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Volatile Metabolites from Actinomycetes

CHARLOTTE E. G. SCHÖLLER,[†,‡,§] HANNE GÜRTLER,[#] RITA PEDERSEN,[#] SØREN MOLIN,[‡] AND KEN WILKINS*[,†]

Department of Indoor Environment, National Institute of Occupational Health, Lersø Parkallé 105, DK-2100 Copenhagen Ø, Denmark; Novo Nordisk A/S, DK-2880 Bagsværd, Denmark; and Section of Molecular Microbiology, BioCentrum-DTU, The Technical University of Denmark, DK-2800 Lyngby, Denmark

Twenty-six *Streptomyces* spp. were screened for their volatile production capacity on yeast starch agar. The volatile organic compounds (VOCs) were concentrated on a porous polymer throughout an 8-day growth period. VOCs were analyzed by gas chromatography with flame ionization detection and identified or characterized by gas chromatography−mass spectrometry. A total of 120 VOCs were characterized by retention index and mass spectra. Fifty-three compounds were characterized as terpenoid compounds, among which 18 could be identified. Among the VOCs were alkanes, alkenes, alcohols, esters, ketones, sulfur compounds, and isoprenoid compounds. Among the most frequently produced compounds were isoprene, acetone, 1-butanol, 2-methyl-1-propanol, 3-methyl-3-buten-1-ol, 3-methyl-1-butanol, 2-methyl-1-butanol, cyclopentanone, dimethyl disulfide, dimethyl trisulfide, 2-phenylethanol, and geosmin. The relationship between the excretion of geosmin and the production of spores was examined for one isolate. A good correlation between headspace geosmin and the number of spores was observed, suggesting that VOCs could be used to indicate the activity of these microorganisms in heterogeneous substrates.

KEYWORDS: Streptomyces; streptomycetes; actinomycetes; GC-MS; VOC; geosmin; 2-methylisoborneol

INTRODUCTION

Bioaerosols can pose health problems to workers engaged in agriculture, waste collection and handling, sewage treatment, and other professions. The bioaerosols are released into the air with organic dust particles or with moisture droplets during handling of various materials (*1*). *Streptomyces* spp. may form an important part of bioaerosols originating from soil and some plant materials (*2*). Spores from these Gram-positive filamentous bacteria are only ∼1 μm in diameter and can readily become airborne when the substrate is disturbed. Actinomycetes, such as *Saccharopolyspora rectivirgula*, *Thermoactinomyces vulgaris*, *Thermoactinomyces thalpophilus*, and *Saccharomonospora viridis*, are widely recognized as causative agents of allergic alveolitis (farmer's lung disease). Also, streptomycetes (e.g., *S. albus*, *S. olivaceus* and *S. thermohygroscopicus*) have been implicated in the etiology of allergic alveolitis (*2*).

The potential of using the volatile organic compounds (VOCs) excreted from microorganisms as a rapid and perhaps specific method for detecting microbial activity has been investigated in many diverse areas. For example, the concept has been applied to investigations of possible alternative automatic detection methods of pathogenic bacteria in clinical specimens (*3*) and in investigations of methods for detecting microbial spoilage of food (*4*, *5*). Furthermore, VOCs have been investigated for use as indicators of fungal growth in grain during storage (*6*). More recently, VOCs have been studied as a possible aid in the identification of houses contaminated with microbiological growth (*7*, *8*). The extensive systematic characterization of VOCs from penicillia for subsequent chemotaxonomic use is another area of application (*9*).

Actinomycetes, especially the genus *Streptomyces*, are well-known for their capability of producing a variety of secondary metabolites such as antibiotics. Despite this, there has been little systematic investigation of the production of volatile organic compounds by these organisms (*10*, *11*); however, much attention has been paid to the production of off-odor, musty, aroma compounds produced by these organisms, mainly geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) and 2-methylisoborneol, due to the detrimental effects of these compounds on the quality of fresh water sources and aquaculture-raised fish (*12*). Nutritional and physical factors influencing the odor production (*13*) and the biochemical pathway leading to geosmin have been examined (*14*, *15*). Furthermore, one streptomycete has been reported to possess a strong and characteristic odor, which originated from geosmin and albaflavenone. The latter is an unusual, odorous, volatile metabolite with antibacterial activity (*16*).

* Corresponding author (telephone +45 39 16 52 00; fax +45 39 16 52 01; e-mail CKW@AMI.dk).
[†] National Institute of Occupational Health.
[‡] Technical University of Denmark.
[§] Present address: New Products, Alpharma ApS, Dalslandsgade 11, 2300 Copenhagen, Denmark.
[#] Novo Nordisk A/S.

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Table 1. Overview of Strain Numbers and Distribution of VOCs among the Strains

