A Novel Group of *Rhizobium tumorigenes*-Like Agrobacteria Associated with Crown Gall Disease of Rhododendron and Blueberry

Nemanja Kuzmanović,^{1,†} Patric Behrens,¹ Elke Idczak,² Stefan Wagner,² Monika Götz,² Cathrin Spröer,³ Boyke Bunk,³ Jörg Overmann,^{3,4} and Kornelia Smalla^{1,†}

¹Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, 38104 Braunschweig, Germany

²Institute for Plant Protection in Horticulture and Forests, Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, 38104 Braunschweig, Germany

³Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, 38124 Braunschweig, Germany

⁴Microbiology, Braunschweig University of Technology, 38106 Braunschweig, Germany

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ABSTRACT

Crown gall is an economically important and widespread plant disease caused by tumorigenic bacteria that are commonly affiliated within the genera *Agrobacterium*, *Allorhizobium*, and *Rhizobium*. Although crown gall disease was reported to occur on rhododendron, literature data regarding this disease are limited. In this study, an atypical group of tumorigenic agrobacteria belonging to the genus *Rhizobium* was identified as a causative agent of crown gall on rhododendron. Genome analysis suggested that tumorigenic bacteria isolated from rhododendron tumors are most closely related to *Rhizobium tumorigenes*, a new tumorigenic bacterium discovered recently on blackberry in Serbia. However, *R. tumorigenes* and novel rhododendron strains belong to separate species and form a homogenous clade within the genus *Rhizobium*, which we named the “tumorigenes” clade. Moreover, tumorigenic bacteria isolated from

rhododendron seem to carry a distinct tumor-inducing (Ti) plasmid, compared with those carried by *R. tumorigenes* strains and Ti plasmids described thus far. To facilitate rapid identification of bacteria belonging to the “tumorigenes” clade, regardless of whether they are pathogenic or not, a conventional PCR method targeting putative chromosomal gene-encoding flagellin protein FlaA was developed in this study. Finally, our results suggested that this novel group of tumorigenic agrobacteria occurs on blueberry but it cannot be excluded that it is distributed more widely.

Keywords: *Agrobacterium*, bacteriology, detection, disease control and pest management, ecology and epidemiology, etiology phylogeny, Ti plasmid, tumorigenic bacteria

Tumorigenic agrobacteria are recognized as causal agents of crown gall disease worldwide (Kuzmanović et al. 2018a; Otten et al. 2008; Puławska 2010). Crown gall occurs in nurseries and plantations on various agriculturally important plants, primarily fruit crops (stone, pome, and cane fruit, and nuts), grapevine, and ornamental plants. Tumorigenic agrobacteria carry tumor-inducing (Ti) plasmids that are indispensable for pathogenicity.

Crown gall is caused by tumorigenic species of the family Rhizobiaceae, which are thus far found within the genera *Agrobacterium*, *Allorhizobium*, and *Rhizobium*. A novel, distinct plant-tumorigenic bacterium, *Rhizobium tumorigenes*, was described recently (Kuzmanović et al. 2018b). It comprised bacterial strains isolated from cane gall tumors on thornless blackberry in Serbia. Based on polyphasic taxonomic characterization, it was shown that *R. tumorigenes* was distantly related to *R. rhizogenes* (i.e., *Agrobacterium rhizogenes* or *Agrobacterium biovar 2*), which is another tumorigenic species within the genus *Rhizobium* (Kuzmanović et al. 2018b).

Rhododendron is a widely cultivated woody ornamental plant belonging to the family Ericaceae. Crown gall disease was reported to occur on rhododendron (Moore 2014), although data regarding this disease are limited. A similar phenomenon, termed “tissue proliferation” (TP), was described on rhododendron in the United States, and was characterized by abnormal tissue overgrowth at the crown, above or below the soil line (Linderman 2014; Zimmerman 1997). Although different bacterial strains were isolated from gall-like tissues, inoculation experiments suggested that these strains were not associated with TP in rhododendron (LaMondia et al. 1997; McCulloch and Britt 1997). Furthermore, Schulz (1998) isolated tumorigenic agrobacteria from tumors on rhododendron and demonstrated their tumor-inducing ability in the pathogenicity assays; however, details regarding the identity of the isolated strains and experimental methods performed are unpublished. Considering data available in literature, little is known about the pathogen causing crown gall disease of rhododendron and therefore etiology of this disease remained to be systematically resolved.

Blueberry (*Vaccinium corymbosum*) is another member of the family Ericaceae observed to be affected by crown gall (Bristow and Moore 1995). In blueberry, strains of *Agrobacterium biovar 1* (i.e., *Agrobacterium tumefaciens* species complex), *R. rhizogenes*, and *A. rubi* were reported as causal agents of the disease (Alippi et al. 2012; Canfield et al. 1995). However, in some cases, as observed in Poland, only nontumorigenic strains were isolated from blueberry plants showing crown or cane gall symptoms (J. Puławska, unpublished).

During the last 10 to 15 years, recurrent outbreaks of crown gall disease of rhododendron were observed in commercial nurseries in Northern Germany. The disease did not cause high economic losses because it occurred in a limited geographical area on several

[†]Corresponding authors: N. Kuzmanović; kuzmanovic1306@gmail.com; and K. Smalla; kornelia.smalla@julius-kuehn.de

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rhododendron cultivars. Aerial tumors were observed on shoots, mostly at cutting injuries, as well as on leaf petioles of affected plants (Supplementary Fig. S1). In our initial investigations, the causative agent was not isolated from tumor samples, despite repeated attempts. PCR-Southern blot analysis of tumor samples did not result in amplification or hybridization, regardless of the use of multiple primers and probes generated with Ti plasmid of the reference strain *Agrobacterium* sp. C58. Moreover, the crown gall disease was observed on blueberry propagation material originating from another nursery in Lower Saxony (Germany) in 2013. Tumor development occurred on blueberry twigs and was recorded on two different cultivars. Similarly as for rhododendron, the pathogen was not detected in tumor tissue by using PCR-Southern blot analysis and disease etiology remained unresolved. Therefore, the primary objective of this study was to detect and characterize bacteria associated with crown gall disease of rhododendron and blueberry.

