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A New *Agrobacterium* Strain Isolated from Aerial Tumors on *Ficus benjamina* L.†

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Crown gall tumors, collected from branches of 1-year-old weeping fig (*Ficus benjamina* L.) trees, yielded both tumorigenic and nonpathogenic agrobacteria. On the basis of classical diagnostic tests, the nonpathogenic strains were identified as *Agrobacterium tumefaciens*, whereas the tumorigenic strains could not be assigned to any of the known terrestrial *Agrobacterium* spp. The tumorigenic strains also differed from other members of the genus by producing more acid from mannitol. According to cluster analysis of carbon substrate oxidation (GN Microplate; Biolog, Inc.) and fatty acid content, the tumorigenic fig strains were distinct from strains of *A. tumefaciens*, *Agrobacterium rhizogenes*, *Agrobacterium vitis*, and *Agrobacterium rubi*. Furthermore, they had unusual opine metabolism, inducing tumors that synthesized nopaline and three recently discovered opines: chrysopine (d-lactone of *N*-1-deoxy-D-fructosyl-L-glutamine), *N*-1-deoxy-D-fructosyl-L-glutamine, and *N*-1-deoxy-D-fructosyl-5-oxo-L-proline. The nonpathogenic *A. tumefaciens* strains present in the same tumors were unable to degrade any of the opines tested. The phylogenetic position of the tumorigenic fig strain AF3.10 was inferred from comparing its *rrs* (i.e., 16S rRNA gene) sequence with those from the type strains of *Agrobacterium* and *Rhizobium* species. The analysis showed that strain AF3.10 clustered with *A. tumefaciens* and *A. rubi* but not with *A. vitis* and was far removed from *A. rhizogenes*. However, the sequence was sufficiently different from those of *A. tumefaciens* and *A. rubi* to suggest that the tumorigenic fig strain may be a new *Agrobacterium* species that is as different from *A. tumefaciens* and *A. rubi* as these two species are from one another.

The genus *Agrobacterium* Conn (13) is a member of the family *Rhizobiaceae* (35) which has been included in the alpha-2 subclass of *Proteobacteria* on the basis of ribosomal characteristics (63). Until recently, the delineation of species within the genus *Agrobacterium* was based mainly on plasmid-borne pathogenicity characters and included *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, *Agrobacterium rubi*, and the type species *Agrobacterium tumefaciens* (35, 53). However, according to chromosome-encoded characteristics, most *Agrobacterium* strains are grouped into biovars not corresponding to pathogenicity (reviewed in reference 35). Not including the five marine species recently described as a distinct subdivision within the genus *Agrobacterium* (47), the genus is composed of at least four distinct and separate taxa (5, 16, 30, 34, 52, 60, 61, 64). DNA-DNA homology studies showed that these taxa corresponded to genospecies (15, 17, 28, 43): the *Agrobacterium* biovar 1 species, *A. rhizogenes* (formerly biovar 2) (52), *Agrobacterium vitis* (formerly biovar 3) (43), and *A. rubi*. The choice of species epithets for the biovar 1 taxon remains controversial; in this report, we will refer to biovar 1 strains as *A. tumefaciens*, regardless of pathogenicity (3, 29). The phylogenetic relationships of members of the genus *Agrobacterium* have been determined by *rrs* (i.e., the 16S rRNA gene according to Riley's nomenclature [46]) sequencing (52, 61, 64). Two major lineages were distinguished: one includes *A. rhizogenes* along with most *Rhizobium* species; the second includes *A. tumefaciens*, *A. vitis*, *A. rubi*, and a distinct strain, NCPPB 1650, along with *Rhizobium galegae*. Thus, in the genus *Agrobacterium*, biochemical traits permit the characterization of species which in turn could be identified by *rrs* sequencing.

Agrobacteria are soil microorganisms (6, 7), some of which induce crown gall tumors primarily at the crown or on roots. However, symptoms can develop on aerial parts of systemically infected plants (e.g., *Vitis* spp., *Rosa* spp., and *Rubus* spp.). The ability of strains to cause crown gall depends on the presence of a Ti plasmid (pTi), a fragment of which (i.e., the T-DNA) is transferred during infection into wounded plant cells (reviewed in reference 62). The T-DNA is stably integrated into the nuclear genome of the plant cell, where its expression leads to the synthesis of plant hormones and unusual compounds termed opines (reviewed in references 18 and 62). Opines play a major role in the epidemiology of crown gall and the ecology of *Agrobacterium* spp.: (i) they serve as carbon and/or nitrogen sources for the tumor-inducing bacterium and (ii) some induce conjugal transfer of the pTi to the neighboring nontumorigenic agrobacteria (18, 25, 27). Various opines are synthesized in crown gall tumors incited by different types of pTi's; however, utilization of opines and induction of conjugal transfer are specific to pTi's that belong to the same opine-type as the inciting pTi. These features contribute to the dissemination of the inciting pTi and the growth of its bacterial host.

In the spring of 1991, aerial galls (up to 5 cm in diameter) were observed in 1-year-old weeping fig (*Ficus benjamina* L.)

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trees grown in a Florida nursery where disease incidence was nearly 100%. The galls generally developed at branch extremities which had been pruned. Actively growing galls exhibited a smooth, beige to orange surface, while older ones were wrinkled and gray. The trees originated from cuttings obtained from old hedges in South Florida after the freeze of 1989. Crown gall was reported on fig trees as early as 1919, when extremely large tumors were observed on roots of *Ficus aurea* Nutt, growing in the Florida Everglades (24). Although galls have been reported on stems of weeping fig (9), the casual agent has to our knowledge never been characterized. In this article, we report on the isolation of a new group of agrobacteria associated with these galls.

MATERIALS AND METHODS

Isolation of bacteria. Bacteria were isolated from 10 galls collected from five different trees. Small fragments of living tumor tissue were minced with a sterile scalpel blade in a few drops of sterile deionized water. The suspension was left standing for approximately 15 min, and 100 μl was spread with a loop onto the *Agrobacterium*-selective media; namely, 1A, 2E, and Roy-Sasser (40). The inoculated plates were incubated for 1 week at 28°C. Representative colony types growing on the different media were selected from each gall sample and purified by successive streaking on King's medium B (37) and potato dextrose agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.08% (wt/vol) $CaCO_3$ (4). Those strains which did not fluoresce on King's medium B were kept at 4°C on potato dextrose agar-$CaCO_3$ slants for routine use and frozen at −70°C in 15% (vol/vol) glycerol for long-term storage.

Pathogenicity assay. The ability of presumptive agrobacteria to cause crown gall was examined by stem prick inoculation (40) of 6-week-old tomato (*Lycopersicon esculentum* Mill. cv. MicroTom) and 15-cm-high weeping fig plants kept in a growth chamber at 25°C. Tumor formation was assessed 6 to 8 weeks after inoculation.

Detection of opines in fig and tomato tumors. Fig and tomato tumors, ranging in weight from 16 to 146 mg, were preserved in 3 ml of 70% ethanol until extraction a few days later. The tumors were removed from 70% ethanol, air dried overnight, and then ground in a coffee mill. The resulting powder was then extracted overnight in the original 70% ethanol. Healthy fig (60 mg) and tomato stem (35 mg) tissues were included as negative controls. Each extract was concentrated to 150 μl by vacuum evaporation. The presence of opines in the concentrated tumor extracts was investigated by high-voltage paper electrophoresis and then by the appropriate staining reaction.