strain name	ID no. assigned	ID no. NN[d]	no. of VOCs total	no. of VOCs common[a]	no. of VOCs specific[b]
Streptomyces albidoflavus AMI 246[f]	1		29	15	ND[c]
Streptomyces spp. AMI 243[f]	2		14	9	1
Streptomyces spp. AMI 240	3		28	11	1
Streptomyces rishiriensis AMI 224[f]	4		30	14	2
Streptomyces albus subsp. *pathocidicus* IFO[g] 13812[e]	5	29570	12	7	1
Streptomyces albus IFO 13014[e]	6	29672	18	8	2
Streptomyces antibioticus CBS[h] 659.68[e]	7	29809	23	13	1
Streptomyces antibioticus ETH[i] 22014	8	24873	28	15	4
Streptomyces aureofaciens ETH 13387	9	24852	31	11	3
Streptomyces aureofaciens ETH 28832	10	24901	42	11	10
Streptomyces coelicolor ATCC[j] 21666	11	29821	16	9	ND
Streptomyces coelicolor DSM 40233[e]	12	29561	29	13	7
Streptomyces diastatochromogenes IFO 13814	13	29664	17	10	ND
Streptomyces diastatochromogenes ETH 18822	14	24863	17	13	1
Streptomyces griseus ATCC 23345[e]	15	29771	36	13	3
Streptomyces griseus IFO 13849	16	29674	29	11	5
Streptomyces hirsutus ATCC 19773[e]	17	29360	30	13	3
Streptomyces hirsutus ETH 1666	18	24869	22	15	1
Streptomyces hygroscopicus IFO 13255	19	29554	30	12	2
Streptomyces hygroscopicus ATCC 27438[e]	20	29313	41	12	13
Streptomyces murinus NRRL[l] 8171	21	29689	37	14	8
Streptomyces murinus DSM 40091[e]	22	29711	37	13	3
Streptomyces olivaceus ETH 6445	23	24851	12	10	1
Streptomyces olivaceus ETH 7437	24	24835	21	11	ND
Streptomyces thermoviolaceus IFO 12382[e]	25	29685	14	6	2
Streptomyces thermoviolaceus CBS 111.62	26	29830	21	11	ND

[a] Found from half or more than half of the strains. [b] Found from <2% of the strains. [c] None detected. [d] Novo Nordic strain collection number. [e] Type strain. [f] Identified by DSM. [g] Institute of Fermentation, Osaka, Japan. [h] Central Bureau für Schimmelkulturen, Baarn. [i] Confederated Technical University of Zürich, Switzerland, via Tübingen University. [j] American Type Culture Collection. [k] Deutche Sammlung von Mikroorganismen. [l] Northern Regional Research Laboratory, USA.

In some investigations, the appearance of odorous compounds from actinomycetes has coincided with the observation of aerial mycelium and spores (*17*). Such findings imply that these VOCs may be useful as indicators of the differentiation and/or sporulation process. The objectives of this investigation were to carry out a systematic study of a larger selection of *Streptomyces* spp. in order to obtain an overview of the VOCs produced by these organisms and to investigate the possibilities of using VOCs as a means of their detection and possible characterization of physiological state (i.e., differentiation) in mixed microbial flora.

MATERIALS AND METHODS

Cultures. Of the 26 actinomycete cultures shown in **Table 1**, 22 isolates were obtained from the culture collection at Novo Nordisk A/S. The four AMI isolates were obtained from the collection at the Danish Institute of Occupational Health. These actinomycetes had been isolated from air during garden waste collection. AMI 246 was classified as *Streptomyces albidoflavus*, AMI 224 as *Streptomyces rishiriensis*, and AMI 243 as belonging to the genus *Streptomyces* on the basis of morphologic, chemotaxonomic and molecular genetic results at DSMZ (German Collection of Microoganisms and Cell Cultures). Isolate 240 was a *Streptomyces* spp. with rectiflexible spore chains.

Culture Methods, VOC Collection, and Screening. The growth medium was selected with the aim of high expression of VOCs and low background levels. The medium was derived from Emmerson's yeast starch agar (DSM) and contained (per liter) the following: starch (Merck), 15 g; yeast extract (Difco), 4 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H2O$, 1 g; and agar (Merck), 20 g. One hundred milliliters of medium in 500-mL conical flasks was inoculated with a 3-mL aqueous spore suspension in 0.05% Tween 80 from a 6-day-old preculture grown on Emmerson's yeast starch agar at 30 °C. The flasks were closed with cotton plugs and incubated for 8 days at 30 °C for gas chromatography−flame ionization dectection (GC-FID) analysis and for 12 days for gas chromatography−mass spectrometry (GC-MS) analysis. VOCs were collected by diffusive sampling throughout the incubation period with a stainless steel Tenax tube containing 200 mg of the porous polymer Tenax TA (Chrompack). The tubes were closed at one end with a Swagelok Union cap fitted with a Teflon ferrule (Swagelok Co., Solon, OH). The Tenax tube was inserted into the flask and held in place by the cotton plug. Results are the means from two successive series except for AMI 243 (strain 2) and *S. hirsutus* ATCC 19773 (strain 17), which were set up in parallel due to contamination in the first series. Two uninoculated control media in each series were analyzed for background VOCs.

Culture Methods, VOC Collection, and Geosmin Recovery in Relation to Spore Formation. Isolate *S. albidoflavus* AMI 246 was grown on 15 mL of Emmerson's yeast starch agar [as mentioned above, with yeast extract (Sigma) and agar (Oxoid)] in 60-mm glass Petri dishes. The medium was covered with a sterile 50-mm polycarbonate membrane (Poretics Corp., Livermore, CA) with a mean pore size of 0.05 μm. Twelve plates were inoculated with 50 μL of a stock spore solution of 7×10^9 colony-forming units (CFU)/mL prepared as above and filtered through sterile cotton wool. Prior to inoculation, the spore solution was washed in sterile physiological NaCl solution. The plates were placed separately in 5-L Tedlar polyvinyl fluoride gas sampling bags (SKC Inc., Eighty-Four, PA), which were then closed with sealing clips (SKC Inc.). After the bags had been emptied of ambient air with an Alpha 1 pump (www.Ametek.com), they were filled with 4.5 L of medical grade air, filtered through a Chrompack charcoal filter. The bags were incubated at 30 °C for 15−129 h. Two 1-L samples from each bag were concentrated on Tenax TA through a polypropylene fitting present in the bags employing the Alpha 1 pump at 50 mL/min. Previous experiments showed that growth on plates in bags with 4.5 L of air resulted in 1% carbon dioxide in the bags after incubation at 30 °C for 1 week when measured with a Kitagawa gas detector tube for CO_2 (Komyo, Tokyo, Japan). The growth on the plates inside bags resembled the growth on plates incubated traditionally in an incubator. The recovery of geosmin from the bags was determined to be 38 ± 10% for the range from 0.025 to 0.66 μg. This was found by comparing the response factors from two linear standard curves obtained from a series of six standard solutions placed either directly onto the Tenax tubes or onto sterile media including membranes in glass Petri dishes

