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ARTICLE

Production of Volatile Metabolites by Grape-Associated Microorganisms

Markus Verginer,[†] Erich Leitner,[‡] and Gabriele Berg*,[†]

[†]Institute of Environmental Biotechnolog and [‡]Institute of Analytical Chemistry and Food Chemistry, Graz University of Technology, A-8010 Graz, Austria

Plant-associated microorganisms fulfill important functions for their hosts. Whereas promotion of plant growth and health is well-studied, little is known about the impact of microorganisms on plant or fruit flavor. To analyze the production of volatiles of grape-associated microorganisms, samples of grapes of the red cultivar 'Blaufraenkisch' were taken during harvest time from four different vineyards in Burgenland (Austria). The production of volatiles was analyzed for the total culturable microbial communities (bacteria, yeasts, fungi) found on and in the grapes as well as for single isolates. The microbial communities produced clearly distinct aroma profiles for each vineyard and phylogenetic group. Furthermore, half of the grape-associated microorganisms produced a broad spectrum of volatile organic compounds. Exemplarily, the spectrum was analyzed more in detail for three single isolates of *Paenibacillus* sp., *Sporobolomyces roseus*, and *Aureobasidium pullulans*. Well-known and typical flavor components of red wine were detected as being produced by microbes, for example, 2-methylbutanoic acid, 3-methyl-1-butanol, and ethyl octanoate.

KEYWORDS: Volatile organic compounds; VOCs; microorganisms; grape

INTRODUCTION

All plant-associated microenvironments, for example, rhizosphere, phyllosphere, and carposphere, are highly colonized by microorganisms (*1*). Bacterial and fungal communities associated with plants are specific for each plant species (*2*−*4*). The majority of microorganisms are known for positive interactions with their host plants and fulfill important functions for them (*5*−*8*). One of these functions is plant growth promotion (*6*, *10*−*12*); another important one is pathogen defense (*13*−*15*). Often, water-soluble chemicals such as enzymes or antibiotics are the causal agent of the interaction between microorganisms and their environment, but also volatile organic compounds (VOCs) can repress growth of fungi (*16*, *17*) and enhance plant growth. Furthermore, VOCs serve as inter- and intraorganismic communication signals in general (*17*, *18*) and have an important influence on microbial ecology (*19*−*21*). There are some specific studies about microbial functions in plants. For example, Hornschuh et al. (*22*) provided evidence that hormone-producing methylobacteria are essential for the germination and development of protonema of bryophytes. The same group of endophytic bacteria is involved in the synthesis of important flavor compounds of strawberry (*23*). Endophytic methylobacteria produce 2-hydroxypropanal, which works as precursor of the flavor compounds 2,5-dimethyl-4-hydroxy-2*H*-furanone and 2,5-dimethyl-4-methoxy-2*H*-furanone. However, although this example exists, little is known about the influence of microbes on fruit quality and flavor.

Grapevine (*Vitis vinifera* L.) is one of the oldest and most important cultivated plants. According to the Food and Agriculture Organization (FAOSTAT), 7.5 million hectares of agricultural area worldwide are dedicated to grapes. Generally, they can be grouped in varieties with a strong varietal aroma and in neutral vine varieties. In the fruits of cultivars of the first group, some key compounds or chemical precursors of those occur in high amounts. These key compounds have a strong impact on the aroma of the produced wines and give them their characteristic flavor. For example, linalool is predominantly in the aroma profile of Muscat varieties, and methoxypyrazine derivatives are typical for Sauvignon blanc and Cabernet Sauvignon (*24*). In contrast, the aroma of wines of neutral varieties is much more influenced by the vinification process. Amino acids from the grapes, which vary in their concentration during the process of fruit ripening, are important precursors for aroma compounds (*24*); other aroma compounds occur in their glycosidic form, and the aglycon is released by hydrolytic activity of the yeast (*25*). In addition, metabolic properties of the yeasts, which are involved in wine fermentation, have a strong impact on the resulting wine (*26*). To produce wines with defined sensory properties, which satisfy the expectations of the customers, in recent decades a great variety of different yeast strains have been developed using classical breeding techniques but also genetic engineering of the target strains (*27*). These yeasts are used to inoculate the must after a sulfur dioxide treatment, which is applied to kill naturally occurring microorganisms on grapes. Using this technique, wines with a defined flavor can be produced and the influence of different parameters on wine quality (vintage, climate, quality of grapes) can be reduced (*28*). On the other hand, some winemakers do not want to follow this trend of universalization of wines. They produce wines that are specific for their region of provenience and that are influenced by several biotic

*Corresponding author (phone +43 316 873 8310; fax +43 316 873 8819; e-mail gabriele.berg@TUGraz.at).

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and abiotic parameters of the growing region of the wine. Autochthonous yeasts that naturally occur on the surface of grapes are responsible for the fermentation process. It is well-known that such spontaneous fermentations underlie changes in the microbial community during the fermentation, which follow a defined pattern (*29*). In the first stage of fermentation the production of VOCs, which are responsible for the flavor of the resulting wine, is highest during the whole fermentation process (*30*). It is also known that a sequential fermentation of a must with different yeast strains increases the content of VOCs in wine and that non-*Saccharomyces* yeasts contribute to a more complex aroma profile (*31*−*33*). In contrast, VOCs of naturally occurring autochthonous grape-associated bacteria and microfungi are still not fully known.

