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Short communication

Rapid identification of *Agrobacterium* species by staircase electrophoresis of low molecular weight RNA profiles

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Abstract

Several plant species are infected by different species of the genus *Agrobacterium*. One problem is that no rapid and sensitive method is available for the identification of isolates of *Agrobacterium* at the species level. The usefulness of LMW RNA profiles for the identification of *Agrobacterium* species was examined. The profiles of strains belonging to the proposed species were identical to those of the type strain of each species except in two cases. In *A. radiobacter*, two groups of strains with different tRNA profiles were detected and in *A. rhizogenes* two groups with different 5S rRNA zones were found. Nevertheless, with the LMW RNA profiles it was possible to assign any isolate to one of each group within these species. The results obtained showed that all isolates studied here can be assigned to a species of *Agrobacterium* and hence that LMW RNA profiles offer a suitable method for the identification of tumor-inducing bacteria.

Abbreviations: LMW RNA – low molecular weight RNA.

Currently, 11 species are described in the genus *Agrobacterium*, which includes terrestrial and marine bacteria (Rüger and Höfle, 1992). Pathogenic species of *Agrobacterium* induce crown gall tumors at the crown, on roots or on the aerial parts of plants (Kersters and de Ley, 1984). The ability of *Agrobacterium* strains to cause these infections depends on the presence of a tumor-inducing plasmid (pTi). Currently, five pathogenic species belonging to the genus *Agrobacterium* are accepted: *A. tumefaciens*, *A. rhizogenes*, *A. vitis*, *A. rubi* and *A. larrymoorei* (Bouzar and Jones, 2001) which includes the strains isolated from *Ficus benjamina* (Bouzar et al., 1995). The taxonomic position of the former species *A. radiobacter* is subject to controversy. Since the taxonomic revision of *Agrobacterium* by Sawada et al. (1993), *A. radiobacter* has not been considered to be separate from *A. tumefaciens*.

According to the recommendation of Bergey's Manual of Determinative Bacteriology (1994), the identification of strains isolated from plant tumors should be based on physiological and biochemical tests and completed by pathogenicity test in plants such as *Lycopersicon esculentum, Nicotiana tabacum* or *Helianthus annuus*. However, some strains isolated from plant tumors are not able to reproduce the symptoms and their identification is difficult. Identification based on plant symptoms, which is dependent on the bacterial plasmid content (pTi), is only useful for virulent strains. For non-pathogenic strains, other criteria related to the chromosomal background are necessary.

LMW RNA profiles have been used in the description of marine species from *Agrobacterium* (Rüger and Höfle, 1992). The method for separating LMW RNA molecules was modified to increase resolution using a new electrophoretic technique: staircase electrophoresis (Cruz-Sánchez et al., 1997). This new technique has been applied to the separation of LMW RNA profiles for the identification of genera and species of Gram-negative and -positive bacteria and yeast (Palomo et al., 2000; Velázquez et al., 1998a,b; 2000a; 2001a,b). In these profiles, three zones were clearly distinguished: 5S rRNA, class 1 tRNA and class 2 tRNA. According to these results, the 5S rRNA zone is characteristic of each genus and the tRNA profiles are characteristic of each species. In this way, the different genera have a distinctive 5S rRNA zone. All species of the same genus displaying the same 5S rRNA zone, whereas each species has a different tRNA profile. All strains from the same species have an identical LMW RNA profile.

In previous work, isolates of the genus *Agrobacterium* were distinguished from other genera of the family Rhizobiaceae in the 5S rRNA zone and different species of *Agrobacterium* were distinguished by their tRNA zone (Velázquez et al., 1998b). In ensuing works, using rhizobial isolates, the plasmid content was shown not to be related to the LMW RNA profiles (Velázquez et al., 2001b). As a result, these profiles allowed the rapid and reliable identification of nodulating legume species. In these bacteria, the capacity for nodulation and nitrogen fixation is coded in plasmids and the ability to induce plant tumors is related to the plasmid content.