through a Teflon septum in the bags during inflation followed by incubation for 48 h at 30 °C with subsequent sampling on Tenax TA as described under VOC Analysis, GC-FIC, and GC-MS.

Culture Method, Growth on Grass. *S. albidoflavus* AMI 246 was grown on 4 g of grass (~2-cm length) cut from a lawn, which had been autoclaved for 1 h at 120 °C and placed in a 60-mm glass Petri dish. The ability of this isolate to degrade cellulose was determined on Cellulose Congo Red agar (*18*). Inoculation, incubation (7 days), and sampling procedure were as described for geosmin in relation to spore formation. The production of geosmin was verified by GC-MS and compared to a negative blank.

Spore Assay. After collection of VOC samples from the Tedlar bags, the polycarbonate membrane including cells was transferred to a tube filled with 5 mL of a 50 mM KH_2PO_4 buffer (pH 7.0) containing 0.001% Triton X-100 detergent and subjected to homogenization for 1.5 min at room temperature with an Ultra-Turrax homogenizer (Janke & Kunkel), operating at 24000 rpm. Subsequent microscopic examination of the samples showed single spores and fragmented mycelia. Spores were counted as acid-resistant units (ARU) (*19*) by taking advantage of the difference in susceptibility of mycelia and spores to dilute acid. A sample of 0.1 mL was treated with 0.4 mL of 0.125 M HCl for 5 min at room temperature. After neutralization with 0.5 mL of 0.1 M NaOH, the sample was diluted in the potassium phosphate buffer and plated in triplicate on a complex medium for enumeration (*20*). This medium contained (per liter) the following: KH_2PO_4, 1.36 g; morpholinepropanesulfonic acid (MOPS, Sigma), 5.78 g; glucose, 9 g; $(NH_4)_2SO_4$, 0.66 g; $MgSO_4$, 0.125 g; casamino acids (Difco), 1 g; yeast extract (Sigma), 5 g; agar (Oxoid), 15 g. After autoclaving of the medium, 2.5 mL of a trace salts solution was added. The concentrated trace salts solution consisted of $CaCl_2$, 27.2 mM; $FeSO_4$, 7.19 mM; $MnSO_4$, 11.8 mM; and $ZnSO_4$, 0.174 mM, in 0.1 N HCl. Colonies were counted after incubation at 30 °C for 2 days. A preliminary experiment showed that this treatment killed >99% of the mycelium while preserving ~90% of the spores.

VOC Analysis, GC-FID, and GC-MS. Volatiles were desorbed from the Tenax tubes in a Perkin-Elmer ATD-400 automatic thermal desorber and transferred to an HP 5890A GC fitted with a 60 m × 0.32 mm i. d., 0.25 μm film, CP 19CB silica capillary column (Chrompack) and an FID. Conditions were as follows: ATD inlet pressure, 16 psi, He 40 mL/min; desorption, 20 min at 250 °C; cooling trae, −30 to 300 °C for 1 min; transfer valve, 175 °C; transfer line, 150 °C. The GC program was as follows: 1 min at 30 °C increasing at 4 °C/min to 220 °C with a hold for 13 min. The carrier gas was He at 2.3 mL/min and the split ratio 0.16. FID conditions were as follows: H_2, 37 mL/min; N_2 makeup gas, 23 mL/mi; and air, 400 mL/min. The VOCs collected were quantified as equivalents of toluene from parallel run toluene standard curves analyzed under conditions similar to those used for the samples. Five microliters of the toluene standard solutions were injected onto the Tenax tube with a gastight syringe and distributed by a helium flow of 60 mL/min for 3 min. When the chromatographic system was in statistical control (i.e., subject only to random variation), the standard curves were pooled. The chromatographic data were collected, stored, and processed with Turbochrom (Perkin-Elmer). No carry-over from one sample to the next was observed in the thermal desorption system used. The detection level in toluene units was 3.3 ng in total, determined as 3 times the standard deviation of the area obtained from 10 successive injections of a standard toluene solution on Tenax TA. When a compound was found in the medium blank, the detection level was defined as the mean value from the blanks plus 3 times the standard deviation of the blank values. If the concentration of a compound then turned out to be <3.3 ng upon subtraction of blank values, the compound was classified as not detected.

