tomato seedlings were also nonpathogenic on broad-bean plants and on carrot disks.

Computerized numerical analysis of phenotypic characters. Forty-nine phenotypic characters were used for calculating coefficients of association (percent *S*-level) between the different strains. Only two characters were redundant: growth at 30 C and growth on medium containing sorbitol (Table 2).

The phenotypic similarities among 90 strains are shown in a dendrogram (Fig. 1). All strains grouped in one of three phenotypic clusters formed at the 65% *S*-level. At the 90% *S*-level, 17 different phenotypic groups were distinguished (Fig. 1). Cluster-1 comprised three groups, cluster-2 four groups, and cluster-3 ten groups.

The characteristics of agrobacteria that grouped in the three different phenotypic clusters and 17 different phenotypic groups are shown in Table 2. Cluster differentiation was based mainly on a combination of the different test reactions. The most discriminating tests were: colony morphology and pigmentation on glucose-yeast extract agar; 3-ketolactose reaction; acid production from media containing melezitose and erythritol; growth on the selective media of Schroth et al (20) and New and Kerr (19); and growth and color on media containing dulcitol and L-sorbose.

Cluster-1, which comprised 52 South African and four reference strains, was the largest (Fig. 1). Three groups were distinguished at the 90% *S*-level (groups-1, -2, and -3). The three groups were distinguished from each other by the following (Table 2): colony morphology; acid or alkaline reaction in litmus milk; growth at 30, 35, or 37 C; growth in 2% NaCl; alkali production from media containing malonate and tartrate; anaerobic growth in the presence of nitrate; and growth on Stonier's medium.

Cluster-2 was relatively small and heterogeneous, comprising three South African (2160SA, 2221SA, and 2164SA) and three reference strains (NCPPB 1650, ICPB TR2, and NCPPB 1771). Groups 4, 5, 6, and 7 were distinguished at the 90% *S*-level. The four groups differed from each other in the following (Table 2): colonies white, beige, smooth, rough, or umbilicate on glucose-yeast extract medium; growth at 30 or 35 C; growth in 2% NaCl; alkali production from malonate or tartrate; growth on media containing ethanol and sorbitol; anaerobic growth in the presence of nitrate; and growth on Stonier's medium.

Cluster-3 comprised 29 strains (17 South African and 12 reference strains). It consisted of 10 groups. At the 90% *S*-level, all seven genetic groups of De Ley (6) (B6, M2/1, TT111, TT9, F/1, 0362, and RV3), as well as three other groups (groups 10, 12, and 16), were distinguished from each other within cluster-3. Group-16 was the largest and consisted of 14 South African strains. The 10 different groups were distinguished from each other by the following (Table 2): 3-ketolactose production; colonies smooth, rough, or umbilicate on glucose-yeast extract medium; growth at 35 or 37 C; acid production from medium containing erythritol; growth and pigmentation on media containing dulcitol, L-sorbose, ethanol, and sorbitol; and anaerobic growth in the presence of nitrate.

DISCUSSION

Computer-assisted numerical analysis of 49 biochemical and physiological characters of 90 strains of *Agrobacterium* grouped the strains into two large and one smaller phenotypic cluster at the 65% similarity level (*S*-level). At 90% *S*-level, 17 different phenotypic groups were distinguished from each other.

Most of the 71 South African strains of *Agrobacterium* were grouped in phenotypic cluster-1. According to the test reactions of the four reference strains (KERR38, 84, ICPB TR101, and 27), group-1 in our cluster-1 corresponds to cluster-2 of Kersters et al (15), to the cluster of *A. rhizogenes* of Holmes and Roberts (11), and to biotype-2 strains of Kerr and Panagopoulos (13). Cluster-2 of Kersters et al (15) was found to be phenotypically and genetically homogeneous, consisting of only one genetic group (6). However, at the 90% *S*-level, three distinct phenotypic groups were recognized in our cluster-1 (Fig. 1), suggesting the existence of two additional genetic groups (groups 2 and 3, Fig. 1) of biotype-2 agrobacteria. These results were confirmed by numerical analysis of soluble protein patterns (*unpublished*).

