The Coexistence of Symbiosis and Pathogenicity-Determining Genes in *Rhizobium rhizogenes* Strains Enables Them to Induce Nodules and Tumors or Hairy Roots in Plants

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Bacteria belonging to the family *Rhizobiaceae* may establish beneficial or harmful relationships with plants. The legume endosymbionts contain *nod* and *nif* genes responsible for nodule formation and nitrogen fixation, respectively, whereas the pathogenic strains carry *vir* genes responsible for the formation of tumors or hairy roots. The symbiotic and pathogenic strains currently belong to different species of the genus *Rhizobium* and, until now, no strains able to establish symbiosis with legumes and also to induce tumors or hairy roots in plants have been reported. Here, we report for the first time the occurrence of two rhizobial strains (163C and ATCC11325^T) belonging to *Rhizobium rhizogenes* able to induce hairy roots or tumors in plants and also to nodulate *Phaseolus vulgaris* under natural environmental conditions. Symbiotic plasmids (pSym) containing *nod* and *nif* genes and pTi- or pRi-type plasmids containing *vir* genes were found in these strains. The *nodD* and *nifH* genes of the strains from this study are phylogenetically related to those of *Sinorhizobium* strains nodulating *P. vulgaris*. The *virA* and *virB4* genes from strain 163C are phylogenetically related to those of *R. tumefaciens* C58, whereas the same genes from strain ATCC 11325^T are related to those of hairy root-inducing strains. These findings may be of high relevance for the better understanding of plant-microbe interactions and knowledge of rhizobial phylogenetic history.

The family *Rhizobiaceae* contains bacteria able to induce nodules in legumes as well as bacteria able to produce hairy

roots or plant tumors. Initially, these two types of bacteria were classified in two different genera, named *Rhizobium* and *Agrobacterium*, respectively. The recent reclassification of genus *Agrobacterium* into genus *Rhizobium* (Young et al. 2001) has changed the taxonomic structure of family *Rhizobiaceae*, although many authors do not agree with the new classification because there are molecular and phenotypic data which support the separation of both genera (Farrand et al. 2003). The current taxonomic classification of the family *Rhizobiaceae* members is based on their 16S rRNA sequences and is not related with their ability to establish different plant-microbe relationships. Nevertheless, in the current genus *Rhizobium*, the strains able to induce tumors or hairy roots are classified in different species than those able to induce nodules (Young et al. 2001). The strains nodulating legumes from the genera *Rhizobium* and *Sinorhizobium* carry plasmids (pSym) containing *nod* and *nif* genes responsible for the nodulation and nitrogen fixation, respectively. The plant-pathogenic strains carry plasmids containing *vir* genes, named pTi and pRi in tumorigenic and hairy root-inducing strains, respectively. The *nodD* and *nifH* genes present a suitable degree of conservation among rhizobia and have been used for several phylogenetic studies (Hauka et al. 1998; Laguerre et al. 2001; Rivas et al. 2002; Suominen et al. 2001). The *nodD* gene is involved in the regulation of *nod* genes by the nod-box (van Rhijn et al. 1993), and the *nifH* codifies for the ferroprotein monomer of nitrogenase. The *virA* is a regulatory gene whose sequence is well conserved in pathogenic strains of *Rhizobium tumefaciens* and *R. rhizogenes* (Endoh et al. 1990; Jin et al. 1990; Lee et al. 1995; Satuti et al. 2000). The *virB4* is also an essential gene for virulence (Berger and Christie 1993, Dang and Christie 1997) included in the *virB* operon present in pathogenic species of the genus *Rhizobium*.

Until now, natural strains carrying symbiotic and pathogenicity-determining genes and able to produce nodules and pathogenic symptoms were unknown. In this article, we report for the first time two strains, 163C and *R. rhizogenes* ATCC11325^T, able to produce tumors in *Lycopersicon esculentum* (Velázquez et al. 2001b) and to induce hairy roots in plants (Kerstens et al. 1973), respectively, which also can nodulate *Phaseolus vulgaris*.

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Nucleotide sequence data is available in the GenBank database under the following accession numbers: 16S rRNA gene sequences, AY206687 (strain 163c) and AY945955 (*Rhizobium rhizogenes* ATCC 11325^T); *virA* sequences, AY945951 (strain 163C) and AY945952 (*R. rhizogenes* ATCC 11325^T); *virB4* sequences, DQ092358 (strain 163C) and DQ092359 (*R. rhizogenes* ATCC11325^T); *nifH* sequences, AY945950 (strain 163C) and AY945954 (*R. rhizogenes* ATCC 11325^T); and *nodD* sequences, AY945949 (strain 163C) and AY945953 (*R. rhizogenes* ATCC11325^T).