For GC-MS the VOCs were desorbed from the Tenax tubes in an ATD-400 thermal desorber coupled to the Kratos Profile double-focusing magnetic scanning system employing the same type of column and temperature program as above. Conditions were as follows: ATD inlet pressure, 8.2 psi; He, 45.6 mL/min; desorption and trap programming as above; transfer valve and transfer lines, 225 °C; electron impact, 70 eV; source temperature, 180 °C; split ratio ~0.12. Kovats indices from GC-FID were calculated from a homologue series of straight-chain alkanes (C_7−C_{17}) and extrapolated to extend the range from C_6 to C_{18}, which was within the linear temperature programming area. For compound identification, mass spectra were compared to the Wiley Library. The following compounds were identified by comparison with reference compounds: isoprene, acetone, 1-hexene, 2-propanol, 2-hexene, 2-butanone, 2-butanol, 2-methyl-1-propanol, 1-butanol, dimethyl disulfide, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-hexanone, cyclopentanone, α-pinene, styrene, 1-hexanol, β-pinene, β-myrcene, limonene, benzonitrile, α-terpinolene, dodecane, methylbenzoate, 2-methylisoborneol (synthesized according to the method described in ref *21*), α-copaene, β-elemene, α-cedrene, β-cedrene, (+)-calarene, geosmin, α-muurolene, δ-cadinene, β-sesquiphellandrene, hexadecane, and heptadecane.

Statistical Analysis. Principal component analysis (PCA) was carried out using Unscrambler, version 6.11.

RESULTS AND DISCUSSION

Survey of VOCs from the Actinomycetes. A total of 120 VOCs were characterized using the retention index and mass spectra. These included 11 alkanes and alkenes, 12 alcohols, 2 aromatic hydrocarbons, 14 esters, 6 ketones, 8 sulfur-containing compounds, 1 carboxylic acid, 53 terpenoids, and 13 miscellaneous compounds. The 19 most common VOCs identified are listed in **Table 2**. The most commonly produced VOCs were alcohols, esters, ketones, sulfur compounds, and terpenoid compounds. Among these, the following 15 compounds were found from half or more of the strains (with the number of positive strains in parentheses): isoprene (22), acetone (24), 1-butanol (20), 2-methyl-1-propanol (16), 3-methyl-3-buten-1-ol (16), 3-methyl-1-butanol (23), 2-methyl-1-butanol (23), cyclopentanone (22), dimethyl disulfide (26), dimethyl trisulfide (24), 2-phenylethanol (17), geosmin (21), and the unidentified terpenoid compounds MW 164 B (19), MW 164 C (14), and MW 164 E (13). There were large variations among the strains. Thus, *S. albidoflavus* AMI 246 was among the strains producing VOCs in the largest concentrations and also showed the most abundant growth and sporulation, whereas *S. thermoviolaceus* IFO 12382 and CBS 111.62 showed more meager growth and fewer VOCs in smaller concentrations. Many of the volatile compounds were detected from only one or a few strains. This was especially true for the VOC profile from *S. hygroscopicus* ATCC 27438 (see **Table 1**, which shows the distribution of common and specific VOCs between the strains). Sunesson et al. (*22*) reported that *S. albidoflavus* grown on tryptone glucose extract agar (TGEA) produced geosmin and dimethyl disulfide as the major terpenoid and sulfur compounds, respectively. This is in agreement with the results from *S. albidoflavus* AMI 246 (strain 1) used in this study. Harris et al. (*10*), who grew *S. griseus* ATCC 10137 on solidified actinomyces broth and bread, found both 2-methylisoborneol and geosmin, which is in accordance with our results for *S. griseus* ATCC 23345 (strain 15). The results from a previous study of VOCs from growth of *S. griseus* ATCC 23345 on nutrient agar (*11*) were in accordance with the present results from *S. griseus* ATCC 23345 (strain 15) with respect to the production of dimethyl di- and trisulfide, isoprene, β-pinene, α-copaene, geosmin, and 2-methylisoborneol. In a previous study of VOCs from *S. albus* grown on DG-18, 2-methyl-1-propanol, 1-butanol, 3-methyl-13-buten-1-ol, 2- and 3-methyl-1-butanol, cyclopentanone, geosmin, and sesquiterpenes among others were produced, in agreement with the results reported here (*23*).

Among the most commonly produced esters were the methyl esters of butanoic acid, 3-methylbutanoic acid, 2-methylbutanoic acid, and 4-methylpentanoic acid. This may reflect the degradation of L-methionine, L-valine, L-isoleucine, and L-leucine

Table 2. Common VOCs Identified from the 26 Isolates (in Both Runs), Characterized by Retention Index (RI) and Mass Spectrum

		strain no.																									
RI[a]	compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
	Alcohols																										
734	2-methyl-1-propanol	x			x			x	x	x		x	x	x	x	x	x		x			x		x	x		x
773	1-butanol	x	x	x	x		x		x	x		x	x	x		x	x	x	x		x	x	x	x	x		x
842	3-methyl-3-buten-1-ol	x			x		x	x				x	x	x		x	x	x	x			x	x		x		x
844	3-methyl-1-butanol	x		x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
845	2-methyl-1-butanol	x		x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
1278	2-phenylethanol	x		x				x							x	x	x	x	x	x	x		x	x	x		x
	Esters																										
742	2-methylpropanoic acid, methyl ester					x			x	x	x	x								x		x			x		x
784	methyl butyrate								x	x		x						x		x	x	x	x				x
834	2-methylbutanoic acid, methyl ester					x			x	x	x	x					x			x		x	x				x
836	3-methylbutanoic acid, methyl ester								x	x	x							x			x						
	Ketones																										
638	acetone	x	x	x	x	x	x	x	x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
901	cyclopentanone	x		x	x	x			x		x	x	x		x	x	x	x	x	x	x	x	x					x
	Sulfur Compounds																										
765	ethanethioic acid, *S*-methyl ester	x				x	x						x			x	x		x							x	
808	dimethyl disulfide	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
1046	dimethyl trisulfide	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			x		x	x
1328	dimethyl tetrasulfide												x			x	x	x	x	x	x						
	Terpenoid Compounds																										
621	isoprene	x	x	x	x			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			x	x
1282	2-methylisoborneol			x						x	x			x		x					x	x	x		x		
1513	geosmin	x	x	x	x		x	x		x	x		x		x	x		x	x	x	x	x	x		x	x	

derived from the yeast extract, thus leading to the formation of the corresponding activated acyl-CoA derivatives (*24*).