In the light of the above, we have analysed the LMW RNA profiles of different strains of *Agrobacterium* isolated from plant tumors in order to evaluate the usefulness of these profiles in their identification. The strains used are listed in Table 1. Strains were isolated from young tumors that were washed with water and soap. Several pieces from each tumor were crushed in sterile water and each suspension was plated on YMA medium (Bergersen, 1961). The cultures were incubated for 72 h at 25 °C.

To identify the strains isolated, the following tests were performed according to Bergey's Manual of Determinative Bacteriology (1994) and Ridé et al. (2000): Gram's stain, urease, glucose oxidation and fermentation (Hugh-Leifson's medium), growth in 2% NaCl, β-glucosidase, β-galactosidase and acid production from melezitose, meso-erithrytol and ethanol. The results of these tests allow the

Table 1. Characteristics of strains used in this study

Strain	Host or source	Origin	Tumorigenicity (in this study)	LMW RNA profile type
AT1, AT2	*Phaseolus vulgaris*	This study	Negative	*A. radiobacter* type B
AT7	*Phaseolus vulgaris*	This study	Negative	*A. rhizogenes* type B
AT8, AT9, AT10, AT11	*Beta vulgaris*	This study	Positive	*A. tumefaciens*
AT14	*Beta vulgaris*	This study	Negative	*A. radiobacter* type B
163C, 97/546	*Prunus persica*	This study	Positive	*A. rhizogenes* type A
AV30, AV12, AV20	*Vitis vinifera*	This study	Positive	*A. vitis*
A. tumefaciens	*Malus* sp.	ATCC 23308^T	Positive	*A. tumefaciens*
A. tumefaciens		CECT 4365 (LMG 185)		*A. tumefaciens*
A. tumefaciens	*Rubus idaeus*	CECT 4364 (LMG 181)		*A. tumefaciens*
A. tumefaciens	Soil	CECT 4363 (LMG 169)		*A. tumefaciens*
A. radiobacter	Soil	CECT 4362 (LMG 142)		*A. radiobacter* type B
A. radiobacter		CECT 4360 (LMG 139)		*A. radiobacter* type A
A. radiobacter		ATCC 19358^T		*A. radiobacter* type A
A. rhizogenes		ATCC 13332		*A. rhizogenes* type B
A. rhizogenes	*Malus sylvestris*	ATCC 25818		*A. rhizogenes* type B
A. rhizogenes		ATCC 11325^T		*A. rhizogenes* type A
A. rubi	*Rubus* sp.	ATCC 13335^T		*A. rubi*
A. rubi	*Rubus* sp.	ATCC 13334		*A. rubi*
A. vitis	*Vitis vinifera*	CECT 4799^T	Positive	*A. vitis*
AV 550–25, AV 550–28	*Vitis vinifera*	IVIA	Positive	*A. vitis*
AV 63/85e, AV 339–26	*Vitis vinifera*	IVIA	Positive	*A. vitis*

ATCC: American Type Culture Collection, Manassas, VA, USA; LMG, Collection of bacteria of the Laboratory voor Microbiologie, Gent, Belgium; CECT, Spanish Type Culture Collection, Valencia, Spain; IVIA: Instituto Valenciano de Investigaciones Agrarias.,Valencia, Spain.

Table 2. Physiological and biochemical characteristics of strains isolated in this study

	AT1	AT2	AT14	AT7	AT8	AT9	AT10	AT11	163C	97/546	AV30	AV12	AV20
Growth in presence of 2% NaCl	+	+	+	−	+	+	+	+	−	−	+	+	+
Acid reaction produced from													
Meso-erythritol	−	−	−	+	−	−	−	−	+	+	−	−	−
Melezitose	w	w	w	−	+	+	+	+	−	−	−	−	−
Ethanol	+	+	+	−	+	+	+	+	−	−	w	w	w
Enzyme production													
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+
PNP-β-Dglc	+	+	+	+	+	+	+	+	+	+	+	+	+
PNP-β-Dgal	+	+	+	+	+	+	+	+	+	+	+	+	+

(+), positive result; (−), negative result; (w), weakly postive. PNP-β-Dglc: Paranitrophenyl-β-D-glucopyranoside; PNP-β-Dgal: Paranitrophenyl-β-D-galactopyranoside.

differentiation of the species from *Agrobacterium* and are shown in Table 2.