RESULTS

16S rRNA sequence analysis.

In a previous study, we showed that the low molecular weight (LMW) RNA profile of strain 163C resembles that of ATCC 11325^T and that, therefore, these two strains were considered as belonging to the same species (Velázquez et al., 2001b). Nevertheless, because the current bacterial phylogeny and taxonomy are based on 16S rRNA gene sequences, these sequences were obtained from strains 163C and ATCC 11325^T. The phylogenetic analysis based on the nearly complete 16S

rRNA sequences (Fig. 1) shows the placement of strain 163C in the phylogenetic group of *R. rhizogenes*. A pairwise analysis of the 16S rRNA gene sequences of strains 163C and ATCC 11325^T displayed 99.9% identity indicating that, in agreement with the results previously obtained by LMW RNA profiling, strain 163C should belong to *R. rhizogenes*.

Plasmid profiling and DNA hybridization.

As explained above, the symbiosis and pathogenicity-determining genes in species from genus *Rhizobium* are codified in plasmids. The plasmid analysis of strains 163C (Fig. 2, lane 2)

Fig. 1. Neighbor-joining tree based on nearly complete 16S rRNA gene sequences of species belonging to the family *Rhizobiaceae*. Bootstrap values calculated for 1,000 replications are indicated. Bar, 1 nucleotide substitution per 100 nucleotides.

Fig. 2. Plasmid profile in horizontal 0.7% agarose gel: *Sinorhizobium meliloti* GR4 (lane 1), strain 163C (lane 2), and ATCC 11325^T (lane 3). Results of the hybridization using *virA* probe: strains 163C (lane 4) and ATCC 11325^T (lane 5); *nodD* probe: strain GR4 (lane 6), strain 163C (lane 7), and ATCC 11325^T (lane 8); and *nifH* probe: strain GR4 (lane 9), strain 163C (lane 10), and ATCC 11325^T (lane 11).

and ATCC 11325^T (Fig. 2, lane 3) revealed three megaplasms in each strain. Both strains have a megaplasmid of similar size, greater than 1,700 kb (pRh163Cc and pRh11325c). The plasmids pRh163Cb and pRh163Ca present in strain 163C are approximately 320 and 190 kb in size, respectively. Strain ATCC 11325^T has two plasmids of approximately 180 kb (pRh11325b) and 120 kb (pRh11325a).

To test the presence of *virA* loci in strains 163C and ATCC 11325^T, we performed a Southern blot using a *virA* probe. The hybridization data revealed that the pRh163Ca and pRh11325a plasmids present in the strains studied contain this gene (Fig. 2, lanes 4 and 5). No hybridization was found using this probe in the remaining plasmids of the strains analyzed or in *Sinorhizobium meliloti* GR4 used as reference (data not shown). Because this probe hybridized with only one of the plasmids present in the strains considered and they are phylogenetically related with nodulating-legume species of the genus *Rhizobium*, we also tested whether any of the plasmids contain symbiotic genes. The results of Southern blot hybridization using *nodD* and *nifH* probes show that homologous genes (Fig. 2) are located in the plasmids pRh163Cb and pRh163Cc carried by strain 163C and pRh11325b and pRh11325c carried by ATCC 11325^T. Thus, it seems that these strains harbor both types of plasmids, pTi and pSym (strain 163C), or pRi and pSym (strain ATCC 11325^T).

Sequencing of *virA*, *virB4*, *nodD*, and *nifH* genes from strain 163C.

Partial sequences of these genes were obtained and analyzed in order to define their phylogenetic position in relation to those of other related species. The *virA* gene sequence of strain 163C was 99.9% similar to the *virA* gene from *R. tumefaciens* C58, a tumorigenic strain whose complete genome has been recently sequenced (Goodner et al. 2001). The *virA* gene of strain ATCC 11325^T was phylogenetically divergent from that of strain 163C (Fig. 3) and closely related (89.6% similarity) to that of strain *R. rhizogenes* A4, which is able to induce hairy roots in plants (Endoh et al. 1990, Liang et al. 1998). The *virB4* gene of strain ATCC 11325^T showed 89.6% similarity to that of *R. rhizogenes* A4 (Fig. 4). The *virB4* sequence of strain 163C again was closely related to that of strain C58 (99.7% similarity).

