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Characterization of Plasmid-Borne and Chromosome-Encoded Traits of *Agrobacterium* Biovar 1, 2, and 3 Strains from France

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We collected 111 *Agrobacterium* isolates from galls of various origins (most of them from France) and analyzed both their plasmid-borne and chromosome-encoded traits. Phenotypic analysis of these strains allowed their classification in three phena which exactly matched the delineation of biovars 1, 2, and 3. A fourth phenon was identified which comprises three atypical strains. The phenotypic analysis has also allowed us to identify 12 additional characteristics which could be used to identify the three biovars of *Agrobacterium*. Our results also suggest that biovar 1 and 2 represent distinct species. Analysis of plasmid-borne traits confirmed that tartrate utilization is a common feature of biovar 3 strains (now named *Agrobacterium vitis*) and of *Agrobacterium* grapevine strains in general. Among pathogenic strains of *Agrobacterium*, several exhibited unusual opine synthesis and degradation patterns, and one strain of biovar 3 induced tumors containing vitopine and a novel opine-like molecule derived from putrescine. We have named this compound ridéopine.

Agrobacterium sp. is a pathogenic bacterium responsible for two plant diseases: crown gall and hairy root. As these names suggest, the visible symptoms at the infection site are the appearance of tumorous overgrowths and roots for crown gall and hairy root, respectively. Both diseases are examples of natural interkingdom genetic exchange, because the infectious process relies on the transfer of a DNA fragment(s) from the prokaryote *Agrobacterium* to the eukaryotic plant cells. This transferred DNA, or T-DNA, is borne on extrachromosomal bacterial replicons. These replicons are the Ti (tumor-inducing) plasmid found in bacteria responsible for crown gall disease, and the Ri (root-inducing) plasmid found in bacteria responsible for hairy root disease. Once transferred to the plant, the T-DNA integrates into the nuclear genome of the cell, where T-DNA genes are transcribed. The molecular mechanism underlying the transfer of DNA has been extensively reviewed (e.g., see references 11, 27 and 41).

Genes located on the T-DNA fall into two groups. The first one includes genes responsible for tumor or root formation (for reviews, see references 4 and 18). The second group of T-DNA genes encode enzymes catalyzing the synthesis of the low-molecular-weight compounds specific for the crown gall or hairy root cells. These compounds, termed opines, generally result from the condensation of amino acids and alpha-ketoacids, or aminoacids and sugars; they play a key role in the ecology of the plant-*Agrobacterium* interaction (for reviews, see references 12 and 13). The combination of opines, the synthesis and the degradation of which are due to genes borne on Ti and Ri plasmids, provides the basis for a simple classification of the pathogenic plasmids of *Agrobacterium* (4, 13). However, data collected from the analysis of Ti plasmids isolated from grapevine isolates strongly suggest that these plasmids are mosaic plasmids, with conserved and variable regions (30, 31, 52).

It appears that the type of disease induced by *Agrobacterium* depends on the type of plasmid hosted by the bacteria. In this respect, the former delineation of *Agrobacterium* species based on the disease symptoms, hence on traits due to plasmid-borne genes, is of little value (for a review, see reference 51). A stronger classification of *Agrobacterium* species has been performed using numerical taxonomy of phenotypic properties (22, 54), analysis of fatty acid methyl ester profiles (20, 44), or comparison of electrophoregrams of soluble proteins (23). These results indicate clearly that the genus *Agrobacterium* can be divided into three different clusters which correspond to biovars 1, 2, and 3, as termed by Keane et al. (21). Biovar 3 is now regarded as the *Agrobacterium* species *A. vitis*, which includes strains isolated from grapes (29). Similarly, biovars 1 and 2 could define different species of *Agrobacterium*. Further studies will be crucial to confirm or refute this hypothesis. Such studies may lead to a deep reorganization of the *Rhizobium*-*Agrobacterium* clusters within the family *Rhizobiaceae*, since some *Agrobacterium* strains have more characteristics in common with *Rhizobium* than with *Agrobacterium* (51).

Among commonly infected plants, grapevine is of major commercial importance. In France, grapevine galls have been reported in cold parts of the Rhone Valley, but also in the Bordeaux and Loire Valley regions (39). The spread has resulted from a combination of cold climatic conditions and the poor sanitary status of the cultivated material (3, 7, 8, 17, 25, 26, 28, 33, 40, 46, 48, 50, 55, 56; for a review, see reference 14). A better characterization of the *Agrobacterium* strains would facilitate their routine identification and subsequent control of plant sanitary conditions. To this end, we have collected 61 isolates from grapevine galls and analyzed their traits due to both plasmid-borne and chromosome-encoded genes with respect to other *Agrobacterium* strains, including reference strains. The results of this study are reported below.

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MATERIALS AND METHODS

Bacterial strains. Out of 111 *Agrobacterium* strains used in this study, 88 were isolated in France between 1976 and 1989, and 23 were of various origins and deposited in the French Collection of Phytopathogenic Bacteria (CFBP). Two clinical isolates were obtained from the Pasteur Institute (Paris, France) (Table 1). *Agrobacterium* isolates were grown on LPGA medium (38) which consisted of yeast extract (Difco Laboratories, Detroit, Mich.), 5 g/liter; Bacto Peptone (Difco), 5 g/liter; glucose, 10 g/liter; and 15 g/liter (pH adjusted to between 7 and 7.2).

Biochemical characters for presumptive diagnosis of *Agrobacterium*. Gram strain response was determined using the aminopeptidase test from Merck (Darmstadt, Germany). The following conventional biochemical characteristics were assessed according to the method of Popoff et al. (36): presence of esculin-β-glucosidase, urease (in urea-indol medium; Diagnostics Pasteur, Marne-la-Coquette, France), orthonitro-phenyl-β-D-galactopyranoside (ONPG) β-galactosidase, gelatinase, Tween 80 esterase, DNase on DNA agar (Diagnostics Pasteur). 3-Ketolactose production (according to Bernaerts and De Ley [2]) and phenylalanine desaminase (PAD) activity were also assayed. PAD detection was carried out on phenylalanine agar, which was made of DL-phenylalanine, 2 g/liter; yeast extract (Difco), 3 g/liter; NaCl, 5 g/liter; K_2HPO_4, 1 g/liter; and agar, 12 g/liter. *Agrobacterium* strains were streaked on this medium to a high density and kept at 26 to 27°C. After 40 to 48 h, the culture was covered with a few drops of $FeCl_3$ (density, 1.26) diluted 1/3 (vol/vol) with distilled water. A positive assay is indicated by an olive-green coloration appearing rapidly and remaining stable for 1 to several hours. Characteristics presumptive for *Agrobacterium* species were confirmed for all assayed strains using the identification system for *Pseudomonas* and related bacteria (Diagnostics Pasteur). This system also gave data on nitrate and arginine metabolism.

