Crown galls of grapevine (Vitis vinifera) host distinct microbiota

Running title: Crown galls of grapevine host distinct microbiota

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ABSTRACT
Crown gall disease of grapevine is caused by virulent Agrobacterium strains and establishes a suitable habitat for agrobacteria and potentially, other bacteria. The microbial community associated with grapevine plants has not been investigated with respect to this disease, which frequently results in monetary losses. This study compares the endophytic microbiota of organs from grapevine plants with or without crown gall disease and the surrounding vineyard soil over the growing seasons of one year. Amplicon-based community profiling revealed that the dominating factor causing differences between the grapevine microbiota is the sample site, not the crown gall disease. The soil showed the highest microbial diversity, which decreased with the distance from the soil over the root, graft union of the trunk to the cane. Only the graft union microbiota was significantly affected by crown gall disease. The bacterial community of graft unions without a crown gall hosted transient microbiota with the three most abundant bacterial species changing from season to season. In contrast, graft unions with a crown gall had a higher species richness, which in every season, was dominated by the same three bacterial species (Pseudomonas sp., Enterobacteriaceae sp., and Agrobacterium vitis). For in vitro cultivated grapevine plantlets, A. vitis infection alone was sufficient to cause crown gall disease. Our data show that microbiota in crown galls are more stable over time than graft union microbiota of healthy trunks, and that the microbial community is not essential for crown gall disease outbreak.

IMPORTANCE
The characterization of bacterial populations in animal and human diseases using high throughput deep sequencing technologies such as 16S amplicon sequencing will sooner or later result in the identification of disease-specific microbiota. We analysed the microbiota of the crown gall...
disease of grapevine, which is caused by infection with the bacterial pathogen *Agrobacterium vitis*. All other *Agrobacterium* species were found to be avirulent even though they lived together with *A. vitis* in the same crown gall tumour. As has been reported for human cancer, the crown gall tumour also hosted opportunistic bacteria that are adapted to the tumour microenvironment. Characterization of the microbiota in various diseases using amplicon sequencing may help in early diagnosis, so serve as a preventative measure of disease in the future.
INTRODUCTION

Agrobacterium vitis infects domesticated as well as wild grapevines (1) and is the most common cause of crown gall disease in grapevine (2, 3). As well as A. vitis, other virulent Agrobacterium species are known to induce grapevine crown gall development (4). A. vitis is known to persist in debris from infested grapevine material in soil (5) and can enter grapevines via the root and move through the xylem (6) to wounded parts of the plant where it transforms the cells (7-9). The pathogen has been detected in the xylem sap of canes, so propagation material of grapevine nurseries serves as an additional risk for distributing A. vitis (10, 11). Virulent A. vitis strains harbour a tumour-inducing (Ti)-plasmid, which enables the transfer of T-DNA into the plant genome, a process supported by virulence (Vir) genes of the Ti-plasmid (12, 13). The T-DNA encoded genes express enzymes for opine and plant hormone production (3, 14, 15). Opines are a nutrient source for virulent A. vitis and for other bacterial species that express opine-metabolizing enzymes (16-20). The altered auxin and cytokinin levels at the transformation site induce uncontrolled cell division and finally, crown gall development. Crown gall development gives rise to an altered tissue morphology and physiology (21).

In nature, both abiotic and biotic factors influence the A. vitis-mediated infection process and consequently, crown gall disease outbreak. Crown gall disease on grapevine occurs preferentially in regions with cold winters, indicating that cold temperatures are beneficial for disease outbreak (3). In addition, treatments that cause wounding such as farming devices and the grafting procedure can also promote outbreak of the disease. Biotic factors that influence crown gall disease in grapevine are both pathogenic and non-pathogenic bacteria. Antagonistic bacteria, which are known as biocontrol agents (e.g., the A. vitis strain F2/5), prevent transformation of grapevine cells by virulent A. vitis strains (22-24). Among the bacteria isolated from grapevine xylem sap, Pseudomonas sp. for example showed inhibitory effects on crown gall growth (25).
In recent years, more and more research groups have studied the bacterial community and structure of grapevine-associated microbiota, focusing on different aspects of viticulture and the taste of the resulting wine. Techniques such as isolation of cultivable bacteria (26) in combination with analysis of fluorescently-labelled terminal-restriction fragment analysis (27) and taxon or genus specific real-time PCR (28) have been used. The 16S rRNA gene amplicon high throughput sequencing technique provides a detailed overview of the microbiota and has been employed to resolve bacterial communities to the species level (29). This technique has been used to describe the above- and belowground microbiota of grapevines. (30-34). Grapevines from vineyards in Europe (Italy (30, 31), Portugal (32)) and the USA (New York State (33), California (34)) were sampled to analyse differences in the microbiota resulting from pest management (fungicide vs. biocontrol (30), integrated vs. organic (31)), vegetative cycle (32), climate (34), and edaphic factors (33). The leaf and grape microbiota correlate respectively with the vegetative cycle of the plant and the temperature (32, 34). Moreover, the study on grapevine from Long Island (Suffolk County, NY, USA) observed that the vineyard soil serves as a source for grapevine- and grape must-associated microbiota (33). A better understanding of the microbiota-plant interaction would help improve applications that promote plant growth and protection against pathogens (35-37).