According to the results obtained, and following Bergey's identification schemes, strains AT1, AT2, AT8, AT9, AT10, AT11, AT14 and AT7 were identified as *A. tumefaciens* (biovar 1) or *A. radiobacter* (biovar 1), which according to Bergey's Manual are not distinguishable. Strains 163C and 97/546 were identified as *A. rhizogenes* (biovar 2). The phenotypic data of *A. vitis* are not included in Bergey's Manual of Determinative Bacteriology, but do appear in Ophel and Kerr (1990). The strains isolated from *Vitis vinifera* were identified as *A. vitis* (biovar 3). The production of β-glucosidase and β-galactosidase (included in the tests performed by Ridé et al., 2000) was positive in all strains.

The pathogenicity tests of strains isolated from *Phaseolus*, *Beta* and *Prunus* were performed on tomato plants (*Lycopersicon esculentum*, cultivar Roma). Five plants per strain were inoculated between the cotyledons and the first true leaves with a syringe, applying 10 µl of a suspension of ca. 10^8 cfu/ml prepared from a 48 h Nutrient Agar plate culture. A positive control with the type strain *A. tumefaciens* ATCC23308^T and a negative control with sterile water were also included. The plants were maintained in a greenhouse for 30 days at a temperature of 18–25 °C. Strains isolated from *Vitis vinifera* were inoculated on green shoots of grapevine plants (*Vitis vinifera* cultivar Alicante Bouschet) between the first and third internodes. To do this, an incision was made with a sterile cutter and then the shoots were inoculated with a syringe, applying 15 µl of a suspension of ca. 10^8 cfu/ml prepared from a 48 h Nutrient Agar plate culture. The plants were maintained in the greenhouse for 8 weeks. A positive control with the type strain *A. vitis* CECT 4799^T and a negative control with sterile water were also included.

According to the results obtained, strains AT1, AT2, AT7 and AT14 did not cause tumors in tomato plants. Strains AT8, AT9, AT10, AT11, 163C and 97/546 were pathogenic. All strains isolated from tumors in *Vitis vinifera* were able to reproduce the symptoms in this plant.

LMW RNA extraction was accomplished following the technique described by Höfle (1988). LMW RNA profiles were obtained using staircase electrophoresis in 14% polyacrylamide gels under denaturing conditions in steps of 10 min, rising through a constant ramp with 50 V increases from 100 to 2300 V (Cruz-Sánchez et al., 1997). The following commercial molecules from Boehringer Manheim (Manheim, Germany) and Sigma (St. Louis, MO, USA) were used as references: 5S rRNA from *Escherichia coli* MRE 600 (120 and 115 nucleotides) (Bidle and Fletcher, 1995); tRNA specific for tyrosine from *E. coli* (85 nucleotides), and tRNA specific for valine from *E. coli* (77 nucleotides) (Sprinzl et al., 1985). Samples were prepared as reported elsewhere (Cruz-Sánchez et al., 1997). After electrophoresis, the gels were silver-stained as described by Haas et al. (1994).