Nutritive characteristics. Utilization (with acid formation) of melizitose, dulcitol, erythritol, and ethanol and utilization (with alkali formation) of L-(+)-tartrate and malonate were assayed. These compounds were added at 1% (vol/vol or wt/vol) to the minimal medium, which consisted of $NH_4H_2PO_4$, 1 g/liter; KCl, 2 g/liter; $MgSO_4 \cdot 7H_2O$ 0.2 g/liter; yeast extract (Difco), 0.1 g/liter; and bromothymol blue, 0.08 g/liter (pH 7.2) (1). Five milliliters of this medium inoculated with *Agrobacterium* strains using 48-h precultures performed on LPGA medium (38), and incubated in a shaker (120 rpm) at 27°C. Growth and acid production were generally stopped after 72 h of incubation but for some strains were stopped after 5 days of incubation.

The assimilation of 49 carbohydrates, 49 organic acids, and 49 amino acids was studied using API 50 CH, LRA 50 AO, and LRA 50 AA strip tests (BioMérieux, La Balme Les Grottes, France). The inoculated strips were maintained at 26°C, and growth was assessed after 5 days.

Digital-numerical taxonomy. A total of 167 characteristics (based on 20 biochemical and physiological tests plus assimilation of carbon sources) were included in the digital-numerical taxonomy analysis. A distance matrix was calculated using the Jaccard coefficient (47). Cluster analysis was done by using the unweighted pair group method of average with arithmetic mean (47).

Pathogenicity assays. Three plant species were used: *Kalanchoe tubiflora*, *Datura stramonium*, and *Lycopersicon esculentum* (var. Montfavet 63/5). These were kept in a growth chamber at a day temperature of 23°C and a night temperature of 18°C, with a 16-h light, 8-h dark photoperiod and a relative humidity of 80 to 85%. Suspensions of the bacteria to be assayed were made in sterile water and adjusted to ca. 10^8 CFU/ml. Of these suspensions, 50-μl aliquots were used to inoculate the plants wounded at the second, fifth, and sixth internodes starting from the apex (*K. tubiflora*) or at the second and fourth internodes (*D. stramonium* and *L. esculentum*) at the stage when four leaves had expanded. The reactivity to inoculation was estimated after 40 days to differentiate the various types of reaction, particularly on *K. tubiflora*. Appearance of tumorous outgrowths was assessed by visual inspection of the inoculated plants.

Opine detection in the tumors and opine utilization by the bacteria. Detection of opines in tumorous tissues and their utilization by the inducing bacteria were performed by using high-voltage paper electrophoresis, as reviewed by Dessaux et al. (12).

RESULTS AND DISCUSSION

Analysis of phenotypic, chromosome-encoded characteristics. (i) Identification of *Agrobacterium* strains. All assayed strains ($n = 111$) exhibited ONPG-hydrolase (β-galactosidase) and urease activities, and were able to degrade esculin. This confirmed that these strains belonged to the genus *Agrobacterium* (24). Additionally, the assayed strains were not able to degrade gelatin or to reduce tetrathionate. It is noteworthy that a negative response for the DNase and Tween esterase assays cannot be used as an orientation test for identifying *Agrobacterium* strains because, out of 111 assayed strains, 12 exhibited DNase activity while 6 produced a Tween esterase.

(ii) Numerical taxonomy. The dendrogram displaying the distance relationships amongst the 111 strains included in this study is shown in Fig. 1. At a phenotypic distance of 0.3, three major and one minor phena were delineated. The major phena 1, 2, and 3 precisely group strains of the three biovars, 1, 2, and 3, respectively. Phenon 4 included three strains, CFBP 2724, 2725, and 2771. Although these strains clustered with biovar 2 strains at a distance of 0.354, they must be regarded as atypical since they exhibit many characteristics which are not common to those of biovar 2 strains (Table 2). Whether the three above-mentioned strains are related to those described by Bouzar et al. (6) remains to determined. At a shorter distance (0.254), phenon 3 divided into two subphena (3a and 3b) which comprised, respectively, 36 and 9 strains, leaving 2 isolated strains (CFBP 2617 and CFBP 2678). At the same distance (0.254), phenon 2 divided into two subphena (2a and 2b) which comprised, respectively, 31 and 2 strains. Strains isolated from grapevines clustered as follows: 10 strains in phenon 1 (which includes 28 strains), 1 strain in phenon 2 (which includes 33 strains), 47 strains in phenon 3 (which includes 47 strains), and 3 strains in phenon 4 (which includes 3 strains). Overall, and except for the three strains CFBP 2724, 2725, and 2771, biovar determination yields clear-cut results. The perfect correspondence between phena 1 and 2 and biovars 1 and 2, respectively, strongly suggests that biovars 1 and 2 could correspond to two distinct species. Bouzar (5) and Sawada et al. (45) previously made this proposal.

(iii) Differential characteristics. The characteristics that differentiate the three phena and the three strains of phenon 4 are shown in Table 2. Ten assays (3-ketolactose production, presence of oxidase, presence of PAD, and utilization of dulcitol, melezitose, L-rhamnose, malonate, propionate, citrate, and L-ornithine) have been used previously by Kersters and De Ley (24) to differentiate among biovars of *Agrobacterium*. As shown in Table 2, 12 additional characteristics could be used to identify the biovars and the three isolated strains. Interestingly, our results confirm the validity of using the 3-ketolactose criterion to identify biovar 1 strains, since all these strains produced this lactose derivative (Table 2).

The 47 grapevine strains (clustered in phenon 3), strains CFBP 2724 and 2725 (biovar undetermined, phenon 4), and the two clinical isolates CFBP 2243 and 2884 (biovar 1, from human origin) produce a PAD. This result is in agreement with those of Popof et al. (36), who previously reported on clinical isolates harboring PAD activity. Though it is not an absolute criterion, production of PAD therefore might be a useful orientation assay to identify grapevine strains belonging to the species *A. vitis* (biovar 3).

