Agrobacterium属細菌の表現形質による類別

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Phenotypic Characteristics of the Genus *Agrobacterium**

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Abstract

One hundred and eight strains of *Agrobacterium* isolated from soil and from affected plants of 13 species, together with type and representative strains, were subjected to cluster analysis using 28 phenotypic characters out of the 36 examined. Data were analyzed by applying the unweighted average pair-group method (UPGMA) on the simple matching coefficient (S_{SM}). At 80% S_{SM}, 5 clusters were recognized, 3 of which corresponded to biovars 1, 2 and 3. These biovars were each grouped into a tight cluster, indicating that each of them is phenotypically homogeneous. Two additional clusters comprised (a) 3 overseas strains (IFO 13261 and 13260 of *A. rubi*, and NCPPB 1650 of *A. tumefaciens*), and (b) 6 Japanese strains of *A. tumefaciens* isolated from cherry and kiwifruit. The former cluster neighbored biovar 3 and the latter biovar 2, respectively. All strains in each biovar gave uniform reactions for the following 15 characters, which are therefore considered to be useful for the differentiation and the identification of the biovars: production of 3-ketolactose; esculin hydrolysis in Sneath's medium; arbutin hydrolysis; arginine dihydrolase in Thornley's medium; growth factor requirement; litmus milk reaction; pellicle in ferric ammonium citrate solution; growth at 35°C, and on New and Kerr medium; utilization of citrate, and L-tyrosine; oxidase by growth on PPGA; acid from dulcitol, and α-methyl-D-glucoside; and alkali from L-tartrate.

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Key words: *Agrobacterium*, phenotypic characteristics, cluster analysis, biovar, differential characters.

INTRODUCTION

In Bergey's Manual of Systematic Bacteriology (1984)[13], the genus *Agrobacterium* is divided into 4 species [*A. tumefaciens* (Smith and Townsend 1907) Conn 1942, *A. rhizogenes* (Riker, Banfield, Wright, Keitt and Sagen 1930) Conn 1942, *A. radiobacter* (Beijerinck and van Delden 1902) Conn 1942, and *A. rubi* (Hildebrand 1940) Starr and Weiss 1943] mainly on the basis of the phytopathogenicity and the types of symptoms induced on plants. In addition, there are 2-3 genetically and phenotypically different groups in each species except for *A. rubi*[7,9-11,15,23,24], which are assigned to biovars 1, 2 and 3[13].

This nomenclature at the species level does not, however, reflect the natural classification of *Agrobacterium*, because phytopathogenic behaviors used as taxonomic characters are not chromosome-encoded but plasmid-mediated[13]. Moreover, plasmids can be lost or transferred into an another strain[13], which leads to an instable taxonomy. A natural classification would establish species on the basis of genetic and phenotypic characteristics which are related to chromosomal DNA. With this perspective, several proposals have been made to elevate the biovars to the species level[7,15]. However, these proposals have not yet found general acceptance. Further detailed phenotypic, chemotaxonomic and genetic studies to clarify the taxonomic structure of the genus are needed, before the nomenclatural confusion in the genus can be resolved.

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In this paper, we analyzed in detail the phenotypic characteristics of strains of *Agrobacterium* derived from various hosts and localities, as a first step to clarify the taxonomic structure of the genus and to develop a rapid and accurate method for the differentiation and the identification of the taxa (biovars) within the genus.

Table 1. Strains of *Agrobacterium* used in the present experiment and assignment of them to taxa