Figure 1 shows the LMW RNA profiles of reference strains of *Agrobacterium* species. The 5S rRNA zone was variable across the reference strains of *Agrobacterium*. The 5S rRNA profiles were the same in type strains of *A. tumefaciens* and *A. rubi*. (Figure 1A, lanes 1 and 2). Also, the type strain of the former species *A. radiobacter* displayed an identical 5S rRNA zone with respect to these two species (Figure 1A, lane 6). However, the type strains of *A. vitis* (Figure 1A, lane 3) and *A. rhizogenes* (Figure 1A,

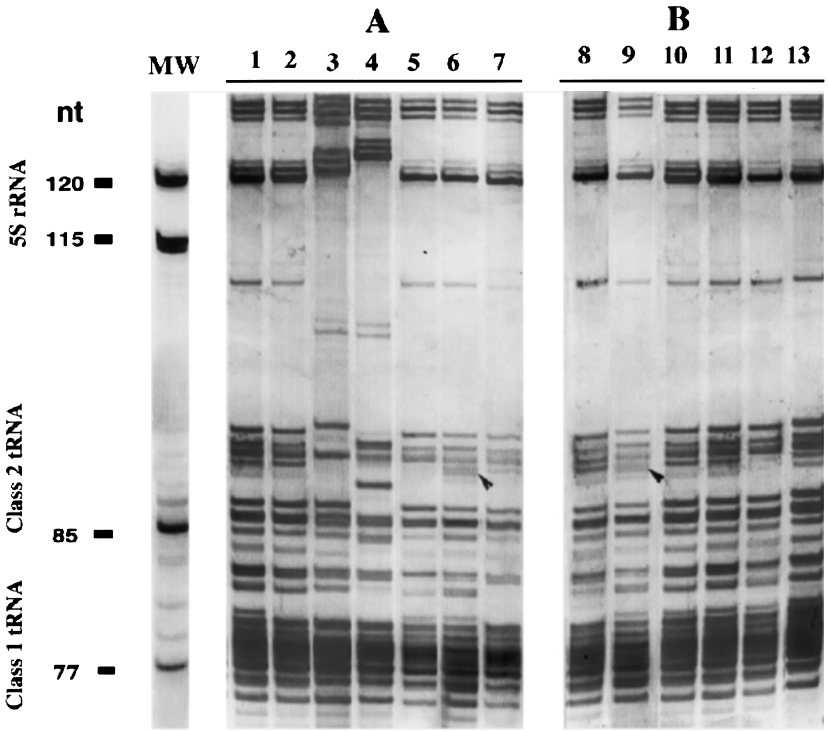

Figure 1. LMW RNA profiles of the reference strains of genus *Agrobacterium*: Lane (1) *A. tumefaciens* ATCC 23308^T, lane (2) *A. rubi* ATCC13335^T, lane (3) *A. vitis* CECT 4799^T, lane (4) *A. rhizogenes* 11325^T (LMW RNA profile type A), lane (5) *A. rhizogenes* ATCC 13332 (LMW RNA profile type B), lane (6) *A. radiobacter* ATCC 19358^T (LMW RNA profile type A), lane (7) *A. radiobacter* CECT 4362 (LMW RNA profile type B), lane (8) *A. rubi* ATCC 13334, lane (9) *A. radiobacter* CECT 4360, lane (10) *A. tumefaciens* CECT 4365, lane (11) *A. tumefaciens* CECT 4364, lane (12) *A. rhizogenes* ATCC 25818, lane (13) *A. tumefaciens* CECT 4363. The arrows marked an additional band present in LMW RNA profile of *A. radiobacter* type A with respect to LMW RNA profile of *A. tumefaciens*.

lane 4) displayed different patterns in this zone both between each other and with respect to *A. tumefaciens* and *A. rubi*. As can be seen in Figure 1, the reference strains from *A. rhizogenes* displayed two types of 5S rRNA zone. In Figure 1A, the type A profile is represented by the type strain of this species (Figure 1A, lane 4) and the type B profile is represented by strains ATCC1332 and ATCC 25818 (Figure 1A, lanes 5 and Figure 1B, lane 12). Only the type A profile differed in the 5S rRNA zone with respect to the type strains of *A. tumefaciens* and *A. rubi*. This had already been observed (Velázquez et al., 1998b) and is related to the differences in 16S rRNA gene sequences among species from *Agrobacterium* (de Lajudie et al., 1998; 1999). In the phylogenetic trees obtained by these authors the type strain of *A. rhizogenes* was more closely related to *Rhizobium* species than to the rest of the species from *Agrobacterium*. Also, the type strain of *A. vitis* was more closely related to *Allorhizobium undicola* than to the other species from *Agrobacterium*.