Cluster-2 consisted of six strains (NCPPB 1650, ICPB TR2, NCPPB 1771, 2160SA, 2221SA, and 2164SA) which comprised four groups at 90% *S*-level. Group-7 consisted of three strains (2160SA, 2221SA, and 2164SA) from grapevines in South Africa. Reference strains of biotype-3 were not included in our investigation, but the diagnostic reactions of the group-7 strains (Table 2) agreed with the biotype-3 strains of Kerr and Panagopoulos (13). The cluster of Holmes and Roberts (11) of strains of *A. rubi* included the biotype-3 strains of Kerr and Panagopoulos (13) as well as strains NCPPB 1650 and NCPPB 1771, which clustered in our groups 4 and 6, respectively. Although strain ICPB TR2 was not included in any cluster of Holmes and Roberts (11), it showed highest similarity to the grouping with strains of *A. rubi.*

Our cluster-3 contained, with the exception of one reference strain (strain 0362), the 3-ketolactose producing strains. This cluster corresponds to cluster-1 of Kersters et al (15) and to biotype-1 strains of Keane et al (12). The seven genetic groups (B6, M2/1, TT111, TT9, F/1, 0362, and RV3) of cluster-1 of Kersters et al (15) were distinguished from each other in our cluster-3 at 90% *S*-level. Strain CIP RV3 was not distinguished from the B6 group by Kersters et al (15), but it formed genotypically a distinct group at 50% DNA homology with all other groups (6). In the present study, strain CIP RV3 was distinguished from the B6 strains (CIP B6 and ATCC 4452) by growth and pigmentation on media containing L-sorbose, ethanol, and sorbitol (Table 2). With the exception of one South African strain (strain K22SA), which clustered with the TT9 group, none of the other South African strains appeared to be phenotypically related to the seven genetic groups of De Ley (6). Fourteen of the South African cluster-3 strains fell into the separate group-16.