Arginine dihydrolase (assayed using the Pasteur gallery of tests) was detected only in biovar 1 strains and in the atypical strains CFBP 2724 and 2771. This characteristic therefore allows the differentiation of biovar 1 strains from strains of the biovars 2 and 3. However, while no arginine dihydrolase was found in strains of biovars 2 and 3, some of them assimilated arginine. These were biovar 2 strains CFBP 1936, 2178, 2688, and 1931 and biovar 3 strains CFBP 2736, 2737, and 2620 (from Australia); CFBP 2621 and 2738 (from Greece); and CFBP 2513 and 2515 from Spain. This feature can be related to the existence of different pathways for assimilation of arginine in this bacterium and to the presence on some *Agrobacterium* plasmids of genes responsible for arginine degradation (15; for a review, see reference 13).

Among the three biovars, reduction of nitrates is a variable character. Only 10 out of 28 biovar 1 strains reduced nitrate to nitrogen. One biovar 2 strain and 12 of the 47 biovar 3 strains reduced nitrate to nitrite.

TABLE 1. Origin of strains; results of pathogenicity tests on various plants; and opine production and utilization of biovar 1, 2, and 3 strains

Biovar	Host plant or source	Yr	Laboratory no.	CFBP strain	Geographical origin[d]	Person who isolated	Pathogenicity on[a]:			Opine[c]	
							T	D	K[b]	Production	Utilization
1	*Vitis vinifera*										
	Danam	1982	143	2407	F34	Ridé	−	NT[e]	+++RE	C, O	C, O
	Cabernet sauvignon 41B	1987	279	2683	F33	Ridé	−	−	−E	NT	NT
	Pinot noir 41B	1987	311	2732	F51	Petit	−	−	−E	C, O	N, C, O
	Merlot 3309	1987	277	2682	F33	Ridé	−	−	−E	C	N, C, O
	Grenache		360-1	2514	S (Navarra)	Lopez	−	−	−E	NT	NT
	Sultana		LBA 649	2883	GR	Hoekema	NT	NT	+	NT	NT
	Sultana		Ag20	1904	GR	Panagopoulos	+	+	+++Re	N	N
	Cabernet sauvignon	1985	224	2642	F33	Ridé	−	−	++rE	C, O	C, O
	Cabernet sauvignon R140	1987	276	2655	F33	Ridé	−	−	++RE	C, O	C, O
	Cabernet sauvignon R140	1987	275	2654	F33	Ridé	+	−	++RE	C, O	C, O
	Prunoideae										
	Prunus × GF677	1987	302	2716	F84	Ridé	+	NT	+++ST	NT	M
	Prunus × GF677	1987	299	2713	F84	Ridé	+	+	+++rS	MA	M
	P. cerasus		C58	1903	USA	Dickey	NT	NT	+++rs	N	NT
	Prunus × GF677	1987	300	2714	F84	Ridé	+	+	+++rS	MA	M
	P. rubiera	1988	317	2741	F13	Ridé	−	NT	−	NT	N, O
	Prunus × GF677	1987	303	2717	F84	Ridé	+	NT	+++rs	NT	M
	Prunus × GF677	1987	298	2712	F84	Ridé	+	−	+++rS	MA	M
	P. rubiera	1988	318	2879	F13	Ridé	+	NT	NT	NT	N
	Pomoideae										
	Malus pumila	1977	96	1933	F02	Lopez	−	−	+++rS	N	NT
	Malus sp.		B6	2413	USA	Braun	NT	NT	+++R	O	NT
	Pyrus syriaca		U85	2747	SYR	Abu-Ghorrah	+	+	++R	NT	NT
	Populus										
	P. tremula × *P. alba* 712-1-856	1988	341	2795	F87	Ridé	+	+	+++Rs	N	NT
	P. tremula	1982	146	2177	F45	Ridé	+	NT	+++rs	N	N
	Populus × (*Leuce*)	1985	10	2517	F45	Michel	+	+	++r	N	NT
	P. alba	1988	347	2885	F87	Ridé	+	+	+	N	NT
	Chrysanthemum	1988	330	2788	F72	Ridé	+	+	+++s	N	NT
	From hospital										
	Vagina		A65-97 (H8)	2243	USA		NT	NT	NT	NT	NT
	Blood		Ag032 (H5)	2884	F	Pasteur Institute	NT	NT	NT	NT	NT
2	*Vitis vinifera* (unknown)		Ag28	1905	GR	Panagopoulos	+	+	+E	N	N
	Prunoideae										
	P. persica	1976	76	1804	F24	Lopez	+	NT	+++rS	N	NT
	P. mahaleb	1977	94	1962	F24	Lopez	+	NT	+++Rs	N	NT
	P. avium F12-1	1982	139	2178	F45	Ridé	+	NT	+++rS	N	N
	P. avium F12-1	1982	145	2719	F45	Ridé	+	+	+++Rs	N	NT
	P. avium F12-1	1982	144	2718	F45	Ridé	+	NT	+++rs	N	NT
	P. avium × *P. cerasus* Colt	1982	149	2326	F34	Audusseau	+	NT	NT	N	NT
	P. avium × *P. cerasus* Colt	1984	207	2417	F30	Audusseau	+	NT	NT	N	NT
	P. avium × *P. cerasus* Colt	1984	210	2420	F30	Audusseau	+	NT	+++Re	N	NT
	P. silvestris	1987	264	2691	F30	Ridé	+	NT	+++rS	NT	NT
	Prunus sp.	1987	265	2692	F30	Ridé	+	NT	+++rS	MA	NT
	Prunus sp.	1987	266	2693	F30	Ridé	+	NT	−E	MA	NT
	Prunus sp.	1987	260	2687	F84	Ridé	+	NT	+++ST	N	NT
	Prunus sp.	1987	261	2688	F84	Ridé	+	NT	+++rST	N	NT
	Prunus sp.	1987	262	2689	F84	Ridé	+	NT	+++rS	N	NT
	P. mariana	1988	356	2942	F47	Ridé	+	NT	+	NT	NT
	P. persica	1988	316	2740	F13	Ridé	+	NT	+E	NT	N
	Prunus × GF677	1988	328	2744	F84	Nesme	+	+	++rS	N	NT
	Pomoideae										
	Malus M9	1977	M9	1931	F24	Lopez	−	−	−E	NT	NT
	M. pumila	1987	310	2728	F49	Ridé	−	−	−E	−	−
	M. pumila	1987	313	2729	F84	Ridé	−	−	−	−	−
	M. pumila	1988	358	2944	F49	Ridé	−	NT	−	NT	NT
	Pyrus communis	1988	359	2945	F49	Ridé	−	NT	−	NT	NT
	Malus M9	1988	327	2880	F49	Ridé	−	−	−E	NT	NT
	Populus										
	P. bolleana	1976	74	1961	F78	Ridé	+	+	+++	N	NT
	P. alba	1976	75	1840	F78	Ridé	+	+	++RSE	N	N
	P. tremula × *P. alba* 709-27	1988	338	2881	F87	Ridé	+	−	++R	N	NT
	P. tremula × *P. alba* 712-8	1988	336	2792	F87	Ridé	+	+	+++R	N	NT