The objective of this study was the analysis of grape berry-associated microorganisms and their production of volatiles, which possibly could influence the flavor of the grape fruit and wine themselves. From four different vineyards in Burgenland (Austria) were taken 16 representative samples of fruits of the red cultivar 'Blaufraenkisch' during the harvest time of the vintage 2006. The production of volatiles was analyzed for the microbial communities (bacteria, yeasts, fungi) on grapes as well as for single isolates. Interestingly, many grape-associated microorganisms produced a broad spectrum of VOCs; some of them are well-known and typical for red wine.

MATERIALS AND METHODS

Sampling. At the time of grape harvest of the vintage 2006, grapes of the cultivar 'Blaufraenkisch' were sampled at four different vineyards in Burgenland (Austria). The sampling sites were located in 7081 Schuetzen am Gebirge (47° 51′ 00.93″ N, 16° 37′ 25.87″ E, Prieler), 7312 Horitschon (47° 35′ 19.19″ N, 16° 32′ 51.21″ E, Iby), 7071 Rust (47° 48′ 2.09″ N, 16° 24′ 21.80″ E, Triebaumer), and 7474 Deutsch-Schuetzen (47° 9′ 50.62′ ′N, 16° 26′ 25.19″ E, Wachter-Wiesler). The sampling date was October 4, 2006, immediately before the vintage of this year. All vineyards were farmed conventionally: this means the wine growers can select from a range of pesticides, which is defined by the legislature, and use them, if necessary. In every vineyard four collective samples were taken; each sample consisted of about 400 g of grapes, which were harvested at four randomly selected different locations in the vineyard. The samples were charged into sterile Stomacher bags and transported to the laboratory in a cool box. For the isolation of microorganisms 5 g of grapes of each sample were transferred into a fresh Stomacher bag, 1 mL of 0.85% NaCl solution was added, and the grapes were broken up by treating the Stomacher bag with a pestle. The liquid supernatant was transferred with a pipet into 2 mL reaction tubes.

Isolation of Microorganisms and Determination of Colony-Forming Units. For the determination of colony-forming units (CFU) the supernatant was sequentially diluted and plated onto synthetic nutrient agar (SNA) and R2A (Difco, Detroit, MI) agar plates. SNA is used especially for the cultivation of fungi and contains per liter 1 g of KH_2PO_4, 1 g of KNO_3, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.2 g of glucose, 0.2 g of sucrose, 0.6 mL of 1 N NaOH, and 22 g of agar (*34*). After the agar had been autoclaved for 20 min and cooled at 50 °C, the following antibiotics were added: 10 mg L^{-1} chlortetracycline, 50 mg L^{-1} dihydrostreptomycinsulfate, and 100 mg L^{-1} penicillin G. Plates were incubated for 3 days at 20 °C, and colony-forming units were counted to calculate the number of colonies per gram of fresh weight of sample. For each vineyard, mean value and standard deviation of the four collective samples were calculated.

To get pure cultures single colonies of bacteria, yeasts, and fungi were randomly selected from plates with at most 50 (SNA plates) to 100 (R2A plates) colonies. Bacteria were transferred onto fresh nutrient agar (NA) plates (Sifin, Berlin, Germany) and yeasts and fungi onto malt extract agar plates (MA, Merck, Darmstadt, Germany). The isolates were purified and stored at −70 °C, bacterial cultures in a liquid nutrient broth (NB, Sifin, Berlin, Germany) with 30% (v/v) glycerol and yeasts and fungi in a storage medium prepared by blending 60 mL of glycerol, 20 mL of glucose (50% w/v), 10 mL of peptone (20% w/v), and 10 mL of yeast extract (10% w/v) (all components were autoclaved separately).