Taxon[a)]	Host	Strain[b)]	Locality (Supplier)[c)]
Biovar 1			
A. tumefaciens	Chrysanthemum	MAFF 03-01276, 01278	Shizuoka, Japan (MAFF)
		CH3, 5	Shizuoka, Japan (T. Makino)
	Marguerite	MAFF 03-01222	Shizuoka, Japan (MAFF)
		At-M-19, 22	Shizuoka, Japan (M. Togawa)
	Rose	MAFF 03-01224	Osaka, Japan (MAFF)
	Cherry	MAFF 03-01001	Saitama, Japan (MAFF)
	Unknown	NCPPB 2437** (ATCC 23308)[d)]	Iowa, USA (NCPPB)
A. rhizogenes	Melon	MAFF 03-01724, 01725, 01726, 01727	Chiba, Japan (MAFF)
		ARM-3, melon-1	Shizuoka, Japan (Y. Takikawa)
		MR4, 40	Shizuoka, Japan (T. Makino)
A. radiobacter	Soil	IAM 1526 (ATCC 4718)	USA (IAM)
		Ct-Ag-1	Mie, Japan
	Unknown	IAM 12048** (ATCC 19358, NCPPB 3001)	Netherlands (IAM)
		IAM 1527 (ATCC 6466)	Unknown (IAM)
Biovar 2			
A. tumefaciens	Rose	MAFF 03-01546	Shizuoka, Japan (MAFF)
		R65, 80, 173, 202, 294	Shizuoka, Japan (T. Makino)
		AtR11	Shizuoka, Japan (K. Ohta)
		Ro-Ag-10, 11, 12, 13	Yamagata, Japan
	Cherry	Ch-Ag-2, 3	Yamagata, Japan
		Ch-Ag-6, 9, 10, 11, 12, 13, 14	Okayama, Japan
	Pear	P-Ag-1, 2, 3, 4, 5	Mie, Japan
		P-Ag-6	Nagasaki, Japan
	Peach	Peach CG 8331	Yamagata, Japan (Y. Takikawa)
		Pch-Ag-2, 3, 4, 5, 6	Okayama, Japan
	Plum	P1-Ag-1, 2	Okayama, Japan
	Almond	NCPPB 2303*	Israel (NCPPB)
A. rhizogenes	Apple	IFO 13257** (ATCC 11325, NCPPB 2991)	USA (IFO)
A. radiobacter	Soil	Kerr 84* (NCPPB 2407)	Australia (T. Makino)
Biovar 3			
A. tumefaciens	Grape	NCPPB 2562*	Greece (NCPPB)
		NCPPB 1771	Iran (NCPPB)
		YGAt 32-3, 33-1, 35-2	Yamanashi, Japan (Y. Terai)
		G-Ag-4, 9, 14	Shimane, Japan
		G-Ag-19, 21, 23, 26, 27, 29, 31, 33, 35, 37, 39, 43, 45, 46, 48, 50	Nagano, Japan
		G-Ag-52, 54, 56, 57, 58, 59	Iwate, Japan
		G-Ag-60,61	Aomori, Japan
		G-Ag-62, 63, 64, 65	Yamagata, Japan
		G-Ag-66, 67	Akita, Japan
	Kiwifruit	K-Ag-1, 2	Hiroshima, Japan

Table 1. (Continued)

Taxon[a)]	Host	Strain[b)]	Locality (Supplier)[c)]
A. rubi	*Rubus* sp.	IFO 13261** (ATCC 13335, NCPPB 1854)	USA (IFO)
		IFO 13260 (ATCC 13334, NCPPB 1856)	USA (IFO)
Unclassified			
A. tumefaciens	*Rosa* sp.	NCPPB 1650	South Africa (NCPPB)
	Kiwifruit	K-Ag-3, 4	Hiroshima, Japan
	Cherry	Ch-Ag-4, 5, 7, 8	Okayama, Japan

a) For assignment of strains to taxa, see Discussion and Fig.1.
b) *: representative strain for the corresponding biovar[13)]; **: type strain.
c) Unless a supplier is stated, the strain was isolated in our laboratory.
d) The numbers in parentheses are other strain designations.
Abbreviations for culture collections: ATCC, American Type Culture Collection; IAM, Institute of Applied Microbiology, University of Tokyo; IFO, Institute for Fermentation; MAFF, Ministry of Agriculture, Forestry and Fisheries, Japan; NCPPB, National Collection of Plant Pathogenic Bacteria.

MATERIALS AND METHODS

Bacteria. Strains used in the present experiment (Table 1) comprised 68 strains isolated in our laboratory, 28 strains isolated in other laboratories in Japan, and 12 strains isolated overseas. These strains were derived from 13 host species, expressing gall or hairy-root symptoms, and from soil. Type strains and representative strains for each biovar[13)] were included (Table 1). In this paper, we follow the classical nomenclature of *Agrobacterium* presented in Bergey's Manual of Systematic Bacteriology (1984)[13)] for the moment to avoid confusion, although another valid naming system was proposed[15)].

Media. The cultures for the studies were grown on potato-peptone-glucose agar (PPGA)[18)] slants at 28°C for 24 hr. Nutrient broth was prepared as previously described[18)]. Proteose Peptone No. 3 (Difco) was employed in all appropriate media.