Continued on following page

TABLE 1—*Continued*

Biovar	Host plant or source	Yr	Laboratory no.	CFBP strain	Geographical origin[d]	Person who isolated	Pathogenicity on[a]:			Opine[c]	
							T	D	K[b]	Production	Utilization
	Others										
	Rosa sp.	1979	115	1935	TAH	Ridé	+	+	+++rSe	N	N
	Rosa sp.	1979	116	1936	TAH	Ridé	+	NT	+++rS	N	NT
	Actinidia	1988	319	2742	F64	Ridé	−	NT	−	NT	−
	Soil		K84	1937	AUS	Kerr	−	−	−E	NT	N
3	*Vitis vinifera*										
	Danam	1982	140	2179	F34	Ridé	+	+	+++RST	N	N
	Unknown	1982	339-6	2513	S (Orense)	Lopez	+	+	++rS	C, O	N, C, O
	Ribol	1984	230	2607	F84	Ridé	+	−	+++RE	C, O	C, O
	Cabernet franc	1985	221	2641	F33	Ridé	+	−	+++Re	C, O	N, O
	Cabernet franc	1985	222	2608	F33	Ridé	+	−	+++Rs	C, O	N, C, O
	Cabernet franc	1985	223	2609	F33	Ridé	+	NT	++rST	N	N
	Cabernet sauvignon	1985	228	2610	F33	Ridé	+	NT	+++S	N	N
	Cabernet sauvignon	1985	229	2643	F33	Ridé	+	+	++rSE	N	N
	Cabernet franc	1985	226	2674	F33	Ridé	+	NT	++Se	N	N
	Cabernet franc	1985	225	2673	F33	Ridé	+	+	++rS	N	N
	Unknown	1985	550-2	2515	S (Portevedro)	Lopez	+	+	++rse	C, O	C, O
	Unknown	1985	565-5	2512	S (Bajoz)	Lopez	+	+	++S	N	N
	Unknown		K305	2736	AUS	Kerr	+	−	+++rs	C, O	NT
	Unknown		K308	2737	AUS	Kerr	+	−	+++rs	C, O	NT
	Unknown		K374	2620	AUS	Kerr	+	NT	+++RST	N	N
	Sultana	1963	63-85	2622	GR (Crete)	Panagopoulos	+	−	+++RE	C, O	C, O
	Sultana		Ag82-81	2738	GR	Panagopoulos	+	NT	+++	C, O	C, O
	Ugni blanc	1986	258	2650	F17	Ridé	+	−	++RE	C, O	C, O
	Cabernet franc	1986	243	2644	F49	Ridé	+	−	+++rE	C, O	C, O
	Chenin	1986	254	2617	F49	Ridé	+	−	+++Re	C, O	C, O
	Cabernet franc	1986	242	2675	F49	Ridé	+	−	++RE	C, O	C, O
	Cabernet franc	1986	245	2676	F49	Ridé	+	+	++RE	C, O	C, O
	Chenin	1986	247	2645	F49	Ridé	+	−	+++RSE	C, O	N, C, O
	Chenin	1986	249	2615	F49	Ridé	+	+	++++rS	C, O	N, C, O
	Chenin	1986	250	2616	F49	Ridé	+	−	+++Rse	C, O	C, O
	Chenin	1986	251	2646	F49	Ridé	+	−	+++RS	C, O	N, C, O
	Chenin	1986	253	2648	F49	Ridé	+	−	+++rse	C, O	N, C, O
	Cabernet sauvignon	1986	252	2647	F49	Ridé	+	NT	++RSe	C, O	N, C, O
	Cabernet sauvignon	1986	255	2618	F49	Ridé	+	+	+++rS	C, O	N, C, O
	Cabernet franc (1 yr)	1986	246	2613	F49	Ridé	+	−	++RE	C, O	C, O
	Cabernet franc (1 yr)	1986	248	2615	F49	Ridé	+	+	+++RE	C, O	C, O
	Chenin	1987	259	2651	F44	Ridé	+	−	++RE	C, O	C, O
	Melon	1986	256	2649	F44	Ridé	+	NT	+++RS	C, O	C, O
	Melon	1987	257	2677	F44	Ridé	+	−	++Re	C, O	C, O
	Cabernet sauvignon	1987	273	2653	F33	Ridé	+	−	+++RE	C, O	C, O
	Cabernet sauvignon	1987	280	2656	F33	Ridé	+	NT	+++RE	C, O	C, O
	Grenache	1987	270	2679	F49	Ridé	+	NT	+++RS	C, O	N, C, O
	Grenache	1987	268	2652	F49	Ridé	+	NT	+++rsE	C, O	C, O
	Grenache	1987	269	2678	F49	Ridé	+	NT	+++RS	C, O	C, O
	Cabernet franc	1987	284	2657	F37	Ridé	+	−	+++RS	C, O	N, C, O
	Cabernet sauvignon	1987	285	2668	F37	Ridé	+	NT	+++RS	C, O	N, C, O
	Grenache	1987	272	2680	F49	Ridé	+	−	+++RSe	C, O	C, O
	Sultana	1970	57-81	2621	GR (Crete)	Panagopoulos	+	+	++RE	C, O	C, O
	Unknown		A260 (S4)	2660	H	Szegedi	+	+	++rs	V	NT
	Navanesizu		A258 (NI-1)	2659	H	Szegedi	+	+	+++ST	N	N
	Pinot meunier	1988	332	2770	F02	Ridé	+	+	++rs	C, O	NT
	Cabernet sauvignon	1987	274	2681	F33	Ridé	+	+	+++RE	V, R	NT
	Uncertain										
	Cabernet franc	1987	306	2724	F37	Ridé	−	−	−	C, O	C, O
	Cabernet franc	1987	307	2725	F37	Ridé	−	−	−	N	C, O
	Pinot noir	1988	331	2771	F51	Ridé	−	−	+	N	NT