Analysis of VOCs Emitted by Microorganisms. For the identification of the produced volatile organic substances microorganisms were grown in glass vials for gas chromatography (75.5 × 22.5 mm, Chromtech, Idstein, Germany) filled with 6 mL of SNA or R2A agar (Difco). To increase the agar surface, the vials were canted during the hardening of the agar. The vials prepared in this way were inoculated with 100 μL of the different dilutions of the supernatant over crushed grapes for mixed cultures or with pure isolates from agar plates, closed with a cotton plug, and incubated for 5 days at 20 °C. A comparison of hermetically sealed and open, cotton-plugged vials during the incubation showed a much higher concentration of the volatiles in the open system (data not shown). A reason for this might be the limited oxygen availability in the closed vial. Losses of volatiles in the open system are quite likely but limited by diffusion, which is drastically hindered by the cotton plug. After incubation, the vials were closed hermetically with magnetic crimp caps with a PTFE-lined silicon rubber septum (LaPhaPack, Langerwehe, Germany). The volatile compounds in the headspace of the vials were enriched on a 2 cm stable flex 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane SPME fiber (Supelco, Bellefonte, PA) for 20 min at 40 °C on an automated SPME sampler (CTC, Chromtech, Idstein, Germany). Desorption of the volatiles was performed directly into the hot injection system of a GCD series instrument (Hewlett-Packard) with a mass selective detector. The split/splitless injector, which was operated in the splitless mode during desorption for 2 min, was operated at 270 °C, and a SPME liner with a 0.75 mm inner diameter was installed. The analytical column was a 30 m HP-5 with a 0.32 mm inner diameter and 1 μm film thickness (Agilent) with helium as carrier gas. The temperature program started at 30 °C for 1 min followed by a ramp of 5 °C min^{-1} to 290 °C, which was held for 1 min. The carrier gas flow was 0.8 mL min^{-1} (32 cm s^{-1} linear velocity) in a constant flow mode. The column was coupled to the mass selective detector via a direct interface, which was operated at 280 °C. The mass range for data acquisition was 20−300 amu with a scan rate of 2.8 scans s^{-1}. The electron multiplier voltage was set by the automated tune parameters. For statistical analysis the GC-MS data were directly imported into a software package (MSStat, ANALYT-MTC, Muehlheim/Ruhr, Germany) for principal component analysis (PCA). The experiments were repeated twice, and to determine VOCs emitted from the culture media, blanks containing only sterile medium were analyzed. Single volatiles were identified by comparing their mass spectra with a MS library or by calculating their retention indices according the method of Kovats (*35*). For the identification of substances via the retention index several databases were used. Besides the Flavornet (http://www.flavornet.org/) and LRI databases (http://www.odor.org.uk/lriindex.html), a self-built database containing more than 800 odor-active substances was used.

After gas chromatography, the glass vials were opened, and the odor impression of the headspace inside the vials was judged by a trained person, who has experience in wine tasting and judging of more than two decades and has fulfilled the requirements concerning taste and odor recognition and sensitivity for sensory panelists. In addition, this person is a member of the jury that is responsible for the Austrian wine quality seal. The main purpose of the sensory evaluation in that specific case was to filter out samples which showed fruity, pleasant winelike aromas and no musty, earthy, or moldy ones. In total, more than 120 isolates were prepared and sensorially evaluated.

Exemplarily for three isolates with remarkable sensory properties, *Paenibacillus* sp. (isolate T2B1c.1-B), *Sporobolomyces roseus* (isolate T3B1c.5-H), and *Aureobasidium pullulans* (isolate T4B1c.17-P), the composition of VOCs was analyzed in detail. These three isolates were chosen because of their extraordinary pleasant, winelike odor.

Identification of Dominant Producers of VOCs. Single isolates, which showed interesting sensory properties or which produced a wide spectrum of different VOCs, were identified by their sequences of 16S rRNA genes in the case of bacteria or by ITS regions in the case of fungi and yeasts.

Mycelia or colonies, grown on NA or MA, were transferred into 2 mL reaction tubes with screw caps containing sterile glass beads (Sigma, 0.25−0.5 mm), and 1 mL of extraction buffer (2 mM Tris, 200 mM NaCl, 25 mM EDTA, 0.5% SDS) was added. The tubes were treated with a FastPrep instrument (Qbiogen BIO 101 Systems, Carlsbad, CA) for 30 s at

level 5. One hundred and fifty microliters of 3 M sodium acetate was added, and the samples were shaken for 2 min by hand. After centrifugation at 13000*g* for 5 min, the clear supernatant was transferred to a fresh 1.5 mL reaction tube and cleaned by phenol−chloroform extraction, and DNA was precipitated at 0 °C for 1 h by adding an equal amount of ice-cold isopropanol. The precipitated DNA was centrifuged for 15 min at 13000*g* and 4 °C, and the resulting pellet was washed with 500 μL of ice-cold 70% ethanol, dried, resuspended in 50 μL of TE buffer (100 mM, pH 8), and stored at −20 °C.

The 16S rDNA of bacterial isolates was amplified in a PCR reaction with the universal primers Eub1 (5′ GAG TTT GAT CCT GGC TCA G 3′) (*36*) and 907r (5′ CCG TCA ATT C(AC)T TT(AG) AGT TT 3′) (*37*). The PCR conditions consisted of an initial denaturation cycle (95 °C, 5 min), 9 amplification cycles (95 °C, 30 s; 52 °C, 30 s; 72 °C, 1 min 40 s), 19 amplification cycles (95 °C, 30 s; 52 °C, 30 s; 72 °C 1 min 30 s + 10 s/cycle), and a final elongation cycle (72 °C, 5 min). ITS regions of fungi and yeasts were amplified with the primer pair ITS1f (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4r (5′ TCC TCC GCT TAT TGA TAT GC 3′) (*38*). PCR conditions consisted of an initial denaturation cycle (95 °C, 5 min), followed by 35 amplification cycles (95 °C, 30 s; 54 °C, 34 s; 72 °C, 40 s) and a final elongation cycle (72 °C, 10 min). The success of the PCR reaction was checked in an agarose gel. Fragments of the expected size were purified using a Geneclean Turbo Kit (MP Biomedicals, Irvine, CA) and sequenced with the Applied Biosystems 3130 L Genetic Analyzer sequencer Data Collection v. 3.0, Sequencing Analysis v. 5 (Foster City, CA) at the sequencing core facility ZMF, Medical University of Graz, Austria. The obtained sequences were aligned with reference gene sequences from GenBank using the BLAST algorithm according to the method of Altschul et al. (*39*).