Identification. The identity of all strains as *Agrobacterium* spp. was confirmed using the determinative tests for the genus[13)], *viz.* aerobic requirement, Gram reaction, morphology, flagella insertion, oxidative metabolism of glucose, production of catalase, extracellular polysaccharide slime on sucrose-containing media, gas from glucose and fluorescent pigment on King's B medium. These tests were conducted as described earlier[18)].

Pathogenicity. Pathogenicity of strains used was tested by prick inoculation[18)] into stems of tomato (*Lycopersicum esculentum*), sunflower (*Helianthus annuus*), grapevine (*Vitis vinifera*) and *Kalanchoe daigremontiana*. Strains were considered pathogenic if they produced a tumorigenic or rhizogenic reaction in any one plant species.

Phenotypic characteristics. Thirty-six characters (Table 2) were examined for the characterization of the taxa (biovars) within the genus. Arbutin hydrolysis was performed according to the method of Crosse and Garrett[2)]. Production of arginine dihydrolase was examined in the media[1)] of Moeller and of Thornley. The method of Moore *et al.*[14)] was employed to test oxidase production by using 16-hour-old cultures grown on both PPGA[18)] and nutrient agar slants as inocula. The other tests (Table 2) were conducted as described earlier[18)].

Cluster analysis. Cluster analysis was used here to confirm groupings of agrobacteria and to assist in the selection of useful differential characters. Of the 36 characters examined, 6 were rejected from the analysis, because all the strains tested gave the same reaction in them (Table 2): acid from raffinose; production of urease, oxidase (nutrient agar), β-galactosidase (ONPG), and arginine dihydrolase (Moeller); and esculin hydrolysis (Dye)[6)]. The data for arbutin hydrolysis and the Nile Blue test[21)] were also excluded from the analysis, since the former is biologically correlated with esculin hydrolysis and the latter was found to be unreliable for the taxonomic characters. One hundred and eight strains, confirmed as *Agrobacterium* spp., were subjected to cluster analysis using the remaining 28

characters.

The data were coded as follows: + (positive), $+^{W}$ (weakly positive), (+) (delayed positive) and K (alkali production in the litmus milk reaction) were coded as 1; − (negative), ± (false positive), NG (no growth) and A (acid production in the litmus milk reaction) were coded as 0. The similarities were