[a] Pathogenicity tests were performed with on tomato (T), datura (D), and kalanchoe (K) plants. Reactions: +, positive; −, negative.
[b] Reactions observed on the tumors: R, r: roots (many, some); S, s: shoots (many, some); T: teratogenic tumor; E, e: embryo-like organs (many, some).
[c] Opine names are indicated as follows: C, cucumopine; O, octopine; N, nopaline; MA, mannopine; V, vitopine; R, ridéopine.
[d] Abbreviations for French departments are indicated by an F followed by the number of the department: 02 (Aisne), 13 (Bouches du Rhône), 17 (Charente-Maritime), 24 (Dordogne), 30 (Gard), 33 (Gironde), 34 (Hérault), 37 (Indre-et-Loire), 44 (Loire-Atlantique), 45 (Loiret), 47 (Lot-et-Garonne), 49 (Maine-et-Loire), 51 (Marne), 64 (Pyrénées-Atlantiques), 72 (Sarthe), 78 (Yvelines), 84 (Vaucluse), or 87 (Haute-Vienne). Other abbreviations: AUS, Australia; S, Spain; GR, Greece; SYR, Syria; USA, United States; TAH, Tahiti.
[e] NT, not tested.

Analysis of traits due to plasmid-borne genes. (i) Utilization of L-(+)-tartrate. Utilization of L-(+)-tartrate yielded positive results for all biovar 2 and 3 strains and only for the biovar 1 strains isolated from grapevine tumors. Though it has been demonstrated that utilization of L-(+)-tartrate is characteristic of many plant-pathogenic bacteria, Szegedi (49) suggested that the degradation of this compound by *A. vitis* (biovar 3) strains might be due to their adaptation to grapevines. Indeed, tartaric

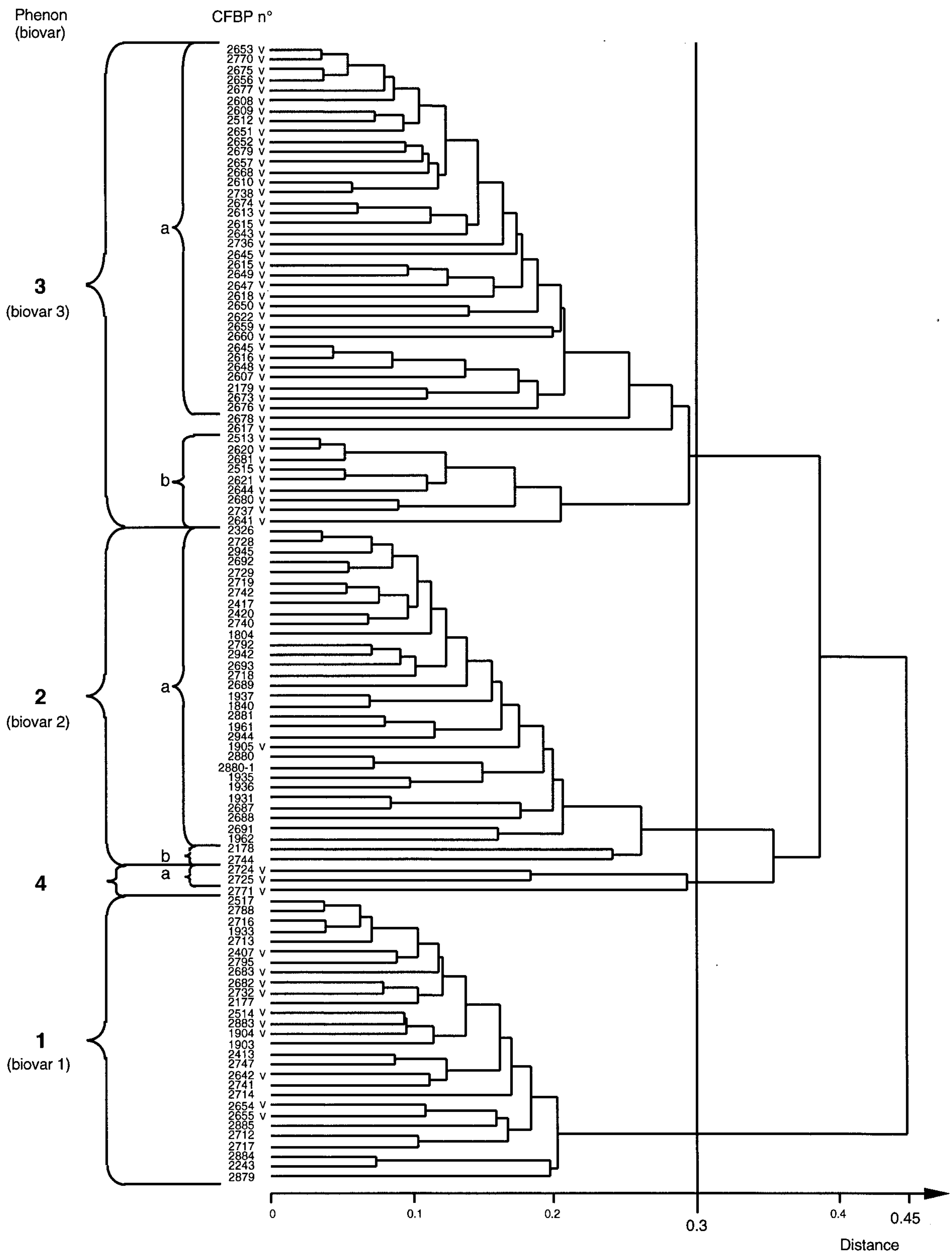

FIG. 1. Phenotypic analysis of 111 *Agrobacterium* strains. Results are presented as a dendrogram based on phenotypic distance value calculation using the Jaccard coefficient and the unweighted pair group method of average with arithmetic mean method. V, from grapevine.

TABLE 2. Phenotypic characteristics that differentiate biovars and subphena and strains[a]

Characteristic	Biovar (phenon)					Strain		
	1	2		3		2771	2724	2725
		a	b	a	b			
Dulcitol	+	+	+	−	−	+	+	+
β-Methyl-D-xyloside	+	+	−	−	−	+	−	−
PAD	−	−	−	+	+	−	+	+
Erythritol	−	+	+	−	−	+	+	+
Oxydase	+	−	−	+	+	+	+	−
Malonate	−	+	+	+	+	−	−	−
L-Ornithine	+	−	−	−	−	−	−	−
Sarcosine	+	−	−	−	−	−	−	−
Arginine dihydrolase	+	−	−	−	−	+	+	−
3-Ketolactose	+	−	−	−	−	−	−	−
Melezitose	+	−	−	−	−	+	+	+
D-Fucose	+	−	−	−	−	+	+	+
Proprionate	+	−	−	−	−	−	−	−
L-Arabitol	+	−	−	−	−	−	−	−
Citrate	−	+	+	+	+	−	−	−
Xylitol	+	+	+	−	−	−	+	+
D-Arabinose	+	+	+	−	−	+	+	−
Aconitate	+	d	d	+	−	−	−	−
α-Methyl-glucoside	+	−	+	+	−	+	−	−
L-Rhamnose	+	−	+	−	+	−	−	−
L-Arginine	+	−	+	+	−	+	−	−
D-Tagatose	+	−	+	+	−	+	−	−

[a] +, 90 to 100% of the strains are positive; −, 0 to 10% of the strains are positive; d, 11 to 89% of the strains are positive.

acid is a major chemical component of grapevines (37, 42). Our results are consistent with Szegedi's hypothesis.

