Volatile compounds from Actinobacteria as mediators of microbial interactions

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CHAPTER 5

Exploring the function of volatile terpene compounds in *Streptomyces griseus* DSM40236.

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ABSTRACT

Terpenes are the largest groups of natural products known to date, synthesized by almost every living organism. *Streptomyces* are recognized for the production of the earthy terpene odorants geosmin and 2-methylisoborneol. These molecules are highly conserved amongst many *Streptomyces* strains, but their function so far remained largely unknown. Here we show that *Streptomyces griseus* is a prolific producer of a diverse collection of terpene compounds dominated by 2-methylisoborneol (2-MIB) and its dehydrogenated products 2-methylenebornane and 2-methyl-2-bornene. To understand the function of these compounds in *Streptomyces* biology, we constructed mutants lacking one or more genes for terpene synthases. Morphological changes were seen in the mutants, and their volatile profile changed substantially. Increased production of sulfur compounds was observed when 2-methylisoborneol was no longer produced. Volatile compounds from *S. griseus* showed inhibitory activity against *Fusarium culmorum,* particularly when exposed to the volatiles from the mutant unable to produce 2-methylisoborneol. This phenotype correlates with the higher production of dimethyl disulfide and dimethyl trisulfide. Finally, when volatile terpene compounds were no longer produced the whole volatile profile from *S. griseus* was modified and very few volatile compounds were produced suggesting a regulatory role of terpene molecules in the overall synthesis of volatile compounds.

INTRODUCTION

Terpenes are the largest class of natural products with approximately 75,000 compounds known to day produced by a great diversity of organisms including plants (Degenhardt et al 2009), fungi (Schmidt-Dannert 2015), bacteria (Dickschat 2016, Harris et al 2015, Yamada et al 2015) and protists (Chen et al 2016). Terpene compounds have diverse biotechnological applications as flavors and fragrances (limonene, menthol) and colorants (carotenoids) in the food industry, as perfumes (geraniol) in the cosmetic industry, as biofuels (bisabolane) or for human health (anticancer compound taxol). Terpenes have been mostly studied

in plants due to their ecological role as defense against herbivores, or signals to beneficial organisms such as pollinators and mycorrhiza among others (Pichersky and Raguso 2018). Interestingly, despite the diversity of molecules and functions, they all arise from the two functional isoprene units: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These basic building blocks condensate into the linear geranyl diphosphate (GPP, precursor of monoterpenes) and farnesyl diphosphate (FPP, precursor of sesquiterpenes).

Well-known examples are the sesquiterpene geosmin, which lends the typical earthy smell to soil, and the musty odor caused by 2-methylisoborneol. Both terpenes are produced by many different microorganisms like fungi (Boerjesson et al 1993, Mattheis and Roberts 1992), cyanobacteria (Izaguirre et al 1982) and bacteria (Dickschat et al 2007, Schöller et al 2002). Their presence in water is a sign of contamination and toxicity, therefore, their biosynthesis has been thoroughly studied (Dickschat et al 2005, Freeman 2010, Gust et al 2003, Jiang et al 2007, Komatsu et al 2008). However, their ecological role in both aquatic and terrestrial remains unknown.

Streptomyces are Gram-positive soil-dwelling bacteria well known for their biotechnological potential. They produce around half of the antibiotics used in clinic (Hopwood 2007). Amongst the secondary metabolites produced by these bacteria are the volatile compounds (VCs) found in bouquets of up to 200 compounds (Schöller et al 2002). Release of VCs has a major impact on the development of *Streptomyces* (Bentley and Meganathan 1981), suggesting a direct relationship between terpenes like geosmin and 2-methylisoborneol with the sporulation process. However, the link of 2-MIB and geosmin to sporulation varies between strains. *Streptomyces albidoflavus* AMI 246 and *Streptomyces rishiriensis* AMI 224 produce neither geosmin nor 2-methylisoborneol and fail to sporulate, whereas *Streptomyces griseus* IFO13849 that also fails to produce these molecules developed normally (Schöller et al 2002). In some cases, terpenes are only induced under specific growth conditions (Schmidt et al 2017). Clearly, better understanding of the biological function of these molecules is required.