The LMW RNA profiling results are also consistent with the results of phylogenetic analyses of 23S rRNA (Pulawska et al., 2000). The results obtained in this study revealed the genetic heterogeneity of *Agrobacterium* species, but do not support the recent classification of the whole genus *Agrobacterium* as *Rhizobium* (Young, 2001), because it may be inferred, from the 5S rRNA zone, that the genus *Agrobacterium* is genetically heterogeneous and may include several genera. In this sense, all species from *Agrobacterium* included in this study were distinguished by their tRNA profiles, including the reference strains from the former species *A. radiobacter*. Therefore, a taxonomic revision of tumor-inducing bacteria is necessary, in the same way as has been done for rhizobial isolates, to establish the correct affiliation of species currently included in *Agrobacterium*.

All reference strains from *A. tumefaciens* (Figure 1B, lanes 10, 11 and 13) displayed the same tRNA profile as the type strain of this species (Figure 1A, lane 1). Also,

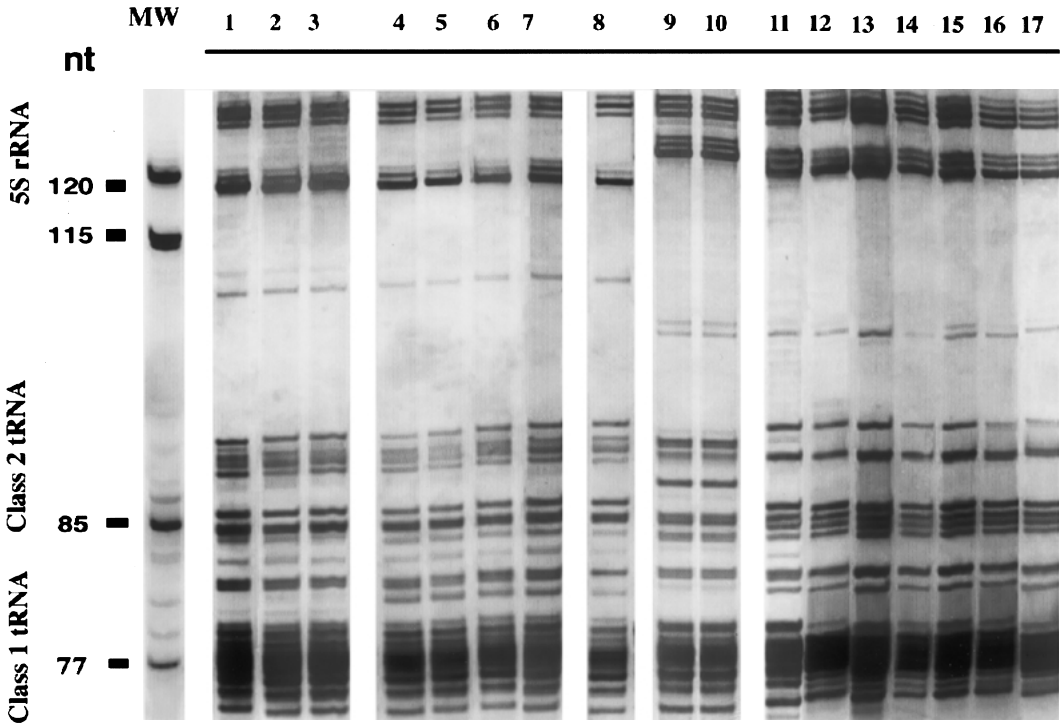

Figure 2. LMW RNA profiles of tumor-inducing strains isolated in this study: Lane (1) AT1; lane (2) AT2, lane (3) AT14, lane (4) AT8, lane (5) AT9, lane (6) AT10, lane (7) AT11, lane (8) AT7, lane (9) 163C, lane (10) 97/546, lane (11) AV 30, lane (12) AV 550–25, lane (13) AV 63/85e, lane (14) AV 12, lane (15) AV 339–26, lane (16) AV 550–28 lane (17) AV 20.