To enhance the sensitivity for detection of leucinopine or succinamopine (if present), the tumor extract was enriched for iminodiacid opines by fractionation on an ion-exchange column. Extracts of eight fig tumors (three field tumors and five tumors incited by fig strains AF1.72, AF3.10, AF3.44, AF3.53, and AF3.9) were pooled and adsorbed onto a 1.5-ml column of Dowex $50H^+$. Sugars and other nonretained metabolites were washed out with 8 ml of water. The column was then eluted with 28 ml of deionized water, a fraction in which the iminodiacids are known to elute (11, 12). Following this, chrysopine (d-lactone of *N*-1-deoxy-D-fructosyl-L-glutamine), DFG (*N*-1-deoxy-D-fructosyl-L-glutamine), nopaline, and amino acids were eluted with 1 M ammonium hydroxide. Both the iminodiacid fraction and the amino acid fractions were concentrated on the rotary evaporator to 200 μl and analyzed by high-voltage paper electrophoresis. A volume of 15 to 30 μl of concentrated tumor extract or column eluate was deposited on 1 cm of Whatman 3MM paper and electrophoresed for 30 to 60 min at 2,000 V (40 V/cm) in the appropriate buffer. Nopaline and octopine were analyzed at pH 1.8 and detected with phenanthrenequinone reagent (66). The presence of nopaline was also confirmed by analysis at pH 4.0. Examination for the presence or absence of succinamopine and leucinopine was conducted by electrophoresis at pH 2.8, and standards were detected with $AgNO_3$-mannitol (12). Cucumopine and mikimopine were analyzed at pH 1.8 with diazotized *p*-aminobenzene sulfonic acid as the detection reagent (66). Chrysopine and DFG were analyzed at pH 1.8 and detected as brown spots with silver nitrate reagent (55). Chrysopine and DFG comigrate with agropine and mannopine, respectively; however, only chrysopine and DFG react with triphenyltetrazolium reagent (66). Chrysopine was also analyzed at pH 2.8, at which it moves slightly cationically. Opine standards used in this study were synthesized by methods described previously (10–12, 14, 31, 32). The synthetic mixture of threo- and *erythro*-succinamopine was biochemically resolved by use of *A. tumefaciens* A281(pTiBo542) to catabolize *S*,*S*-succinamopine. The remaining *R*,*S*-succinamopine was recovered by absorption onto Dowex $50H^+$ ion-exchange resin and elution with 20 bed volumes of distilled water.

A feature associated with nopaline pTi is the sensitivity of the bacterium to agrocin 84, a bacteriocin produced by the biological control agent *A. rhizogenes* K84 (21). Strains were tested for agrocin sensitivity on agar plates by the method of Stonier (54). The agrocin-sensitive strain C58 and its agrocin-resistant derivative C58C1 were used as references.

Diagnostic tests. The identity of each suspected *Agrobacterium* sp. strain was determined by the appropriate biochemical and physiological tests (40): reaction on litmus milk, alkali production from malonic acid and L-tartaric acid, oxidase reaction, growth at 35°C and on 2% (wt/vol) NaCl, growth and pigmentation on ferric ammonium citrate, utilization of Simmons citrate, and oxidation of lactose to 3-ketolactose. In addition, the strains were tested for acid production on potato dextrose agar-$CaCO_3$ (4) and mannitol-$CaCO_3$ medium (containing [in grams per liter]: mannitol, 10; Difco nutrient broth, 2; yeast extract, 0.5; $CaCO_3$, 0.8; agar, 15).

Carbon substrate utilization. The ability of the fig strains to oxidize 95 substrates from the Biolog GN MicroPlate (Biolog, Inc., Hayward, Calif.) was compared with that of strains from other taxa by use of our *Agrobacterium* database (5). Inoculation, incubation, data acquisition, and cluster analysis were performed as described previously (5).

Fatty acid analysis. The procedures used to prepare, extract, and differentiate fatty acids by gas-liquid chromatography have been described previously (51). The fatty acid profiles of the fig strains were compared with those of our *Agrobacterium* database (5) and used to determine the Euclidian distance to other *Agrobacterium* spp.

DNA extraction and hybridization. Extraction of genomic DNA was performed as described by Dhaese et al. (19) on 10 ml of overnight bacterial cultures grown in nutrient broth medium. Endonuclease treatments of genomic DNA and analysis of restriction fragments in 0.8% low-electroendosmosis agarose (Boehringer Mannheim Co., Indianapolis, Ind.) gels were carried out by standard procedures (49). Southern hybridization was performed on genomic DNA digested with the endonuclease *Bam*HI. The restriction fragments were transferred onto a nylon membrane (Hybond N; Amersham Co., Arlington Heights, Ill.) by standard protocols (2). Hybridization and detection of the DNA-DNA duplex were performed with digoxigenin-labelled probes as described by the manufacturer (Boehringer Mannheim) and with modifications described elsewhere (58). To obtain probes generated from tumorigenic fig strain DNA, *Hin*dIII restriction fragments obtained from genomic DNA of strain AF3.9 were randomly cloned into pUC18 and propagated into *Escherichia coli* strain DH5α by standard procedures (49, 65). Two of these randomly cloned *Hin*dIII fragments, with apparent sizes of 4.8 and 5.5 kb, were gel purified (GeneClean; Bio 101, Inc., La Jolla, Calif.), labelled with digoxigenin-modified nucleotides, and used as fig-generated probes. Because strain AF3.9 contains at least one plasmid, these two fig-generated probes were hybridized to both plasmid and chromosomal DNA sequences of strain AF3.9; the probes detected only chromosomal DNA sequences of this strain. In Southern hybridization assays, these two fig-generated probes were always used as a mixture. By similar procedures, a 5.5-kb DNA fragment was randomly cloned from *Hin*dIII-digested genomic DNA of the plasmid-free, nonpathogenic *A. tumefaciens* strain 3.1 isolated from a weeping fig tumor. This fragment was used as a probe for nonpathogenic *A. tumefaciens* strains.

PCR-restriction fragment length polymorphism typing. To identify the chromosomal genotypes of each *Agrobacterium* strain, primers FGPS6-63 (GGA GAG TTA GAT CTT GGC TCA G) and FGPL132′-38 (CCG GGT TTC CCC ATT CGG) were used to amplify the *rrs*+IGS region consisting of *rrs* (i.e., 16S rRNA gene) and the intergenic spacer (IGS) located between the *rrs* and the *rrl* (i.e., 23S rRNA gene) as described previously (45). The amplified products were digested with *Taq*I, *Rsa*I, *Hae*III, or *Sau*96I. Restriction fragments were separated by electrophoresis on a 3% (wt/vol) agarose gel and stained with ethidium bromide (0.4 mg/liter).

Sequencing and phylogenetic analysis. Amplification of *rrs* (16S rRNA gene) of strain AF3.10 was performed with primers FGPS6-63 and FGPS1509′-153 (AAG GAG GGG ATC CAG CCG CA) (47), which contain restriction sites *Bgl*II and *Bam*HI, respectively. The 1.5-kb fragments (corresponding to 95% of the gene), purified with the GeneClean kit (Bio 101), were restricted with the appropriate restriction endonucleases, ligated into the *Bgl*II-*Bam*HI-restricted pBluescript II SK vectors, and cloned into *E. coli* DH5αF′ (Bethesda Research Laboratories, Cergy-Pontoise, France). Plasmid DNA from *E. coli* was prepared by the alkaline lysis procedure (38). Three clones were sequenced by the dideoxy chain termination method of Sanger et al. (50). Twelve primers (FGPS310-20, GAG ACA CGG CCC AGA CTC CT; FGPS485-292, CAG CAG CCG CGG TAA; FGPS747-293, AAC AGG ATT AGA TAC; FGPS1047-295, ATG TTG GGT TAA GTC; FGPS1156-39, GAC GTC AAG TCA CAT GCC C; FGPS305′-78, CCA GTG TGG CCG GTC GCC CTC TC; FGPS505′-313, GTA TTA CCG CGG CTG CTG; FGPS659′-64, CAC CGC TAC ACC AGG AAT TC; FGPS910′-270, AGC CTT GCG GCC GTA CTC CC: FGPS1176′-112, GGG GCA TGA TCA CTT GAC GTC; and primers T7 and T3 from Pharmacia [LKB Biotechnologies, Uppsala, Sweden]) were necessary to determine the whole *rrs* molecule on both strands. The dideoxy chain termination sequencing procedure was performed with the T7 sequencing kit (Pharmacia). The EMBL accession number of the AF3.10 *rrs* sequence is Z30542. Sequences of *rrs* genes of *A. rhizogenes*, *A. rubi*, *A. vitis*, *R. galegae*, *Rhizobium tropici*, *Rhizobium leguminosarum*, *Rhizobium fredii*, *Rhizobium meliloti*, *Rhizobium loti*, *Bradyrhizobium japonicum* type strains, strains of *A. tumefaciens* (B6 = ATCC 23308 = NCPPB 2437 = IAM 13129 = LMG 187, and ICPB TT111 = LMG 196 identical to the sequence of DSM 30150 = IAM 14141), and *Agrobacterium* sp. strain NCPPB 1650 were obtained from the EMBL and GenBank databases (52, 61, 64). Sequences were aligned with SeqApp (Internet resource available via anony-

