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Plasmid Heterogeneity in Spanish Isolates of *Agrobacterium tumefaciens* from Thirteen Different Hosts

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Plasmid DNA was isolated from 80 Spanish isolates of *Agrobacterium tumefaciens* from 13 hosts of several geographical and temporal origins. One to five plasmids occurred in all of the isolates studied. Plasmid sizes varied between 5 and greater than 1,000 MDa. Generally, there was no correlation between plasmid number or size and geographical origin, host, biovar, sensitivity to agrocin 84, or opine-catabolizing ability of the different isolates.

Crown gall, caused by *Agrobacterium tumefaciens*, is widespread in Spain on different hosts (18). Symptoms of the disease are common in fruit tree and rose nurseries; however, sometimes symptoms do not occur in plants until 1 or 2 years after being planted in the field, and it is difficult to determine if the pathogen was introduced via infected plant material or was already present in the soil.

Plasmid profile analysis has been used in epidemiological studies of clinical gram-positive and -negative bacteria. The method has been applied to *Clostridium* (23), *Salmonella* (14), *Klebsiella* (15), *Enterobacter* (19), *Shigella* (32), and *Legionella* spp. (25). In plant pathogenic bacteria, indigenous plasmids have been analyzed in *Corynebacterium* (12, 6). *Pseudomonas* (2, 7, 22), and *Xanthomonas* (5, 17, 28) spp., but only in studies with plasmids of *Xanthomonas campestris* pv. *citri* and *X. campestris* pv. *pruni* was there any attempt to correlate the origins of the isolates with their plasmid contents (5, 17, 28).

Plasmids of *A. tumefaciens* have been analyzed in detail, but no extensive plasmid profiling has been done. One, two, or three plasmids have been reported to occur (8, 26, 29, 30, 33–35), but only a limited number of reference strains have been assayed. There is no information available about the plasmid profile of *A. tumefaciens* isolates from different areas in one country or from different hosts in the same area.

The purposes of this article were (i) to study the distribution of plasmids in a collection of Spanish isolates of *A. tumefaciens* from different hosts of several geographical and temporal origins and (ii) to evaluate the usefulness of plasmid profile analysis to determine the geographical origin of the isolates implicated in epidemic outbreaks of crown gall disease.

Eighty isolates of *A. tumefaciens* from tumors on 13 different hosts were used. The origins and characteristics of the isolates are listed in Table 1. Biovar, agrocin 84 sensitivity, and opine utilization of most of the isolates included in this article have been reported previously by Lopez et al. (18). The same methods were used for the isolates described here, but not included in the previous article. Isolates with the same three-digit prefix came from the same nursery or orchard. The molecular sizes of the plasmids were estimated from their relative mobilities in agarose gels (20) as compared with those of molecular size markers: *Escherichia coli* V517 plasmids pVA517B (4.8 MDa) and pVA517A (35.8 MDa), *Rhizobium meliloti* L5-30 plasmids pRme-L530 (91 MDa) and pRme-MVII-1 (994 MDa), *Rhizobium leguminosarum* T83K3 plasmids pRL7J1 (100 MDa) and pRL12J1 (310 MDa), and *A. tumefaciens* C58 plasmids pTi58 (130 MDa) and pAtC58 (273 MDa). Plasmids were identified by the in-well lysis and electrophoresis procedure of Eckhart (9), with minor modifications. Briefly, 1×10^8 to 6×10^8 cells were grown with shaking at 28°C in 5 ml of L broth (1% tryptone, 0.5% yeast extract, 1% NaCl) for *A. tumefaciens* and *E. coli*. For some *A. tumefaciens* biovar 2 isolates, 0.004% biotin and 0.2% sodium glutamate were added. TY medium (0.5% tryptone, 0.1% yeast extract, 0.084% $CaCl_2$) was used for *Rhizobium* spp. Bacterial cells were centrifuged at 5,000 × *g* for 15 min at 4°C. The pellet was carefully resuspended in 20 μl of a lysis mixture containing 20% Ficoll 400, 0.05% bromophenol blue, 10 μl of RNase A per ml, and 1 mg of lysozyme per ml in Tris-borate-EDTA buffer (TBE) (89 mM Tris, 2.5 mM Na_2 EDTA, 89 mM boric acid [pH 8.2]). The suspension was immediately transferred into the well of a vertical 0.8% agarose (type A, Pharmacia) gel (3 by 137 by 140 mm) in TBE. The lysozyme was omitted for some *Agrobacterium* isolates (Table 1). The number of cells per well was critical for visualization of the large plasmids by the Eckhart technique (9). The presence of more than 6×10^8 cells resulted in gel overloading with high-chromosomal DNA background and reduced the yield of intact plasmid DNA. For *Agrobacterium* species and *E. coli*, 30 μl of a sodium dodecyl sulfate (SDS) mixture (0.2% SDS, 10% Ficoll 70 in TBE) was carefully layered on top of the bacterial sample, and 100 μl of an overlay mixture (0.2% SDS, 5% Ficoll 70 in TBE) was added without disturbing the SDS-lysozyme layers. For *Rhizobium* spp., 40 μl of an SDS mixture (2% SDS, 10% Ficoll 70 in TBE) was added, and the overlay mixture was 2% SDS and 5% Ficoll 70 in TBE. The slots were sealed with molten agarose, and the plasmid DNA was separated by electrophoresis at 4 mA for 30 min, 33 mA for 15 min, and 15 mA for 3 h. The gel was stained with 0.5 μg of ethidium bromide per ml of TBE and photographed under UV light. The identification of the three possible forms of plasmid DNA in bands visualized in the gels was determined by the method of Hintermann et al. (13). Bands were considered to be L DNA when their mobilities on agarose gels were not altered by treatment with ethidium bromide and UV light (13) and their presence, absence, or proportion varied with each plasmid DNA preparation. The relative plasmid mobilities were determined with a laser densitome-