MATERIALS AND METHODS

Sampling. Grafted and potted 2-year-old rhododendron plants with visible tumors were received from a phytosanitary service (Landwirtschaftskammer Niedersachsen, Lehr- und Versuchsanstalt für Gartenbau Bad Zwischenahn, Bad Zwischenahn-Rostrup, Germany; location A) in 2012 or were collected from a production greenhouse in a nursery in Lower Saxony in Germany (location B) in 2017 (Table 1). Rhododendron cuttings containing fresh tumor tissue were collected at the same nursery (location B) from scion and rootstock mother plants in 2017 (Table 1).

Blueberry propagation material was received from a phytosanitary service (Landwirtschaftskammer Niedersachsen, Hannover, Germany) in 2013 (Table 1). These samples originated from another, geographically unrelated, nursery in Lower Saxony (Germany). Unlike rhododendron tumors from which isolation also was performed, blueberry samples were only analyzed by using cultivation-independent methods (see below).

Bacterial isolation, strains, and growth conditions. Bacteria were isolated on yeast mannitol agar (YMA) (Kuzmanović et al. 2015a) from internal tumor tissue fragments of 11 rhododendron samples collected in 2017 (Table 1) following the procedure described before (Kuzmanović et al. 2014). Isolation plates were incubated at 28°C for 3 to 5 days. In total, 40 bacterial colonies with morphology typical of Rhizobiaceae spp. were purified and preserved in a -80°C freezer in nutrient broth with 20% glycerol for further analysis. The collection of reference agrobacterial

strains was used in PCR analysis for testing specificity of primers designed in this study (Supplementary Table S1).

The growth of isolated strains was evaluated on the following culture media: D1M (Perry and Kado 1982), YMA, yeast extract-dextrose-CaCO₃ (YDC) (Lelliott and Stead 1987), potato dextrose agar supplemented with 0.08% CaCO₃ (PDA-CaCO₃) (Bouzar et al. 1995), tryptone-yeast (TY) medium (tryptone at 5 g liter⁻¹, yeast extract at 3 g liter⁻¹, and CaCl₂ × 2H₂O at 0.9 g liter⁻¹; pH 6.8), tryptic soy agar (TSA; Bacto tryptic soy broth at 30 g liter⁻¹ and Bacto agar at 20 g liter⁻¹) (BD Biosciences), and King's medium B (King et al. 1954).

DNA extraction. DNA extraction was performed from all tumor tissue collected (Table 1), and from bacterial strains isolated from rhododendron samples collected in 2017 (Table 2). For samples Rho2 and Rho3, total DNA was extracted from 0.25 g of plant tissue using PowerLyzer Power Soil DNA Isolation Kit (MoBio Laboratories), according to the manufacturer's instructions. For the other plant samples, total DNA was extracted from 0.1 g of tumor tissue by using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. For PCR-based analysis, total genomic DNA was extracted from bacteria grown on TY medium at 28°C for 24 to 48 h, according to the procedure described by Aljanabi and Martinez (1997). For whole-genome sequencing, genomic DNA was extracted from selected bacterial strains using a Qiagen genomic tip 100/G and Qiagen Genomic DNA Buffer Set (Qiagen).

Enterobacterial repetitive intergenic consensus PCR. The genetic diversity among strains isolated from rhododendron tumors and differentiation of representative ones were assessed by enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprinting (Versalovic et al. 1991). PCR amplifications were carried out as previously described (Kuzmanović et al. 2015b), using GoTaq Flexi buffer and GoTaq Flexi DNA polymerase (Promega Corp.).

Pathogenicity assay. Pathogenicity of isolated strains was tested by inoculating rhododendron cultivar Cunningham's White, tomato (*Lycopersicon esculentum* L.), sunflower (*Helianthus annuum* L.), and *Bryophyllum delagoense* (syn. *Kalanchoe delagoensis*) plants. Rhododendron was inoculated 9 months after transplanting of stem cuttings to pots. Tomato plants were inoculated at the stage of two to three true leaves, whereas the sunflower seedlings were inoculated at the stage of two true leaves immediately before transplanting to pots. *Bryophyllum* plants were vegetatively propagated by cuttings and were inoculated approximately 10 weeks after their planting in pots. Test plants were inoculated as described before (Kuzmanović et al. 2014), with the exception that inoculation sites were not covered with wet cotton and aluminum foil. At least five test plants were inoculated per bacterial strain. Inoculated plants were maintained in a greenhouse at

TABLE 1. Plant samples analyzed in this study

Samples ^b	Type	Source	Collection year	PCR-Southern blot detection ^a		
				<i>virD2</i> (C58)	<i>virD2</i> (rho-6.2)	<i>flaA</i> (rho-6.2)
Rho2, Rho3, Rho4-Rho7	Grafted and potted young plants	Rhododendron, unknown cultivars, location A	2012	–	+	+ ^c
1R, 3R, 4R, 6R, 7R	Scion mother plants grown in an open field	<i>Rhododendron</i> 'Jens Jörgen Sörensen', location B	2017	NT	+	+ ^d
8R, 10R, 11R	Rootstock mother plants grown in an open field	<i>Rhododendron</i> 'Rhodunter 37', location B	2017	NT	+	+
13R, 14R, 15R	Grafted and potted 2-year-old plants grown in a greenhouse	<i>Rhododendron</i> 'Jens Jörgen Sörensen' grafted onto 'Rhodunter 37', location B	2017	NT	+	+ ^d
Hei1, Hei3, Hei5, Hei6, Hei7	Propagation material	<i>Vaccinium corymbosum</i> 'Duke'	2013	– ^e	+ ^f	+ ^g
Hei10, Hei12	Propagation material	<i>V. corymbosum</i> 'Draper'	2013	– ^e	+	+

^a NT = not tested.

^b Geographical origin of all samples was Lower Saxony, Germany.

^c Samples Rho4 and Rho6 were tested.

^d Samples 1R, 6R, 7R, and 15R were tested.