In the present study, we investigated the microbiota of grapevines with and without crown gall disease because the microbiota of diseased and non-diseased grapevines have, to our knowledge, have not yet been studied. Employing high throughput sequencing of 16S rRNA gene amplicons, we analysed the microbiota of the soil, root and graft union of the trunk, and one-year-old canes of grapevine plants with and without crown gall over the growing seasons of a year. We also established an infection assay using in vitro cultivated grapevine plantlets. This assay allowed us
to investigate the capability of environmental *Agrobacterium* isolates to induce crown gall growth and to analyse the role of the microbiota associated with crown gall disease.

**MATERIAL AND METHODS**

**Sample collection**

Grapevine material was collected from four individual plants (Fig. 1A) growing in one row in a stretch of 22 meters located at a vineyard at Himmelstadt, Franconia, Germany (49°55´23.78N, 9°49`05.22O). The grapevines of the variety Carbernet Dorsa had been grafted on the rootstock SO4, and planted in 2008 in loamy sand. Four different sampling sites of each grapevine plant were analysed (Fig. 1B): (i) one-year-old cane (c), (ii) graft union of the trunk (g), (iii) root (r), and (iv) soil (s) from the root environment. The samples were collected before noon on 2013-October-30 (autumn), 2014-April-04 (spring), and 2014-July-23 (summer). At each time point, the weather was sunny and the soil dry. Three replicates were harvested per sample site and season, resulting in a total of 144 samples (Fig 1C). Roots were washed with tap water and the periderm of all plant material was discarded. Half of the wooden grapevine material and the soil was stored at -80°C for DNA extraction and the other half at 4°C for isolation of bacteria.

**DNA extraction and amplicon sequencing**

The frozen plant and soil samples (-80°C) were shredded in a ball mill MM2000 (Retsch, Haan, Germany) and DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). DNA extractions with the kit components and no added sample material served as negative controls. For PCR of the 16S rRNA gene, the primers 515F and 806R including 2 x 8 bp multiplexing indices and Illumina Adapters attached to their 5’ end were used to amplify the 6
variable region V4 of the 16S rRNA gene (38). The sequence of the forward primer was: 5' –
AAT GAT ACG GCG ACC ACC GAG ATC TAC ACX XXX XXX XTA TGG TAA TTG TGT
GCC AGC MGC CGC GGT AA – 3' and the reverse primer: 5' – CAA GCA GAA GAC GGC
ATA CGA GAT XXX XXX XXA GTC AGT CAG CCG GAC TAC HVG GGT WTC TAA T –
3'. XXX XXX XX indicate the index sequences.

Each sample was processed in three technical replicates to reduce random PCR effects (39). PCR
was performed in 10 µl reactions, each containing 5 µl 2 x Phusion® High Fidelity PCR Master
Mix (New England Biolabs, Ipswich, MA, USA), 0.33 µl of the 10 µM forward and reverse
primers (Eurofins MWG Operon, Huntsville, AL, USA), 3.34 µl PCR grade water, and 1 µl
template DNA. PCR conditions comprised an initial denaturation step at 95°C for 4 min, 35
cycles of denaturation at 95°C for 40 sec, annealing at 55°C for 30 sec, and elongation at 72°C
for 1 min, followed by a final extension step at 72°C for 5 min. We combined the three technical
PCR replicates to a 30 µl PCR pool. Successful amplification was verified with agarose gel
electrophoresis using 5 µl of the PCR pool. The remaining 25 µl were further processed using the
SequalPrep™ Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA) to remove excess
primers and nucleotides as well as for normalizing the PCR product to quantities of 25 ng. 5 µl of
normalised DNA were used for pooling with the samples of other projects for parallel sequencing
(38). This final DNA pool was verified for DNA fragment size of the library with a High
Sensitivity DNA Chip (Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) and
quantified with the dsDNA High Sensitivity Assay (Life Technologies GmbH, Darmstadt,
Germany). The final DNA pool was diluted to 2 nM, and 3 µl of each of the custom sequencing
and indexed primers were added to the cartridge of a 2 x 250 bp v2 paired-end sequencing MiSeq
sequencing kit (Illumina, San Diego, CA, USA). 16S rRNA amplicons were sequenced according
to the manufacturer’s protocol for the Illumina MiSeq instrument using a 2 x 250 bp v2 paired-end sequencing run.

**Amplicon sequencing data analysis**

The quality of the sequences was analysed using FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The program fastq-join v.1.8.0 (https://code.google.com/p/ea-utils/wiki/FastqJoin) was used to join forward and reverse reads.

The reads were filtered with USEARCH v8 (40), which included quality filtering according to the phred score (> Q20) and the sequence length (> 250bp). Clusters of operational taxonomic units (OTUs) were built, chimeras removed and taxonomically classified using the UCLUST (40) and UCHIME (41) algorithms, as implemented in USEARCH (42) v.7.0.1090. Using the Ribosomal Database Project, RDP-classifier v.2.2 (43), we assigned the taxonomy for each OTU. A phylogenetic tree was calculated using FastTree (44) v.2.1.3. Plastids and mitochondrial 16S rRNA gene sequences were removed from the OTU table before continuing the analyses.