Nucleotide Sequence Accession Numbers. Sequence accession numbers for 16S rDNA and ITS sequences submitted to GenBank sequence database are FJ490618−FJ490629 and FJ999710−FJ999728.

Chemicals. For the identification of the volatiles in the samples, reference compounds were injected under the same chromatographic conditions into the GC-MS system. Stock solutions of 1 g/L were prepared in methanol and further diluted for the identification experiment. Aliquots of 10 μL of the diluted stock solution were pipetted into the 20 mL headspace vials and sealed. The final concentration of the individual components was 100 ng each in the headspace of the vial. To avoid chromatographic interferences several mixes were prepared and analyzed separately. The aldehyde mixture was analyzed within a few days after preparation of the stock solution to avoid the formation of methyl acetals. Methanol was used as a solvent because it shows only low affinity to ad/absorb on the selected SPME fiber. The following reference substances were used for the preparation of the stock and working solutions. Alcohols: 1-propanol (Roth 9169.1), 3-methylbutan-1-ol, (Fluka 59091), 2-methylbutan-1-ol (Merck 979), 2,3-butanediol (Aldrich B8.490-4), 2-heptanol (Fluka 51800), 2-ethyl-1-hexanol (Schuchardt 6932), 1-octen-3-ol (Aldrich W28,051-8), 2-nonanol (Aldrich W37.200-5), 3,7-dimethyl-1-octanol (Fluka 41011), and 2-phenylethanol (Fluka 141821 31). Aldehydes: 2-methylbutanal (Aldrich W26.910-7), hexanal (Aldrich W25571-8), heptanal (Alfa Aesar B23830), *trans*-2-octenal (Aldrich W32150-8), *trans*-2-nonenal (Aldrich W32.130-3-K), undecenal (Aldrich W30.920-6), *trans*, *trans*-2,4-decadienal (Aldrich 18.051-3), *trans*-2-dodecenal (Aldrich W26150-5), and phenylacetaldehyde (Aldrich 10739-5). Ketones: 2-butanone (Aldrich W21.701-8), 2-pentanone (Aldrich W28420-3), 2-heptanone (Aldrich W25440-1), 6-methyl-5-hepten-2-one (Aldrich W27.070-9), 2-nonanone (Aldrich 10873-1), and 2-undecanone (Aldrich W30930-2). Esters: ethyl acetate (Promochem 3427), methyl 3-methylbutanoate (SAFC W27530-1), ethyl 3-methylbutanoate (SAFC W24.630-1K), 3-methyl-1-butyl acetate (Fluka 17880), ethyl octanoate (Aldrich W24490-2), 2-phenylethyl acetate (Fluka 46030), ethyl benzoate (Fluka 12360), and ethyl hexadecanoate (Sigma P9009). Other compounds: dimethyl disulfide (Aldrich 15,031-2), dimethyl trisulfide (Aldrich W32750-6), acetic acid (Sigma-Aldrich 242853), 3-methylbutanoic acid (Fluka 59850), 2,3,5-trimethylpyrazine

Table 1. Sensory Description of Mixed Cultures

culture	sampling site/vineyard	sensory description
bacteria	Prieler	bread, clammy carton, yeast
bacteria	Iby	weak, inconspicuous
bacteria	Triebaumer	slightly fruity, roasty
bacteria	Wachter-Wiesler	mushrooms
yeasts, fungi	Prieler	weak, inconspicuous
yeasts, fungi	Iby	weak, inconspicuous
yeasts, fungi	Triebaumer	earthy, moldy, beetroot,
yeasts, fungi	Wachter-Wiesler	inconspicuous