the reference strain *A. rubi* ATCC 13334 (Figure 1B, lane 8) displayed the same tRNA profile as the type strain of this species (Figure 1, lane 2). The strains of *A. rhizogenes* that displayed a type A profile can be distinguished from the strains of the same species displaying type B profile in tRNA zone as well as in 5S rRNA zone (Figure 1A, lanes 4 and 5).

In the case of the former species *A. radiobacter*, differences in the tRNA profiles were found among the strains classified as this species. In Figure 1A, the type A profile is represented by the type strain of the former species *A. radiobacter* (Figure 1A, lane 6) and by strain CECT 4362 (Figure 1B, lane 9). The type B profile is represented by strain CECT 4360 (Figure 1A, lane 7). *A. radiobacter* and *A. tumefaciens* are not currently considered as separate species, but according to our results their chromosomal backgrounds are indeed closely related, but are not identical: *A. radiobacter* has an additional band in class 1 tRNA with respect to *A. tumefaciens* (marked with an arrow in Figure 1). These results suggest that these two former species may be two subspecies of the same species, although further studies involving polyphasic taxonomy are necessary to establish a more definitive conclusion.

The same method that was used to determine LMW RNA profiles of reference strains was also used to identify isolates from tumors of different plants. The plasmid profiles of these strains were also analysed (Figure 3), because to be able to identify non-pathogenic strains using LMW RNA profiles, it was necessary to establish that they were not dependent on the plasmid profile. Among the isolates from tumors present in *Phaseolus vulgaris* and *Beta vulgaris*, three different LMW RNA profiles were observed (Figure 2). Strains AT8, AT9, AT10 and AT11 had the same profile as *A. tumefaciens* (lanes 4–7). Strains AT1, AT2 and AT14 (lanes 1–3) had the same LMW RNA profile as *A. radiobacter* type B. Finally, strain AT7 (lane 8) had the type B profile from *A. rhizogenes.* Therefore, LMW RNA profiles have a potential in species differentiation that phenotypic tests fail to provide.

The two isolates from *Prunus* (Figure 2) had the same LMW RNA profile as *A. rhizogenes* type A (lanes 9 and 10). The type strain of *A. rhizogenes* is