The most frequently produced alcohols from the strains in this study were 1-butanol, 2-methyl-1-propanol, and 3-methyl-1-butanol, and 2-methyl-1-butanol (**Table 2**), which could be formed by transesterification (or reduction) of the activated acyl-CoA derivatives from the amino acids mentioned above (butyryl CoA, methyl malonyl CoA, β-methylbutyryl CoA, and α-methylbutyryl CoA) followed by additional reduction reactions (*24*). The latter two, in addition to 3-methylfuran, have been proposed as indicators of fungal growth in cereal grain (*25*). Other studies of volatiles from fungi and bacteria have shown that these alcohols are frequently produced microbial metabolites (*4*, *9*, *26*).

Among the ketones, acetone and cyclopentanone were produced most frequently by the strains in this study. This general production of cyclopentanone may be a special feature of actinomycetes (*10*, *11*, *23*), although the concentration found in the present survey was close to the detection level for many of the strains. This ketone has also been found from an isolate of *Penicillium commune* and *Phialophora fastigata* (*27*). Acetone has been identified earlier from bacteria and fungi (*25*, *28*).

The production of the sulfur compounds methyl thioacetate, dimethyl disulfide, dimethyl trisulfide, and dimethyl tetrasulfide probably reflects the breakdown of growth medium as dimethyl disulfide is known to be produced by degradation of methionine and subsequent oxidation of methanethiol (*29*). Dimethyl disulfide, dimethyl trisulfide, and methyl thioacetate have been identified from mixed Gram-negative spoilage flora grown on chilled beef and were believed to originate from methionine degradation (*4*).

All isolates, except four, were found by GC-MS to produce isoprene. Bacterial isoprene production has previously been shown for both Gram-negative and Gram-positive bacteria and from fungi (*11*, *30*, *31*). Kuzma et al. (*30*) suggested that dimethyl allyl pyrophosphate, a key compound in the isoprenoid pathway leading to several types of isoprenoid compounds (*24*), was a precursor for enzymatic isoprene production.

The most frequently produced terpenoid compounds identified, apart from isoprene, were geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) and 2-methylisoborneol. These compounds exhibit musty-earthy aromas and have long been recognized as being produced by the actinomycetes (*32*). It has been established that 2-methylisoborneol is produced by the addition of a methyl group to a monoterpene precursor, whereas geosmin is produced by the side-chain cleavage of a sesquiterpene precursor (*17*). Both compounds are produced by fungi also (*9*, *33*); however, the production of geosmin by penicillia is not as widespread as for the streptomycetes in the present study. Only four strains in this survey produced neither 2-methylisoborneol nor geosmin. Three of these strains exhibited only initial stages of aerial mycelium development, and one strain (*S. griseus* IFO 13849, strain 16) produced spores. When *S. albidoflavus* AMI 246 was grown as a monoculture in autoclaved grass, geosmin production was confirmed by GC-MS.

There was a general tendency for the presence of aerial mycelium and spores to coincide with the excretion of terpenoid compounds, whereas nondifferentiating strains either did not excrete such compounds or released them only to a limited extent. The aerial mycelium, which ultimately produces spores, develops from the substrate mycelium accompanied by lysis of the substrate hyphae (*34*). During this transition phase, the streptomycetes are particularly vulnerable to competition from other organisms, and many secondary metabolites (i.e., antibiotics) appear in this growth phase (*35*). Previous studies connect the production of geosmin and 2-methylisoborneol in streptomycetes to differentiation (*17*). To visualize the possible connection with differentiation and excretion of terpenoid compounds observed in the present study, a PCA was carried out with the results from both series. PCA is a useful tool to clarify patterns in a complex data matrix. In PCA a number of principal components (PC) are extracted, representing the largest variation through a swarm of data points. The dominant patterns

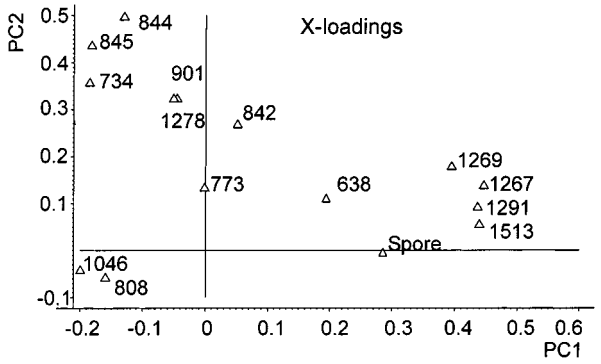

Figure 1. PCA of 14 of the most commonly produced VOCs and a differentiation parameter termed "spore". The numbers refer to the retention indices in **Table 2**: 638, acetone; 734, 2-methyl-1-propanol; 773, 1-butanol; 808, dimethyl disulfide; 842, 3-methyl-3-buten-1-ol; 844, 3-methyl-1-butanol; 845, 2-methyl-1-butanol; 901, cyclopentanone; 1046, dimethyl trisulfide; 1267, MW 164 B; 1269, MW 164 C; 1278, 2-phenylethanol; 1291, MW 164 E; 1513, geosmin. The first PC explained 20% of the variance, and the second explained 16%.