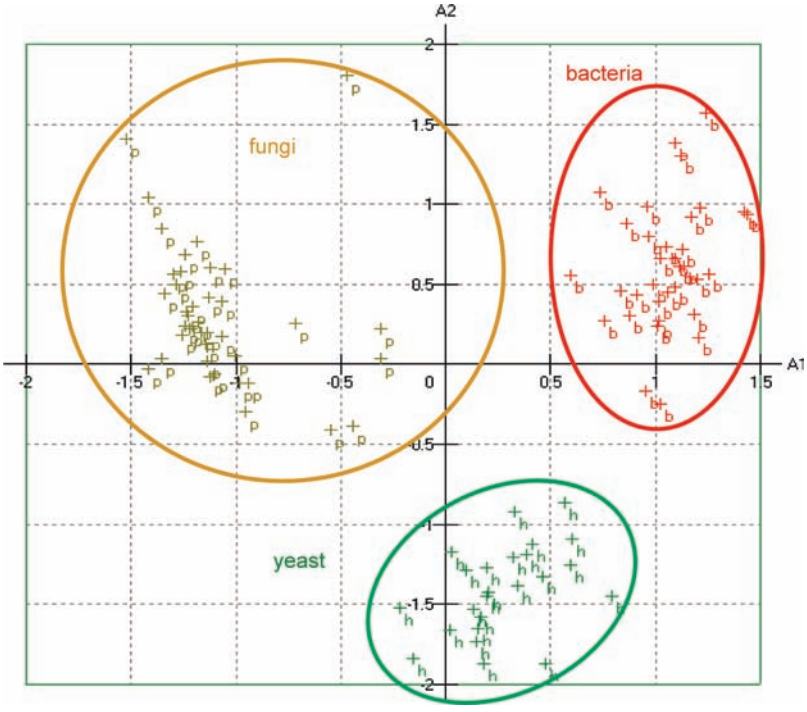

Figure 1. Principle component analysis of the VOCs emitted from isolated cultures from different vineyards (54 bacteria, 47 fungi, and 26 yeast strains). Chromatograms were analyzed with a software package for GC-MS data (MSStat, AnalytMTC, Germany), and results are shown as a 2D pattern. Yeasts, fungi, and bacteria can be clearly distinguished by separate clusters.

Table 2. Identification of Microorganisms with Interesting Sensory Properties

isolate	origin	identification	AN[a]	SI[b]	taxonomic affiliation
T1B1c.1-B	Prieler	*Clavibacter michiganensis*	AM237375.1	100	Actinobacteria
T1B1c.3-B	Prieler	*Exiguobacterium* sp.	FJ348035.1	98	Firmicutes
T1B1c.6-B	Prieler	*Exiguobacterium* sp.	FJ348035.1	99	Firmicutes
T1B1c.9-B	Prieler	*Exiguobacterium* sp.	FJ348035.1	99	Firmicutes
T1B1c.10-B	Prieler	*Exiguobacterium* sp.	FJ348035.1	99	Firmicutes
T1B1c.1-P	Prieler	*Davidiella tassiana*	EU622926.1	100	Ascomycota
T1B1c.2-P	Prieler	*Stereum hirsutum*	EU673089.1	100	Basidiomycota
T1B1c.3-P	Prieler	*Stereum hirsutum*	EU673089.1	100	Basidiomycota
T2B1c.1-B	Iby	*Paenibacillus* sp.	AB110989.1	98	Firmicutes
T2B1c.6-B	Iby	*Exiguobacterium* sp.	FJ348035.1	100	Firmicutes
T2B1c.1-H	Iby	*Sporidiobolus pararoseus*	EU002958.1	99	Basidiomycota
T2B1c.3-H	Iby	*Cryptococcus magnus*	EU871517.1	100	Basidiomycota
T2B1c.7-H	Iby	*Sporidiobolus pararoseus*	EU409803.1	98	Basidiomycota
T2B1c.10-H	Iby	*Sporidiobolus pararoseus*	EU409803.1	98	Basidiomycota
T2B1c.2-P	Iby	*Aureobasidium pullulans*	FJ820762.1	100	Ascomycota
T2B1c.4-P	Iby	*Aureobasidium pullulans*	FJ820762.1	100	Ascomycota
T2B1c.10-P	Iby	*Aureobasidium pullulans*	EU52999.1	100	Ascomycota
T3B1c.10-P	Triebaumer	*Aureobasidium pullulans*	EU622924.1	100	Ascomycota
T3B1c.5-H	Triebaumer	*Sporobolomyces roseus*	AM190644.1	99	Basidiomycota
T3B1c.8-H	Triebaumer	*Bulleromyces albus*	AF444662.1	99	Basidiomycota
T3B1c.3-P	Triebaumer	*Aureobasidium pullulans*	AM160630.1	99	Ascomycota
T3B1c.7-P	Triebaumer	*Davidiella tassiana*	EU529999.1	100	Ascomycota
T4B1c.5-B	Wachter-Wiesler	*Bacillus simplex*	FJ644693.1	100	Firmicutes
T4B1c.7-B	Wachter-Wiesler	*Paenibacillus illinoisensis*	EU218535.1	99	Firmicutes
T4B1c.8-B	Wachter-Wiesler	*Paenibacillus* sp.	EF612326.1	99	Firmicutes
T4B1c.9-B	Wachter-Wiesler	*Paenibacillus illinoisensis*	AB073192.1	99	Firmicutes
T4B1c.13-P	Wachter-Wiesler	*Aureobasidium pullulans*	EU555310.1	100	Ascomycota
T4B1c.17-P	Wachter-Wiesler	*Aureobasidium pullulans*	EU529999.1	100	Ascomycota
T4B1c.2-P	Wachter-Wiesler	*Aureobasidium pullulans*	EU529999.1	100	Ascomycota
T4B1c.8-P	Wachter-Wiesler	*Aureobasidium pullulans*	AY251074.2	99	Ascomycota

[a] GenBank accession number. [b] Similarity index.