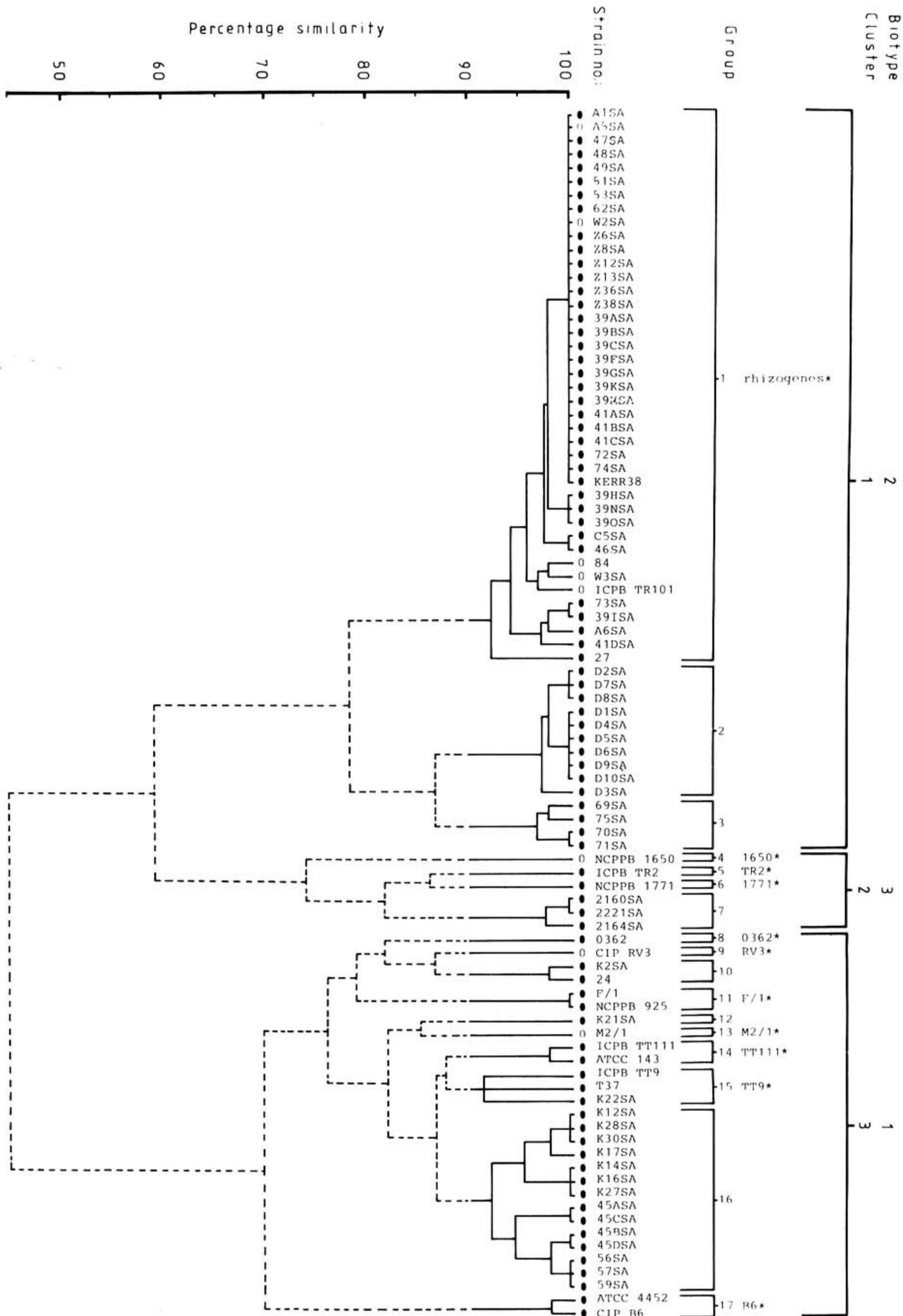

Fig. 1. Dendrogram of the S_{sm} similarity coefficients (×100), grouped by unweighted average linkage cluster analysis, showing the phenotypic similarities among 71 South African and 19 reference strains of *Agrobacterium*. Symbols: * = genetic groups identified by De Ley (6,7), ● = pathogenic (causes crown gall), 0 = nonpathogenic.

The results obtained in this study were paralleled by numerical analysis of polyacrylamide gel electrophoresis (PAGE) of soluble proteins (*unpublished*). However, PAGE showed that strains NCPPB 1650, NCPPB 1771, and ICPB TR2 were genetically not closely related to each other or to strains 2160SA, 2221SA, and 2164SA, although these six cluster-2 strains were phenotypically similar to each other. Furthermore, PAGE also indicated that strains 2160SA, 2221SA, and 2164SA were genetically dissimilar. Protein profiles of cluster-3 strains also revealed that all except one South African 3-ketolactose positive strain belong to the genetic group TT111 of De Ley (6). The South African 3-ketolactose positive strain (strain K2SA) clustered by PAGE in the TT9 group.

Our results confirm the earlier findings of Kersters et al (15) and White (25) that there are two major groups of strains of *Agrobacterium*; these are regarded as two different biotypes (12) and two different species (11). We also agree with the existence of a third cluster, considered as biotype-3 (13) or *A. rubi* (11).

Nonpathogenic strains of *Agrobacterium* were found in each of the three clusters (Fig. 1). They were indistinguishable from other members of the group or cluster, except for phytopathogenicity. Our results, therefore, agree with those of other reports (8–11,14,18) which were unable to distinguish strains of *A. tumefaciens* and *A. radiobacter* except on the basis of phytopathogenicity. The proposal of Holmes and Roberts (11) to adopt the species names *A. tumefaciens* and *A. rhizogenes* for the two major groups of agrobacteria, and to reject *A. radiobacter*, seems justified. We also support the use of the terms tumorigenic, rhizogenic, and saprophytic states when referring to agrobacteria. PAGE indicated that none of our phenotypic cluster-2 strains were genetically closely related to each other (*unpublished*); therefore, we prefer not to include strains NCPPB 1650 and NCPPB 1771, and biotype-3 strains such as 2160SA, 2221SA, and 2164SA in the

TABLE 2. Diagnostic reactions[a] of 90 strains of *Agrobacterium* discerned by computer analysis in three clusters at the 65% similarity level[b] and 17 groups at the 90% similarity level[b] (Fig. 1)