mous ftp to ftp.bio.indiana.edu) and Seqboot from the Phylip package (22a). Matrix pairwise comparisons were corrected for multiple base substitutions by the method of Juke and Cantor (33). Phylogenetic trees were constructed by the neighbor-joining method (48). A bootstrap confidence analysis was performed with 100 replicates to determine the reliability of the tree topology obtained (22).

RESULTS AND DISCUSSION

Isolation of phenotypically distinct tumorigenic and nontumorigenic agrobacteria from aerial fig tumors. Of 32 putative *Agrobacterium* strains recovered from the weeping fig tumors sampled, 15 were randomly selected for analysis. Seven strains (AF1.72, AF3.43, AF3.44, AF3.53, AF3.81, AF3.9, and AF3.10) when inoculated into weeping fig and tomato plants induced tumor formation on both plant species. Six of the tumorigenic strains were originally isolated on Roy-Sasser medium, which is semiselective for *A. vitis*, whereas strain AF1.72 was isolated on medium 1A, which is semiselective for *A. tumefaciens*. Eight nonpathogenic strains present in the same tumors were identified as *A. tumefaciens* on the basis of carbon substrate utilization (Table 1; Fig. 1), fatty acid content (Table 2; Fig. 2), and traditional diagnostic tests (Table 3). In contrast, the tumorigenic strains could not be assigned to any of the previously reported *Agrobacterium* spp. on the basis of the diagnostic tests (Table 3). With the exception of a few *A. vitis* strains, the tumorigenic fig strains produced larger quantities of acid from mannitol than the other agrobacteria tested (see list of strains in reference 4); this characteristic of the tumorigenic fig strains was easily recognized after 4 days at 28°C by the production of a clear zone around the bacterial growth on mannitol-$CaCO_3$ medium.

Phenotypic differences of the tumorigenic fig strains from other *Agrobacterium* spp. were confirmed by cluster analyses of carbon substrate oxidation patterns and, to a lesser extent, fatty acid profiles. Both assays segregated the tumorigenic fig strains from other agrobacteria (Fig. 1 and 2). Analysis of the carbon sources oxidized by tumorigenic fig strains revealed that this group of strains oxidized the carbon compounds previously reported to be common substrates for other agrobacteria (5). However, tumorigenic fig strains were differentiated from other agrobacteria by their inability to oxidize *cis*-aconitic acid (Table 1), a substrate utilized by other agrobacteria. Several other carbon substrates differentiated the tumorigenic fig strains from the other *Agrobacterium* spp. (Table 1). Analysis of the fatty acid composition of the tumorigenic fig strains revealed the predominance of *cis*-vaccenic acid ($18:1_{\omega7}$ *cis*), which accounted for about 71% of the total fatty acid profile (Table 2). The high level of *cis*-vaccenic acid is typical of agrobacteria (5). Other major fatty acids shared with other agrobacteria were palmitic acid (16:0), 3-hydroxymyristic acid (3-OH-14:0), 3-hydroxypalmitic acid (3-OH-16:0), and lactobacillic acid (19:0 $cyc_{\omega8}$ *cis*). Palmitoleic acid ($16:1_{\omega7}$ *cis*) was also present in significant amounts. With the exception of *A. tumefaciens* K15/73 (5), the tumorigenic fig strains contained more palmitoleic acid than any other *Agrobacterium* strain tested (Table 2).

Screening for the presence of opines in tumors incited by the fig strains on tomato and weeping fig allowed for the detection of nopaline and two recently discovered opines; namely, chrysopine and DFG. Additional investigation (58) allowed us to detect a fourth opine, *N*-1-deoxy-D-fructosyl-5-oxo-L-proline. These opines were found to be metabolized by *A. tumefaciens* Chry9 isolated from galls of chrysanthemum (*Dendranthemum* × *grandiflorum* [Ramat] Kitamura) grown in Florida (8, 20, 58). These opines were also detected in four naturally infected fig tumors, whereas nopaline was detected in two of these tumors. As reported elsewhere (58), octopine, leucinopine, and succinamopine were not detectable in unfractionated extracts of any of the tomato or fig tumors. These results were confirmed by a more sensitive opine detection method based on the analysis of pooled tumor extract fractions enriched for iminodiacids (see Materials and Methods). Investigating the presence of agropine and mannopine is complicated by the fact that agropine and mannopine comigrate with chrysopine and DFG, respectively. All four opines are oxidized by silver nitrate, but only chrysopine and DFG react with triphenyltetrazolium. With this reagent, it was possible to demonstrate the presence of chrysopine and DFG in tumors but not to analyze independently for agropine and mannopine. The absence of agropine in the tumors was inferred from catabolic data reported elsewhere (58). Resistance of the tumorigenic fig strains to agrocin 84 is further evidence that the nopaline-chrysopine

TABLE 1. Differential oxidation of substrates (Biolog GN MicroPlate) by tumorigenic fig (AF) strains and other *Agrobacterium* species

Substrate	Differential oxidation[a] by:				
	AF	*A. tumefaciens*[b,c]	*A. rhizogenes*[b]	*A. vitis*[b]	*A. rubi*[b]
Glycogen	+	+	v	+	−
Tween 40 and Tween 80	−	+	v	+	+
N-Acetyl-D-galactosamine	−	v	−	v	+
N-Acetyl-D-glucosamine	+	+	−	+	+
i-Erythritol	−	−	+	−	+
α-Lactose	−	+	+	+	−
α-D-Lactose lactulose	+	v	+	+	−
D-Melibiose	+	+	+	+	−
β-Methylglucoside	−	+	+	v	−
D-Raffinose	−	+	+	+	−
Xylitol	+	+	+	−	−
Acetic acid	−	+	−	−	+
cis-Aconitic acid	−	+	+	+	+
Citric acid	−	−	+	+	+
Formic acid	−	v	+	v	−
D-Galactonic acid lactone	−	+	+	−	+
D-Gluconic acid	−	+	v	−	+
D-Glucosaminic acid	−	−	+	−	+
D-Glucuronic acid	−	+	−	−	−
α-Hydroxybutyric acid	v	v	−	−	+
β-Hydroxybutyric acid	−	v	v	+	+
α-Ketobutyric acid	v	v	−	−	+
α-Ketoglutaric acid	−	−	v	−	+
Malonic acid	−	−	+	+	−
Propionic acid	v	+	−	−	+
D-Saccharic acid	+	−	+	v	−
Succinamic acid	−	−	+	+	−
D-Alanine	−	v	−	−	+
Glycyl-L-aspartic acid	−	+	−	−	+
Hydroxy-L-proline	−	+	−	−	+
L-Leucine	−	−	v	−	+
L-Pyroglutamic acid	−	+	+	+	−
L-Threonine	+	+	−	v	+
DL-Carnitine	−	−	v	−	+
γ-Aminobutyric acid	−	−	−	−	+
Uroncanic acid	−	−	−	−	+
Inosine	−	−	−	−	+
Gycerol	+	+	+	+	−
Glucose-1-phosphate	+	+	−	−	−
Glucose-6-phosphate	+	+	−	−	−

[a] Symbols: +, substrate oxidized by more than 80% of the strains; −, substrate oxidized by less than 20% of the strains; v, substrate oxidized by 20 to 80% of the strains.