The R script of the following downstream analyses, using the packages phyloseq (45) and vegan (http://CRAN.R-project.org/package=vegan) are provided in the supplemental material. Using the OTU table without any normalization (46), bacterial community dissimilarities between each of the individual samples were estimated using the Bray Curtis distance and the resulting beta diversity was visualized through non-metric multidimensional scaling (NMDS). Four outliers were excluded according to the NMDS. The various influential factors (sample site, season and crown gall disease) were fitted onto the ordination axes, representing the differences in the microbiota, so as to identify significant correlations. A general linear model with the coefficients, soil, root, graft union, cane, and the scores was generated and the relevance of this model for our data was tested using an ANOVA statistical test (NMDS axis one). Fold changes of the sample
types were calculated using the R package EdgeR (46, 47). Significant fold changes had a False Discovery Rate (FDR) <0.001. Only OTUs with a mean abundance \( \geq 20 \) sequences per sample in at least one group were considered for analysis.

We determined the bacterial species richness as raw counts of the OTUs and calculated the alpha diversity using the Shannon Index (48) based on the OTUs. Significant differences in the alpha diversity and bacterial species richness between sample types were calculated using the Wilcoxon test (49). For taxonomic analysis of the microbiota, all samples from one site and all OTUs of the same taxonomic rank were merged. To calculate the relative abundance of a taxonomic rank, the sequences of a taxonomic rank were divided by all sequences of one sample site. We merged the taxonomic ranks that were less abundant than 0.5% to one group called “other”. The relative abundance of each OTU within one sample was calculated by using Random Forest (50), a supervised learning analysis, with 1,500 decision trees. The relative sample counts of the OTUs were used as predictors with season, sample site or crown gall disease as class labels. The percentage of calculated and actual sample class labels resulted in the out-of-bag error (OOB). A small OOB indicates distinctive microbiota according to the class labels. The VennDiagram package (51) of the R software was used to calculate shared OTUs between the sample sites of galled and non-galled grapevines. Within each season, we randomly paired a galled and a non-galled grapevine plant for a paired Wilcoxon test. This allowed us to calculate any significant differences between the amounts of shared OTUs between, for example, canes and graft unions of plants with and without a crown gall. This calculation was repeated for soils and roots.

**Isolation and PCR screening of agrobacteria**

We isolated bacteria from the graft union material used in this study for amplicon sequencing. The wooden parts of the grapevine material were shredded using a ball mill (Retsch, Hannover,
Purified water (Rotisol® HPLC Gradient Grade, Roth) was added to 300 mg of the processed grapevine material or soil. After 2 h at 28°C, the supernatant was used for tenfold serial dilutions and 100 μl were incubated for five days on agar plates supplemented with 213 μM cycloheximide (CHX, Sigma-Aldrich, St. Louis, USA) to prevent growth of fungi. Either YEB-CHX agar plates [0.5% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 0.5% (w/v) Sucrose, 1.23% (w/v) MgSO4 (AppliChem, Darmstadt, Germany), 1.5% (w/v) Agar-Agar Kobe I (Carl-Roth, Karlsruhe, Germany)] or LB-CHX agar plates [1% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 1% (w/v) NaCl, (AppliChem, Darmstadt, Germany), 1.5% (w/v) Agar-Agar Kobe I, (Carl-Roth, Karlsruhe, Germany)] were used for bacterial growth. Single colonies with an Agrobacterium-like morphology were sub-cultured on YEB-CHX or LB-CHX agar plates. PCR-based screening for Agrobacterium colonies was performed as follows: (i) Two different fragments of the 16S rRNA gene was amplified to identify Agrobacterium, (ii) a RecA gene fragment (52) served for differentiation between Agrobacterium vitis and other Agrobacterium species, and (iii), a fragment of the VirD2 was PCR amplified to screen for the presence of the Ti-plasmid (11). The following primer sequences were used: (i) 16S rRNA gene (27F: 5' AGR GTT YGA TYM TGG CTG AG 3' and 1492R: 5' GGY TAC CTT GTT ACG ACT T 3'; or 515F: 5' GTG YCA GCM GCC GCG GTA A 3' and 806R: 5' GGA CTA CNV GGG TWT CTA AT 3'), (ii) A. spp. specific RecA (F8360: 5' AGC TCG GTT CCA ATG AAA 3'; F8361: 5' GCT TGC GCA GCG CCT GGC T 3'), A. vitis specific RecA (G0004F: 5' GAT ATC GCG CTC GGC ATT GGT 3'; G0005R: 5' CCT TCG ATT TCA GCT TTC G 3') (52), and (iii) virD2 (virD2F: 5' TTG GAA TAT CTG TCC CGG AAG 3'; virD2R: 5' CTT GTA CCA GCA GGG AAG CT T A 3') (11). A 50 μl PCR reaction mixture contained: 1 x HF-buffer (New England BioLabs, Ipswich, USA), an experimentally determined amount of custom-made Phusion Polymerase (53), 0.2 μM of each primer, 400 μM of dNTPs (Fermentas, Waltham, USA), and 2 μl of a bacterial colony.
resuspended and boiled in 100 µl HPLC-grade water (RotisolV HPLC Gradient Grade, Roth) for 10 minutes. The 16S rRNA gene sequences of the PCR-products were analysed by Sanger sequencing (GATC, Konstanz, Germany) followed by a nucleotide search using the BLAST algorithm (54) from the NCBI database (55).