^e Samples Hei1, Hei5, and Hei12 were tested.

^f Samples Hei1, Hei3 and Hei7 were tested.

^g Samples Hei1, Hei3, Hei6, and Hei7 were tested.

approximately 22°C. The first 3 to 5 days after inoculation, the relative humidity was set at 90%. Tumor formation was scored 4 to 6 weeks (tomato, sunflower, and bryophyllum) and 10 weeks (rhododendron) after inoculation.

PCR detection of Ti plasmid. The detection of the Ti plasmid was initially conducted by PCR using primers amplifying fragments of *virC1-virC2* (VCF3 and VCR3) (Suzaki et al. 2004), *virD2* (A and C') (Haas et al. 1995), and *tms2* (*tms2F1* and *tms2R2*) (Pulawska and Sobiczewski 2005) genes, as well as the intergenic region between *virB11* and *virG* genes (F749 and F14) (Mougel et al. 2001; Picard et al. 1992). PCR protocols were as reported before (Kuzmanović et al. 2015b). All primer pairs were used for analysis of bacterial strains isolated from rhododendron, while *virD2* gene-specific primers were also used for Ti plasmid detection in tumor samples.

Amplification and sequencing of *recA* housekeeping gene. The bacterial strains isolated from rhododendron tumors were preliminary identified by sequence analysis of the housekeeping gene *recA*. For sequencing and phylogenetic analysis, the *recA* gene fragment was amplified by using primer pair F2898/F2899 (Costechareyre et al. 2010; Shams et al. 2013), as described before (Kuzmanović et al. 2015b). The PCR products obtained were sequenced with standard T7 and T3 primers by Macrogen Europe.

PCR-Southern blot analysis of plant samples. For PCR amplification, primer pair A/C' targeting the *virD2* gene located on Ti plasmid (Haas et al. 1995) was used (see above, subsection "PCR detection of Ti plasmid"). Moreover, TFP_F and TFP_R primers targeting chromosomal gene *flaA* specific for *R. tumorigenes*-like bacteria were used (see below, subsection "Development of the PCR protocol for detection and identification of "tumorigenes" group of bacteria"). To produce probes, the following reference strains were used: *Agrobacterium* sp. C58 for *virD2* probe and *Rhizobium* sp. rho-6.2 (Table 2) for *virD2* and *flaA* probes. Southern blotting and hybridization were performed as previously described (Binh et al. 2008; Gomes et al. 2007). Hybridization was performed under conditions of medium stringency, following the protocol published by Fulthorpe et al. (1995), that typically allow detection of DNA fragments having >75% nucleotide identity to the hybridization probe. All analyses were performed at least twice.

Cloning and sequencing of *virD2* gene fragments amplified from tumor samples. Amplification of *virD2* gene fragments from representative tumor samples on rhododendron and blueberry was performed as described above. The amplicons were purified using the Silica Bead DNA Gel Extraction Kit (Thermo Scientific). Purified DNA fragments were cloned into *Escherichia coli* JM109 Competent Cells (Promega Corp.) using the pGEM-T Easy Vector System I (Promega Corp.), according to the manufacturer's instructions. Plasmid extraction was performed

from transformed bacteria grown in liquid Luria-Bertani medium at 37°C overnight using the Gene JET Plasmid Miniprep Kit (Thermo Scientific). Sequencing was performed using standard T7 and SP6 primers by Macrogen Europe.

Genome sequencing, assembly, and annotation. Draft whole-genome sequences were obtained for *Rhizobium* sp. strains rho-1.1, rho-6.2, and rho-13.1. DNA libraries (paired-end) with insert length of approximately 300 bp were obtained with Nextera XT DNA Library Prep Kit (Illumina). Paired-end sequencing (2 × 151 bp) was performed on an Illumina NextSeq 500 platform generating 3,891,960 (rho-1.1), 1,692,866 (rho-6.2), and 3,964,991 (rho-13.1) paired reads. Adapter clipping was done using bcl2fastq2 conversion software. De novo sequence assembly was performed using the SPAdes genome assembler (Bankevich et al. 2012) (Galaxy version 3.12.0+galaxy1) implemented on the Galaxy Web server (Afgan et al. 2018). The genome sequence was annotated by the RASTtk pipeline at the Rapid Annotation Using Subsystem Technology (RAST) server (Aziz et al. 2008) and NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) (Tatusova et al. 2016).

Phylogenetic, phylogenomic, and whole-genome comparison analyses. The phylogenetic analysis was performed on the *recA* housekeeping gene sequence. Sequences of related Rhizobiaceae strains were retrieved from NCBI GenBank and included in the analysis (Fig. 1). The obtained sequences were aligned using the ClustalW algorithm (Higgins et al. 1996) in the MEGA 7.0.21 software package (Kumar et al. 2016). Maximum-likelihood trees were generated with PhyML 3.0 by using 1,000 bootstrap replicates (Guindon et al. 2010). The most suitable substitution model was determined by the Smart Model Selection tool (Lefort et al. 2017), according to the Akaike information criterion (Akaike 1974).

Moreover, genome-wide phylogeny was reconstructed based on 385 conserved protein sequences extracted from genome sequences of *Rhizobium* sp. strains rho-1.1, rho-6.2, and rho-13.1, and strains of related Rhizobiaceae strains using PhyloPhlAn pipeline, version 0.99 (Segata et al. 2013).

Genome sequences of *Rhizobium* sp. strains rho-1.1, rho-6.2, and rho-13.1 were compared between each other and with genome sequences of related *Rhizobium* spp. For this purpose, average nucleotide identity (ANI) values were calculated using the JSpecies Web Service (Richter et al. 2016), as well as in silico DNA-DNA hybridization (isDDH) values by the Genome-to-Genome Distance Calculator (GGDC 2.1; <http://ggdc.dsmz.de/distcalc2.php>) using the recommended BLAST + alignment and formula 2 (identities/HSP length) (Meier-Kolthoff et al. 2013).