[b] Carbon substrate oxidation data for *A. tumefaciens*, *A. rhizogenes*, *A. vitis*, and *A. rubi* are presented elsewhere (5).

[c] Substrate oxidation patterns of the eight nonpathogenic strains isolated from aerial weeping fig tumors were similar to those of other *A. tumefaciens* strains.

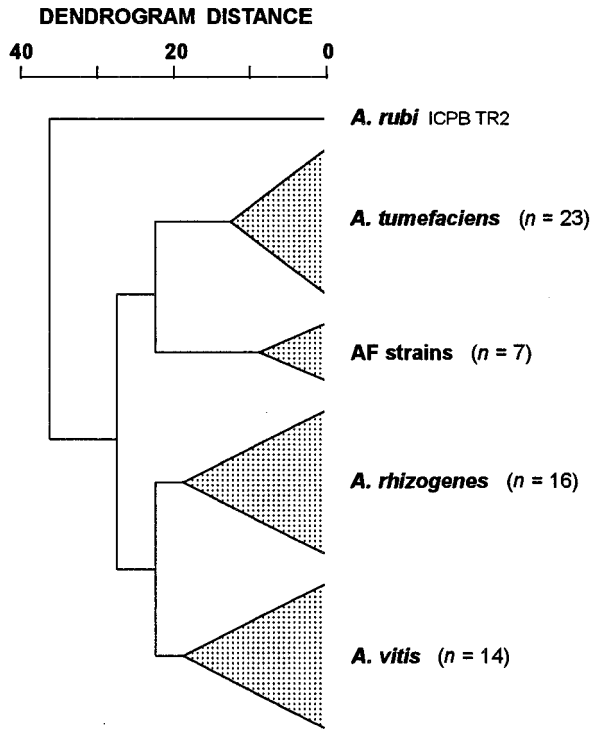

FIG. 1. Dendrogram showing the relationships between the tumorigenic fig (AF) strains and other *Agrobacterium* spp. based on differential oxidation of the 95 carbon substrates available in the Biolog GN Microplate. Strains of *A. tumefaciens*, *A. rhizogenes*, *A. vitis*, and *A. rubi* are from the *Agrobacterium* database (5).

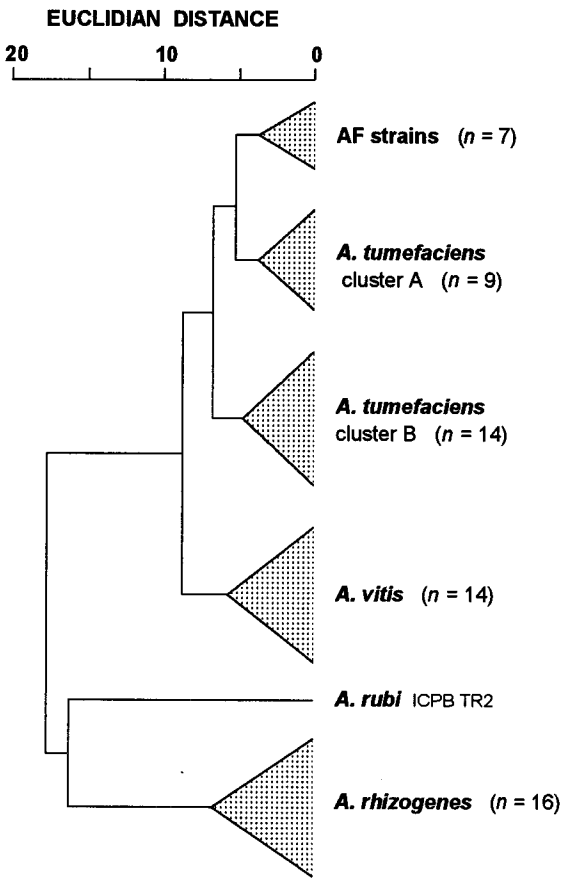

FIG. 2. Dendrogram showing the relationships between the tumorigenic fig (AF) strains and other *Agrobacterium* spp. based on fatty acid composition data. Strains of *A. tumefaciens*, *A. rhizogenes*, *A. vitis*, and *A. rubi* are from the *Agrobacterium* database (5).

pTi harbored by these strains is not closely related to the common nopaline or agrocinopine pTi found in agrocin 84-sensitive strains. Resistance to agrocin 84 also suggests that strain K84 may not be an effective biological control for crown gall outbreaks on weeping fig trees.

Interestingly, none of the nonpathogenic *A. tumefaciens* strains isolated from the fig tumors could catabolize any of the opines present in these tumors (unpublished data). The presence of nontumorigenic agrobacteria in crown or root tumors is common (40); they are thought to be opportunistic bacteria from soil invading the nutrient-rich tumor environment. However, the origin of these nontumorigenic *A. tumefaciens* strains and the reason for their presence in aerial fig tumors remain to be determined.

Genotypic analysis of the tumorigenic fig strains. The tumorigenic fig strains appeared very closely related as indicated by their identical DNA patterns obtained by (i) *Bam*HI diges-

TABLE 2. Mean percent fatty acid composition of tumorigenic fig (AF) strains and other *Agrobacterium* strains[a]

Fatty acid	Mean % fatty acid composition of:					
	AF	*A. tumefaciens*		*A. rhizogenes*	*A. vitis*	*A. rubi*
		Cluster A	Cluster B			
3-OH-14:0[b]	6.9 ± 0.5	6.5 ± 1.0	7.8 ± 1.2	5.5 ± 0.8	6.6 ± 0.9	tr[c]
16:1$_{\omega 7}$*cis*	6.2 ± 0.2	2.8 ± 0.8	3.8 ± 0.9	tr	4.9 ± 0.7	tr
16:0	8.1 ± 0.6	8.1 ± 1.4	9.2 ± 1.0	4.8 ± 0.8	4.6 ± 0.7	3.8
16:0				3.6 ± 0.5		
3-OH-16:0	4.1 ± 0.2	4.1 ± 0.7	4.6 ± 0.7	6.6 ± 1.0	tr	
18:1$_{\omega 7}$*cis*[d]	71.0 ± 1.4	75.2 ± 3.1	67.4 ± 1.9	62.6 ± 2.2	74.4 ± 2.3	63.6
18:0				tr	tr	3.4
19:0 cyc$_{\omega 8}$*cis*	2.5 ± 0.6	2.3 ± 0.9	5.0 ± 1.3	8.5 ± 2.2	tr	22.7
19:0 10-methyl	tr	tr	tr	tr	2.5 ± 0.4	

[a] Fatty acid data for *A. tumefaciens* (clusters A and B), *A. rhizogenes*, *A. vitis*, and *A. rubi* are presented elsewhere (5).
[b] Identified as Summed Feature 3 of the Microbial ID library (trypticase soy broth agar, version 3.70).
[c] tr, trace amounts (<2.0%) detected.
[d] Identified as Summed Feature 7 of the Microbial ID library (trypticase soy broth agar, version 3.70).