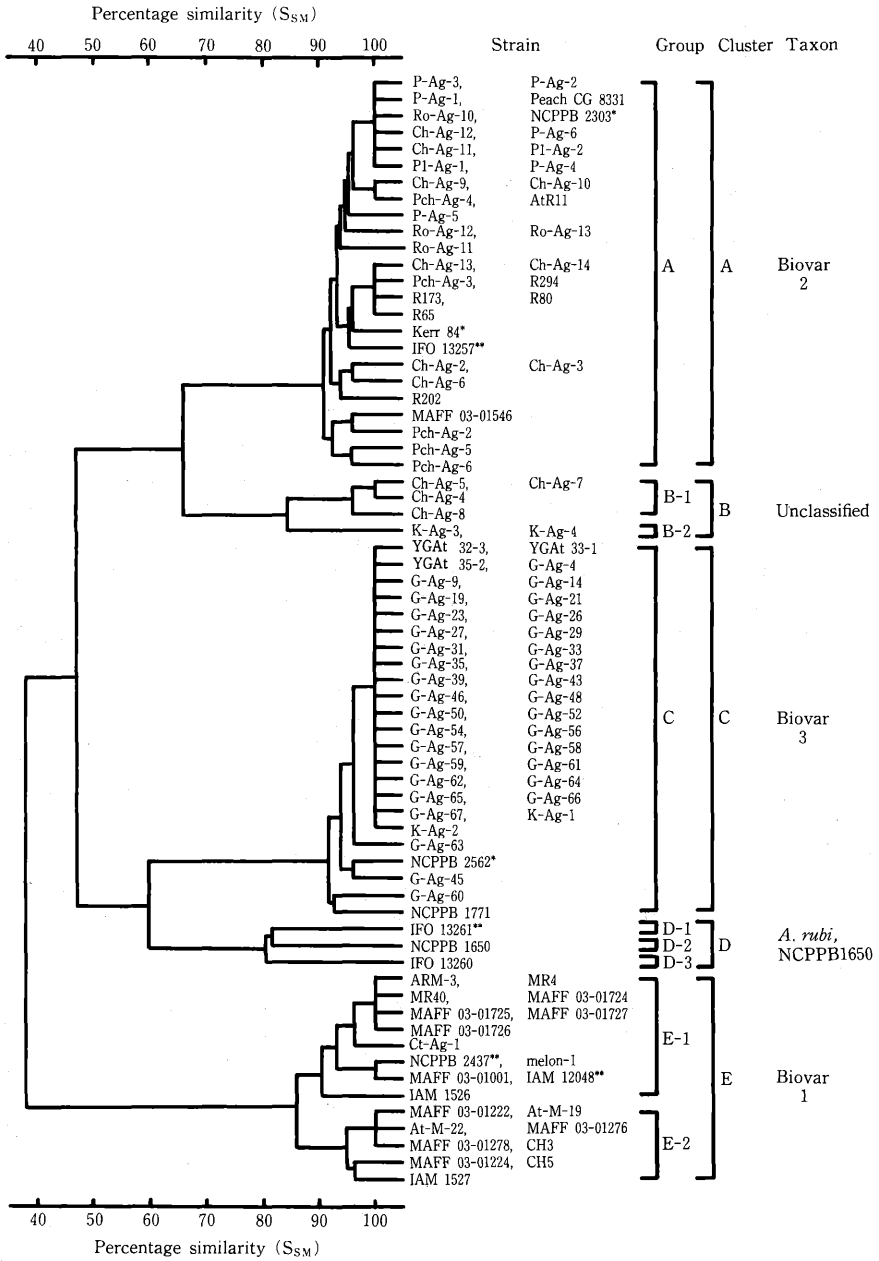

Fig. 1. Dendrogram of the simple matching coefficient (S_{SM}), clustered by the unweighted average pair-group method (UPGMA), showing the phenotypic similarities among 108 strains of *Agrobacterium*, including representative strains (*) for the corresponding biovar[13] and type strains (**). Five clusters and nine groups were recognized at 80 and 90% S_{SM}, respectively. For assignment of clusters to taxa, see Discussion.

Table 2. Phenotypic characteristics of *Agrobacterium* biovars (clusters) discerned by the cluster analysis

Characteristics	Cluster[a]						Incubation period (day)
	E	A	C	D-1,3	D-2	B	
	Taxon[b]						
	Biovar			*A. rubi*	Unclassified		
	1 (22)[d]	2 (37)	3 (40)	(2)	NCPPB 1650	Others[c] (6)	
Production of 3-ketolactose	21, 1[w e]	0	0	0	0	0	2
Esculin hydrolysis:							
in Dye's medium[6]	22	37	40	2	1	6	7
in Sneath's medium[22]	22	37	0	1, (1)	1	6	7
Arbutin hydrolysis	22	36	0	2	1	2, 4[w]	10
β-Galactosidase (ONPG)	22	37	40	2	1	6	0.7
Nitrate reduction	13	1[w]	0	0	0	2	3
Nitrate respiration	13	0	0	0	0	2	7
Arginine dihydrolase:							
in Thornley's medium[1]	22	0	38	0	0	0	28
in Moeller's medium[1]	0	0	0	0	0	0	28
Urease	22	37	40	2	1	6	0.7
H_2S formation (Pb acetate paper)	22	0	0	1	0	0	14
Growth factor requirement	0	37	40	2	1	6	14
Litmus milk reaction:							
alkali production	22	0	40	2	1	6	28
acid production	0	37	0	0	0	0	28
Phosphatase	19	31	0	2	0	6	3
Nile Blue test[21]	12, 1[w]	8	2	0	0	1	21
Pellicle in ferric ammonium citrate solution	20, 2[w]	0	0	0	0	0	21
Growth:							
in 2% NaCl	22	0	39	1	0	2	7
at 30°C	22	15	39	1	1	6	7
at 35°C	22	0	0	0	0	6	7
on Schroth *et al.* medium[14]	21	5	0	0	0	2, 4[w]	14
on New and Kerr medium[14]	0	36	0	0	0	6	14
Utilization of:							
citrate	20	37	40	0	0	6	14
L-tyrosine	0	37	0	0	0	6	14
Oxidase by growth on:							
nutrient agar	22	37	40	2	1	6	0.7
PPGA[18]	22	0	40	2	1	6	0.7
Acid from:							
dulcitol	22	37	0	0	0	6	14
melezitose	22	(1)	0	2[w]	0	2, (4)	14
meso-erythritol	2, 1[w], (5)	37	0	1	0	6	14
α-methyl-D-glucoside	22	37	0	2[w]	1	6	14
raffinose	22	37	40	2[w]	1	6	14
ethanol	22	1, (3)	40	0	0	0	14
Alkali from:							
malonate	0	37	40	2	0	2	14
propionate	22	(3)	(37)	0	0	(5)	14
L-tartrate	0	37	39	0	0	0	14
mucic acid	7, (1)	37	0	1	1	6	14