Actinobacteria are one of the largest bacterial phyla present in soil (Barka et al 2016, Cordovez et al 2015) as such, their volatile compounds (VCs) likely play an important role in intra- and inter-species communication. VCs can act as antibacterials or antifungals, inhibitors or enhancers of plant growth, as triggers of plant resistance to disease and as signals for other micro- and macro-organisms to sense each other (quorum sensing) or regulate various physiological responses such as drug resistance (Kai et al 2010, Kim et al 2013, Park et al 2015, Schulz-Bohm et al 2017b, Strobel et al 2001). Many classes of VCs exist, including alkanes, alkenes, alcohols, esters, ketones, terpenoids, sulfur-containing compounds and a range of small inorganic compounds. One of the most studied compounds are terpenes, however, their function has mostly remained elusive. In this study, we explored the function of VCs in the biology of *Streptomyces griseus* DSM40236, in their development and in interspecies interactions.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains used in this work are listed in Table 1. *E. coli* strain JM109 (Sambrook 1989) was used for routine cloning. The specific site-methylating *E. coli* IR539 (Suzuki 2011) was used for DNA isolation and introduction to *S. griseus*. *E. coli* transformation was carried out as described elsewhere (Sambrook 1989), selected on LB agar media containing the relevant antibiotics and grown O/N at 37°C except for *E. coli* IR539 which was grown in LB with the appropriate antibiotics and IPTG (100µM) and incubated at 30°C. *Streptomyces griseus* DSM40236 was used as the parent strain to construct all the mutants. *Streptomyces* strains were grown on SFM (soya flour mannitol) solid media. The techniques for culture and transformation of *Streptomyces* are described in (Kieser et al 2000). YEME:TSBS (Yeast extract-malt extract and tryptone soy broth with 20% sucrose and 2.5 mM $MgCl_2$) were used for growth of *Streptomyces* in liquid cultures and for generating protoplasts. Regeneration agar with yeast extract (R2YE) was used for recovery of

protoplasts and for selection of recombinants using the appropriate antibiotic (Kieser et al 2000). The fungal strain *Fusarium culmorum* PV was isolated from a sandy dune soil in the Netherlands (De Boer et al 1998), pre-cultured on 0.5 Potato Dextrose Agar plates (PDA) (Fiddaman and Rossall 1993) and incubated for 6 days at 20 °C before use.

Terpene synthase gene knockouts in *Streptomyces*

Gene deletion mutants of the terpene synthases from *S. griseus* were constructed as individual, double, triple or quadruple gene deletions, as described previously (Świątek et al 2012). Details of plasmids, constructs and primer pairs used for the construction of the mutants are listed in Tables 2 & S1. Briefly, around 1500 nt of the gene flanking regions (upstream and downstream) were amplified by PCR and cloned with *Eco*RI/*Hind*III into the unstable pWHM3 (Vara et al 1989). Following this, the apramycin resistance cassette (*aac(3)IV*) (Blondelet-Rouault et al 1997) flanked by *loxP* sites was introduced between the flanking regions via the engineered XbaI site. Constructs in Table 2 were created. The presence of an *aac(3)IV-loxP* site allows an efficient removal of the apramycin cassette from the chromosome after introduction of the pUWLCre plasmid expressing the Cre recombinase (Fedoryshyn et al 2008). This methodology allowed us to use the same antibiotic disruption cassette for the double and triple gene knockouts. After a triple gene replacement with the apramycin resistance cassette it was no longer possible to remove it using the Cre-lox recombination system since the genome of *S. griseus* already contained 3 *loxP* sites. For this reason, a new construct with a different resistance cassette was designed. The apramycin resistance cassette from pEDP2 was replaced by a kanamycin resistance cassette isolated from the plasmid pKD4 (Datsenko and Wanner 2000) using XbaI. A list of the mutants created can be found in Table 1.

Table 1. Bacterial strains used in the present study.