in the matrix are thus extracted in terms of a complementary set of scores and loading plots (*36*). The strains were objects, and 14 compounds, which had been identified from half or more of the strains, were chosen as variables together with a variable expressing the differentiation of the cultures. Although isoprene had been detected from more than half of the strains, this compound was excluded due to coeluting peaks for *S. albidoflavus* AMI 246, *S. antibioticus* CBS 659.68, *S. hirsutus* ATCC19773, and *S. hygroscopicus* IFO 13255 (strains 1, 7, 17, and 19). The differentiation variable was expressed in values ranging from 0 (bald culture, i.e., without aerial mycelium), 1 (beginning aerial mycelium formation), 2 (aerial mycelium formation), 3 (slight sporulation), 4 (medium sporulation), to 5 (heavy sporulation), which was assessed by visual examination of the cultures. Prior to the analysis, all data were log transformed, standardized, and centered. **Figure 1** shows the loading plot of the first two PCs. The first component was found to express the differentiation. The cluster of compounds, which have positive values on the PC1 axis, consisted of the secondary metabolites geosmin and three terpenoid compounds designated MW 164 B, C, and E, which appear to be C_{12} terpene derivatives. In the opposite direction with negative values, dimethyl di- and trisulfide and the three commonly produced microbial alcohols 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol are clustered.

Relationship of Geosmin to Sporulation. *S. albidoflavus* AMI 246, which sporulated well on Emerson's yeast starch agar, was used to examine the time course of VOC excretion to the headspace in relation to the number of spores formed employing 12 independent Tedlar bags (**Figure 2**). Headspace geosmin, expressed as micrograms of geosmin collected on a Tenax tube, and spore number, expressed as ARU, were strongly correlated (Spearmann's correlation coefficient, $r = 0.86$, $p < 0.01$). ARU (spores) also correlated with other terpenoid VOCs; however, geosmin gave the best correlation. After 15 h, the cultures were still completely bald, and the geosmin concentration was below the detection level in the headspace. No ARU were detected either. After 20 h, aerial mycelium started to appear, and after 35 h, the cultures were covered with aerial mycelium. Sporulation was clearly visible at 44 h.

This study gives an indication of the biosynthetic potential of a selection of streptomycetes in terms of volatile compounds from a single substrate. The observed VOC variation supports

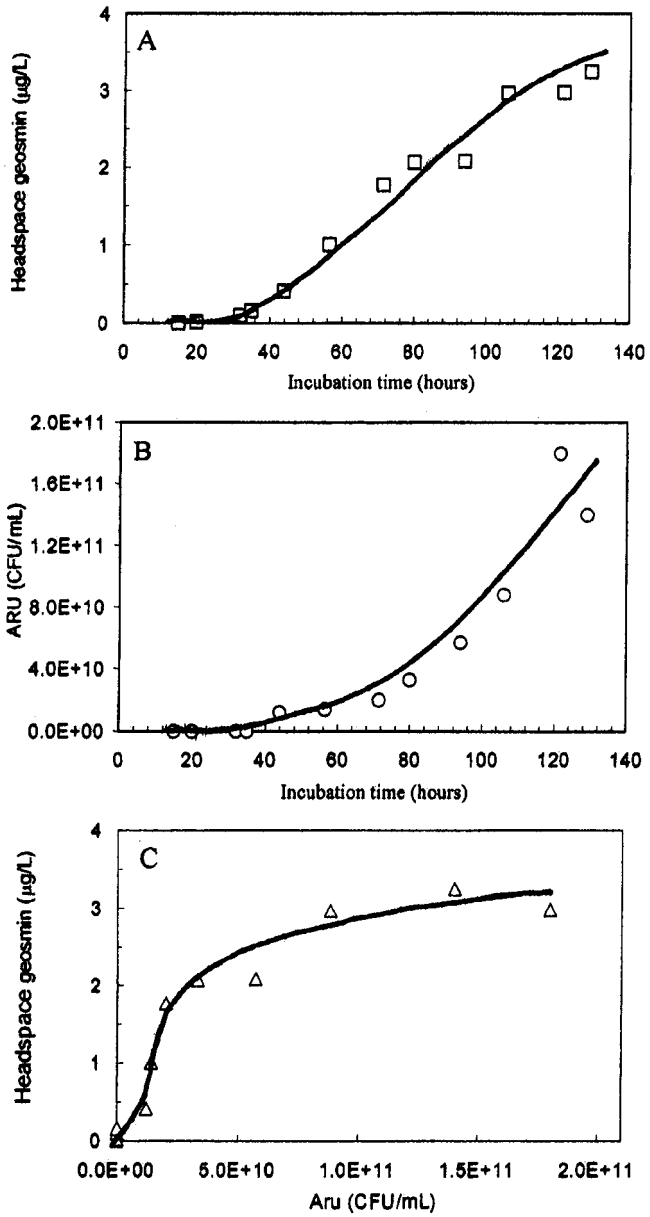

Figure 2. Excretion of geosmin to headspace by *S. albidoflavus* AMI 246 and sporulation measures as acid-resistant units: (A) headspace geosmin as a function of incubation time; (B) ARU as a function of incubation time; (C) geosmin as a function of ARU.

the idea that VOCs can be used as indicator substances for this particular genus and other actinomycetes. However, it appears to be unlikely that one or a few compounds can be used as indicators of certain classes of microorganisms in mixed microflora. Cyclopentanone is interesting, especially because the widespread production of this compound may be a unique feature of actinomycetes. Other volatiles may be quantitatively connected to microbial differentiation, as seen in the case of geosmin from *S. albidoflavus* AMI 246. Thus, the connection between headspace geosmin and ARU implies that it may be possible to detect recent sporulation by means of volatile secondary metabolites such as geosmin in the headspace.