TABLE 3. Reaction of tumorigenic fig (AF) strains to biochemical and physiological tests used to distinguish agrobacteria

Test[a]	Reaction[b] of:				
	AF	*A. tumefaciens*[c]	*A. rhizogenes*	*A. vitis*	*A. rubi*
3-Ketolactose	−	+	−	−	−
Ferric ammonium citrate	−	+	−	−	−
Growth at 35°C	−	+	−	v	v
Growth on 2% NaCl	+	+	−	+	
Oxidase	+	+	−	+	
Litmus milk	Alkali	Alkali	Acid	Alkali	Alkali
Citrate utilization	−	−	+	+	−
Malonic acid	−	−	+	+	+
Tartaric acid	+	−	+	+	
PDA-$CaCO_3$[d]	v	−	+	−	−
Mannitol-$CaCO_3$	+	−	−	v	−

[a] Diagnostic tests are described in Materials and Methods and elsewhere (4, 40).
[b] Symbols: +, positive reaction by more than 80% of the strains; −, positive reaction by less than 20% of the strains; v, positive reaction by 20 to 80% of the strains.
[c] The reaction profiles of the eight nonpathogenic strains isolated from aerial weeping fig tumors were similar to those of other *A. tumefaciens* strains.
[d] PDA, potato dextrose agar.

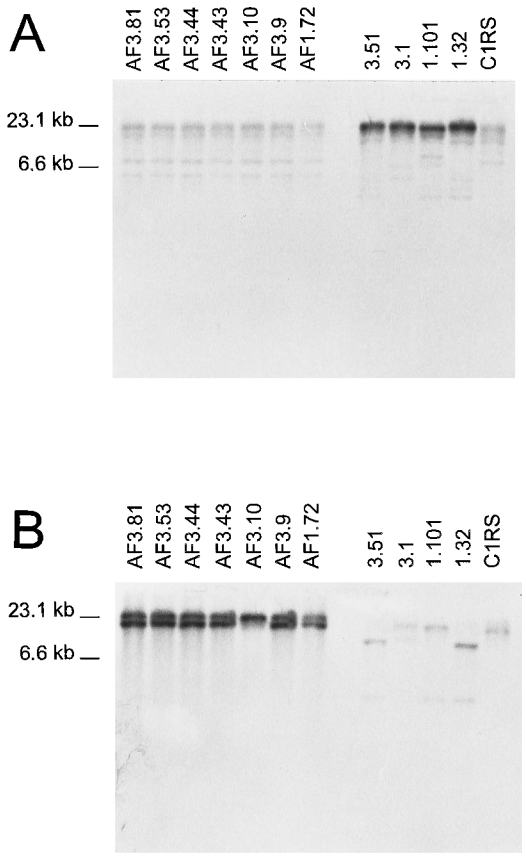

FIG. 3. Restriction fragment length polymorphism analysis of *Bam*HI digestions of genomic DNA from tumorigenic fig strains (AF3.81, AF3.53, AF3.44, AF3.43, AF3.10, AF3.9, and AF1.72), nonpathogenic *A. tumefaciens* strains (3.51, 3.1, 1.101, and 1.32), and the reference strain C58C1RS (C1RS). (A) The probe used was a 5.5-kb *Hin*dIII fragment, randomly cloned from the plasmid-free *A. tumefaciens* strain 3.1. (B) Two probes, 5.5- and 4.8-kb *Hin*dIII fragments randomly cloned from the tumorigenic fig strain AF3.9, were used together.

tion (not shown), (ii) Southern hybridization with the *A. tumefaciens* probe obtained from the nonpathogenic strain 3.1 (Fig. 3A), and (iii) PCR amplification of the *rrs*+IGS region and its subsequent digestion with restriction endonucleases (not shown). All of these patterns were different from those obtained with the nonpathogenic *A. tumefaciens* strains (Fig. 3). These results suggest that most of the tumorigenic fig strains are very closely related, perhaps clones of the same strain. However, the restriction fragment length polymorphism patterns obtained by hybridization with a mixture of the two AF3.9 probes distinguished strain AF3.10 from the other tumorigenic fig strains; AF3.10 had one band missing (Fig. 3B). Differences were also observed in the plasmid profiles of the tumorigenic fig strains (58). These data confirmed those obtained from the cluster analyses of fatty acid and carbon substrate utilization assays, which suggested that strain AF3.10 was distinct but closely related to the other tumorigenic fig strains. Thus, the tumorigenic fig strains did not belong to the same clone. This close relatedness among the tumorigenic population isolated from aerial fig tumors is in sharp contrast to the situation encountered in other crown gall outbreaks, where different tumorigenic strains are commonly found in the same tumor (1, 42) and where the same pTi has been found harbored by strains belonging to different species (39, 42). This may be due to a particular affinity between the fig pTi and the chromosomal background of the tumorigenic fig strains as reported elsewhere (7, 44).

Phylogenetic position of the tumorigenic fig strain AF3.10. Cluster analyses of carbon substrate utilization and fatty acid composition inferred that the tumorigenic fig strains were closely related to the *Agrobacterium* group. To determine the relationship of the tumorigenic fig strains to other closely related bacteria, strain AF3.10 was selected for *rrs* sequencing and phylogenetic analysis. No sequence presently available in data banks was found to be identical to those of strain AF3.10. However, the sequence of AF3.10 could be aligned with sequences from strains of *A. tumefaciens*, *A. rubi*, and strain NCPPB 1650, while gaps were required for alignment of sequences of *A. vitis* and *A. rhizogenes* (Fig. 4). By use of all pairwise comparisons, a phylogenetic tree was constructed from the *rrs* sequences of members of the *Rhizobiaceae* family, which confirmed the position of strain AF3.10 in a cluster containing *A. tumefaciens*, *A. rubi*, and NCPPB 1650 strains (100% of the bootstraps) (Fig. 5). However, the grouping of AF3.10, NCPPB 1650, and *A. rubi* was not significant (52% of the bootstraps), indicating that AF3.10 is no more closely related to *A. rubi* and NCPPB 1650 than to *A. tumefaciens*. The concept of a bacterial genus is not as well defined as the concept of bacterial species (59); species which appear closely related by various criteria are nevertheless generally maintained in the same genus. Several authors reported that *Rhizobium* and *Agrobacterium* species are phylogenetically intertwined (52, 61, 64), and a revision of the genera will probably occur soon. However, whatever the future revision, the tumorigenic fig strains should be maintained in the same genus as *A. rubi* and *A. tumefaciens* because of the phylogenetic position of the strain AF3.10 (Fig. 5), especially if the distantly related species *A. vitis* and *R. galegae* are also included in the same genus.

At the species level, *rrs* data alone are not sufficient to draw formal conclusions, but some important comments can be made. The correlation between *rrs* diversity and DNA-DNA homology depends upon the genus considered. Fox et al. (23) showed that distinct *Bacillus* genospecies have identical *rrs*, indicating that *rrs* identity is not a clue to species identity in this genus. However, species in other bacterial genera can be dif-