Infection assay of in vitro cultivated grapevine

Four to eight-week-old in vitro cultivated grapevine plantlets (variety Mueller Thurgau and 5BB) provided by the vine nursery Steinmann, Sommerhausen, Germany, were used in a virulence assay (Fig. S1). In vitro plantlets originate from one-year-old cane pieces with one node of cuttings from environmental grapevine plants from the year 2000. The cane pieces were surface sterilized (ethanol 96%, sodium hypochloride 6%) and after root and shoot induction, the plantlets were sub-cultivated every 8 to 12 weeks in plastic boxes filled with 3 cm of a grapevine-specific agar growth medium. Plantlets were incubated in a growth chamber with a 14 h photoperiod (light, 23°C; dark, 21°C) and a light intensity of 180 µmol s⁻¹ m⁻² using universal white lamps (L 36W/25, Osram, Munich, Germany). Agrobacterium isolates were inoculated to induce crown gall development at the second or third internode of the grapevine stems using a sterile needle dipped into a colony. The known virulent Agrobacterium vitis strain S4 (13) and the non-virulent disarmed Agrobacterium tumefaciens strain GV3101 (56) served as positive and negative controls respectively. At least eight plantlets were inoculated with the same Agrobacterium strain/isolate. We visually screened the plantlets for the appearance of crown galls on a weekly basis for up to eight weeks. Four-week-old infection sites were used for 16S rRNA gene amplicon sequencing as described in the paragraphs “DNA extraction and amplicon sequencing” and “Amplicon sequencing data analysis”.

11
Data availability

Raw 16S rRNA gene sequencing data are deposited at the European Nucleotide Archive (ENA), http://www.ebi.ac.uk/ena, accession number PRJEB12040.

RESULTS

Each sampling site harbours a distinct microbiota

Material of two grapevines with and two without a crown gall (Fig. 1A) from four sampling sites each were collected at three different time points over a year (Fig. 1B), resulting in 144 samples (Fig. 1C). A total of 4,572,415 16S rRNA gene sequences were analysed. After removing the sequences belonging to chloroplasts and mitochondria, 1,201,593 sequences remained. These were grouped into 8,674 different operational taxonomic units (OTUs). The non-metric multidimensional scaling (NMDS) ordination shows that the structural differences in the microbial community composition were determined first and foremost by the sample site (Fig. 2, environmental fit: r²=84%, p-value<0.001). The calculation of a general linear model of the values of NMDS1 resulted in a significant influence of each sampling site on the microbiota (soil: t-value=32, p-value<0.001; root: t-value=12, p-value<0.001; graft union: t-value=8, p-value<0.001; cane: t-value=-18, p-value<0.001). The ANOVA test for the general linear model (F= 357, p-value<0.001, residuals degrees of freedom= 136) and Random Forest analysis (Table S1) illustrates that the sample site accounts for the main difference in microbial community composition.

Microbial structure of the sampling sites
In terms of richness, the soil microbiota harboured a greater diversity of bacterial taxa than the other sampling sites (Fig. 3A, avg±SD richness=2712±673). The richness in bacterial taxa decreased with the distance from the soil over the root (richness=253±170), to the graft union (richness=166±50), and the cane (richness=76±41). Similarity analyses of the microbiota from the different sites showed that 410 OTUs (5%) were identical in the soil, root, graft union, and the cane. Each sample site shared most of the OTUs with the soil (root: 88%, graft union: 82%, and cane: 79%). The alpha biodiversity (Shannon index) also changed with the distance from the soil (Fig. 3B) in that the microbiota of the soil was most diverse (mean±sd: Shannon Index=6.8±0.2), followed by the root (mean±sd: Shannon Index=4.0±0.7), graft union (mean±sd: Shannon Index=3.2±0.9), and the cane (mean±sd: Shannon Index=3.0±1).

A detailed analysis of the bacterial phyla composition revealed that the relative number of Proteobacteria sequences increased with the distance from the soil (Fig. 3C). Proteobacterial sequences comprised 22% of all OTUs in the soil, 62% in the root, 89% in the graft union, and 89% in one-year-old cane samples. In contrast, Actinobacteria decreased along the plant axis from 20% in root, 5% in graft union, with 2% in cane samples. Sequences of the class Acidobacteria were only present in the soil (17%) and root (2%) microbiota. In addition, Bacteroidetes (soil: 9%, root: 9%), Planctomycetes (soil: 6%, root: 1%), and Verrucomicrobia (soil: 5%, root: 1%) were more represented in soil and root samples than in the graft union and cane microbiota. At the genus level (Fig. 3D), Pseudomonas dominated the aboveground microbiota (graft union: 46%, cane: 72%), while in the soil it was “Nitrososphaera” (24%), and in the root Methylobacterium (12%). Agrobacterium-related sequences were mainly present in roots (2%) and graft unions (17%, Fig. 3D), while in soil (0.3%) and canes (0.7%), the relative sequence numbers were very low. Taken together, the sample site-specific grapevine-associated
microbiota changed with the distance from the soil in diversity, richness, shared operational
taxonomic units, composition, and structure.