a) See Fig. 1.
b) For assignment of clusters to taxa, see Discussion, Table 1 and Fig. 1.
c) Others: strains K-Ag-3, 4 and Ch-Ag-4, 5, 7, 8 (see Table 1 and Discussion).
d) Number of strains used belonging to that biovar (cluster) (see Table 1 and Fig. 1).
e) Number of strains which gave positive reactions.
w: weak reaction; ( ): delayed positive reaction.

calculated by using the simple matching coefficient (S_{SM}), which includes positive and negative matches. The strains were clustered by the unweighted average pair-group method (UPGMA), and the levels of association were used to construct a dendrogram.

RESULTS

Identification and pathogenicity

All the strains tested were aerobic, Gram negative, motile rods with peritrichous flagella. They metabolized glucose oxidatively ; produced catalase and abundant extracellular polysaccharide slime on glucose- or sucrose-containing media, but no gas nor pigment on any of the media used.

Five strains of *A. radiobacter* were not pathogenic on any plants used. The other 103 strains caused tumors or hairy roots on tomato, grapevine, sunflower or *Kalanchoe daigremontiana*.

Cluster analysis

The dendrogram (Fig. 1) of 108 strains tested was structured on 28 nutritional, physiological and biochemical characters. At 80% S_{SM}, they were grouped into 5 distinct clusters (Clusters A-E). At 90% S_{SM}, 2 groups were recognized within each of Clusters B and E, and 3 groups, comprising a single strain each, were distinguished within Cluster D.

Characterization of clusters

The number of strains within each cluster, which gave positive reactions in each test, is listed in Table 2. Fifteen characters shown in Table 3 were found to be useful to characterize the clusters (biovars).

Cluster A (Fig.1) consisted of 34 Japanese strains, and 3 reference strains of biovar 2 (NCPPB 2303, Kerr 84, and IFO 13257 ; the type strain of *A. rhizogenes*). *A. tumefaciens*, *A. rhizogenes* and *A. radiobacter* were found in this cluster. These were indistinguishable from one another except for their pathogenic differences.

Cluster B (Fig. 1) contained 6 Japanese strains of *A. tumefaciens*. Two groups (Groups B-1 and B-2) were distinguished at 90% S_{SM}. Group B-1 comprised 4 strains isolated from cherry, and Group B-2 consisted of 2 strains from kiwifruit. These groups differed from each other in tests for nitrate

Table 3. Selected characteristics for the differentiation of *Agrobacterium* biovars

Characteristics	Biovar			*A. rubi*	Unclassified	
	1	2	3		NCPPB 1650	Others[a)]
Production of 3-ketolactose	+[b)]	−	−	−	−	−
Esculin hydrolysis in Sneath's medium[22)]	+	+	−	+	+	+
Arbutin hydrolysis	+	+*	−	+	+	+
Arginine dihydrolase in Thornley's medium[1)]	+	−	+**	−	−	−
Growth factor requirement	−	+	+	+	+	+
Litmus milk reaction	KR	AC	KR	K	K	K
Pellicle in ferric ammonium citrate solution	+	−	−	−	−	−
Growth at 35°C	+	−	−	−	−	+
Growth on New and Kerr medium[14)]	−	+*	−	−	−	+
Utilization of citrate	+**	+	+	−	−	+
Utilization of L-tyrosine	−	+	−	−	−	+
Oxidase by growth on PPGA[18)]	+	−	+	+	+	+
Acid from dulcitol	+	+	−	−	−	+
Acid from α-methyl-D-glucoside	+	+	−	+	+	+
Alkali from L-tartrate	−	+	+*	−	−	−

a) Others: strains K-Ag-3, 4 and Ch-Ag-4, 5, 7, 8 (see Tables 1 and 2).

b) +: positive; −: negative; K: alkali production; A: acid production; R: reduction of litmus; C: clot formation; *: one exception obtained in the present experiment; **: two exceptions.

reduction, nitrate respiration, growth in 2% NaCl and alkali from malonate (Table 2).