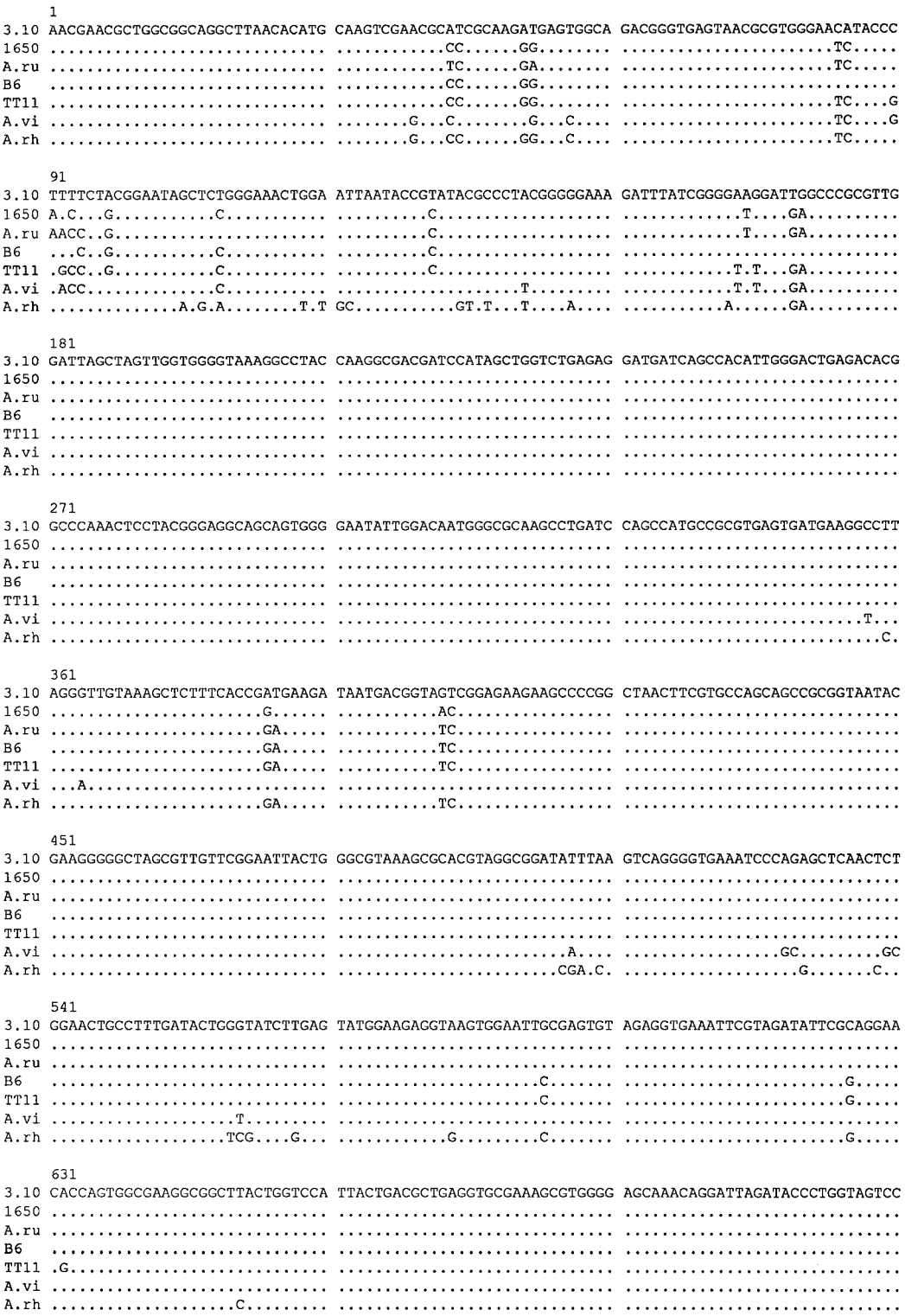

FIG. 4. Sequence of *rrs* of *Agrobacterium* sp. strain AF3.10 (EMBL accession number Z30542) and alignment with *rrs* sequences of six *Agrobacterium* strains. Only the nucleotides that differ from those of AF3.10 are shown; identical nucleotides are indicated by dots, and gaps are indicated by dashes. Abbreviations: 3.10, *Agrobacterium* sp. strain AF3.10; 1650, *Agrobacterium* sp. strain NCPPB 1650; A. ru, *A. rubi*; B6, *A. tumefaciens* NCPPB 2437; TT111, *A. tumefaciens* ICPB TT111; A. vi, *A. vitis*; A. rh, *A. rhizogenes*. Sequences are those of type strains unless a strain number is indicated.

ferentiated on the basis of *rrs* differences (26, 41, 45, 56, 57). In the present study, *rrs* sequences of several strains belonging to the same phylogenetic cluster provided a tool to evaluate the relationships between nucleotidic substitutions in *rrs* and differences shown by other methods, including biochemical and physiological assays (36) and DNA-DNA homology studies (28, 35). The rates of nucleotidic substitutions (inferred from Fig. 4) between B6 and TT111 and between *A. rubi* and

```
     721
3.10 ACGCCGTAAACGATGAATGTTAGCCGTCGG GCAGTATACTGTTCGGTGGCGCAGNTAACG CATTAAACATTCCGCCTGGGGAGTACGGTC
1650 ............................. .....C.................C..... .............................
A.ru ............................. .....TG................C..... .............................
B6   ............................. ........................C..... .............................
TT11 ............................. ........................C..... .............................
A.vi ...TT........................ CA...TG...TG............C..... .............................
A.rh ............................. ........................C..... .............................

     811
3.10 GCAAGATTAAAACTCAAAGGAATTGACGGG GGCCCGCACAAGCGGTGGAGCATGTGGTTT AATTCGAAGCAACGCGCAGAACCTTACCAG
1650 ............................. ............................... .............................
A.ru ............................. ....-.......................... .............................
B6   ............................. ............................... .............................
TT11 ............................. ............................... .............................
A.vi ............................. ............................... .............................
A.rh ............................. ....-.......................... .............................

     901
3.10 CTCTTGACATTCGGGGTATGGTCATTGGAG ACGATGACCTTCAGTTCGGCTGGCCCTAGA ACAGGTGCTGCATGGCTGTCGTCAGCTCGT
1650 ................T...G..G..... ..AT..T.........A.........C... .............................
A.ru ................T...G..G..... ..AT..T.........A.........C... .............................
B6   ................T...G..G..... ..AT..T.........A.........C... .............................
TT11 ................T...G..G..... ..AT..T.........A.........C... .............................
A.vi ..........C.T.T.--ACCG.CAC.... ...TG.TT-...C-..TC.GG.A.A.AGAG .............................
A.rh .C........C.T.T..TACCCGTAGA..T .T.GG.T..---.C.....-....G.AGAG .............................

     991
3.10 GTCGTGAGATGTTGGGTTAAGTCCCGCAAC GAGCGCAACCCTCGCCCTTAGTTGCCAGCA TTTGGTTGGGCACTCTAAGGGGACTGCCGG
1650 ............................. ............................... .............................
A.ru ............................. ............................... .............................
B6   ............................. ............................... ...A.........................
TT11 ............................. ............................... ...A.........................
A.vi ............................. ............................... ..CA.........................
A.rh ............................. ............................... ..CA.........................

     1081
3.10 TGATAAGCCGAGAGGAAGGTGGGGATGACG TCAAGTCCTCATGGCCCTTACGGGCTGGGC TACACACGTGCTACAATGGTGGTGACAGTG
1650 ............................. ............................... .............................
A.ru ............................. ............................... .............................
B6   ............................. ............................... .............................
TT11 ............................. ............................... .............................
A.vi ............................. ............................... .............................
A.rh ............................. ............................... .............................

     1171
3.10 GGCAGCGAGACAGCGATGTCGAGCTAATCT CCAAAAGCCATCTCAGTTCGGATTGCACTC TGCAACTCGAGTGCATGAAGTTGGAATCGC
1650 ............................. ............................... .............................
A.ru ............................. ............................... .............................
B6   ............................. ............................... .............................
TT11 ............................. ............................... .............................
A.vi ..........C....G............ ............................... .............................
A.rh .........CAC....GTGT......... ............................... .............................

     1261
3.10 TAGTAATCGCAGATCAGCATGCTGCGGTGA ATACGTTCCCGGGCCTTGTACACACCGCCC GTCACACCATGGGAGTTGGTTTTACCCGAA
1650 ............................. ............................... .............................
A.ru ............................. ............................... .............................
B6   ............................. ............................... .............................
TT11 ............................. ............................... .............................
A.vi ............................. ............................... .............................
A.rh ..........G...........C..... ............................... .............................

     1351
3.10 GGCGCTGCGCTAACCGCAAGGGGGCAGGCG ACCACGGTAGGGTCAGCGACTGGGGTGAAG TCGTAACAAGGTAGCCGTAGGGGAACC
1650 ............................. ............................... ...........................
A.ru ............................. ............................... ...........................
B6   ..TAG..............A.....CTA ............................... ...........................
TT11 ..TAG..............A.....CTA ............................... ...........................
A.vi ..TCG..............A.....CGA ............................... ...........................
A.rh ..TAG..............A.....CTA ............................... ...........................
```