**Impact of the seasons and crown gall disease on the microbiota**

We next analysed the amplicon data of each sample site with respect to the seasons (Fig. 4). Separate NMDS ordinations for each sample site (Fig. 4A) demonstrated the effects of the season on the microbiota of soil (environmental fit: $r^2=29\%$, p-value<0.001), graft unions (environmental fit: $r^2=52\%$, p-value<0.001), and canes (environmental fit: $r^2=65\%$, p-value<0.001). The seasons had no significant influence on the root microbiota (root, environmental fit: $r^2=13\%$, p-value≤0.067). Computable classification by Random Forest of the samples taking the seasons into account revealed the highest error rate for the root samples (Table S2, out-of-bag estimated error, OOB: 21%) followed by the soil (OOB: 11%) and finally the aboveground samples, graft union (OOB: 8%) and cane (OOB: 9%). Both, the NMDS ordinations and the Random Forest classifications indicated a greater influence of the seasons on aboveground than on belowground microbiota.

With respect to the presence/absence of crown gall disease, the data showed neither a significant effect on the soil microbial community composition (Fig. 4B, environmental fit: $r^2=0\%$, p-value≤0.91), on the root (B: $r^2=1\%$, p-value≤0.76) nor on the cane (D: $r^2=4\%$, p-value≤0.23). However, the microbiota differed significantly between the graft unions with a crown gall and those without (Fig. 4B, environmental fit, $r^2=25\%$, p-value<0.001). Computable classification using Random Forest revealed the smallest out-of-bag estimated error rate in the microbiota of the graft unions (Table S3, OOB, graft unions= 8%) as compared to the soil (OOB: 40%), root (OOB: 59%), and cane (OOB: 37%).
We then compared the microbiota of the two types of graft unions (without and with a crown gall) from each season (spring, summer, autumn) to each other (Fig. 5). The bacterial richness was higher in the graft unions with a crown gall in spring (p-value≤0.065), summer (p-value≤0.092), and autumn (p-value≤0.065) compared to those without (Fig. 5A). Furthermore, the higher richness in the microbiota of the graft unions with a crown gall did not change significantly over the seasons. In contrast, in graft unions without a crown gall, the richness was significant lower in autumn compared to spring and summer (Wilcoxon test, spring-autumn: p-value≤0.004, summer-autumn: p-value≤0.005). The richness analysis indicates that the microbial community in graft unions with a crown gall contains additional bacterial taxa and is more stable over the seasons than those without. The alpha diversity (Shannon index) did not change prominently in graft unions without a crown gall over the seasons. In contrast, the alpha diversity differed significantly between the seasons in graft unions with a crown gall (Fig. 5B, Wilcoxon-test, p-value: spring-summer≤ 0.002, summer-autumn≤ 0.002) and was highest in summer.

**Bacterial taxa that are affected by the crown gall disease**

We recovered 23 *Agrobacterium* isolates from the grapevine and soil material used for amplicon sequencing. The screening for agrobacterial virulence resulted in identification of six virulent *Agrobacterium vitis*. The remaining 17 non-*A. vitis* isolates were classified as non-virulent agrobacteria. *A. vitis* isolates were only found in crown galls and roots of the diseased grapevine plants together with non-virulent *Agrobacterium* species. According to the RDP classifier, *A. vitis* is also one of the three most abundant OTUs in graft unions with a crown gall, the others being *Pseudomonas sp.*, OTU_0005 and *Enterobacter*, OTU_0008. In graft unions with a crown gall, these three most abundant OTUs (*A. vitis*, OTU_0003; *Pseudomonas sp.*, OTU_0005; *Enterobacter*, OTU_0008) amounted to 53% and 58% of all sequences in spring and autumn,
respectively, although in summer this dropped to 19% of all obtained sequences (Fig. 5C).

Nevertheless, these three OTUs still remained the most abundant ones in summer. In contrast, the three most abundant OTUs in graft unions without a crown gall differed in every season (Fig. 5C): in spring *Pseudomonas* species (OTU_0055, OTU_2368, OTU_4255); in summer *Pseudomonas* sp. (OTU_0005), *Sphingomonas* sp. (OTU_0052), and *Curtobacterium* sp. (OTU_0011), and in autumn, *Pseudomonas* sp. (OTU_0055), *Ralstonia* sp. (OTU_0021), and *Erwinia* sp. (OTU_7832).

To record the bacterial taxa that are significantly affected by the crown gall disease, we calculated the fold changes of the sequence numbers for the OTUs detected in graft unions with and without a crown gall separately for each season (EdgeR, FDR<0.001, Table S4). Of the 28 different OTUs with significant changes in sequence numbers, 24 increased in graft unions with a crown gall compared to those without. Among the latter, nine OTUs comprised zero sequences in graft unions without a crown gall; hence, these were exclusively present in graft unions with a crown gall. Of the four OTUs of which the sequence numbers decreased in graft unions with a crown gall, three (OTU_0005, OTU_0011, OTU_0052) were less abundant in summer. At this time of the year, two other OTUs showed a significant increase: an unknown member of the *Proteobacteria* phylum (OTU_3436) and *A. vitis* (OTU_0003). These two were significantly enriched in all seasons and are part of the core microbiota in graft unions with a crown gall. Four additional OTUs contributed to the core microbiota of crown galls: OTU_0005 (Pseudomonas sp.), OTU_0007 (*Burkholderiales*), OTU_0008 (*Enterobacteriales*), and OTU_0032 (*Agrobacterium* sp.). These represented more than 20 sequences per sample in at least 80% of the graft union samples with a crown gall. In graft unions without a crown gall, no OTU met this definition; in graft unions of healthy trunks, the microbiota were more fluctuating.
Crown gall induction without core microbiota