Cluster C (Fig. 1) contained 38 Japanese and 2 overseas strains (NCPPB 2562 and 1771) of *A. tumefaciens*. Strain NCPPB 2562 is a representative strain of *A. tumefaciens* biovar 3[13].

Cluster D (Fig. 1) consisted of 3 diverse strains; IFO 13260 and 13261 of *A. rubi*, and NCPPB 1650 of *A. tumefaciens* isolated from *Rosa* sp.

Cluster E (Fig. 1) contained 18 Japanese strains, and 4 overseas strains including 2 reference strains of biovar 1 (NCPPB 2437; the type strain of *A. tumefaciens*, and IAM 12048; the type strain of *A. radiobacter*). *A. tumefaciens, A. rhizogenes* and *A. radiobacter* were present in this cluster. Groups E-1 and E-2 were separated by results obtained in nitrate reduction and nitrate respiration (Table 2).

DISCUSSION

One hundred and eight strains from a variety of host species and localities (Table 1) were confirmed as members of the genus *Agrobacterium*. Their status at the species level[13] was also confirmed according to type of symptoms induced (Table 1).

The following 7 characters were chosen for the characterization of the taxa (biovars) within the genus, because they were reported to differentiate the biovars in various taxonomic studies: β-galactosidase (ONPG)[7]; nitrate reduction[7,9,16]; nitrate respiration[4,5,11,17]; urease[7]; utilization of citrate[7,9,11,14,23]; acid from raffinose[7,16]; and arginine dihydrolase (Moeller)[7]. Esculin hydrolysis in Dye's medium[6] and oxidase production by using cultures on nutrient agar slants were examined to compare with the other method for testing the same character, respectively. The remaining 27 characters of the 36 listed in Table 2 were also chosen, because differences had been observed for these characters among the biovars in our previous report[18].

The Nile Blue test, which was considered to differentiate *Agrobacterium* from *Rhizobium*[21], appeared to have no differential value between them, because many agrobacteria which were reported to reduce the dye[21] gave negative reactions (Table 2). Moreover, variable results were obtained within each biovar (Table 2), suggesting that it is not adequate for the characterization of the biovars, either.

Cluster analysis of 28 characters selected from the 36 examined grouped strains into 5 clusters at 80% S_{SM} (Fig. 1). These clusters corresponded to the biovars[13] well as mentioned below. On the other hand, no correlation existed between these clusters and 4 species distinguished by phytopathogenicity (Table 1). Namely, strains of *A. tumefaciens*, *A. rhizogenes* and *A. radiobacter* occurred in both Clusters A and E, and strains of *A. rubi* and *A. tumefaciens* were found in Cluster D. These results support earlier claims that species classifications and nomenclature based on plasmid-mediated phytopathogenicity are inadequate[7,13,15,17]. In the following, the relationship between the clusters established here (Fig. 1) and the biovars[13] is examined.

Cluster E (22 strains) included 2 reference strains of biovar 1 (NCPPB 2437; the type strain of *A. tumefaciens*, and IAM 12048; the type strain of *A. radiobacter*)[13], and the test reactions of the Cluster-E strains (Table 2) agreed with those of biovar 1 described in Bergey's Manual[13]. Therefore, Cluster E corresponds to biovar 1.

Cluster A (37 strains) included 3 reference strains of biovar 2 (NCPPB 2303, Kerr 84, and IFO 13257; the type strain of *A. rhizogenes*)[13] and the test reactions (Table 2) coincided with those of biovar 2 recorded in Bergey's Manual[13]. Thus, Cluster A corresponds to biovar 2.

The taxonomic status of biovars 1 and 2 has been thoroughly studied by means of phenotypic characteristics[7,9–11,17,24], serology[9], electrophoregrams of soluble proteins[12], thermal stability of DNA/rRNA hybrids[3,8] and DNA/DNA hybridizations[3,13]. The results obtained by these methods corroborate each other, and indicate that each of biovars 1 and 2 is a distinct taxon. In our experiments also, each of them was found to be phenotypically (Fig. 1) and chemotaxonomically[20] homogeneous. These data support the proposal to treat biovars 1 and 2 as separate species[7].