The *nifH* genes of strains 163C and ATCC 11325^T showed 100% identity between them and with respect to the *nifH* of *Sinorhizobium* sp. GR-06 and GR-X8, two strains isolated from *P. vulgaris* in Spain (Herrera-Cervera et al. 1999) (Fig. 5). The sequences of *nodD* genes from strains 163C and ATCC 11325^T were closely related, but not identical (96.3% similarity), their closest relative being the *nodD* gene of *Sinorhizobium* sp. BR816, a strain with broad host range able to nodulate *P. vulgaris*, and *Leucaena* and *Trifolium* spp. (Hernández-

Fig. 3. Neighbor-joining tree of *virA* gene sequences of the strains analyzed in this study and representative related strains from GenBank. Bootstrap values calculated for 1,000 replications are indicated. The analysis was based on 300 nucleotides. Bar, 1 nucleotide substitution per 100 nucleotides.

Fig. 4. Neighbor-joining tree of *virB4* gene sequences of the strains analyzed in this study and representative related strains from GenBank. Bootstrap values calculated for 1,000 replications are indicated. The analysis was based on 1,130 nucleotides. Bar, 5 nucleotide substitutions per 100 nucleotides.

Fig. 5. Neighbor-joining tree of *nifH* gene sequences of the strains analyzed in this study and representative related strains from GenBank. Bootstrap values calculated for 1,000 replications are indicated. The analysis was based on 344 nucleotides. Bar, 5 nucleotide substitutions per 100 nucleotides.

Fig. 6. Neighbor-joining tree of *nodD* gene sequences of the strains analyzed in this study and representative related strains from GenBank. Bootstrap values calculated for 1,000 replications are indicated. The analysis was based on 300 nucleotides. Bar, 5 nucleotide substitutions per 100 nucleotides.

Lucas et al. 1995; van Rhijn et al. 1996) (Fig. 6). The close phylogenetic relationship of the *nodD* and *nifH* genes from the analyzed strains to those of symbiotic rhizobial strains raises the possibility that these strains nodulate legumes.

Nodulation of *P. vulgaris*.

In order to test the ability to nodulate legumes, we inoculated *P. vulgaris* with strains 163C and ATCC 11325^T. The re-

sults (Fig. 6) indicate that both strains nodulated this legume. The external morphology of the nodules induced by these strains (Fig. 7A and B) was similar to that obtained with strain *R. etli* CFN42^T (Fig. 6C). The external color of these nodules was white and, in a transversal section, only a light pink color was detected (data not shown). The plants inoculated with strains 163C and ATCC 11325^T have significantly lower numbers of nodules, dry matter, and N content than those inoculated with strain *R. etli* CFN42^T, used as positive control (Table 1). The plants inoculated with strains 163C and *R. rhizogenes* showed a lighter green color than those inoculated with *R. etli* CFN42^T, but had significantly higher dry matter and N content than uninoculated plants. Therefore, strains 163C and ATCC 11325^T seem to be able to fix low amounts of N in *P. vulgaris*.

DISCUSSION

The genus *Rhizobium* currently includes fast-growing species able to induce hypertrophies in plants that may be nitrogen-fixing nodules, hairy roots, or tumors. The induction of these structures is determined by plasmid-borne genes that can be transferred in the rhizosphere. Nevertheless, to our knowledge, this transference has been reported among pathogenic and nonpathogenic strains from the same species (López-López et al. 1999; Stockwell et al. 1996) and from rhizobial species to nonrhizobial species (Chen et al. 2001; Moulin et al. 2001; Rivas et al. 2002; Sy et al. 2001; Trujillo et al. 2005; van Berkum et al. 2002; Vandamme et al. 2002). Therefore, until now, there are no reports on the transfer of virulence genes to a symbiotic species or vice versa under natural environmental conditions. Although some artificial transconjugants of *R. tumefaciens* carrying plasmids from the rhizobial strain CFN299 may nodulate *Phaseolus* spp. (Martínez et al. 1987), most of the artificial strains carrying pSym and pTi or pRi plasmids do not induce typical nodules and hairy roots or tumors (Hooykaas et al. 1981; Truchet et al. 1984; van Veen et al. 1989). Broothaerts and associates (2005) have just reported the induction of tumors by transconjugant strains of *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*; nevertheless, they did not test the nodulation of legumes by these strains.