We also analysed an amplicon-sequencing data set of in vitro cultivated grapevine plantlets to address the question as to whether crown gall development requires a core microbiota and if this in turn profits from the crown gall environment. The in vitro cultivated plantlets were inoculated with either the virulent A. vitis S7, an isolate from a grapevine crown gall of the same vineyard used for sampling in this study (Fig. S1A and B), or with the disarmed Agrobacterium tumefaciens strain GV3101. Uninoculated plantlets served as controls (Fig. S1C). Altogether, amplicon sequencing was performed on 18 samples (Fig. S1D), resulting in a total of 568,855 sequences. After removing the plastid and mitochondrial related 16S rRNA gene sequences, 42,700 sequences remained and were grouped into 612 OTUs. In non-inoculated in vitro cultivated grapevines, no OTU was detected with more than 15 amplicon sequences, suggesting an extremely low abundance of bacteria. The stems inoculated with the avirulent A. tumefaciens GV3101 contained an enriched number of sequences of this strain (OTU_0507), another Agrobacterium (OTU_0032) and Curtobacterium (OTU_0011; Table 1, EdgeR, FDR<0.001). In crown galls of the plantlets inoculated with the virulent A. vitis S7 strain (OTU_0003), no other OTU was significantly increased (Table 1). This experiment indicates that the virulent A. vitis S7 can induce crown gall disease on grapevine without any requirement of a core microbiota.

Bacterial taxa shared between crown galls and the other sample sites

To identify the source of the additional bacterial taxa found in native crown galls, we analysed the OTUs of the graft unions shared with the other sample sites (soil, root, cane) separately for diseased and non-diseased native grapevines. We randomly paired a diseased with a non-diseased plant sample from the same season using a paired Wilcoxon test. The microbiota of the graft unions with a crown gall shared significantly more bacteria with the root (p-value≤0.024) and the...
soil (p-value≤0.003) than with the healthy graft unions without a crown gall. In contrast, the latter
shared more OTUs with the cane (p-value≤0.009). Thus, the soil and root rather than the cane
serve as a source for bacteria in grapevine crown galls.

**DISCUSSION**

To understand the infection ecology of the crown gall disease, we investigated the endophytic
microbial community of grapevines with and without a crown gall. Amplicon-based community
profiling revealed a distinct microbial community for each of the sample sites (soil, root, graft
union of the trunk, cane). Distinct microbiota have previously been published for grapevines from
vineyards in Long Island (Suffolk county, NY, USA) for soil, root, leaf, flower, and grape berry
(33) and from Lussac St. Emillion (Gironde, France) for soil, bark, leaf, and grape berry samples
(27). *Proteobacteria, Acidobacteria, Actinobacteria,* and *Bacteroidetes* dominated the microbiota
in our soil samples and in those from Long Island, while in the samples from Lussac St. Emillion,
no *Bacteroidetes* colonies were isolated. Furthermore, our root samples and those from Long
Island (33) as well as our cane samples and those from Trentino, Italy (26), were similar on the
phylum level. The microbiota of our sample sites, those from Long Island (33), and Lussac St.
Emillion (27) all showed a gradient from belowground to aboveground. In all cases, the structure
of the soil microbiota was most complex (highest richness and alpha diversity) with that of the
aboveground least complex (lowest richness and alpha diversity). This gradient in the microbial
structure and composition is most likely the result of changing environmental factors such as
humidity, distance from the soil, organic substrates, and UV exposure (57-60). The factor season
had an additional impact on the microbial structure, which was stronger on our aboveground than
belowground samples. Not only do the seasons have an impact on the microbial composition of
the leaf microbiota of grapevine (28, 32), but the time of year also influences endophytic bacteria
in woody material, as shown in this study. Thus, we conclude that grapevines have similar phyla compositions in distinct locations and that the crown gall disease does not substantially affect the microbial structure of the soil, root, and cane.

Crown gall disease affected the microbiota only in graft unions. The microbiota of graft unions with a crown gall contained a higher number of different bacterial species in all seasons compared to graft unions without a crown gall. *Agrobacterium vitis* together with eight other bacterial species caused the difference in the microbial community between graft unions with and without a crown gall. These were exclusively found in graft unions with a crown gall. Likewise, *Arabidopsis* leaves infected with the fungus *Albugo* significantly enriched a subset of bacterial endophytes (61). This suggests that both pathogens (*A. vitis* and *Albugo*) promote colonization with certain endophytic microbes at the infection site. Compared to healthy graft unions, crown galls share more bacteria with the belowground microbiota and fewer with canes. Therefore, the source for the invasive bacteria in crown galls seems to be the soil and root. This finding supports the idea of the soil as a microbial seed bank for grapevine-associated microbiota, as previously postulated (33).