Cluster C (40 strains) contained a representative strain of *A. tumefaciens* biovar 3 (NCPPB 2562)[13] and produced reactions (Table 2) that agreed with those of biovar 3[13,18], which showed that Cluster C conforms to biovar 3. We also assigned strain NCPPB 1771, hitherto treated as an aberrant strain[11,13],

to biovar 3, since it was involved in Cluster C (Fig. 1), coincided with biovar 3 phenotypically, and also gave similar responses to those of biovar 3 with respect to serology and fatty acid methyl ester profiles in our experiments[19,20].

Although phenotypical studies of biovar 3 had been conducted[5,7,10,16,17,23], characterization was not considered adequate, because insufficient numbers of strains and characters were examined[13]. Afterwards, the discrimination of biovar 3 from biovars 1 and 2 has been confirmed by DNA homology[15], serology[15,19] and phenotypic characteristics[18], supporting the observation of the homogeneous cluster obtained in the present experiment (Fig. 1), and Ophel and Kerr[15] proposed *A. vitis* for biovar 3 isolated from grapevine. However, it is necessary to determine the boundary of biovar 3 definitely, by comparing with *A. rubi* and strain NCPPB 1650 mentioned below.

Cluster D (3 strains) comprised 2 strains of *A. rubi*, including the type strain, and strain NCPPB 1650 treated as an aberrant strain[11,13]. These strains were phenotypically diverse and neighbored Cluster C (biovar 3). Although the relationships among strain NCPPB 1650, *A. rubi* and biovar 3 have been investigated by phenotypic characteristics[7,11,17], fatty acid methyl ester profiles[20] and DNA homology[15], different groupings were obtained among investigators. A large collection of strains of *A. rubi* is necessary for detailed chemotaxonomic and genetic work to clarify the affinity among them and the relationship with biovar 3 (*A. vitis*).

Six Japanese strains (K-Ag-3, 4 and Ch-Ag-4, 5, 7 and 8) of *A. tumefaciens* were phenotypically unique, and formed Cluster B (Fig. 1), suggesting that they should be treated as unclassified for the moment. Thorough comparison with Cluster A (biovar 2), which neighbored Cluster B, is needed to determine their position. We are in the process of further chemotaxonomic[20] and genetic studies of the genus *Agrobacterium* to refine its classification and to clarify anomalies in it.

Fifteen characters (Table 3) gave uniform results within each biovar and clear differences among the biovars. By using these differential characters, strains of *Agrobacterium* can be efficiently separated into three biovars and *A. rubi*. And these 15 characters may form a significant part of minimal standards for the genus *Agrobacterium*.

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和 文 摘 要

澤田宏之・家城洋之：*Agrobacterium* 属細菌の表現形質による類別

土壌および13種類の植物から分離された108菌株（外国産の12菌株を含む）の *Agrobacterium* 属細菌について36項目の表現形質を調査した。このうちの28項目のデータを利用し，単純一致係数と群平均法によるクラスター分析を行ったところ，供試菌は80％の類似度において五つの cluster に類別された。biovar 1, 2 および 3 に相当する cluster はいずれも高い類似度で一つにまとまっており，表現形質に関して均質な分類群であることがわかった。*A. rubi* (IFO 13261 および IFO 13260) と NCPPB 1650 の計3菌株，ならびにキウイフルーツとサクラから分離した *A. tumefaciens* の計6菌株は，それぞれ一つの cluster として独立した。前者は biovar 3, 後者は biovar 2 と最も近いものの，類似度は比較的低かった。3-ケトラクトースの生成，エスクリンの分解 (Sneath)，アルブチンの分解，アルギニンジヒドロラーゼ活性(Thornley)，生長素要求性，リトマスミルク培養，クエン酸鉄アンモニウム培地における薄膜の形成，35°C および New and Kerr 培地での生育，クエン酸および L-チロシンの利用，オキシダーゼ活性(PPGA)，ズルシットおよび α-メチル-D-グルコシドからの酸の産生，および L-酒石酸からのアルカリの産生の15項目では，biovar 間に明瞭な違いが認められることから，biovar の識別性状として有効である。