FIG. 4—*Continued.*

NCPPB 1650 are relatively low (i.e., 0.6%). This low rate occurs between strains which were considered either as belonging to readily different species (NCPPB 1650 and *A. rubi*) or to strains included in the same species but distantly related (B6 and TT111). Between the clearly distinct species *A. tumefaciens* and *A. rubi*, the rate of nucleotidic substitutions ranged from 1.5 to 1.6%. Strain AF3.10 has a much greater rate of nucleotidic substitutions, ranging from 1.8 to 2.7%, with *A. rubi*, *A. tumefaciens*, and NCPPB 1650 strains. This clearly demonstrates that the tumorigenic fig strains constitute a species as

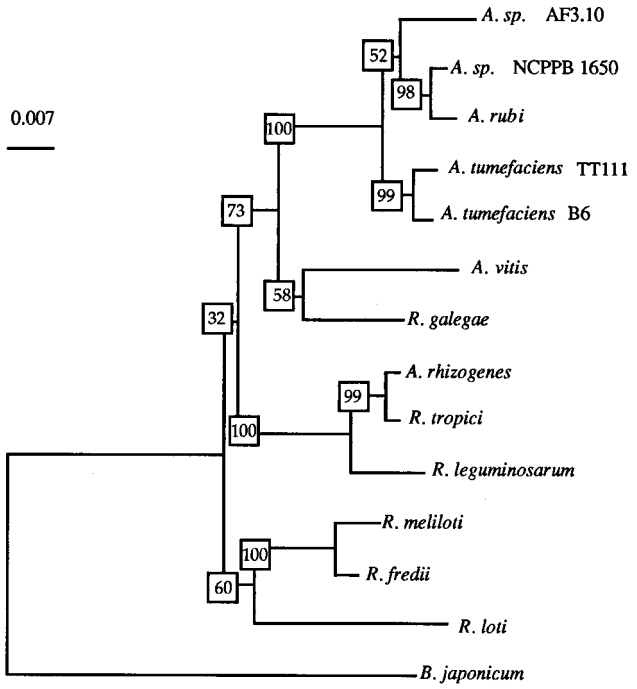

FIG. 5. Phylogenetic relationships between *Agrobacterium* sp. strain AF3.10 and members of the genera *Agrobacterium*, *Bradyrhizobium*, and *Rhizobium* based on *rrs* sequencing. All pairwise comparisons were done after correction for multiple substitutions by the method of Juke and Cantor (33). Numbers indicate percentages of grouping confidence calculated by bootstrap analysis (22). The scale bar represents the rate of nucleotidic substitutions per nucleotide site.

distinct from *A. tumefaciens* and *A. rubi* as the two latter species differ from one another, confirming results obtained by the phenotypic tests. Thus, the tumorigenic fig strains belong to a new species which should be included in the same genus of the alpha-2 subclass that includes *A. tumefaciens*, *A. rubi*, and strain NCPPB 1650.

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REFERENCES

1. **Anderson, A. R., and L. W. Moore.** 1979. Host specificity in the genus *Agrobacterium*. Phytopathology **69:**320–323.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Bouzar, H.** 1994. Request for a judicial opinion concerning the type species of *Agrobacterium*. Int. J. Syst. Bacteriol. **44:**373–374. (Letter to the editor.)
4. **Bouzar, H., and J. B. Jones.** 1992. Distinction of biovar 2 strains of *Agrobacterium* from other chromosomal groups by differential acid production. Lett. Appl. Microbiol. **15:**83–85.
5. **Bouzar, H., J. B. Jones, and N. C. Hodge.** 1993. Differential characterization of *Agrobacterium* species using carbon-source utilization patterns and fatty acid profiles. Phytopathology **83:**733–739.
6. **Bouzar, H., and L. W. Moore.** 1987. Isolation of different *Agrobacterium* biovars from a natural oak savanna and tallgrass prairie. Appl. Environ. Microbiol. **53:**717–721.
7. **Bouzar, H., D. Ouadah, Z. Krimi, J. B. Jones, M. Trovato, A. Petit, and Y. Dessaux.** 1993. Correlative association between resident plasmids and the host chromosome in a diverse *Agrobacterium* soil population. Appl. Environ. Microbiol. **59:**1310–1317.
8. **Bush, A., and S. G. Pueppke.** 1991. Characterisation of an unusual new strain of *Agrobacterium tumefaciens* from *Chrysanthemum morifolium* Ram. Appl. Environ. Microbiol. **57:**2468–2472.
9. **Chase, A. R.** 1987. Compendium of ornamental foliage plant diseases. APS Press, St. Paul, Minn.
10. **Chilton, W. S., and M. D. Chilton.** 1984. Mannityl opine analogs allow isolation of catabolic pathway regulatory mutants. J. Bacteriol. **158:**650–658.
11. **Chilton, W. S., E. Hood, and M. D. Chilton.** 1985. Absolute stereochemistry of leucinopine, a crown gall opine. Phytochemistry **24:**221–224.
12. **Chilton, W. S., K. L. Rinehart, and M. D. Chilton.** 1984. Structure and stereochemistry of succinamopine. Biochemistry **23:**3290–3297.
13. **Conn, H. J.** 1942. Validity of the genus *Alcaligenes*. J. Bacteriol. **44:**353–360.
14. **Davioud, E., J.-C. Quirion, M. E. Tate, J. Tempé, and H. P. Husson.** 1988. Structure of cucumopine, a new crown gall and hairy root opine. Heterocycles **27:**2423–2429.
15. **De Ley, J.** 1972. *Agrobacterium*: intrageneric relationships and evolution, p. 251–259. *In* H. P. Maas Geesteranus (ed.), Proceedings of the Third International Conference on Plant Pathologenic Bacteria. University of Toronto Press, Toronto.
16. **De Ley, J., M. Bernaerst, A. Rassel, and J. Guilmot.** 1966. Approach to an improved taxonomy of the genus *Agrobacterium*. J. Gen. Microbiol. **43:**7–17.
17. **De Ley, J., R. Tijtgat, J. De Smedt, and M. Michiels.** 1973. Thermal stability of DNA-DNA hybrids within the genus *Agrobacterium*. J. Gen. Microbiol. **78:**241–252.
18. **Dessaux, Y., A. Petit, and J. Tempé.** 1992. Opines in *Agrobacterium* biology, p. 109–136. *In* D. P. S. Verma (ed.), Molecular signals in plant-microbe communications. CRC Press, Inc., Boca Raton, Fla.
19. **Dhaese, P., H. De Greve, H. Decraemer, J. Schell, and M. van Montagu.** 1979. Rapid mapping of transposon insertion and deletion mutations in the large Ti plasmids of *Agrobacterium tumefaciens*. Nucleic Acids Res. **7:**1837–1845.
20. **Dransart, V., A. Petit, C. Poncet, H. Bouzar, J. B. Jones, W. S. Chilton, and Y. Dessaux.** 1994. Identification and characterization of new pathogenic *Agrobacterium tumefaciens* strains, p. 221–228. *In* M. Lemattre, S. Freigoun, K. Rudolph, and J. G. Swings (ed.), Les colloques INRA Editions, Paris.
21. **Ellis, J. G., A. Kerr, M. Van Montagu, and J. Schell.** 1979. *Agrobacterium*: genetic studies on agrocin 84 production and the biological control of crown gall. Physiol. Plant Pathol. **15:**311–319.
22. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.