Table 3. Identification and Sensory Description of Volatile Compounds Produced by *Paenibacillus* sp. (Isolate T2B1c.1-B)[a]

substance	RI (WAX)	RI (HP-5)	rel peak area (%)	sensory description	sensory threshold in water[b] (mg/kg)
2-butanone	893	610	4.1	ether[c]	7
2-methyl-1-propanol	1097	621	0.1	obtrusive, wine[d]	0.36
methyl-3-methylbutanoate	1022	674	0.2	apple[e]	0.0044
2-pentanone	980	685	0.1	sweet, ether, fruity[d]	0.055
3-methyl-1-butanol	1205	736	69.0	whiskey, malt, burnt[e]	0.071
2-methylbutan-1-ol	1208	755	1.1	wine, onion, malt[e]	0.25
dimethyl disulfide	1074	785	13.4	onion, cabbage, putrid[e]	0.000205
2-heptanone	1186	890	0.4	fruity, spicy, cinnamon[d]	0.0055
6-methyl-5-hepten-2-one	1336	974	2.3	herbaceous, green, oily, pungent[d]	0.05
dimethyl trisulfide	1377	974	1.7	sulfur, fish, cabbage[e]	0.0000075
trimethylpyrazine	1395	1000	0.1	roasted nuts, cocoa, peanuts[d]	0.023
2-ethyl-1-hexanol	1487	1032	0.1	oily, sweet, rose[d]	0.198
phenylacetaldehyde	1609	1043	0.1	bitter, withethorn[d]	na
3-methylbutanoic acid	1255	1047	0.2	rancid cheese, sweaty, putrid[c]	0.02
2-phenylethanol	1918	1113	0.4	rose[c]	0.0000015

[a] Substances were detected in the headspace above the microorganism in closed culture vials by GC-MS. [b] VanGemert, Compilations of Odor Threshold Values in Air, Water & other Media. [c] Smells Database (http://mc2.cchem.berkeley.edu/Smells/). [d] Flavors & Fragrances product catalog. [e] Flavornet (http://www.flavornet.org/flavornet.html).

(Aldrich 19.941-9), 2,5-dimethylpyrazine (Janssen 17.542-0), and 2-pentylfuran (Aldrich W33170-8). Two substances were identified by GC-MS only tentatively due to missing reference substances (4-pentenal, 3-ethyl-2,5-dimethylpyrazine).

RESULTS

Microbial Abundances. The abundances of microorganisms in the samples of grape berries showed a wide range: bacterial abundances were 7.5×10^1 CFU g^{-1} of fresh weight in the sample Prieler, 3.2×10^2 in the sample Wachter-Wiesler, 3.3×10^3 in the sample Iby, and 1.2×10^5 in the sample Triebaumer. For fungi and yeasts, abundances of 1.0×10^1 CFU g^{-1} of fresh weight in the sample Prieler, 3.8×10^2 in the sample Wachter-Wiesler, 2.8×10^4 in the sample Iby, and 1.0×10^5 in the sample Triebaumer were determined. All differences between the microbial abundances in the samples were statistically significant (t test, $p = 0.05$).

Sensory Evaluation of Mixed Cultures. A sensory evaluation of mixed cultures of cultivatable microorganisms isolated from the different samples showed preliminary interesting results (**Table 1**). The sensory impressions of the different mixed cultures ranged from inconspicuous to very specific flavors that can be attributed to single chemical compounds. One of the interesting findings of that trial was that even with the use of synthetic media, winelike

Table 4. Identification and Sensory Description of Volatile Compounds Produced by *Sporobolomyces roseus* (Isolate T4B1c.17-P)[a]

substance	RI (WAX)	RI (HP-5)	rel peak area (%)	sensory description	sensory threshold in water[b] (mg/kg)
1-propanol	947	<500	0.3	alcohol, pungent[c]	5.7
acetic acid	1450	600	0.5	sour[c]	10
2-butanone	893	610	0.1	ether[c]	7
2-methylbutanal	915	658	0.6	cocoa, almond[c]	0.001
3-methyl-1-butanol	1205	736	20.8	whiskey, malt, burnt[d]	0.071
2-methyl-1-butanol	1208	755	4.2	wine, onion, malt[d]	0.25
ethyl 3-methylbutanoate	1060	849	0.1	fruits[c]	0.00001
3-methyl-1-butyl acetate	1070	876	0.1	banana[c]	0.002
2,5-dimethylpyrazine	1031	925	0.1	cocoa, roasted nuts, roast beef[c]	na
ethyl acetate	1358	1010	26.8	glue, fruity, pineapple[c]	0.0085
3-ethyl-2,5-dimethylpyrazine[e]	1439	1083	0.1	potato, roasty[c]	na
2-phenylethanol	1925	1118	4.2	rose[d]	0.000015
2-phenylethyl acetate	1810	1192	0.7	rose, honey, tobacco[d]	3

[a] Substances were detected in the headspace above the microorganism in closed culture vials by GC-MS. [b] VanGemert, Compilations of Odor Threshold Values in Air, Water & other Media. [c] Flavornet (http://www.flavornet.org/flavornet.html). [d] Smells Database (http://mc2.cchem.berkeley.edu/Smells/). [e] Tentatively identified only by mass spectrum due to missing reference compound.