Genome comparisons were conducted using the BLAST Ring Image Generator (BRIG) program, version 0.95 (Alikhan et al. 2011). BRIG analysis involved the calculation and visual representation

TABLE 2. Tumorigenic bacterial strains isolated from rhododendron tumors in this study

Strain	Species	Pathogenicity	Host plant ^a	Sample ^b	Year of isolation	PCR analysis				
						<i>flaA</i> (TFP_F/TFP_R)	<i>virD2</i> (A/C')	<i>virC1-virC2</i> (VCF3/VCR3)	<i>tms2</i> (<i>tms2F1/tms2R2</i>)	<i>virB11-virG</i> (F749/F14)
rho-1.1	<i>Rhizobium</i> sp.	Tumorigenic	Rhododendron, JJS	1R	2017	+	+	–	–	(+) ^c
rho-3.1	<i>Rhizobium</i> sp.	Tumorigenic	Rhododendron, JJS	3R	2017	+	+	–	–	(+) ^c
rho-4.1	<i>Rhizobium</i> sp.	Tumorigenic	Rhododendron, JJS	4R	2017	+	+	–	–	(+) ^c
rho-6.2	<i>Rhizobium</i> sp.	Tumorigenic	Rhododendron, JJS	6R	2017	+	+	–	–	(+) ^c
rho-13.1	<i>Rhizobium</i> sp.	Tumorigenic	Rhododendron, JJS+37	13R	2017	+	+	–	–	(+) ^c
rho-14.3	<i>Rhizobium</i> sp.	Tumorigenic	Rhododendron, JJS+37	14R	2017	+	+	–	–	(+) ^c

^a Cultivars: JJS = *Rhododendron* 'Jens Jörgen Sørensen' and JJS+37 = *Rhododendron* 'Jens Jörgen Sørensen' grafted onto *Rhododendron* 'Rhodunter 37'.

^b Isolation source. Geographical origin of all samples was Lower Saxony, Germany.

^c Weak DNA fragment of a size similar to the amplicon specific for nopaline-type (e.g., pTiC58) tumor-inducing plasmids was amplified. Please see section "Isolation and identification of the pathogen in rhododendron" for more details.

of percentage of sequence identity and sequence coverage, in respect to the reference genome. The analysis was done by using the blastn option. The genome sequence of *R. tumorigenes* 1078^T was used as a reference sequence, whereas the genome sequences of *Rhizobium* sp. strains rho-1.1, rho-6.2, and rho-13.1 were used as query sequences.

Furthermore, the OrthoVenn web server (Wang et al. 2015) was used for genome comparisons and determination of orthologous gene clusters on the basis of coding sequences (CDSs). To ensure similar annotation quality, genomes annotated using RAST were used. The identification of orthologous clusters was performed with default parameters, 1e-5 e-value cut-off for all protein similarity comparisons and 1.5 inflation value for the generation of orthologous clusters.

Particular Ti plasmid-specific sequences were retrieved from draft genome sequences of strains rho-1.1, rho-6.2 and rho-13.1 and compared with corresponding sequences of reference Ti or hairy root-inducing (Ri) plasmids characterized thus far. The NCBI blastn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) tool was used for individual sequence comparisons at the nucleotide level.

Development of the PCR for detection and identification of “tumorigenes” group of bacteria. By genome sequence comparison and orthology analysis (see above) of different Rhizobiaceae species, genes specific for *R. tumorigenes* 1078^T and *Rhizobium* sp. strains rho-1.1, rho-6.2, and rho-13.1 were identified. A specific primer set, TFP_F (5'-GTTTGAAAGTCGCTTCCG CATCC-3') and TFP_R (5'-CTCAGCATGTTGACGCCGTTG-3'), targeting this group of bacteria was designed based on the sequence of the putative gene-encoding flagellin protein FlaA using Primer 3.0 software (Untergasser et al. 2012). The size of the amplification product was 304 bp. The putative gene *flaA* was considered as a chromosomal gene, because it belonged to contigs that carried genes involved in basic cellular functions (e.g., genes encoding ribosomal proteins), as determined by examination of genome sequences of *R. tumorigenes* 1078^T and *Rhizobium* sp. strains rho-1.1, rho-6.2, and rho-13.1. Specificity of the primer set was tested with 30 reference strains and additionally checked in silico by performing NCBI Primer-BLAST against the nonredundant (nr) and “Refseq representative genomes” databases (Ye et al. 2012).

The PCR amplifications were performed in a 15-μl mixture containing 1× colorless GoTaq Flexi buffer (Promega Corp.), 1.5 mM MgCl₂, 200 μM each dNTP, 0.5 μM each primer, 0.3 U of GoTaq Flexi DNA polymerase (Promega Corp.), and 1 μl of template DNA. The thermal profile was as follows: initial denaturation at 94°C for 1 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 30 s; and final extension at 72°C for 5 min.

Accession numbers. The DDBJ/ENA/GenBank accession numbers for the partial *recA* gene sequences of the *Rhizobium* sp. strains rho-1.1, rho-3.1, rho-4.1, rho-6.2, rho-13.1, and rho-14.3 are MK414443 to MK414448, respectively.

The *virD2* gene sequences rho2_w10, rho2_w8, rho2_w5, hei3_w7, hei3_w6, hei3_w3, 10R_w9, 10R_w6, 10R_w4, 6R_w9, 6R_w5, and 6R_w1 obtained by cloning and sequencing the PCR products from tumor samples were submitted to GenBank under accession numbers MK423918 to MK423918, respectively.

This Whole-Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under the accessions SCFJ000000000 (rho-1.1), SCFK000000000 (rho-6.2), and SCFL000000000 (rho-13.1). The versions described in this article are versions SCFJ01000000, SCFK01000000, and SCFL01000000.

RESULTS

Isolation and identification of the pathogen. Bacteria showing the morphology typical of Rhizobiaceae species were isolated from rhododendron tumors on YMA medium. Colonies on YMA were white to cream colored, circular, and glistening. However, at least two types of colonies were observed; the first

group had a faster growth and formed larger colonies, while the second group comprised colonies that grew more slowly and, in some cases, were overgrown by the former group and saprophytic bacteria. In total, 40 isolates from both groups of colonies were purified from most of the isolation plates and subjected to further analyses.