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TABLE 1. Characteristics of the 80 *Agrobacterium* Spanish strains and grouping by their plasmid profiles

Strain[a]	Origin	Host[b]	Biovar	Agrocin 84[c]	Opine[d]			Plasmid size(s) (MDa)[e]	Plasmid pattern[f]
					O	N	M		
369-3	Guadalajara	Plum	2	R	−	+	−	200, 115	1
251-11	Badajoz	Plum	2	S	−	+	−	200, 115	1
545-2	Castellón	Quince	2	S	−	+	−	200, 115	1
369-4	Guadalajara	Plum	2	R	−	+	−	200, 115, 33, 10	2
308-3	Zaragoza	Plum	2	S	−	+	−	115, 80	3
388-78*	Zaragoza	Almond	2	S	−	+	−	115, 80	3
388-7*	Zaragoza	Almond	2	S	−	+	−	115, 80	3
020-9	Valencia	Peach	2	S	−	+	−	115, 80	3
261-1	Badajoz	Poplar	2	R	−	+	−	180, 125, 120	4
012-6	Badajoz	Plum	2	R	−	+	−	180, 125, 120	4
014-15	Zaragoza	Peach	2	R	−	+	−	180, 125, 120	4
206-2	Zaragoza	Peach	2	R	−	+	−	180, 125, 120	4
251-15	Badajoz	Plum	2	R	−	+	−	130, 100	5
308-4	Zaragoza	Plum	2	S	+	+	−	130, 100	5
388-1*	Zaragoza	Almond	2	S	−	+	−	130, 100	5
325-4	Tarragona	Peach × almond	1	S	−	+	−	130, 100	5
251-22	Badajoz	Plum	2	S	−	+	−	130	6
251-3	Badajoz	Plum	2	S	−	+	−	130	6
251-21	Badajoz	Plum	2	R	−	+	−	300, 115, 105, 6	7
018-72	Logroño	Almond	2	R	−	+	−	300, 115, 105, 6	7
020-3	Valencia	Peach	2	S	−	+	−	300, 115, 105, 6	7
576-71	Cuenca	Osier	1	S	−	+	−	300, 115, 105, 6	7
576-72	Cuenca	Osier	1	S	−	+	−	300, 115, 105, 6	7
017-24	Valencia	Plum	2	R	−	+	−	180, 120	8
017-23	Valencia	Plum	2	R	−	+	−	180, 120	8
024-56*	Zaragoza	Peach	2	S	−	+	−	180, 120	8
024-51*	Zaragoza	Peach	2	S	−	+	−	180, 120	8
206-6*	Zaragoza	Peach	2	S	−	+	−	180, 120	8
339-37	Orense	Grapevine	3	R	+	+	−	180, 120	8
339-6	Orense	Grapevine	3	R	+	+	−	180, 120	8
565-52*	Badajoz	Grapevine	3	R	−	+	−	180, 120	8
565-51*	Badajoz	Grapevine	3	R	+	−	−	180, 120	8
565-11*	Badajoz	Grapevine	3	R	−	+	−	180, 120	8
014-3*	Zaragoza	Peach	2	R	−	+	−	180, 125	9
085-1	Castellón	Walnut	1	S	−	+	−	270, 130	10
347-4	Valencia	Peach	1	R	−	+	−	120, 110	11
347-8	Valencia	Peach	1	S	−	+	−	240, 135, 125, 105	12
354-14	Valencia	Almond	1	S	−	+	−	240, 130	13
325-7	Tarragona	Peach × almond	2	S	−	+	−	240, 130	13
545-3	Castellón	Quince	2	S	−	+	−	240, 130	13