Table 5. Identification and Sensory Description of Volatile Compounds Produced by *Aureobasidium pullulans* (Isolate T3B1c-5H)[a]

substance	RI (WAX)	RI (HP-5)	rel peak area (%)	sensory description	sensory threshold in water[b] (mg/kg)
2-methylbutanal	915	658	0.5	cocoa, almond[c]	0.001
3-methyl-1-butanol	1205	736	39.8	whiskey, malt, burnt[c]	0.071
4-pentenal[d]	1131	754	0.6	strawberry, tomato, fruity[c]	0.31
hexanal	1085	800	4.7	grass, tallow, fat[c]	0.0045
2,3-butanediol	1583	806	0.1	fruits, onion[c]	4.5
heptanal	1189	901	0.5	citrus, fatty, rancid[c]	0.003
2-heptanol	1469	969	1.0	earthy, oily[e]	0.94
1-octen-3-ol	1464	979	0.8	fungi[c]	0.000005
2-pentylfuran	1240	993	2.0	green beans, vegetable[e]	0.006
ethyl acetate	1358	1010	0.5	glue, fruity, pineapple[c]	0.0085
2-ethyl-1-hexanol	1487	1032	0.8	rose, green[c]	0.198
trans-2-octenal	1431	1058	0.7	nuts, green, fatty[c]	0.003
2-nonanone	1394	1092	0.9	hot milk, soap, green[c]	0.005
2-phenylethanol	1925	1118	0.6	rose[f]	0.000015
trans-2-nonenal	1538	1159	0.5	fatty, cucumber[c]	0.0002
ethyl benzoate	1648	1185	0.2	camomile, flower, celery, fruit[c]	0.06
2-nonanol	1535	1187	2.1	fatty, green[c]	0.058
3,7-dimethyl-1-octanol	1664	1196	0.3	sweet, rose[e]	na
ethyl octanoate	1436	1198	0.5	fruity, fatty[c]	0.005
trans-2-undecenal	1666	1300	0.2	fatty, waxy, rose, citrus[e]	0.0004
2-undecanone	1609	1308	0.3	orange, fresh, green[c]	0.004355
trans,*trans*-2,4-decadienal	1812	1317	0.4	fried, wax, fat[c]	0.00007
trans-2-dodecenal	1715	1410	0.3	green, fatty, sweet[c]	0.00053
ethyl hexadecanoate	2250	1902	2.0	wax[c]	2

[a] Substances were detected in the headspace above the microorganism in closed culture vials by GC-MS. [b] VanGemert, Compilations of Odor Threshold Values in Air, Water & other Media. [c] Flavornet (http://www.flavornet.org/flavornet.html). [d] Tentatively identified only by mass spectrum due to missing reference compound. [e] Flavors & Fragrances product catalog. [f] Smells Database (http://mc2.cchem.berkeley.edu/Smells/).

aroma impressions were observed. A clear discrimination between aroma profiles emitted from isolates of different phylogenetic branches (fungi and yeasts and from bacteria) was observed (**Figure 1**). This was analyzed by PCA of the VOCs emitted from isolated cultures from different vineyards (54 bacteria, 47 fungi, and 26 yeast strains).

Identification of Single Isolates. All together, 30 pure cultures of grape-associated microorganisms with interesting sensory properties were identified according to their partial 16S rDNA or ITS sequences (**Table 2**). Interestingly, the phylogenetic diversity of VOC producers was relatively low. All identified bacterial isolates belong to the genera *Exiguobacterium* and *Paenibacillus*, whereas the genera *Aureobasidium* and *Cladosporium* dominate the fungal isolates. These dominating genera were not specific for single sampling sites; they could be found in different vineyards.

Identification of VOCs. Exemplarily for three isolates, *Paenibacillus* sp. (isolate T2B1c.1-B), *Sporobolomyces roseus* (isolate T3B1c.5-H), and *Aureobasidium pullulans* (isolate T4B1c.17-P), the composition of VOCs was analyzed in detail. In the headspace in the culture vials above microorganism cultures between 100 and 150 different volatile organic compounds were found. To distinguish between volatiles that were produced from microorganisms and volatiles that were emitted from the culture medium, chromatograms from vials inoculated with isolates were compared with chromatograms measured from vials filled with sterile culture medium without any inoculum. After subtraction of the medium background, between 34 and 45 different substances per isolate remained. Substance peaks were identified by comparing their mass spectra with a MS Library or by calculating their retention indices according the method of Kovats (*35*). Those substances for which could be found a description of their sensory activity in the literature are summarized in **Tables 3**−**5**.

For the *Paenibacillus* isolate, a total of 43 different substances could be identified. For 15 of them in the literature was found an

odor description (**Table 3**). Alcohols, aldehydes, and ketones dominate the fraction of the odor-active volatiles of *Paenibacillus* sp. Also, two sulfur compounds, dimethyl disulfide and dimethyl trisulfide, were found in the aroma profile. The substances 2-methylbutan-1-ol, 3-methylbutan-1-ol, and phenylethanol in the literature are described as aroma compounds occurring in red wine (*40*).