This investigation shows that the excretion and composition of terpenoid compounds varies considerably among strains. Previous investigations reported that the excretion of sesquiterpenes from microfungi was very dependent on the medium composition with respect to carbon, nitrogen, and phosphorus (*37*, *38*), although it was not reported whether the growth was

comparable on the different media. However, sesquiterpene patterns (profiles) from some penicillia, grown on three different laboratory media, were very similar (*9*). Although the concentration of headspace geosmin during growth has been shown to be dependent on temperature, pH, oxygen concentration, and medium composition (*13*, *39*, *40*), geosmin will still be a useful qualitative indicator if the production is general for streptomycetes.

Interestingly, the often-reported fungal metabolite, 3-methylfuran, was found only from three strains in the present study and always at low concentrations. This compound has not been found in previous studies on smaller selections of actinomycetes (*10*, *11*, *22*). Neither 1-octen-3-ol, 3-octanone, nor 3-octanol was found in the present work. These compounds were previously identified from fungi (*9*, *25*). 3-Octanone has been reported from actinomycetes (*12*). These eight-carbon oxygenated compounds, which cannot be expected in viable undamaged cells, were not detected in the previously mentioned studies on VOCs from actinomycetes (*10*, *11*, *22*).

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LITERATURE CITED

(1) Dutkiewicz, J. Bacteria and fungi in organic dust as a potential health hazard. *Ann. Agric. Environ. Med.* **1997**, *4*, 11−16.

(2) Lacey, J.; Dutkiewicz, J. Bioaerosols and occupational lung disease. *J. Aerosol. Sci.* **1994**, *25*, 1371−1404.

(3) Zechman, J. M.; Aldinger, S.; Labows, J., Jr. Characterization of pathogenic bacteria by automated headspace concentration-gas chromatography. *J. Chromatogr. B., Biomed. Appl.* **1986**, *377*, 49−57.

(4) Dainty, R. H.; Edwards, R. A.; Hibbard, C. M.; Marnewick, J. J. Volatile compounds associated with microbial growth on normal and high pH beef stored at chill temperatures. *J. Appl. Bacteriol.* **1989**, *66*, 281−289.

(5) Viehweg, S. H.; Schmitt, R. E.; Schmidt-Lorenz, W. Microbial spoilage of refrigerated fresh broilers. Part VII. Production of off odors from poultry skin by bacterial isolates. *Lebensm.-Wiss. Technol.* **1989**, *22*, 356−367.

(6) Tuma, D.; Sinha, R. N.; Muir, W. E.; Abramson, D. Odor volatiles associated with microflora in damp ventilated and non-ventilated bin-stored bulk wheat. *Int. J. Food Microbiol.* **1989**, *8*, 103−119.

(7) Miller, J. D.; Laflamme, A. M.; Sobol, Y.; Lafontaine, P.; Greenhalgh, R. Fungi and fungal products in some Canadian houses. *Int. Biodeterior.* **1988**, *24*, 103−120.

(8) Wilkins, K.; Larsen, K.; Simkus, M. Volatile metabolites from mould growth on building materials and synthetic media. *Chemosphere* **2000**, *41*, 437−446.

(9) Larsen, T. O.; Frisvad, J. C. Characterization of volatile metabolites from 47 *Penicillium* taxa. *Mycol. Res.* **1995**, *99*, 1153−1166.

(10) Harris, N. D.; Karahadian, C.; Lindsay, R. C. Musty aroma compounds produced by selected molds and actinomycetes on agar and whole wheat bread. *J. Food Prot.* **1986**, *49*, 964−970.

(11) Wilkins, K. Volatile metabolites from actinomycetes. *Chemosphere* **1996**, *32*, 1427−1434.

(12) Wood, S.; Williams, S. T.; White, W. R. Microbes as a source of earthy flavours in potable water—a review. *Int. Biodeterior. Bull.* **1983**, *19*, 83−97.

(13) Dionigi, C. P.; Athen, T. S.; Wartelle, L. H. Effects of several metals on spore, biomass, and geosmin production by *Streptomyces tendae* and *Penicillium expansum*. *J. Ind. Microbiol.* **1996**, *17*, 84−88.

(14) Dionigi, C. P.; Millie, D. F.; Johnsen, P. B. Effects of farnesol and the off-flavor derivative geosmin on *Streptomyces tendae*. *Appl. Environ. Microbiol.* **1991**, *57*, 3429−3432.

(15) Pollak, F. C.; Berger, R. G. Geosmin and related volatiles in bioreactor-cultured *Streptomyces citreus* CBS 109.60. *Appl. Environ. Microbiol.* **1996**, *62*, 1295−1299.

(16) Gürtler, H.; Pedersen, R.; Anthoni, U.; Christophersen, C.; Nielsen, P. H.; Wellington, E. M. H.; Pedersen C.; Bock, K. Albaflavenone, a sesquiterpene ketone with a zizaene skeleton produced by a streptomycete with a new rope morphology. *J. Antibiot.* **1994**, *47*, 434−439.