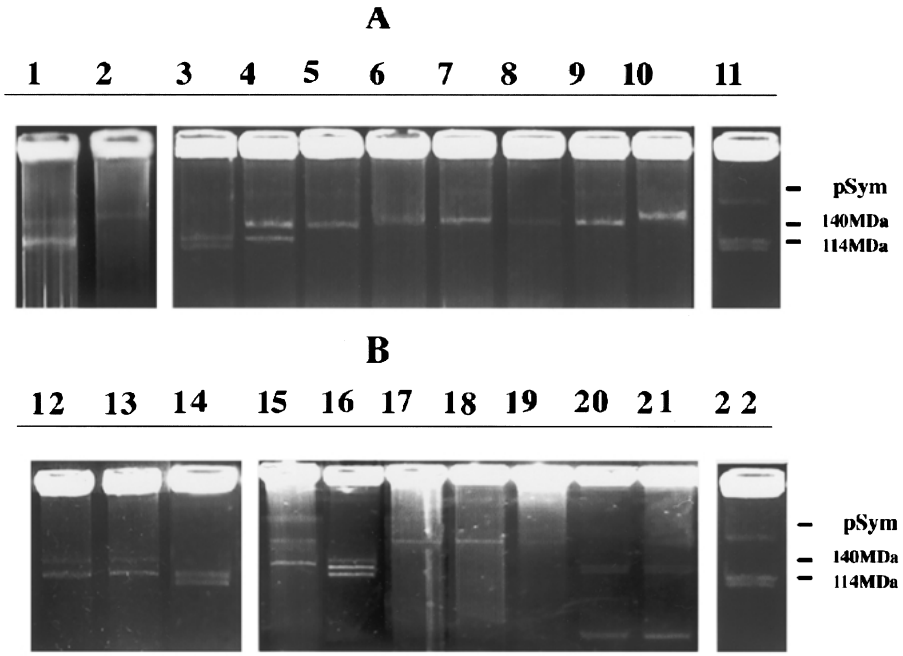

Figure 3. Plasmid profiles of strains used in this study. A: Lane (1) *A. tumefaciens* ATCC 23308^T, lane (2) *A. radiobacter* ATCC 19358^T, lane (3) AT1, lane (4) AT2, lane (5) AT8, lane (6) AT9, lane (7) AT10, lane (8) AT11, lane (9) AT14, lane (10) AT7, lane (11) *Sinorhizobium meliloti* GR4. B: lane (12) 163C, lane (13) 97/546, lane (14) *A. rhizogenes* 13332^T, lane (15) *A. vitis* CECT 4799^T, lane (16) AV12, lane (17) AV30, lane (18) AV 63/85e, lane (19) AV339–26, lane (20) AV550–25, lane (21) AV550–28, lane (22) *Sinorhizobium meliloti* GR4.

able to induce hairy roots in plants, but this species also included tumorigenic strains harbouring a Ti plasmid (Sawada et al., 1993). Here, the ability of these two strains to induce hairy roots was not tested because LMW RNA profiling clearly identified the strains as *A. rhizogenes*. The isolates from *Vitis vinifera* (Figure 2, lanes 11–17) had the same LMW RNA profiles that was identical to that of the type strain of *A. vitis* (Figure 1, lane 3).

To compare the LMW RNA profiles and plasmid profiles, the strains isolated from tumors were also subjected to plasmid profile analysis (Plazinski et al., 1985). The electrophoretic procedure was modified as follows: electrophoresis was carried out at 30 V for 90 min, at 60 V for 60 min, and at 40 V for 3 h. The plasmids of *Sinorhizobium meliloti* GR4, which has a cryptic plasmid of 140 MDa and another of 114 MDa (Toro and Olivares, 1986), were used as size markers.

Figure 3A,B shows the plasmid profiles of all strains isolated in this study and those of type strains from tumor-inducing *Agrobacterium* species. The three non-pathogenic strains identified as *A. radiobacter* (type B), (AT1, AT2 and AT14 (lanes 3, 4 and 9)) had different plasmid contents. However, they had the same LMW RNA profile. Strains AT8, AT9, AT10 and AT11 (lanes 5–8) only had one plasmid of identical size and this was not the same size as the plasmids of the type strain of *A. tumefaciens* (lane 1). Strains 163C and 97/546 had the same plasmid profile (lanes 12–13). This profile is different from the plasmid profile of the type strain of *A. rhizogenes* (lane 14). Nevertheless, the three strains displayed the same LMW RNA profile. Finally, the strains isolated from tumors on *Vitis vinifera* (lanes 16–21) also had several plasmid profiles that differed with respect to the plasmid profile of the type strain of *A. vitis*. (lane 15). Despite this, the LMW RNA profile of all these strains (including the type strain) was the same.

It may thus be concluded that the plasmid content of agrobacterial strains, as well as of rhizobial strains (Velázquez et al., 2001b), was not reflected in the LMW RNA profiles. Neither are the LMW RNA profiles dependent on physiological, biochemical or pathogenic characteristics of the strains. These profiles therefore allow the identification of pathogenic and non-pathogenic strains of genus *Agrobacterium*

and can thus be used in rapid and sensitive identification of species from this genus. Also, the LMW RNA profiles showed that a more extensive taxonomic revision of the new Family *Rhizobiaceae* should be made before establishing the taxonomic level of each genus and species included in this Family.

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