In graft unions with a crown gall, three OTUs, *A. vitis* (OTU_0003), *Pseudomonas* sp. (OTU_0005), and *Enterobacteriaceae* sp. (OTU_0008) were most abundant in every season. These three, together with three additional OTUs, were present in 80% of graft union samples with a crown gall, indicating that the crown gall microbiota is relatively stable. In summer the percentage of the three most abundant bacterial species, which included *A. vitis*, decreased in crown galls, thereby increasing the species evenness in the bacterial communities at this time of year. Other studies have reported that in summer, the colony forming units of *A. vitis* are reduced in grapevines (62) and that the isolation of *A. vitis* from grapevine samples is more difficult (7). A reason for the reduction in species abundance in summer could be higher temperatures and
drought stress. It is known from the model plant *Arabidopsis* (63, 64) and *Ricinus communis* (65) that crown gall growth is affected by drought stress. In graft unions without a crown gall, the diversity of the bacterial species was only marginally affected by the season. The three most abundant OTUs encompassed 57% of all sequences in summer, which was only marginally higher than the three in spring (42%) and autumn (36%). However, the three most abundant bacterial species varied between the seasons, indicating that, unlike in graft unions with a crown gall, no core microbiota exists in graft unions without a crown gall. Thus, crown gall disease seems to stabilize the bacterial composition over the seasons, as previously reported for phytoplasma infected grapevine leaves (28). Nonetheless, the striking decrease in abundance of bacterial species in graft unions with a crown gall in summer seems to be specific for crown gall tissue.

The bacterial species exclusively found or enriched in graft unions with a crown gall may profit from the crown gall environment. Indeed, it is well known from the literature that this habitat provides opines and other accumulating metabolites, as well as additional living space (14, 66). It has been shown that opines serve as common nutrients and cause an increase in the local population of opine metabolising bacteria (19). This has also been demonstrated by transgenic opine-producing legume species, which harboured an altered bacterial composition, including an increase in opine degrading *Pseudomonas* (67). We also found a *Pseudomonas* strain (OTU_0005) clearly enriched in spring and autumn in crown galls. *Pseudomonas* is able to cause wounds by producing ice crystals (68, 69). Wounds induce *Agrobacterium*-mediated processes such as plant cell transformation, production of opines, and phytohormones (70). For example, indole-3-acetic acid (IAA) is involved in plant and crown gall developmental processes, enriched in crown galls, and can serve as a source of carbon for *Pseudomonas putida* 1290 (71).

Furthermore, *Pseudomonas* sp. (72, 73), *Enterobacteriaceae* sp. and many other endophytic...
grapevine bacteria (26) are able to produce IAA. Interactions of non-pathogenic with pathogenic bacteria are known for tumours of olive trees, induced by *Pseudomonas savastanoi* (pv. *Savastanoi*), which host a non-pathogenic *Erwinia* species (74). An *Erwinia* species (OTU_7832) was enriched in graft unions with a crown gall and it seems to profit from the crown gall environment.

We used *in vitro* cultivated grapevine plantlets to investigate the mechanisms of the infection process and development of crown gall disease. This infection assay demonstrated that *A. vitis* and no other *Agrobacterium* species of the environmental isolates, including *A. tumefaciens*, caused crown gall disease. This indicates that in grapevines, *A. vitis* retains its virulence machinery. This finding is in accordance with a high throughput isolation study of agrobacteria from crown galls of herbaceous and woody hosts (75). In this study, only seven out of 5419 isolates became non-virulent-mutants after being inoculated into host plants to induce crown galls. Furthermore, induction of crown gall growth on *in vitro* cultivated grapevine plantlets proved that *A. vitis* does not require a microbial community for disease outbreak. This observation suggests that the crown gall-specific bacterial species and those that strongly multiply in crown galls appear to benefit from the crown gall environment provided for them by *A. vitis* infection.

**CONCLUSION**

Grapevine organs and the vineyard soil harbour a distinct microbial community, which is not affected by crown gall disease, except at the site of graft union and gall formation. Graft unions with a crown gall stabilise core microbiota and host opportunistic bacteria over the seasons. These however, are not essential for the induction of crown gall growth. Our *in vitro* assay showed that induction of crown gall growth requires no other bacterium than *Agrobacterium*.
This finding suggests that none of the invasive endophytic bacteria including *A. tumefaciens* is obligate for crown gall development. Nonetheless, a supportive role in the performance of crown gall development cannot be excluded and will be addressed in future studies. The invasive bacterial species most likely profit from the crown gall environment in that they have an advantage, nutritional or otherwise, by living within crown gall tissues. Unravelling the role of the opportunistic bacteria in crown gall performance may help to support disease management in the future.
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REFERENCES


54. **Altschul SF, Lipman DJ.** 1990. Protein Database Searches for Multiple Alignments. Proc Natl Acad Sci U S A **87:**5509-5513.


56. **Koncz C, Schell J.** 1986. The Promoter of T1-DNA Gene 5 Controls the Tissue-Specific Expression of Chimeric Genes Carried by a Novel Type of Agrobacterium Binary Vector. Mol Gen Genet **204:**383-396.


FIGURE LEGENDS
FIG 1  Grapevine plants and sampling procedure used for comparison of grapevine microbiota. (A) Graft unions of the trunk of the two grapevine plants with (1, 2) and two without (3, 4) a crown gall. (B) Illustration of the sampling sides; c, one-year-old cane, g, graft union, r, root, and s, soil. (C) Scheme of the experimental setup.

FIG 2  Distribution of the 144 grapevine associated microbiota within a non-metric multidimensional scaling (NMDS) ordination. Analysis is based on the Bray Curtis distance. The factor sample site explains 84% ($R^2$) of the variation among the microbiota. Significance (p-value) was calculated using a permutation test. Colours indicate sample sites.