(17) Bentley, R.; Meganathan, R. Geosmin and methylisoborneol biosynthesis in streptomycetes. Evidence for an isoprenoid pathway and its absence in non-differentiating isolates. *FEBS Lett.* **1981**, *125*, 220−222.

(18) Hendricks, C. W.; Doyle, J. D.; Hugley, B. A new solid medium for enumerating cellulose-utilizing bacteria in soil. *Appl. Environ. Microbiol.* **1995**, *61*, 2016−2019.

(19) McBride, M. J.; Ensign, J. C. Metabolism of endogenous trehalose by *Streptomyces griseus* spores and by spores or cells of other actinomycetes. *J. Bacteriol.* **1987**, *169*, 5002−5007.

(20) McBride, M. J.; Ensign, J. C. Effects of intracellular trehalose content on *Streptomyces griseus* spores. *J. Bacteriol.* **1987**, *169*, 4995−5001.

(21) Wood, N. F.; Snoeyink, V. L. 2-methyl isoborneol, improved synthesis and a quantitative gas chromatographic method for trace concentrations producing odor in water. *J. Chromatogr.* **1977**, *132*, 405−420.

(22) Sunesson, A.-L.; Nilsson, C.-A.; Carlson, R.; Blomquist, G.; Andersson, B. Production of volatile metabolites from *Streptomyces albidoflavus* cultivated on gypsum board and tryptone glucose extract agar: Influence of temperature, oxygen and carbon dioxide levels. *Ann. Occup. Hyg.* **1997**, *41*, 393−413.

(23) Kiviranta, H.; Tuomainen, A.; Reiman, M.; Laitinen, S.; Liesivuori, J.; Nevalainen, A. Qualitative identification of volatile metabolites from two fungi and three bacteria species cultivated on two media. *Centr. Eur. J. Public Health* **1998**, *6*, 296−299.

(24) Luckner, M. *Secondary Metabolism in Microorganisms, Plants, and Animals*, 3rd ed.; Springer-Verlag: Berlin, Germany, 1990.

(25) Börgesson, T.; Stöllman, U.; Schnürer, J. Volatile metabolites produced by six fungal species compared with other indicators of fungal growth on cereal grains. *Appl. Environ. Microbiol.* **1992**, *58*, 2599−2605.

(26) Urbach, G. Headspace Volatiles from Cold-Stored Raw Milk. *Aust. J. Dairy Technol.* **1990**, *11*, 80−85.

(27) Sunesson, A.-L.; Vaes, W. H. J.; Nilsson, C.-A.; Blomquist, G.; Andersson, B.; Carlson, R. Identification of volatile metabolites from five fungal species cultivated on two media. *Appl. Environ. Microbiol.* **1995**, *61*, 2911−2918.

(28) Lee, M. L.; Smith, D. L.; Freeman, L. R. High-resolution gas chromatographic profiles of volatile organic compounds produced by microorganisms at refrigerated temperatures. *Appl. Environ. Microbiol.* **1979**, *37*, 85−90.

(29) Segal, W.; Starkey, R. L. Microbial decomposition of methionine and identity of the resulting sulfur products. *J. Bacteriol.* **1969**, *98*, 908−913.

(30) Kuzma, J.; Nemecek-Marshall, M.; Pollock, W. H.; Fall, R. Bacteria produce the volatile hydrocarbon isoprene. *Curr. Microbiol.* **1995**, *30*, 97−103.

(31) Schöller, C.; Molin, S.; Wilkins, K. Volatile metabolites from some Gram-negative bacteria. *Chemosphere* **1997**, *35*, 1487−1495.
(32) Gerber, N. N.; Lechevalier, H. A. Geosmin, an earthy-smelling substance isolated from actinomycetes. *Appl. Microbiol.* **1965**, *13*, 935−938.
(33) Börgesson, T.; Stöllmann, U. M.; Schnürer, J. L. Off-odorous compounds produced by molds on oatmeal agar. Identification and relation to other growth characteristics. *J. Agric. Food Chem.* **1993**, *41*, 2104−2111.
(34) Méndez, C.; Braña, A. F.; Manzanal, M. B.; Hardisson, C. Role of substrate mycelium in colony development in *Streptomyces*. *Can. J. Microbiol.* **1985**, *31*, 446−450.
(35) Chater, K. F. Morphological and physiological differentiation in *Streptomyces*. In *Microbial Development*; Losick, R., Shapiro, L., Eds.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1984; pp 89−116.
(36) Wold, S.; Esbensen, K.; Geladi, P. Principal component analysis. *Chemom. Intell. Lab. Syst.* **1987**, 2, *37*−*52*.
(37) Bjurman, J.; Kristensson, J. Volatile production by *Aspergillus versicolor* as a possible cause of odor in houses affected by fungi. *Mycopathology* **1992**, *118*, 173−178.
(38) Sprecher, E.; Hanssen, H.-P. Influence of strain specificity and culture conditions on terpene production by fungi. *Planta Med.* **1982**, *44*, 41−43.
(39) Bjurman, J.; Kristensson, J. Production of volatile metabolites by the soft rot fungus *Chaetomium globosum* on building materials and defined media. *Microbios* **1992**, *72*, 47−54.
(40) Blevins, W. T.; Schrader, K. K.; Saadoun, I. Comparative physiology of geosmin production by *Streptomyces halstedii* and *Anabaena* spp. *Water Sci. Technol.* **1995**, *31*, 127−133.

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