Among strains of *A. vitis*, two independent pathways for tartrate metabolism exist. In the model *A. vitis* strain AB3, the enzymes defining a first pathway are encoded by genes located on pTrAB3 at the TARI region while enzymes defining a second pathway are encoded by genes located on pTiAB3 at the TARII region (10, 32, 43). Because tartrate utilization in biovar 1 strains is restricted solely to the strains isolated from grapevines, it is tempting to speculate that utilization of L-(+)-tartrate by these strains is due to the in planta transfer of a plasmid bearing the genes encoding utilization of L-(+)-tartrate, possibly from biovar 3 to biovar 1 strains. Moreover, biovar 3 and 1 strains were indeed isolated from the same grapevine plant.

(ii) Pathogenicity assays. The results of the pathogenicity assays are summarized in Table 1. Only ca. 60% of the biovar 1 strains induced tumors upon inoculation of tomato plants and daturas. Among the strains which were nonpathogenic on tomato plants, seven were isolated from grapevines. Out of these seven strains, three induced overgrowths on daturas, suggesting a possible host range limitation. On the other hand, biovar 1 strains isolated from other host plants (*Prunoideae*, *Pomoideae*, *Populus* sp. and *Chrysanthemum* sp.) were pathogenic on most if not all test plants, with the exception of strain CFBP 2741 isolated from *Prunus rubiera* tumors.

Interestingly, biovar 2 strains CFBP 1931, 2728, 2729, 2944, 2945, and 2880 isolated from rootstocks of apple trees and *Pyrus communis* and strain CFBP 2742 isolated from kiwi plants did not induce tumors on the test plants. The results obtained with the *Agrobacterium* strains isolated from apple rootstock are reminiscent of those reported by Picard (35). All the other biovar 2 strains, isolated from *Prunoideae*, *Populus* sp., and *Rosa* sp., induced tumor formation on tomato plants daturas or kalanchoes. On kalanchoes, ca. 70% of biovar 2 strains induced large tumors.

Biovar 3 strains always induced tumors on both tomato plants and kalanchoes, but most of them did not induce tumors on daturas. On kalanchoes, ca. 65% of the strains incited large tumors.

In addition to variation affecting the size of tumors, we also observed a wide range of tumor morphologies upon inoculations of kalanchoes. To take into account all these results, we utilized the following traits (Table 1): presence of roots at the lower part of the tumors, presence of shoots at the upper part of the tumors, teratogenic organization defined as tumors covered with fasciated shoots and hypertropic roots, and presence of embryolike organs defined as plantlets growing on leaf edges of the inoculated plants. Six strains incited only tumors: two from each biovar 1 and 2, one from biovar 3, and one from the unidentified biovar. The presence of embryolike organs only (assessed with respect to the uninoculated control plants) was observed on plants inoculated with four biovar 1 grapevine strains, six biovar 2 strains (including one grapevine strain), strains isolated from *Prunoidae* and *Pomoidae*, and strain K84 (though this strain is nonpathogenic). The different response patterns described above (also see Table 1) may be attributed to particular phytohormone balances, sensitivity of the transformed gall cells, or production of limited amounts of phytohormones by the bacterium itself (for reviews, see references 9, 18, and 19).

(iii) Production and utilization of opines. Opines synthesized in the tumors and opines degraded by *Agrobacterium* strains were analyzed, and results are summarized in Table 1. Four opine groups can be defined from the analysis of tumors induced by biovar 1 strains: octopine, nopaline, mannopine-agropine, and cucumopine-octopine. However, two opine degradation patterns were unusual. Firstly, some cucumopine-octopine grapevine strains degraded both opines, while others degraded cucumopine, octopine, and nopaline. Two of these strains (CFBP 2732 and 2682) remained nonpathogenic on the three test plants. Though not formally demonstrated, their opine degradation capability suggests that they do, however, harbor a Ti plasmid. The second unusual degradation pattern was detected in strains that induced mannopine-agropine-type tumors (CFBP 2712, 2713, and 2714): these degraded only mannopine. If this result is not artifactual, it could be attributed either to a mutation, as reported for mannopinic acid utilization in strain 89.10 (16), or to plasmid dissociation (34) or cointegration (53).

Two pathogenic biovar 2 strains (CFBP 2692 and CFBP 2693) most probably harbor a mannopine-agropine-type Ti plasmid. The other strains, representing over 90% of the pathogenic biovar 2 isolates, harbored a nopaline-type Ti plasmid. Interestingly, the nonpathogenic biovar 2 *Malus* strains were unable to degrade any assayed opines, suggesting that they do not harbor a Ti plasmid or that they possess a Ti plasmid of an unknown type (35).

The cucumopine-octopine type accounted for ca. 75% of the biovar 3 strains, the remaining strains being either nopaline type (ca. 20%) or vitopine type (ca. 5%) *Agrobacterium* strains. Among the cucumopine-octopine type strains, some degraded both cucumopine and octopine only while others degraded these two opines plus nopaline, as reported above for the biovar 1 grapevine strains. One strain (CFBP 2641) is of particular interest since it induced tumors synthesizing octopine and cucumopine but degraded cucumopine and nopaline.

An interesting outcome of this study is the identification of a new opine-like molecule in the tumors induced by strain CFBP 2681. Aside from containing vitopine, tumors induced by this strain contained a ninhydrin-positive compound which was specifically degraded by strain CFBP 2681. Examination of the electrophoretic mobilities of this compound and its reaction with ninhydrin (presence of a free NH_2 group) indicated that this molecule could result from the condensation of alpha-ketoglutarate and putrescine. Further experiments demonstrated the validity of this hypothesis (Chilton et al., unpublished data). This compound was termed ridéopine and may define a new class of opines (polyamine derivatives).