Strains	Description	Reference
E. coli JM109	See reference	(Sambrook 1989)
E. coli IR539	See reference	(Suzuki 2011)
S. griseus DSM40236	Wild-type strain	Krainsky, 1914(Liu et al 2005)
Δ*mibS*	*S. griseus* DSM40236 Δsgr1269	This work
Δ*gcoA*	*S. griseus* DSM40236 Δsgr2079	This work
Δ*gecA*	*S. griseus* DSM40236 Δsgr6065	This work
Δ*geoA*	*S. griseus* DSM40236 Δsgr6839	This work
Δ*geoA*Δ*mibS*	*S. griseus* DSM40236 Δsgr6839/Δsgr1269	This work
VTN	*S. griseus* DSM40236 Δsgr6065/Δsgr6839/Δsgr1269Δ(::*aac(3) IV*)/ Δsgr2079::*kan*	This work

aac(3)IV: apramycin resistance cassette; kan: kanamycin resistance cassette.

Table 2. Plasmids and constructs used in this study

Plasmids/ Constucts	Description	Reference
pWHM3	*E. coli/Streptomyces* shuttle vector, multi-copy and very unstable in *Streptomyces*	(Vara et al 1989)
pUWLCre	plasmid expressing the *Cre* recombinase	*(Fedoryshyn et al 2008)*
pKD4	Kanamycin resistance cassette	(Datsenko and Wanner 2000)
pEDP1	pWHM3 containing flanking regions -1409/+36 upstream and +2011/+3461 downstream of *S.griseus* SGR6839 with apra*loxP* inserted in-between	This work
pEDP2	pWHM3 containing flanking regions -1359/+3 upstream and +1291/+2728 downstream of *S.griseus* SGR1269 with apra*loxP* inserted in-between	This work
pEDP3	pWHM3 containing flanking regions -1196/+36 upstream and +979/+2467 downstream of *S.griseus* SGR2079 with apra*loxP* inserted in-between	This work

pEDP4	pWHM3 containing flanking regions -1342/+36 upstream and +928/+2409 downstream of *S.griseus* SGR6065 with apra*loxP* inserted in-between	This work
pEDP2_kan	pWHM3 containing flanking regions -1359/+3 upstream and +1291/+2728 downstream of *S. griseus* SGR1269 with kan^R inserted in-between	This work

Morphological analysis

S. griseus strains were grown on SFM plates (90 mm) for four days at 30°C. The strains were imaged after 4 days of growth using a Zeiss Lumar V12 stereomicroscope. Light microscopy images were taken with a Zeiss Axio Lab A1 upright microscope coupled to an Axiocam MRc5 camera.

For the antifungal assays, *F. culmorum* was grown in 0.5 PDA 3.5 cm plates for 3 days at 20°C under the effect of VCs from *S. griseus* strains. Fungal hyphae micro-morphology images were taken with a Leitz DM IRB inverted microscope with 80x magnification using an Axiocam MRc5 camera. Images of fungal aerial hyphae and pigment production of were taken with a Leica M205c stereomicroscope with a 7.8x magnification using a Leica DFC450 camera.

Collection and analysis of volatile compounds

Streptomyces volatile compounds (VCs) were collected from monocultures grown on SFM agar using a glass petri dish designed for headspace volatile trapping (Garbeva et al 2014). The lid of this glass petri dish contains an outlet specially designed to hold a stainless-steel column packed with 200mg Tenax® TA 60/80 material (CAMSCO, Houston, TX, USA). Samples were taken in triplicates from day 3 to day 5 of growth, after that, the Tenax steel traps were sealed and stored at 4°C until GC-Q-TOF analysis.

Trapped volatiles were desorbed using an automated thermodesorption unit (model UnityTD-100, Markes International Ltd., United Kingdom) at 210°C for 12 min (Helium flow 50 ml/min) and trapped on a cold trap at -10°C. The trapped volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, USA) by heating the cold trap for 3 min to 280°C. Split ratio was set to 1:20, and the column used was a 30 × 0.25 mm ID RXI-5MS, film thickness 0.25 µm (Restek 13424-6850, USA). Temperature program used was as follows: 39°C for 2 min, from 39 to 95°C at 3.5 °C/min, then to 165°C at 6°C/min, to 250°C at 15°C/min and finally to 300°C at 40°C/min, hold 20 min. The VCs were detected by the MS operating at 70 eV in EI mode. Mass-spectra were extracted with MassHunter Qualitative Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, USA) using the GC-Q-TOF qualitative analysis module. The obtained mass spectra were exported as mzData files for further processing in MZmine. The files were imported to MZmine V2.14.2 (Pluskal et al 2010) and compounds were identified via their mass spectra using deconvolution function (Local-Maximum algorithm) in combination with two mass-spectral-libraries: NIST 2014 V2.20 (National Institute of Standards and Technology, USA http://www.nist.gov) and Wiley 9th edition mass spectral libraries and by their linear retention indexes (LRI). The LRI values were calculated using an alkane calibration mix before the measurements in combination with AMDIS 2.72 (National Institute of Standards and Technology, USA). The calculated LRI were compared with those found in the NIST and in the in-house NIOO-KNAW LRI database. After deconvolution and mass identification peak lists containing the mass features of each treatment (MZ-value/Retention time and the peak intensity) were created and exported as CSV files for statistical processing via MetaboAnalyst V3.0 (www.metaboanalyst.ca; (Xia et al 2015)).