FIG 3  Comparison of the microbial communities from the soil, the grapevine roots, graft unions, and canes. (A) Number of bacterial taxa (richness) and (B) Shannon Index ($\alpha$-diversity) for each of the sample sites. P-values are calculated according to Wilcoxon rank sum test. (C) Percentages of phyla and (D) genera in the microbial community for each sample site. Phyla or genera with a relative abundance lower than 0.5% in the microbial community are combined into the group "other".

FIG 4  Factors determining the differences between the microbial communities of the sample sites. Non-metric multidimensional scaling (NMDS) ordinations for (A) the factors seasons and (B) graft unions without or with a crown gall. The percentage of variation among the microbiota of a sample site was correlated with the factor seasons or crown gall disease ($R^2$). Significance was calculated using permutation test (p-value).

FIG 5  Comparison of the microbial communities of the graft unions without and with crown gall disease. (A) Number of bacterial taxa (richness) and (B) including abundance within each bacterial taxa ($\alpha$-diversity), calculated according to Shannon for each of the sample sites. P-values are calculated according to the Wilcoxon rank sum test. (C) Percentage of the three most
abundant operational taxonomic units (OTU) in the microbial community for each sample site. All OTUs with a relative abundance lower than 0.5% were merged, forming the group “other”.

TABLES

TABLE 1 Operational taxonomic units (OTUs) with significant differences in 16S rRNA gene sequence numbers from stems without and with a crown gall of in vitro cultivated grapevine plantlets. Stems were inoculated with the virulent Agrobacterium vitis isolate S7 (OTU 0003) and the disarmed Agrobacterium tumefaciens strain GV3101 (OTU 0507) four weeks before analysis. Displayed are mean sequence numbers in the samples with and without a crown gall calculated according to the EdgeR package in R. Log2 fold changes (logFC) and log2 counts per million (logCPM). P-values are adjusted to multiple testing according to Benjamin-Hochberg (FDR, false discovery rate <0.001).

<table>
<thead>
<tr>
<th>bacterial identity</th>
<th>16S sequence numbers</th>
<th>adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without</td>
<td>with</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens GV3101 (OTU 0507)</td>
<td>3591</td>
<td>78</td>
</tr>
<tr>
<td>Agrobacterium (OTU 0032)</td>
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<td>Agrobacterium vitis isolate S7 (OTU 0003)</td>
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<td>2758</td>
</tr>
<tr>
<td>Curtobacterium (OTU 0011)</td>
<td>30</td>
<td>1</td>
</tr>
</tbody>
</table>

SUPPLEMENT LEGENDS

FIG S1 Infection procedure of in vitro cultivated grapevine plantlets. (A) Inoculation of agrobacteria into grapevine stems using a needle. (B) Grapevine stems with a crown gall and (C) mock-inoculated stems without a crown gall. Scale bar distance in B and C is 1 mm. (D) Experimental setup and number of samples used for amplicon sequencing.
TABLE S1 Classification of the grapevine-associated microbiota attained by performing a supervised learning analysis according to the factor sampling site (Random Forest). The predicted sample site classifications (soil, root, graft union, cane; horizontal) were compared to the known classifications of the sample sites (vertical). The percentage of wrongly classified samples within one sample site is called class error while the percentage of wrongly classified samples of all classified samples is termed the out-of-bag error (OOB: 2%).

TABLE S2 Grapevine-associated microbiota were used to perform a supervised learning analysis (Random Forest) according to the factor season. Predicted classifications of the samples into spring, summer and autumn (horizontal) were compared to the known classifications in spring summer and autumn (vertical). The percentage of wrongly classified samples within one season is called class error while the percentage of wrongly classified samples within one sample site (soil, root, graft union, cane) is called out-of-bag error (OOB).

TABLE S3 Classification of the grapevine-associated microbiota according to the factor crown gall from performing a supervised learning analysis (Random Forest). The predicted classification of the sample sites from grapevines with and without a crown gall (horizontal) are compared to the known sample site classifications of grapevines with and without a crown gall (vertical). The percentage of wrongly classified samples of grapevines with or without a crown gall is called class error while the percentage of wrongly classified samples within one sampling site (soil, root, graft union, cane) is termed an out-of-bag error (OOB).

TABLE S4 Operational taxonomic units (OTUs) with significant differences (FDR < 0.001) in the mean number of 16S rRNA gene amplicon sequences. Differences between graft unions with 'A' and without 'B' crown gall disease in spring, summer, and autumn. Fold changes are given as log2FC and log2CPM (average log2 counts per million). Statistics analysis was performed using...
a two-sided test for calculation of the p-values. The false discovery rate (FDR) gives an adjusted p-value for multiple testing according to Benjamin-Hochberg.
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A

B

C

- Pseudomonas sp. OTU55
- Pseudomonas sp. OTU2368
- Pseudomonas sp. OTU4255
- Pseudomonas sp. OTU5
- Sphingomonas sp. OTU52
- Xanthomonas campestris sp. OTU21
- Ralstonia sp. OTU31
- Erwinia sp. OTU832
- Enterobacteriaceae sp. OTU8
- A. vitis OTU3
- other

0.065 0.092 0.065

0.002 0.002

0.004

0.005