For differentiation of representative strains, ERIC-PCR fingerprints were used (data not shown). All 40 strains isolated were also tested for the presence of Ti plasmid by using *virC1-virC2* gene-specific primers VCF3 and VCR3 (Suzaki et al. 2004) to confirm the isolation of tumorigenic agrobacteria. However, all isolates were negative in *virC* gene-specific PCR (Table 2). Nevertheless, the pathogenicity assays were performed with 20 representative strains by inoculating several test plants. Interestingly, only the six slow-growing strains (Table 2) caused tumors on rhododendron, sunflower, and tomato plants. In the case of bryophyllum plants, these strains did not cause typical tumors but, rather, necrotic swellings. To complete Koch's postulates, the pathogen was routinely reisolated from tumor tissue of test plants and reidentified by ERIC-PCR fingerprinting.

In order to detect the Ti plasmid in tumorigenic strains isolated from rhododendron tumors, we employed additional primer sets specific for different Ti plasmid genes (see above, subsection “PCR detection of Ti plasmid”). However, the corresponding DNA fragments were amplified only with *virD2* gene-specific primers (A and C') (Table 2).

Tumorigenic strains isolated from rhododendron did not grow on DIM, TSA, and YDC media after 7 days of incubation, whereas very weak growth was observed on King's medium B. On the other hand, bacteria showed good growth on YMA, TY, and PDA-CaCO₃ media, forming single colonies after 3 to 4 days. Colonies on YMA medium had a diameter of 1 to 2 mm after 72 h of growth at 28°C, and were white to cream colored, circular, convex, and glistening (Supplementary Fig. S2). Furthermore, formation of clear zones was visible around bacterial growth on PDA-CaCO₃ medium.

Draft genome sequences of tumorigenic strains isolated from rhododendron tumors. For a further and more thorough analysis, we obtained draft whole-genome sequences of three representative strains. The genome assembly of strains rho-1.1, rho-6.2, and rho-13.1 resulted in 141 (N50 value = 221,621 bp), 144 (N50 value = 324,337 bp), and 137 (N50 value = 334,709 bp) contigs, respectively. The estimated genome coverages were 153× (rho-1.1), 68× (rho-6.2), and 150× (rho-13.1). The total size of the draft genome sequences of sequenced strains ranged from approximately 5.8 to 6 Mb, with a GC content of 60.0% (Table 3). By NCBI PGAP, in total, 5,602 CDSs, 45 transfer RNAs (tRNAs), and 3 ribosomal RNAs (rRNAs) were predicted for strain rho-1.1, while 5,550 CDSs, 45 tRNAs, and 3 rRNAs were predicted for strain rho-6.2. For strain rho-13.1, 5,734 CDSs, 47 tRNAs, and 3 rRNAs were predicted.

Taxonomic position of the pathogen and relationships with related Rhizobiaceae species. The tumorigenic strains isolated from rhododendron (Table 2) possessed identical *recA* gene sequences, as calculated for the length of 945 bp. Based on *recA* gene phylogeny, the strains studied were grouped together with other members of the Rhizobiaceae family, and were closely related to the species *R. tumorigenes* (Fig. 1A).

Using the whole-genome sequence data, a phylogenomic tree based on amino acid sequences of 385 conserved proteins was obtained, providing a more robust and reliable phylogenetic inference. Similarly as for the *recA*-based phylogeny, the selected representative strains rho-1.1, rho-6.2, and rho-13.1 clustered closely together, with *R. tumorigenes* 1078^T as their closest relative (Fig. 1B). Tumorigenic *Rhizobium* sp. strains isolated from rhododendron and *R. tumorigenes* 1078^T formed a homogenous clade that was named “tumorigenes” clade.

In order to determine whether novel strains isolated from rhododendron belong to *R. tumorigenes* or to a novel species, we

performed whole-genome comparisons by using ANI-Blast (ANiB), ANI-MUMmer (ANIm), and isDDH methods. Obtained values were >99% when comparing strains rho-1.1, rho-6.2, and rho-13.1 among each other, suggesting that they belong to the same species. However, when these strains were compared with *R. tumorigenes* 1078^T, the values obtained were below the proposed threshold for species delineation (95 to 96% for ANI or 70% for DDH), suggesting that they belong to a novel species (Table 3). For instance, the ANiB, ANIm, and isDDH values between strain rho-1.1 and the most closely related *R. tumorigenes* 1078^T were 92.84, 94.14, and 55.1%, respectively. Therefore, because tumorigenic strains isolated from rhododendron could not be assigned to any known *Rhizobium* sp., they are classified as *Rhizobium* sp. in this study.

The genome sequences of the novel *Rhizobium* sp. strains rho-1.1, rho-6.2, and rho-13.1, as well as *R. tumorigenes* 1078^T, were compared using BRIG software. We used *R. tumorigenes* 1078^T as a reference genome and rhododendron strains as query genomes. Circular genome visualization showed a close genetic relatedness of the strains belonging to the “tumorigenes” clade (Supplementary Fig. S3A). Although most regions within the genomes included in the analysis were conserved, we could observe a number of regions that were present in *R. tumorigenes* 1078^T but not in rhododendron strains. It is unclear whether these regions are located on plasmids or chromosomal DNA because only draft, unclosed genomes were available.

Furthermore, genome-wide comparisons of orthologous clusters between the genome sequences of novel *Rhizobium* sp. strains and

TABLE 3. Average nucleotide identity (ANI) and in silico DNA-DNA hybridization (isDDH) comparisons between *Rhizobium* sp. strains rho-1.1, rho-6.2, and rho-13.1 (reference sequences) and related *Rhizobium* spp.^a

Species	Strain	Size (bp)	GC (%)	Accession number ^c	ANI values (%) ^b		
					ANiB	ANIm	isDDH (%)
<i>Rhizobium tumorigenes</i>	1078 ^T	5,899,412	60.0	PCDQ01	92.8	94.2	54.9–55.1
<i>R. tubonense</i>	CCBAU 85046 ^T	6,540,512	59.3	PCDP01	76.2	84 ^d	21.3
<i>R. rhizogenes</i>	ATCC 11325 ^T	7,044,473	59.9	BAYX01	75.1–75.2	83.8 ^d	21.1
<i>R. rhizogenes</i>	K84	6,656,043 ^e	60.1 ^c	CP000629, CP000629	75.3	83.8 ^d	21.1

^a Obtained ANI and isDDH values were >99% when comparing strains rho-1.1, rho-6.2, and rho-13.1 among each other, suggesting that they belong to the same species. The genome size (Size) and GC content (GC) are given for chromosomal and chromid DNA, without plasmids.