206-3*	Zaragoza	Peach	2	S	−	+	−	120	14
018-72	Logroño	Almond	2	S	−	+	−	120	14
07-98*	Valencia	Apricot	2	R	−	+	−	180, 125, 105, 12	15
436-31	Zaragoza	Peach × almond	2	S	−	+	−	160, 120, 100, 10	16
436-32	Zaragoza	Peach × almond	2	S	−	+	−	160, 120, 100, 10	16
436-1	Zaragoza	Peach × almond	2	S	−	+	−	230, 160, 120, 100, 10	17
436-2	Zaragoza	Peach × almond	1	R	−	−	+	230, 160, 120, 100, 10	17
436-3	Zaragoza	Peach × almond	1	R	−	−	+	230, 160, 120, 100, 10	17
325-5	Tarragona	Peach × almond	1	S	−	+	−	270, 125	18
467-7	Cuenca	Osier	1	S	−	+	−	300, 125	19
570-6	Cuenca	Osier	1	S	−	+	−	300, 125	19
570-13	Cuenca	Osier	1	S	−	+	−	300, 125	19
570-11	Cuenca	Osier	1	S	−	+	−	300, 125	19
575-3	Cuenca	Osier	1	S	−	+	−	300, 125	19
576-2	Cuenca	Osier	2	S	−	+	−	300, 125	19
260-21	Badajoz	Poplar	2	S	−	+	−	300, 125	19
260-22	Badajoz	Poplar	2	S	−	+	−	300, 125	19
257-3*	Lérida	Cherry	2	S	−	+	−	160, 120	20
257-9*	Lérida	Cherry	2	S	−	+	−	160, 120	20
257-4*	Lérida	Cherry	2	S	−	+	−	160, 120	20
354-1*	Valencia	Almond	2	R	−	+	−	160, 120	20
354-2*	Valencia	Almond	2	R	−	+	−	160, 120	20
018-63	Logroño	Almond	2	R	−	+	−	160, 120	20
018-93*	Logroño	Almond	2	S	−	+	−	240, 120, 80	21
455-8	Tenerife	Raspberry	2	S	−	+	+	120, 30	22
455-7	Tenerife	Raspberry	2	R	−	−	+	115	23

Continued on following page

TABLE 1—*Continued*

Strain[a]	Origin	Host[b]	Biovar	Agrocin 84[c]	Opine[d]			Plasmid size(s) (MDa)[e]	Plasmid pattern[f]
					O	N	M		
455-9	Tenerife	Raspberry	2	R	−	−	+	115	23
455-69	Tenerife	Raspberry	2	S	−	−	+	115	23
287-7	Sevilla	Rose	1	R	−	+	−	260, 120, 115	24
278-3	Tenerife	Rose	1	S	−	+	−	260, 120, 115	24
282-1	Tenerife	Rose	2	S	−	+	−	230, 115	25
280-11	Tenerife	Rose	2	R	−	+	+	115, 90	26
575-7	Cuenca	Osier	1	S	−	+	−	300, 115, 105, 5	27
570-4	Cuenca	Osier	1	S	−	+	−	300, 115, 105, 5	27
575-8	Cuenca	Osier	1	S	−	+	−	300, 160, 125, 100, 5	28
570-12	Cuenca	Osier	1	S	−	+	−	300, 125, 11	29
565-59	Badajoz	Grapevine	3	R	−	+	−	1,000, 300, 200, 125, 115	30
565-58	Badajoz	Grapevine	3	R	−	+	−	1,000, 300, 200, 125, 115	30
550-22*	Pontevedra	Grapevine	3	R	+	−	−	200, 125	31
550-27*	Pontevedra	Grapevine	3	R	+	−	−	200, 125	31