Strain 163C, isolated several years ago from *Prunus persica*, is closely related to strain ATCC 11325^T on the basis of its LMW RNA profile (Velázquez et al. 2001b). The high similarity (near 100%) of the 16S rRNA sequence of strain 163C to that of the type strain of the species *R. rhizogenes* confirms that it probably belong to this species. *R. rhizogenes* is widely known as a hairy root inducer (Kerstens et al. 1973), although some strains from this species may form plant tumors (Sawada et al. 1993). This species forms a phylogenetic cluster within the genus *Rhizobium* together with species able to nodulate *Phaseolus* spp. (Fig. 1) which is different from that formed by other species of genus *Rhizobium* able to originate tumors, such as *R. tumefaciens*, *R. rubi*, or *R. vitis*. Nevertheless, all these species are able to produce plant hypertrophies due to

Fig. 7. Nodules induced by A, strain *Rhizobium rhizogenes* ATCC 11325^T, B, *Rhizobium* sp. 163C, and C, *R. etli* CFN42^T in *Phaseolus vulgaris* roots. Bars, 0.4 cm.

Table 1. Symbiotic characteristics of the *Rhizobium* strains from this study^z

Treatment	Nodules per plant	Shoot dry matter	Total N (mg)
Control without N	0 a	470 a	9.4 a
<i>R. rhizogenes</i> ATCC11325 ^T	8 b	680 b	15.0 b
163C	9 b	700 b	15.4 b
<i>R. etli</i> CFN42 ^T	170 c	1,163 c	27.7 c

^z Values from each column followed by the same letter are not significantly different from each other at *P* = 0.05 according to Fisher's protected least significant differences.

the presence of plasmids harboring virulence or symbiotic genes.

We reported previously that strains 163C and ATCC 11325^T carry two megapasmids (Velázquez et al. 2001b). In the present study, a third, larger plasmid was detected in both strains by taking the samples in an earlier growth phase than in the previous work. This seems to favor the detection of high plasmids in rhizobial and nonrhizobial species (Rivas et al. 2002). In addition, we have observed that these plasmids remain stable in the strains analyzed after many subcultures performed since their isolation several years ago.

Taking into account that *R. rhizogenes* ATCC 11325^T is able to produce hairy roots (Kerstens et al. 1973) and strain 163C was able to produce tumors in plants (Velázquez et al. 2001b), the presence of *vir* genes in some of the plasmids harbored by these strains was expected. The *virA* gene was located by Southern blot analysis of plasmid profiles in pRh163Ca and pRh11325a plasmids. The sequences of *virA* and *virB* genes are closely related to those of other strains from tumorigenic or hairy root-inducing species of *Rhizobium*. Nevertheless, whereas the *virA* and *virB4* genes of strain ATCC 11325^T showed differences from those of the other strains, in the case of strain 163C these genes were practically 100% homologous to those of strain *R. tumefaciens* C58. Although these results suggest that the pTi plasmid of strain 163C and *R. tumefaciens* C58 would be the same, more studies are necessary to establish this conclusion.

The partial sequences of *nodD* and *nifH* genes showed a close phylogenetic relationship with those of strains nodulating *Phaseolus vulgaris*, a promiscuous host nodulated by several rhizobial species (Herrera-Cervera et al. 1999; Martínez et al. 1987; Martínez-Romero 2003; Martínez-Romero et al. 1991; Michiels et al. 1998; Rogel et al. 2001; Velázquez et al. 2001a). The symbiotic ability of the strains analyzed here was confirmed by inoculation in *P. vulgaris* plants of both strains 163C and ATCC11325^T, resulting in the formation of less effective nodules than those obtained with *R. etli* CFN42^T. The fact that these strains elicited a low number of nodules and fixed only small amounts of nitrogen may indicate that *P. vulgaris* is not their natural host. This conclusion is further supported by the fact that the similarity of the symbiotic genes of our isolates with respect to those of strains nodulating *Phaseolus* spp. is much lower than 100%.

Therefore, the results of this study show that there are natural strains of the genus *Rhizobium* which may be pathogenic for nonlegume plants and also endosymbionts for legumes. These results raise the possibility that an ancestral strain belonging to the genus *Rhizobium* carrying pSym plasmids acquired pTi- or pRi-type plasmids by lateral transfer. These possibilities are in agreement with the hypothesis posed by Paulsen and associates (2002) which postulated that an ancestral strain unable to establish relationships with other organisms evolved into animal pathogens such as *Brucella* spp. and plant pathogens or symbionts such as *Rhizobium* spp.

MATERIALS AND METHODS

Strains and culture conditions.