^b ANiB = ANI-Blast and ANIm = ANI-MUMmer.

^c Accession numbers refer to draft genomes or chromosome sequences.

^d Less than 20% of the genome has been aligned.

^e The size and GC content of the complete genome of the strain K84 are 7,273,300 bp and 59.87%, respectively.

Fig. 1. A, Maximum-likelihood phylogenetic tree based on the partial *recA* gene sequence (945 bp), constructed using a general time-reversible substitution model with a gamma distribution and invariant sites (GTR+G+I), indicates the phylogenetic position of tumorigenic *Rhizobium* sp. strains isolated from rhododendron (marked in bold) and their relationship with related members of the Rhizobiaceae family. B, Maximum-likelihood phylogenetic tree based on concatenated sequences of 385 conserved proteins extracted from whole-genome sequences, constructed using the PhyloPhlAn pipeline, indicates the evolutionary relationships between tumorigenic *Rhizobium* sp. strains rho-1.1, rho-6.2, and rho-13.1. (marked in bold) and related Rhizobiaceae members. Bootstrap values equal to or higher than 60% are shown at nodes. Scale bars represent the estimated number of nucleotide (A) or amino acid (B) substitutions per site. *Bradyrhizobium japonicum* USDA 6^T was used as the outgroup organism. DDBJ/ENA/GenBank accession numbers are given in Supplementary Table S2.

closely related *R. tumorigenes* 1078^T were conducted. In total, 6,083 protein clusters, 6,011 orthologous clusters (contains at least two strains), and 4,416 single-copy gene clusters were identified for these four strains. The strains studied shared 4,452 orthologous protein clusters (Supplementary Fig. S3B). Strains originating from rhododendron (rho-1.1, rho-6.2, and rho-13.1) shared a large number of orthologous protein clusters (1,183) that were not present in strain 1078^T. Congruent with the whole-genome comparisons of rhododendron strains (Table 3), strains rho-1.1 and rho-13.1 were more closely related and shared 238 orthologous protein clusters that were absent in rho-6.2. Although unique orthologous protein clusters were not identified in strains rho-1.1 and rho-13.1, two orthologous protein clusters were found as unique for strain rho-6.2. These two orthologous protein clusters (four loci) belonged to mobile genetic elements and were identified as putative IS66 family transposase.

Analysis of Ti plasmid-specific sequences in tumorigenic strains recovered from rhododendron tumors. Sequences specific for Ti plasmids were retrieved from draft genome sequences obtained in this study and compared with corresponding sequences of Ti plasmids described thus far. For instance, *virD2* gene sequences of strains rho-1.1, rho-6.2, and rho-13.1 were identical and shared <87% nucleotide identity with the most closely related Ti or Ri plasmids pRi1724 (86.43%), pRi2659 (86.43%), and pTiS4 (78.19%) for >99% of sequence query coverages. Furthermore, for *virB11*, *virC1*, *virC2*, and *virG* gene sequences of strains rho-1.1, rho-6.2, and rho-13.1 that were also targeted by PCR analysis (see above, section “Materials and Methods”), relatively low nucleotide identities values (<74%; sequence query coverage <94%) were obtained by comparison with most closely related Ti or Ri plasmids (i.e., pTiBo542, pTiC5.7/pTiC6.5, pTiChry5, pRi1724, pRi2659, and pTiS4). When *vir* gene sequences of strains rho-1.1, rho-6.2, and rho-13.1 were BLASTed against the draft genome sequence of *R. tumorigenes* 1078^T, nucleotide sequence identity values < 82% (sequence query coverage < 94%) were obtained. Interestingly, a gene homologous to T-DNA gene *iaaH* (*tms2*) commonly found on different Ti plasmids was not found in the genome sequences of strains rho-1.1, rho-6.2, and rho-13.1 but was present in the draft genome sequence of *R. tumorigenes* 1078^T. On the other hand, some genes that were commonly found in T-DNA of Ti plasmids (e.g., in pTiC58) such as putative gene-encoding protein C (*c*) and the open reading frame annotated to encode unknown opine synthase were found in draft genome sequences of strains rho-1.1, rho-6.2, and rho-13.1 but were distantly related to corresponding gene sequences of Ti plasmids described thus far (data not shown).

PCR-based detection and identification of “tumorigenes” group of agrobacteria. In this study, new primers targeting a putative chromosomal gene encoding flagellin protein FlaA in *R. tumorigenes* and related tumorigenic *Rhizobium* sp. strains isolated from rhododendron were designed. The sequence of this gene gave no significant hits based on blastn analysis against the nr database in GenBank. Primer-BLAST analysis against the nr database indicated that primer pair TFP-F/R anneals to regions which seem to be specific for the “tumorigenes” clade (Fig. 1). Furthermore, corresponding fragments were not amplified from various agrobacteria, confirming the specificity of this primer system for the representatives of the “tumorigenes” clade, because specific PCR amplicons were only obtained for strains belonging to this phylogenetic group. Therefore, this primer set was used for the cultivation-independent detection of the pathogen in rhododendron and blueberry tumors (see below).