[a] Strains from which lysozyme was omitted in plasmid extraction are indicated by an asterisk.
[b] Hosts: plum, *Prunus domestica*; quince, *Cydonia oblonga*; almond, *P. dulcis* (*P. amygdalus*); poplar, *Populus alba*; peach, *P. persica*; peach × almond, *P. persica* × *P. dulcis*; osier, *Salix americana*; grapevine, *Vitis vinifera*; walnut, *Juglans regia*; apricot, *P. armeniaca*; cherry, *P. avium*; raspberry, *Rubus idaeus*; rose, *Rosa indica*.
[c] Resistant (R) or sensitive (S) to agrocin 84.
[d] Opine utilized: O, octopine; N, nopaline; M, mannopinic acid.
[e] Approximate sizes of plasmid DNA.
[f] Isolates within each plasmid pattern contained plasmids that comigrated on agarose gel.

ter (LKB 2020 Ultroscan) and a model 3390A Hewlett-Packard integrator. There was a linear relationship between the logarithm of the molecular mass and the relative mobility (20) in the range of 4.8 to 273 MDa, as confirmed by a BASIC program based on the model of Plikaytis et al. (27). The errors in the molecular sizes of the plasmids were calculated, and plasmids larger than 30 MDa were affected by an increasing error (30 ± 0.5 to 270 ± 20 MDa). For plasmids with molecular sizes greater than 270 MDa, the estimated values should be considered as minimums rather than exact determinations of molecular weight. These results agree with those obtained with *Rhizobium* plasmids (4). The correlation between the characteristics of each *Agrobacterium* isolate (geographical origin, host, biovar, sensitivity to agrocin 84, and opine-catabolizing ability) and its plasmid profile was evaluated by using the program BMDP.4F (BMDP Statistical Package, University of California at Los Angeles); to study the joint occurrence of plasmid characteristics, DBASE III PLUS (Ashton Tate, Inc.) was used.

Plasmid DNA was observed in the 80 Spanish isolates of *A. tumefaciens*. Some of them contained as many as five plasmids. The isolates were grouped in 31 patterns (Table 1). These patterns were consistently observed in at least three different extractions for each isolate. Some of the patterns are shown in Fig. 1. Generally, there was a high degree of variation in the plasmid profiles among isolates having the same geographical origin or similar characteristics (host, biovar, sensitivity to agrocin 84, and opine-catabolizing ability). All of the isolates studied had one to three plasmids with molecular sizes of 100 to 160 MDa, one of them probably the Ti plasmid. Fifty percent of the isolates had two plasmids with molecular sizes of 100 to 160 mDa and 160 to 270 MDa. Twenty-five percent had plasmids with molecular sizes of greater than 270 MDa, and about 20% of the isolates had small plasmids with molecular sizes of 5 to 10 MDa. There were wide differences in the plasmid sizes among isolates of biovars 1 and 2, whereas all biovar 3 isolates had plasmids of between 100 and 1,000 MDa but no low-molecular-weight plasmids. Variability in plasmid profile also was observed among isolates from the same host. Nevertheless, all of the isolates from osier plants had two plasmids of 300 MDa and 125 MDa, as did two poplar isolates. All of the isolates from raspberry plants had one plasmid of 115 to 120 MDa, and isolates from cherry plants had two plasmids of 120 and 160 MDa. On the other hand, isolates with each of