Antifungal assays

To examine the effect of *S. griseus* VCs on the growth of *Fusarium culmorum*, a plate-within-a-plate system was used Figure 1. Two division petri-dishes were used; in one compartment containing SFM medium, *S.*

griseus spores were inoculated (100 µL from a 10^4cfu/mL dilution) and incubated for 2 days at 30°C. After 2 days, *F. culmorum* plugs (6 mm ⌀) were inoculated on 0.5 PDA in a 3.5 cm petri-dish. This small petri-dish was placed in the second compartment of the two-division petri-dish and incubated at 20°C for 3 days after which pictures were taken to record the *F. culmorum* growth. As control, the *F. culmorum* plates were placed in a two-division petri-dish with non-inoculated SFM.

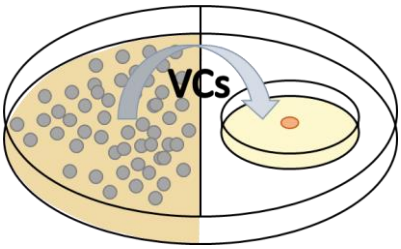

Figure 1. Schematic representation of the antifungal setup.

RESULTS

Volatile compounds released by *Streptomyces griseus*

It was shown previously that *S. griseus* produces volatile antimicrobials, and in particular ammonia, when grown on media containing glycine (Chapter 3). However, this strain is capable of releasing a complex blend of VCs under normal culturing conditions. To identify the complex mixture of VCs in the headspace of *S. griseus,* the strain was grown on SFM agar and incubated at 30°C. The VCs were trapped using stainless steel columns packed with Tenax, further desorbed and analysed with GC-Q-TOF. 46 different VCs were identified from the headspace after 4 days of growth including, alkanes, aldehydes, ketones, aromatic compounds, sulfur and terpenoid compounds, the latter being the most abundant (Figure 2, Table 3). *S. griseus* produced terpenoids in high quantities, particularly 2-methylisoborneol (2-MIB) and its dehydration products 2-

methylenebornane and 2-methyl-2-bornene (see Chapter 3). The production of 2-methylisoborneol as well as geosmin is highly conserved amongst members of the genus *Streptomyces*, and their role in the biology of these bacteria, still largely remains an enigma. The S. *griseus* genome encodes four terpene synthases, namely 2-MIB synthase MibS (SGR1269), (+)-caryolan-1-ol synthase GcoA (SGR2079), (+)-epicubenol synthase GecA (SGR6065) and geosmin synthase GeoA (SGR6839). These terpene synthases are also able to produce other terpenes, so that 36 out of the 46 VCs of *S. griseus* identified by GC-MS are terpenes. To investigate the role of terpenes in the biology of streptomycetes we decided to construct mutants lacking one or more of these terpene synthases. The list of the single and multiple knock-out mutants is shown in Table 1.