Cultivation-independent detection of the pathogen in rhododendron and blueberry tumors. Tumorigenic bacteria were not isolated from samples 8R, 10R, and 11R that were taken from rootstock mother plants (*Rhododendron* ‘Rhodunter 37’). Therefore, PCR amplification using *virD2* gene-specific primers (A and C’) followed by Southern blot analysis were used for detection

of the pathogen in these samples. Although corresponding PCR fragments were amplified in all samples, no hybridization signals were observed by using a probe generated from tumorigenic strain *Agrobacterium* sp. C58 (data not shown). In contrast, specific hybridization signals were obtained when using a probe generated from tumorigenic strain rho-6.2 isolated from rhododendron tumor tissue (Table 1; Supplementary Fig. S4). Using the same approach, the Ti plasmid was detected in DNA extracted from rhododendron and blueberry tumor tissue collected in 2012 to 2013, from which the isolation was not performed (Table 1).

The primer system developed in this study (Table 2), TFP_F/R, targeting the chromosomal *flaA* gene of *R. tumorigenes*-like bacteria, was used for PCR-Southern blot detection of the pathogen. Corresponding DNA fragments were amplified by PCR in all samples analyzed (Supplementary Fig. S5). By using a probe generated from tumorigenic strain rho-6.2, specific hybridization signals were obtained for all rhododendron and blueberry samples, indicating more than 75% sequence identity (Table 1).

Furthermore, we cloned and sequenced *virD2* gene fragments amplified from several representative samples (Rho2, 10R, and Hei3) from which we could not isolate the pathogen or isolation was not performed. Indeed, the *virD2* gene sequences (177 bp) obtained showed a high nucleotide identity (99.4 to 100%) to the corresponding sequences of the strains rho-1.1, rho-6.2, and rho-13.1 isolated from rhododendron. Only one single-nucleotide polymorphism (SNP) at different locations was identified within the sequences 6R_w9, Rho2_w5, Hei3_w6, and 10R_w9 obtained from tumor samples 6R, Rho2, Hei3, and 10R, respectively (Supplementary Table S3). However, only SNPs in sequences Hei3 and 10R resulted in an amino acid change. Furthermore, comparing the *virD2* gene sequence of rhododendron strains (177-bp fragment) with reference plasmids, 26, 28, 32, and 37 SNPs were observed for plasmids pRi1724, pRi2659, pTiS4, and pTiC58, respectively.

DISCUSSION

Although crown gall was observed on rhododendron, to the best of our knowledge, the causal agent of this disease has not been identified thus far. In this study, we fully resolved the etiology of the crown gall disease on rhododendron by analyzing tumor samples originating from Northern Germany. As a causative agent, we isolated and identified a bacterial species belonging to the genus *Rhizobium*. Interestingly, this bacterium was similar to *R. tumorigenes*, a novel tumorigenic bacterium recently discovered on blackberry in Serbia (Kuzmanović et al. 2018b).

More specifically, phylogenomic analysis that involved the use of a large number of conserved protein sequences extracted from whole-genome data clearly showed that *R. tumorigenes* and tumorigenic strains isolated from rhododendron form a separate clade within the genus *Rhizobium*, which we named the “tumorigenes” clade. The “tumorigenes” clade was grouped quite separately from the other *Rhizobium* spp. (Fig. 1). The closest relative, *R. tubonense* (Zhang et al. 2011), a symbiotic bacterium isolated from root nodules of *Oxytropis glabra* in China, was located on the neighboring branch. Although *R. tumorigenes* and the tumorigenic strains from rhododendron were closely related, the causative agent of crown gall of rhododendron represents a novel tumorigenic species, as indicated by whole-genome comparisons (ANI and iDDH) (Table 3). Therefore, it is classified as *Rhizobium* sp. in this study.

Members of the “tumorigenes” clade have been thus far identified exclusively as plant pathogens, associated with crown or cane gall disease of blackberry (Kuzmanović et al. 2018b) and rhododendron (this study). The symptoms caused by these pathogens were similar, and were generally characterized by the development of aerial tumors. It is not excluded that this group of pathogens has wider geographical distribution and significance. Indeed, the results of

molecular analyses of blueberry tumor samples suggested that the *R. tumorigenes*-like pathogen might be associated with blueberry showing crown gall symptoms originating from Lower Saxony. However, further investigations, including the isolation of the pathogen, are needed to confirm this assumption, which was based on the detection and analysis of *virD2* and *flaA* gene-specific sequences in total community DNA from blueberry tumors. Unfortunately, the symptomatic material from this site was not available, because the nursery has ended production. Interestingly, atypical agrobacteria that could not be placed into *Agrobacterium* biovars 1, 2, or 3, or into species *A. rubi*, were isolated from aerial tumors of wild blackberry in the United States (Canfield and Moore 1989). Although these strains remained unidentified, Canfield and Moore (1989) suggested that they grew well only on a mannitol glutamate medium supplemented with a large variety of salts, trace elements, and yeast extract. Similarly, strains belonging to the “tumorigenes” clade showed good growth only on a limited number of nutrient rich media such as YMA or TY medium. The fact that tumorigenic agrobacteria from the novel group (“tumorigenes” clade) seem to have different growth requirements compared with other tumorigenic Rhizobiaceae species might explain why they were not discovered until recently. In fact, various differential, semiselective, and selective media were recommended for isolation of tumorigenic agrobacteria from tumor tissue (Moore et al. 2001). Accordingly, in the initial investigations of this study, D1M medium (Perry and Kado 1982) that should support growth of various tumorigenic agrobacteria (Moore et al. 2001) was used but could not recover the pathogen from rhododendron tumors. Indeed, tumorigenic strains that were subsequently isolated on YMA medium did not develop on D1M medium. Taken together, the standard, nonselective, YMA medium can be recommended for isolation of tumorigenic agrobacteria from fresh galls in which the proportion of other bacteria is not too high.

Notably, tumorigenic bacteria were not isolated from samples 8R, 10R, and 11R that were taken from rootstock mother plants (*Rhododendron* ‘Rhodunter 37’). Possibly, the number of tumorigenic bacteria was low in respect to nonpathogenic and saprophytic members. For this reason, other bacteria inhabiting tumors might overgrow the pathogen. However, by using cultivation-independent methods that involved PCR, Southern blot, molecular cloning, and sequence analysis, the pathogen was detected in tumor samples 8R, 10R, and 11R.