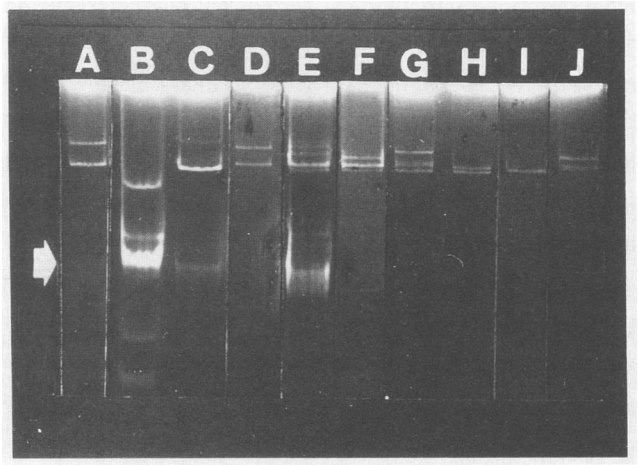

FIG. 1. Representative plasmid patterns of the *A. tumefaciens* isolates. Lanes: A, *A. tumefaciens* 260-22 (300 and 125 MDa, plasmid pattern 19); B, *E. coli* V517 (35.8, 4.8, and 3.7 MDa) (the band observed above chromosomal DNA was L DNA); C, *R. meliloti* (994 and 91 MDa); D, *A. tumefaciens* C58 (273 and 130 MDa); E, *A. tumefaciens* 565-58 (1,000, 300, 200, 125, and 115 MDa, plasmid pattern 30)·(the smaller band observed above chromosomal DNA was considered to be L DNA); F, *A. tumefaciens* 436-31 (160, 120, 100, and 10 MDa, plasmid pattern 16); G, *A. tumefaciens* 347-8 (240, 135, 125, and 105 MDa, plasmid pattern 12); H, *A. tumefaciens* 347-4 (120 and 110 MDa, plasmid pattern 11); I, *A. tumefaciens* 455-7 (115 MDa, plasmid pattern 23); J, *A. tumefaciens* 206-2 (180, 125, and 120 MDa, plasmid pattern 4). Arrow indicates chromosomal DNA.

the patterns 6, 16, 24, 27, 30, and 31 had similar characteristics. Isolates with patterns 3, 4, and 14 had different origins and hosts. Isolates with pattern 23 showed different sensitivity to agrocin 84. Isolates with patterns 1, 5, 7, 8, 13, 19, and 20 differed in more than three characteristics. The rest of the patterns in Table 1 corresponded to unique isolates. The diversity of plasmid profiles obtained indicate that a large number of different *Agrobacterium* strains are present in Spain and that, in most cases, crown gall outbreaks most likely originate from a mixture of strains.

Plasmid profiles did not appear to be a useful tool to identify the origin of *A. tumefaciens*, since isolates from the same origin and host (indicated by the same three-digit prefix in Table 1) showed different profiles. Nevertheless, in some cases, isolates from the same origin and host had identical profiles (i.e., isolates 017-23 and 017-24; 257-3, 257-9, and 257-4; 260-21 and 260-22; 339-37 and 339-6; 550-22 and 550-27). Also, two isolates from different areas but from rose plants previously grown in the same nursery had the same characteristics and plasmid profiles (i.e., isolates 278-3 and 287-7).

To our knowledge, this is the first survey of the comparative plasmid distribution in a large number of *Agrobacterium* isolates from different hosts. The number of plasmids observed in this work (five in some isolates) and their sizes (greater than 1,000 MDa in some isolates) were surprising and not previously reported. Plasmid heterogeneity could be due to plasmid loss, deletions, gain, or insertions. The high number of plasmids observed in some isolates could be due to conjugation between *A. tumefaciens* and other species sharing the same habitat, like *Agrobacterium radiobacter*. Plasmid transfer between *A. tumefaciens* and *A. radiobacter* has been observed in vitro (10, 11) and in vivo (16, 31, 33a). Natural conjugation between *A. tumefaciens* and soil bacteria of other genera has not been reported. Different authors have described variability in serological characteristics (1, 24), antibiotic sensitivity (24), total DNA (3), and chromosomal DNA, *vir* DNA, and T-DNA (21) among *Agrobacterium* strains. The general presence of many plasmids in *A. tumefaciens* suggests that they may have important functions in the survival and/or ecological properties of the bacterium. The molecular relatedness among these plasmids remains to be determined. However, their detection and characterization is essential for ecological, epidemiological, and genetic studies.

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