Though further studies involving DNA-DNA hybridization will be necessary to precisely organize the taxonomy of biovar 1 and 2, the survey of a large collection of original strains belonging to several biovars has proved very useful. It has enabled us to isolate strains of unidentified biovars, to propose new phenotypic properties that can be used to define biovar-discriminating markers, and to identify a novel opine-like molecule.

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REFERENCES

1. **Ayers, S. H., P. Rupp, and W. T. Johnson.** 1919. A study of alcali-forming bacteria in milk. Bulletin no. 782. U.S. Department of Agriculture, Washington, D.C.
2. **Bernaerts, M. J., and J. De Ley.** 1963. A biochemical test for crown-gall bacteria. Nature (London) **197:**406–407.
3. **Bien, E.** 1988. Isolierung und Charakterisierung von *Agrobacterium tumefaciens* Biovar 3, dem Erreger der Mauke an Reben. Ph.D. thesis. University of Kaiserslautern, Kaiserslautern, Federal Republic of Germany.
4. **Binns, A. N., and P. Costantino.** 1998. The *Agrobacterium* oncogenes, p. 251–266. *In* H. P. Spaink, A. Kondorosi, and P. Hooykaas (ed.), The *Rhizobiaceae*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
5. **Bouzar, H.** 1994. Request for a judicial opinion concerning the type species of *Agrobacterium*. Int. J. Syst. Bacteriol. **44:**373–374.
6. **Bouzar, H., W. S. Chilton, X. Nesme, Y. Dessaux, V. Vaudequin, A. Petit, J. B. Jones, and N. C. Hodge.** 1995. A new *Agrobacterium* strain isolated from aerial tumors on *Ficus benjamina* L. Appl. Environ. Microbiol. **61:**65–73.
7. **Burr, T. J., and B. H. Katz.** 1983. Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and saf and from vineyard soil. Phytopathology **73:**163–165.
8. **Burr, T. S. A., A. L. Bishop, L. M. Katz, and C. Bazzi.** 1987. A root specific decay of grapevine caused by *Agrobacterium tumefaciens* and *Agrobacterium radiobacter* biovar 3. Phytopathology **77:**1424–1427.
9. **Cervera, M., M. M. Lopez, L. Navarro, and L. Penal.** 1998. Virulence and supervirulence of *Agrobacterium tumefaciens* in woody fruit plants. Phys. Mol. Plant Pathol. **52:**67–78.
10. **Crouzet, P., and L. Otten.** 1995. Sequence and nutritional analysis of a tartrate utilization operon from *Agrobacterium vitis*. J. Bacteriol. **177:**6518–6526.
11. **de la Cruz, F., and E. Lanka.** 1998. Function of the Ti-plasmid Vir proteins. T-complex formation and transfer to plant cell, p. 281–301. *In* H. P. Spaink, A. Kondorosi, and P. Hooykaas (ed.), The *Rhizobiaceae*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
12. **Dessaux, Y., A. Petit, and J. Tempé.** 1992. Opines in *Agrobacterium* biology, p. 109–136. *In* D. P. S. Verona (ed.), Molecular signals in plant-microbe communications. C.R.C. Press, Inc. Boca Raton, Fla.
13. **Dessaux, Y., A. Petit, S. K. Farrand, and P. M. Murphy.** 1998. Opine and opine-like molecules involved in plant-*Rhizobiaceaea* interactions, p. 173–197. *In* H. P. Spaink, A. Kondorosi, and P. Hooykaas (ed.), The *Rhizobiaceae*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
14. **El-Fiki, F., and K. L. Giles.** 1981. *Agrobacterium tumefaciens* in agriculture and research, p. 47–58. *In* K. L. Giles and A. G. Atherly (ed.), Biology of the *Rhizobiaceae*. International Review of Cytology, supplement 13, Academic Press, New York, N.Y.
15. **Ellis, G., A. Kerr, J. Tempé, and A. Petit.** 1979. Arginine catabolism: a new function of both octopine and Nopaline Ti plasmids of *Agrobacterium*. Mol. Gen. Genet. **173:**263–269.
16. **Farrand, S. K., J. Tempé, and Y. Dessaux.** 1990. Localization and characterization of the region encoding catabolism of manopinic acid from the octopine-type Ti plasmid pTi15955. Mol. Plant-Microbe Interact. **3:**259–267.
17. **Ferreira, J. H. S., and F. G. H. Vanzyl.** 1986. Susceptibility of grapevine root-stocks to strains of *Agrobacterium tumefaciens* biovar 3. S. Afr. J. Evol. Vitic. **7:**101–104.
18. **Gaudin, V., T. Vrain, and L. Jouanin.** 1994. Bacterial genes modifying hormone balances in plants. Plant Physiol. Biochem. **32:**11–29.
19. **Gresshoff, P. M., M. L. Skotnicki, and B. G. Rofle.** 1970. Crown gall teratoma formation is plasmid and plant controlled. J. Bacteriol. **137:**1020–1021.
20. **Jarvis, B. W. D., S. Sivakumaran, S. W. Tighe, and M. Gillis.** 1996. Identification of *Agrobacterium* and *Rhizobium* species based on cellular fatty acid composition. Plant Soil **184:**143–153.
21. **Keane, P. J., A. Kerr, and P. B. New.** 1970. Crown gall of stone fruits. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. **23:**585–595.
22. **Kersters, K., J. Deley, P. H. A. Sneath, and M. Sackin.** 1973. Numerical taxonomic analysis of *Agrobacterium*. J. Gen. Microbiol. **78:**227–239.
23. **Kersters, K., and J. Deley.** 1975. Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. J. Gen. Microbiol. **87:**333–342.
24. **Kersters, K., and J. Deley.** 1984. Genus III. *Agrobacterium* Conn 1942, p. 244–254. *In* N. R. Krieg (ed.), Bergey's manual of systematic bacteriology. Williams and Wilkins Co., Baltimore, Md.
25. **Lehoczky, J.** 1978. Root system of the grapevine as a reservoir of *Agrobacterium tumefaciens* cells, p. 239–243. *In* Proceedings of 4th International Conference on Plant Pathogenic Bacteria, 1978. I.N.R.A., Angers, France.
26. **Malenine, I.** 1971. Recherche de l'influence du cancer bactérien sur le cépage Bolgar. Hortic. Vitic. Sci. **3:**101–107.
27. **Nester, E. W., M. P. Gordon, R. M. Amasino, and M. F. Yanofsky.** 1984. Crown gall: a molecular and physiological analysis. Annu. Rev. Plant Physiol. **35:**387–413.
28. **Ophel, K., T. J. Burr, P. Magarey, and A. Kerr.** 1988. Detection of *Agrobacterium tumefaciens* biovar 3 in South Australian grapevine propagation material. Aust. Plant Pathol. **17:**61–86.
29. **Ophel, K., and A. Kerr.** 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. Int. J. Syst. Bacteriol. **40:**236–241.
30. **Otten, L., J. Canaday, J.-C. Gérard, P. Fournier, P. Crouzet, and F. Paulus.** 1992. Evolution of agrobacteria and their Ti plasmids—a review. Mol. Plant-Microbe Interact. **4:**279–287.
31. **Otten, L., and P. De Ruffray.** 1994. *Agrobacterium vitis* nopaline Ti plasmid pTiAB4: relationship to other Ti plasmids and T-DNA structure. Mol. Gen. Genet. **4:**493–505.
32. **Otten, L., P. Crouzet, J. Y. Salomone, P. de Ruffray, and E. Szegedi.** 1995. Agrobacterium vitis strain AB3 harbors two independent tartrate utilization systems, one of which is encoded by Ti plasmid. Mol. Plant-Microbe Interact. **8:**138–146.
33. **Panagopoulos, C., P. G. Psallidas, and A. S. Alivizatos.** 1978. Studies on biotype 3 of *Agrobacterium radiobacter* var. *tumefaciens*, p. 221–228. *In* Proceedings of 4th International Conference on Plant Pathogenic Bacteria, 1978. I.N.R.A., Angers, France.
34. **Petit, A., D. C. Dahl, G. A. Ellis, G. J. Guyon, F. Casse-Delbart, and J. Tempé.** 1983. Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. Mol. Gen. Genet. **190:**204–214.
35. **Picard, C.** 1993. Caractérisation et détection dans le sol des *Agrobacterium* du pommier. Ph.D. thesis. University of Lyon I, Lyon, France.
36. **Popoff, M. Y., K. Kersters, M. Kiredjian, I. Miras, and C. Coynault.** 1984. Position taxonomique de souches de *Agrobacterium* d'origine hospitalière. Ann. Microbiol. (Inst. Pasteur) **135A:**427–442.
37. **Riberau-Gayon, J., and E. Peynaud.** 1971. Sciences et techniques de la vigne. Dunod ed., Paris, France.
38. **Ridé, M.** 1963. Our present knowledge of bacterial canker on poplar caused by *Aplanobacterium populi*, p. 1–11. *In* FAO/Internat. Poplar Commission, Casale Monferrato, Italy.
39. **Ridé, M.** 1991. Galle du collet, broussin: une maladie spectaculaire. Vigne **3:**26–27.
40. **Ridé, M.** 1993. Crown gall: vigilance dès la pépinière. Viticulture **3:**47–49.
41. **Rossi, L., B. Tinland, and B. Hohn.** 1998. Role of virulence proteins of *Agrobacterium* in the plant, p. 303–320. *In* H. P. Spaink, A. Kondorosi, and P. Hooykaas (ed.), The *Rhizobiaceae*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
42. **Ruffner, H. P.** 1982. Metabolism of tartaric and malic acids in vitis. Vitis **21:**247–259.
43. **Salomone, J. Y., P. Crouzet, P. de Ruffray, and L. Otten.** 1996. Characterization and distribution of tartrate utilization genes in the grapevine pathogen *Agrobacterium vitis*. Mol. Plant-Microbe Interact. **9:**401–408.
44. **Sawada, H., Y. Takikawa, and H. Ieki.** 1992. Fatty acid methyl ester profiles of the genus *Agrobacterium*. Ann. Phytopathol. Soc. Jpn. **58:**46–51.
45. **Sawada, H., H. Ieki, H. Oyaizu, and S. Matsumoto.** 1993. Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. Int. J. Syst. Bacteriol. **43:**694–702.
46. **Schroth, M. N., A. H. McCain, J. H. Foott, and O. C. Huisman.** 1988. Reduction in yield and vigor of grapevine caused by crown gall disease. Plant Dis. **72:**241–246.

47. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical taxonomy. The principle and practice of numeral classification. W.H. Freeman and Co., San Francisco, Calif.
48. **Süle, S.** 1978. Biotypes of *Agrobacterium tumefaciens* in Hungary. J. Appl. Bacteriol. **44:**207–213.
49. **Szegedi, E.** 1985. Host range and specific L+ tartrate utilization of biotype 3 *Agrobacterium tumefaciens*. Acta Phytopathol. Acad. Sci. Hung. **20:**1–2, 17–22.
50. **Szegedi, E., J. Korbuly, and L. Otten.** 1989. Types of resistance of grapevine varieties to isolates of *Agrobacterium tumefaciens* biotype 3. Physiol. Mol. Plant Pathol. **35:**35–45.
51. **van Berkum, P., and B. D. Eardly.** 1998. Molecular evolutionary systematics of the *Rhizobiaceae*, p. 1–24. *In* H. P. Spaink, A. Kondorosi, and P. Hooykaas (ed.), The *Rhizobiaceae*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
52. **Van Nuenen, M., P. de Ruffray, and L. Otten.** 1993. Rapid divergence of *Agrobacterium vitis* octopine-cucumopine Ti plasmids from a recent common ancestor. Mol. Gen. Genet. **240:**49–57.
53. **Vaudequin-Dransart, V., A. Petit, W. S. Chilton, and Y. Dessaux.** 1998. The cryptic plasmid of *Agrobacterium tumefaciens* cointegrates with the Ti plasmid and cooperates for opine degradation. Mol. Plant-Microbe Interact. **11:**583–591.
54. **White, L. O.** 1972. The taxonomy of the crown gall organism *Agrobacterium tumefaciens* and its relationships to rhizobia and other bacteria. J. Gen. Genet. **72:**565–574.
55. **Zinca, N., and E. Rajkov.** 1969. Tumeurs bactériennes de la vigne (*Agrobacterium tumefaciens*). Rôle du gel. Influence d'autres facteurs. Méthodes de lutte contre la maladie. Bull. Off. Int. Vigne Vin **42:**1159–1179.
56. **Zoz, N. N., I. Avdienko, N. B. Lemanova, O. D. Sultanova, M. N. Sakharova, A. B. Puglazov, R. J. Khmel, and L. S. Chernin.** 1986. Biochemical and genetic characteristics of *Agrobacterium tumefaciens* strains isolated on grapevine in Moldavia. Mol. Gen. Mikrobiol. **2:**22–27.