Strain 163C was isolated in a previous work from tumors of *Prunus persica* in Spain (Velázquez et al. 2001b) and strain *R. rhizogenes* ATCC 11325^T was isolated from apple (Riker et al. 1930). Single isolates were used to obtain pure cultures for the analyses. Strains 163C and ATCC 11325^T were grown on YMA medium (Vincent 1970). For DNA extraction and plasmid analysis, these strains and *Sinorhizobium meliloti* GR4 used as reference were incubated in TY liquid medium (Beringer 1974) and grown at 28°C and 180 rpm for 24 h.

DNA extraction and sequence analysis.

DNA was extracted as described previously (Rivas et al. 2001). The polymerase chain reaction (PCR) amplification and the sequencing of 16S rRNA and *nifH* and *nodD* genes of strains 163C and ATCC 11325^T were carried out as described by Rivas and associates (2002) using the primers described by Zurdo-Piñeiro and associates (2004) for sequencing of the 16S rRNA gene. The *virA* and *virB4* genes were amplified and sequenced using the same conditions and the following primers pairs: *virA*1F, 5'-ATGAATGGAAGGTATTCACCG-3' and *virA*2R, 5'-GGCTCAGGCAGCTTCGCTGCG-3' (*R. tumefaciens virA* gene, AE007923, positions 1 to 21 and 1,070 to 1,054, respectively); and *virB4*1F, 5'-CGCTCTWGC GCAATATHGC-3' and *virB4*1R, 5'-GCCGCGATCCTTGTCGAAG-3' (*R. tumefaciens virB4* gene, AE007923, positions 185 to 203 and 1,407 to 1,389, respectively). The sequences obtained were compared with those from GenBank using the BLAST program (Alschul et al. 1990). Sequences were aligned using the ClustalW software (Thompson et al. 1997). The distances were calculated according to Kimura's two-parameter method (Kimura 1980) and phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei 1987). Bootstrap analysis was based on 1,000 resamplings. The MEGA2 package (Kumar et al. 2001) was used for all analyses.

Plasmid profile analysis.

The strains were incubated in TY medium at 25°C and 180 rpm until the culture reached a concentration of 1×10^6 cells/ml and then the cells were collected for plasmid analysis by centrifuging 1.5 ml of culture at $9,000 \times g$ for 5 min. This analysis was performed according to Plazinski and associates (1985), except that the electrophoresis was performed at 2 V cm⁻¹ for 90 min, followed by 3 V cm⁻¹ for 60 min and finally 6 V cm⁻¹ for 4 h. The pRmeGR4b (205-kb) and pRmeGR4a (175-kb) plasmids of *S. meliloti* GR4 (Toro and Olivares 1986) were used as molecular markers.

DNA hybridization.

The probes for Southern hybridization (Southern 1975) were obtained by PCR using oligonucleotide primers designed in this study to amplify a fragment of the *virA* gene (discussed above) and primers designed previously to amplify fragments of the *nodD* and *nifH* genes (Rivas et al. 2002).

The PCR-amplified fragments of *nifH*, *nodD*, and *virA* were digoxigenin (DIG) labeled with the DIG DNA Labeling Kit (Roche Diagnostics Corp., Branchburg, NJ, U.S.A.) following the manufacturer's instructions and used as probes. Hybridization was detected with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Indianapolis, IN, U.S.A.), using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates for alkaline phosphatase.

Nodulation tests.

Surface-sterilized seeds of *Phaseolus vulgaris* were germinated axenically in petri dishes. Seedlings were transferred to pots with sterile vermiculite and watered with autoclaved nitrogen-free Rigaud and Puppo (1975) nutrient solution. Five plants were inoculated with 1 ml each of a suspension of strains 163C and ATCC 11325^T containing 8×10^8 cells/ml. The inoculated plants were placed in a plant growth chamber with mixed incandescent and fluorescent lighting (400 micro-einsteins m⁻² s⁻¹; 400 to 700 nm), programmed for a 16-h photoperiod, day-night cycle, with a constant temperature varying from 25 to 27°C, and 50 to 60% relative humidity. Strain *R. etli* CFN42^T was used as positive control. The negative controls were noninoculated *P. vulgaris* plants. Plants were watered with free-nitrogen Rigaud and Puppo solution. After 6 weeks,

the strains from this study were isolated from nodules that were counted and the dry weight of the aerial part of the common bean plants was determined. Plant N content was measured according to the A.O.A.C. methods (Johnson 1990). The data obtained were analyzed by one-way analysis of variance, with the mean values compared using Fisher's protected least significant differences ($P = 0.05$).

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