In the chromatogram of *S. roseus* a total of 34 substances could be identified. For 13 of them exists an odor description in the literature (**Table 4**). Alcohols and esters represent more than the half of the volatiles. The substances 2-methyl-1-butanol, 3-methyl-1-butanol, acetic acid, ethyl acetate, 2-methylpropyl acetate, and phenylethanol were extracted from red wine by Vas et al. (*40*).

In the headspace above *A. pullulans* a total of 45 volatile organic compounds were found. For 24 of them in the literature can be found an odor description (**Table 5**). Alcohols as well as aldehydes together account for more than two-thirds of the compounds in the aroma profile of *A. pullulans*. Also, different esters play an important role in the aroma profile. 3-Methyl-1-butanol, ethanol, ethyl acetate, ethyl octanoate, hexanal, and phenylethanol are known as aroma compounds of red wine (*40*).

DISCUSSION

Even if VOCs with a total concentration of around 50 mg/L present only a small proportion of the constituents of wine, they are the central object of interest for the producers as well as for the consumers of wine. The volatile fraction of wines can be composed of more than 400 different chemical compounds, the concentrations of which range over 8 orders of magnitude. The impact of an aroma active compound is linked not only to its concentration but also to its odor-activity value (*41*); therefore, also VOCs that occur only in very low concentrations can have a great impact on the final aroma of a wine. This model has been successfully applied to determine compounds with major impact on the flavor of wines (*42*, *43*). This work reveals the potential of the autochthonous microbial populations on grapes to produce VOCs.

Differences in Microbial Communities. Bacterial abundances on the collected grapes showed statistically significant differences between the single vineyards and ranged from 10^1 to 10^5 CFU g^{-1} of fresh weight. These differences may be explained with different abiotic conditions in the vineyards that influence the growth of microorganisms, and the different ways of farming of the vineyards (use of fertilizers, pesticides, cutting of the vines) can influence the microbial populations. The very low abundances of microorganisms in the sample in vineyard Prieler (PRL) can be explained by the fact that the vineyard has been treated with $CuSO_4$ to prevent infections of the ripe berries with *Botrytis cinerea*, the causal agent of gray mold (*44*). In our study we also analyzed the composition of microbial communities in soil and on/in grape berries by cultivation-independent community fingerprints using single-strand conformation polymorphism analysis (PCR-SSCP) of 16S rRNA and ITS fragments (*45*) (data not shown), which again confirmed differences between the investigated vineyards.

Identification of Single Isolates. The identification of bacterial isolates by partially sequencing their 16S rDNA showed that the majority of the bacterial isolates belong to the genera *Exiguobacterium* and *Paenibacillus*. Both genera in the literature are described as potent antagonists against *B. cinerea*, or they are linked to other positive plant−microbe interactions (*40*). The identified fungi and yeasts show a higher diversity, but also here two organisms, *A. pullulans* and *C. cladosporioides*, occur remarkably often. It seems that these genera in general show a high production of different VOCs.

VOC Production of Total Communities. The PCA of the VOCs emitted by the total communities of bacteria or fungi and yeasts isolated from grapes originating from different vineyards resulted in a clear distinction of the VOC spectra of communities with different proveniences.

VOC Production of Single Isolates. Pure cultures of isolated microorganisms were able to produce a large spectrum of different VOCs with a wide variety of sensory properties. In total, the aroma profiles of single isolates consisting of 54 bacteria, 47 fungi, and 26 yeast strains were measured. Nevertheless, the interesting fact is that the isolates of different phylogenetic origin can clearly be differentiated by a PCA of the aroma profiles (**Figure 1**). It appears that all of the different individual isolates produce a volatile pattern, which is typical for bacteria, fungi, or yeasts.

Paenibacillus sp. was able to produce 2-methylbutan-1-ol, 3-methylbutan-1-ol, and phenylethanol. In the literature, these compounds are described to occur in red wine (*40*). From the VOCs emitted by *S. roseus* the 2-methyl-1-butanol, 3-methyl-1-butanol, acetic acid, ethyl acetate, 3-methyl-1-butyl, and phenylethanol could be identified as components of red wine. *A. pullulans* produced 3-methyl-1-butanol, ethyl acetate, ethyl octanoate, hexanal, and phenylethanol as known components of red wine. All of these compounds are sensorially active and typical for the flavor profile of many red wines. For further investigations it would be interesting to quantify the emission of VOCs from microorganisms into grapes or into the must. Also, the survival of microorganisms occurring on the grapes during the process of wine fermentation and their production of VOCs would be an interesting topic of further investigations. Furthermore, the function of VOCs in grape-associated microbial communities and their interactions with plants are important to understand. Our results prove the potential of microorganisms occurring on grapes to produce VOCs. Wines produced via spontaneous fermentation can be subjected to influences of microorganisms that naturally occur on grapes and can therefore potentially influence the character of the wine.

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