In this study, a specific PCR assay targeting putative chromosomal gene-encoding flagellin protein FlaA of bacteria belonging to the “tumorigenes” clade was developed. This assay is intended for rapid identification of Rhizobiaceae strains belonging to this phylogenetic clade, regardless of whether they are pathogenic or not. However, because Ti plasmids are indispensable for pathogenicity of tumorigenic agrobacteria, their detection in isolated strains represents a crucial step in disease diagnosis. Intriguingly, some commonly used primers targeting different Ti plasmid regions such as primer pairs VCF3/VCR3 (Suzaki et al. 2004), tms2F1/tms2R2 (Puławska and Sobiczewski 2005), and F749/F14 (Mougel et al. 2001; Picard et al. 1992) failed to detect a Ti plasmid in the pathogen isolated from rhododendron. For instance, primers VCF3 and VCR3 targeting the *virC1-virC2* gene amplified specific PCR fragments in a wide variety of tumorigenic agrobacteria (Kawaguchi and Inoue 2009; Kumagai and Fabritius 2008; Kuzmanović et al. 2015b, 2016; Suzaki et al. 2004) and were therefore considered to be a reliable tool for detection of crown gall pathogens. Nevertheless, no amplicons were obtained for rhododendron strains when using these primers. In this respect, analysis of draft whole-genome sequences of strains rho-1.1, rho-6.2, and rho-13.1 indicated that *virC1* and *virC2* genes of these strains exhibited relatively low nucleotide identities (<74%) with reference Ti or Ri plasmids, which explains the lack of PCR amplification. Similar values were obtained when comparing sequences of genes *virB11* and *virG* targeted by primers F749 and F14, whereas gene *tms2* targeted by primers tms2F1 and tms2R2 was not identified in

rhododendron strains. The fact that this gene is not indispensable for tumorigenesis and T-DNA without the *tms1* gene was reported before (Ottens and Schmidt 1998).

Furthermore, higher nucleotide identities, reaching 87%, were obtained when comparing *virD2* gene sequences of strains rho-1.1, rho-6.2, and rho-13.1 with related Ti or Ri plasmids. Indeed, primers targeting the *virD2* gene (A and C') (Haas et al. 1995) were able to anneal to the target region and amplified specific fragments in strains causing crown gall of rhododendron, including all tumor samples tested. However, tumor samples tested, including tumorigenic strain *Rhizobium* sp. rho-6.2 used as positive control, were negative by using a hybridization probe generated from tumorigenic strain *Agrobacterium* sp. C58. In contrast, a specific hybridization signal was obtained using the *virD2* probe derived from the strain rho-6.2. Indeed, the *virD2* gene fragments of strains rho-6.2 and C58 that are flanked by primers A and C' (224 bp) and used for PCR-Southern blot analysis shared only 79.5% of nucleotide identity. This is close to the detection limit of hybridization under medium stringency (>75% of nucleotide identity between target and probe), which could explain the fact that no signals were obtained by using a hybridization probe generated from strain C58. Taken together, tumorigenic bacteria isolated from rhododendron seem to carry a Ti plasmid different from those described thus far.

In contrast to tumorigenic strains isolated from rhododendron, *R. tumorigenes* strains associated with cane gall disease of blackberry were positive in PCR using the abovementioned primers targeting genes *virC1-virC2*, *virD2*, *ipt*, and *tms2* (Kuzmanović et al. 2018b). Ti plasmid gene comparisons further suggested that *R. tumorigenes* harbors a different Ti plasmid compared with rhododendron strains. Members of the clade “tumorigenes” certainly have an ability to stably maintain distinct Ti plasmids and efficiently infect and exploit different host plants. It seems that they did not coincidentally acquire Ti plasmid from other known tumorigenic agrobacteria in a limited geographical area but, instead, represent *bona fide* tumorigenic bacteria that remained undiscovered thus far because of different growth requirements. The tumor-inducing ability under natural conditions has been well documented for *Agrobacterium* spp., *A. vitis*, and *R. rhizogenes*. However, isolation of a *Neorhizobium* sp. strain carrying Ti plasmid from a rose gall in Taiwan was recently reported (Haryono et al. 2018). This further extends taxonomic distribution of tumorigenic strains occurring in nature. However, additional investigations are required to provide more conclusive insights into the occurrence, distribution, and phytopathological significance of such strains.

Agrobacteria are generally characterized as soil-inhabiting and plant-associated rhizosphere bacteria. For instance, *A. vitis* strains are able to survive in soil and were thus far detected exclusively in association with grapevine plants. This pathogen can persist in decaying grape roots and canes in soil for at least 2 years (Burr et al. 1995). Accordingly, in order to better understand epidemiology and ecology of tumorigenic agrobacteria belonging to the “tumorigenes” clade, it is certainly necessary to investigate their persistence in soil. It also would be interesting to determine whether this pathogen systemically colonizes its host plants, as previously demonstrated for some other agrobacteria in different host plants (Cubero et al. 2006; Jones and Raju 1988; Lehoczy 1968; Tarbah and Goodman 1987; Yakabe et al. 2012; Zoina et al. 2001). In any case, the symptoms occurring primarily on above-ground plant parts of rhododendron and blueberry (this study), as well as blackberry (Kuzmanović et al. 2018b), suggest that the novel pathogen might be systemically present in host plants. In this regard, infection could occur upon plant tissue wounding, most likely by low winter temperatures or cultural praxis.

In conclusion, this study revealed tumorigenic agrobacteria belonging to the “tumorigenes” clade of the genus *Rhizobium* and carrying an atypical Ti plasmid as causative agents of crown gall on rhododendron. In addition to the recent report (Kuzmanović et al.

2018b), our results suggest that the novel group of tumorigenic agrobacteria is distributed more widely and likely causes crown gall on blueberry. Although significant economic losses caused by these pathogens were not recorded thus far, many outbreaks might have remained undocumented because of difficulties with isolation and identification of the causal agent. The data presented in this article highlight the diversity of tumorigenic agrobacteria and their Ti plasmids. Moreover, this study contributes to the diagnostics of tumorigenic agrobacteria and better understanding of the crown gall disease on some host species and, consequently, could facilitate disease management strategies.

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