22a. **Felsenstein, J., and the University of Washington.** 1993. Phylip package, version 3.52c.

23. **Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk.** 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. **42:**166–170.
24. **Galloway, B. T.** 1919. Giant crowngalls from the Florida Everglades. Phytopathology **9:**207–208.
25. **Gelvin, S. B.** 1992. Chemical signaling between *Agrobacterium* and its host plant, p. 137–167. *In* D. P. S. Verma (ed.), Molecular signals in plant-microbe communications. CRC Press, Inc., Boca Raton, Fla.
26. **Gurtler, V., V. A. Wilson, and B. C. Mayall.** 1991. Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. J. Gen. Microbiol. **137:**2673–2679.
27. **Guyon, P., A. Petit, J. Tempé, and Y. Dessaux.** 1993. Transformed plants producing opines specifically promote growth of opine-degrading agrobacteria. Mol. Plant-Microbe Interact. **6:**92–98.
28. **Heberlein, G. T., J. De Ley, and R. Tijtgat.** 1967. Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium*, and *Chromobacterium*. J. Bacteriol. **94:**116–124.
29. **Holmes, B.** 1988. Taxonomy of *Agrobacterium*. Acta Hortic. (The Hague) **225:**47–52.
30. **Holmes, B., and P. Roberts.** 1981. The classification, identification, and nomenclature of agrobacteria. Incorporating revised descriptions for each of *Agrobacterium tumefaciens* (Smith & Townsend) Conn 1942, *Agrobacterium rhizogenes* (Riker et al.) Conn 1942, and *Agrobacterium rubi* (Hildebrand) Starr & Weiss 1943. J. Appl. Bacteriol. **50:**443–467.
31. **Isogai, A., N. Fukuchi, M. Hayashi, M. Kamada, H. Harada, and A. Suzuki.** 1988. Structure of a new opine, mikimopine, in hairy root induced by *Agrobacterium rhizogenes*. Agric. Biol. Chem. **52:**3235–3237.
32. **Jensen, R. E., W. T. Zdybak, K. Yasuda, and W. S. Chilton.** 1977. A useful synthesis of nopaline. Biochem. Biophys. Res. Commun. **75:**1066–1067.
33. **Juke, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, Inc., New York.
34. **Keane, P. J., A. Kerr, and P. B. New.** 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. **23:**585–595.
35. **Kersters, K., and J. De Ley.** 1984. Genus III. *Agrobacterium* Conn 1942, p. 244–254. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
36. **Kersters, K., J. De Ley, P. H. A. Sneath, and M. Sackin.** 1973. Numerical taxonomic analysis of *Agrobacterium*. J. Gen. Microbiol. **78:**227–239.
37. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44:**301–307.
38. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Michel, M. F., M.-C. Brasileiro, C. Depierreux, L. Otten, F. Delmotte, and L. Jouanin.** 1990. Identification of different *Agrobacterium* strains isolated from the same forest nursery. Appl. Environ. Microbiol. **56:**3537–3545.

40. **Moore, L. W., C. I. Kado, and H. Bouzar.** 1988. *Agrobacterium*, p. 16–36. *In* N. W. Schaad (ed.), Laboratory guide for identification of plant pathogenic bacteria, 2nd ed. APS Press, St. Paul, Minn.
41. **Navarro, E., P. Simonet, P. Normand, and R. Bardin.** 1992. Characterization of natural populations of *Nitrobacter* spp. using PCR-RFLP analysis of the ribosomal intergenic spacer. Arch. Microbiol. **157:**107–115.
42. **Nesme, X., C. Ponsonnet, C. Picard, and P. Normand.** 1992. Chromosomal and pTi genotypes of *Agrobacterium* strains isolated from *Populus* tumors in two nurseries. FEMS Microbiol. Ecol. **101:**189–196.
43. **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.
44. **Paulus, F., M. Ridé, and L. Otten.** 1989. Distribution of two Agrobacterium tumefaciens insertion elements in natural isolates: evidence for stable association between Ti plasmids and their bacterial hosts. Mol. Gen. Genet. **219:**145–152.
45. **Ponsonnet, C., and X. Nesme.** 1994. Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions. Arch. Microbiol. **161:**300–309.
46. **Riley, M.** 1993. Functions of the gene products of *Escherichia coli*. Microbiol. Rev. **57:**862–952.
47. **Rüger, H.-J., and M. G. Höfle.** 1992. Marine star-shaped aggregate-forming bacteria: *Agrobacterium atlanticum* sp. nov.; *Agrobacterium meteori* sp. nov.; *Agrobacterium ferrugineum* sp. nov., nom. rev.; *Agrobacterium gelatinovorum* sp. nov., nom. rev.; and *Agrobacterium stellulatum* sp. nov., nom. rev. Int. J. Syst. Bacteriol. **42:**133–143.
48. **Saitou, R. R., and M. Nei.** 1987. A neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **44:**406–425.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. **Sanger, F. S., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **7:**5463–5467.
51. **Sasser, M.** 1990. Identification of bacteria through fatty acid analysis, p. 199–204. *In* Z. Klement, K. Rudolph, and D. C. Sands (ed.), Methods in phytobacteriology. Akademiai Kiado, Budapest.
52. **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.
53. **Skerman, V. B. D., V. McGowan, and P. H. A. Sneath.** 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. **30:**225–420.
54. **Stonier, L.** 1960. *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. J. Bacteriol. **79:**880–898.
55. **Travelyan, W. E., D. P. Procter, and J. P. Harrisson.** 1950. Detection of sugars on paper chromatograms. Nature (London) **166:**444–445.
56. **Vaneechoutte, M., H. De Beenhouwer, G. Claeys, G. Verschraegen, A. De Rouck, N. Paepe, A. Elaichouni, and P. Portaels.** 1993. Identification of *Mycobacterium* species by using amplified ribosomal DNA restriction analysis. J. Clin. Microbiol. **31:**2061–2065.
57. **Vaneechoutte, M., R. Rossau, P. De Vos, M. Gillis, D. Jansens, N. Paepe, A. De Rouck, T. Fiers, G. Clayes, and K. Kersters.** 1992. Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). FEMS Microbiol. Lett. **93:**227–234.
58. **Vaudequin-Dransart, V., A. Petit, C. Poncet, C. Ponsonnet, X. Nesme, J. B. Jones, H. Bouzar, W. S. Chilton, and Y. Dessaux.** Novel Ti plasmids in *Agrobacterium* strains isolated from weeping fig and chrysanthemum tumors and their opine-like molecules. Mol. Plant-Microbe Interact., in press.
59. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, R. G. E. Murray, E. Stackbrandt, M. P. Starr, and H. G. Truper.** 1987. Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37:**463.
60. **White, L. O.** 1972. The taxonomy of the crown gall organism *Agrobacterium tumefaciens* and its relationship to rhizobia and other bacteria. J. Gen. Microbiol. **72:**565–574.
61. **Willems, A., and M. D. Collins.** 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. **43:**305–313.
62. **Winans, S. C.** 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiol. Rev. **56:**12–31.
63. **Woese, C. R., E. Stackebrandt, W. G. Weisburg, B. J. Paster, M. T. Madigan, V. J. Fowler, C. M. Hahn, P. Blanz, R. Gupta, K. H. Nealson, and G. E. Fox.** 1984. The phylogeny of purple bacteria: the alpha subdivision. Syst. Appl. Microbiol. **5:**315–326.
64. **Yanagi, M., and K. Yamasato.** 1993. Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. **107:**115–120.
65. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M12mp18 and pUC19 vectors. Gene **33:**103–113.
66. **Zweig, G., and J. Sherma.** 1972. Handbook of chromatography, vol. II. CRC Press, Inc., Cleveland.