	Clusters[c] of groups 1–17																
	1			2				3									
Diagnostic character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Colonies[d] white,	+	+	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−
beige,	−	−	−	+	−	−	−	+	+	+	+	+	+	+	+	+	+
smooth,	+	+	+	−	+	+	+	+	+	+	+	+	−	+	+	−	+
rough,	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	+	−
opaque, or	±	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
umbilicate	±	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−
3-Ketolactose reaction	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Litmus milk reaction	Ac	Al	Al	Al	Al	Al	Al	Al	Al	Al	Al	Al	Al	Al	Al	Al	Al
Growth in 2% NaCl	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 30, 35, or 37 C	30	35	37	30	30	30	35	37	37	37	35	37	37	37	37	37	37
Acid from: erythritol	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
melezitose	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+
Alkali from: malonate	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−
tartrate	+	−	±	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Growth on selective media of:																	
New and Kerr (19)	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Schroth et al (20)	−−−	−−−	−−−	−−−	−−−	−−−	−−−	+++	−−−	+++	++−	+−−	+++	−−−	++− +++	++− +++	−−−
Growth and color on media containing:																	
Dulcitol: growth	+++	+++	+++	−−−	−−−	−−−	−−−	+++	+++	+++	+−−	+−−	++−	+++	+++	+++	+++
color	Gr	Gr	Gr	Nc	Nc	Nc	Nc	Gr	PY	PY	Gr	Gr	Gr	Bl	Bl	Bl	PY
L-sorbose: growth	++−	++−	++− +−−	−−−	−−−	−−−	−−−	+−−	+−−	+−−	+−−	+−−	+−−	+−−	+−−	+−−	+++
color	Gr	Gr	Gr	Nc	Nc	Nc	Nc	Gr	Gr	Gr	Gr	Gr	Gr	Gr	Gr	Gr	PY
Ethanol: growth	++−	++−	+−−	+−−	+−−	++−	−−−	++−	++−	++−	+++	+−−	+++	++−	++− +++	++− +−−	++− +++
color	Gr PY	Gr	Gr	Gr	Gr	Gr	Nc	PY	Gr	PY	Gr	Gr	Gr	PY	PY Gr	Gr PY	PY
Sorbitol: growth	++−	++−	++−	+−−	+−−	+−−	++−	+−−	++−	+++	+−−	+++	+++	+−− ++−	++− +++	+++	+++
color	Gr	Gr	Gr	Gr	Gr	PY	PY	Gr	Gr	Gr	Gr	Bl	Gr	Bl	Bl	Bl Gr	PY
Anaerobic growth in presence of nitrate	−−−	+−−	−−−	−−−	−−−	+−−	−−−	+++	+++	+++	+++	++−	++−	++−	++− +++	++−	+++
Growth on medium of Stonier (23)	++−	++−	+++	++−	+−−	++−	+−− −−−	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

[a]Symbols and abbreviations: + = positive, − = negative, +++ = good growth, ++− = moderate growth, +−− = poor growth, −−− = no growth, Nc = no comparison, Ac = acid, Al = alkaline, Bl = blue, Gr = greyish, and PY = pale yellow. Two sets of results for the same character indicate variable reactions among strains within the same group.
[b]Similarity levels determined by unweighted average linkage cluster analysis (see Fig. 1).
[c]Cluster-1 = biotype-2, cluster-2 = biotype-3, and cluster-3 = biotype-1.
[d]Cultured on glucose-yeast extract medium.

A. rubi species proposed by Holmes and Roberts (11).

The heterogeneity of South African agrobacteria, which fall into three biotypes, each consisting of different phenotypic groups, complicates the identification of isolates from tumors and from soil. Diagnostic characters determined in this study (Table 2) can, however, be useful. Strains 69SA, 75SA, 70SA, and 71SA in group-3 of cluster-I can be allocated to biotype-3 according to biotype diagnostic characters proposed by Kerr and Panagopoulos (13). Numerical analysis of 49 phenotypic characters, however, showed that these four strains were phenotypically more related to biotype-2 strains. The phenotypic variation among different members of the same cluster or biotype was so considerable that it is not surprising that traditional identification methods applied earlier (4,22,24) were inadequate to allocate some strains of *Agrobacterium* to any of the three known biotypes.

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