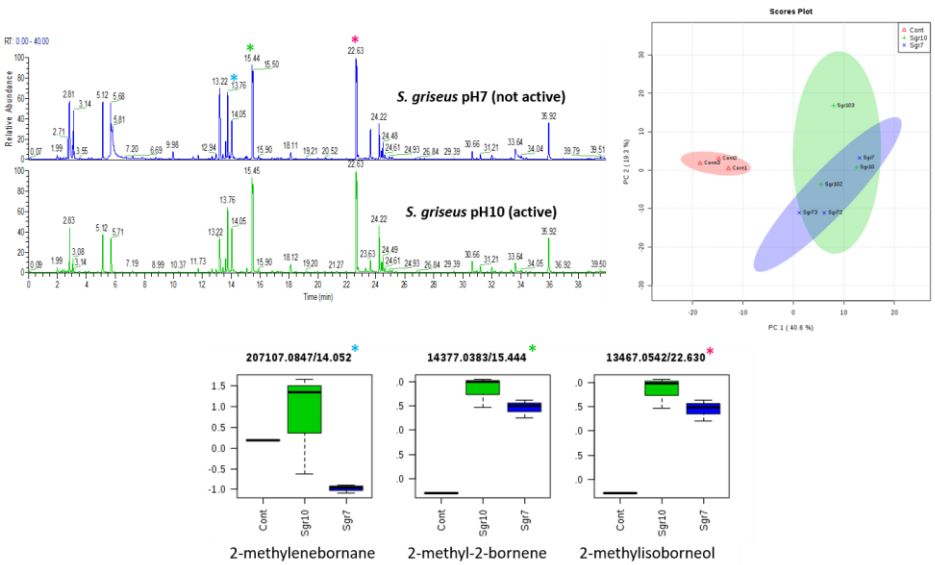

Figure 2. Chromatograms and metabolomic analysis of *S. griseus* grown on agar plates buffered to pH 7 and pH 10 using a glycine/NaOH buffer. Terpene compounds are the main VCs in both conditions.

Table 3. List of putative VCs identified in the headspace of *S. griseus* DSM40236.

No.	RT^	Compound	RI_{exp}	RI_{lit}
1	1.89	sulfur dioxide ∝	577	
2	2.11	acetone	587	
3	2.17	2-methyl-3-methylene-1-pentene	590	
4	2.56	3-methylpentane	609	
5	2.96	methylcyclopentane	629	
6	4.8	dimethyl disulfide	718	734
7	6.04	cyclopentanone	783	796
8	10.94	alpha thujene ∝	924	927
9	11.23	alpha pinene	931	933
10	11.9	camphene	947	950
11	12.41	benzaldehyde ∝	959	959
12	12.64	dimethyl trisulfide	964	968
13	12.9	sabinene	971	978
14	13.08	tp-like	975	-
15	13.5	2-methyl-2-bornene	985	981
16	13.61	beta-pinene ∝	988	
17	13.65	beta-myrcene	989	1989
18	14.87	2-methylenebornane	1017	1017
19	15.33	limonene	1028	1028
20	16.6	gamma terpinene	1057	1059
21	16.73	tp-like ∝	1061	-
22	17.75	terpinolene ∝	1084	1086
23	18.61	6-nonenal ∝	1105	1107
24	19.21	tp-like ∝	1120	-
25	19.4	tp-like	1124	-
26	20.32	camphor ∝	1146	1143
27	21.84	tp-like	1184	-
28	22.01	2-methylisoborneol	1187	1180
29	22.72	decanal ∝	1206	1205
30	23.62	tp-like	1230	-
31	23.77	tp-like	1234	-
32	28.81	copaene	1376	1376
33	29.25	beta-elemene ∝	1389	1390
34	30	geosmin	1412	1403
35	30.37	tp-like	1423	-

36	30.61	tp-like	1431	-
37	30.71	tp-like	1434	-
38	31.4	tp-like	1456	-
39	32	germacreneD	1474	1488
40	32.74	alpha muurulene	1498	1498
41	33	germacrene isomer ∝	1507	-
42	33.34	delta cadinene	1518	1523
43	33.44	calamenene isomer	1521	1522/ 1528
44	33.89	4-Isopropyl-1,6-dimethyl-1,2,3,4,4a,7-hexahydronaphthalene ∝	1536	
45	34.02	alpha calacorene ∝	1541	1541
46	36.46	cubenol	1629	1636

^RT indicates the retention time from each compound. RI exp refers to the experimental retention index. RI lit: Retention index found in literature.
∝ refers to VCs found in very small amounts.

Construction of mutants of *S. griseus* DSM40236 defective in terpene synthases

Single deletion mutants of genes for terpene synthases in *S. griseus* were constructed by replacing them with the apramycin resistance cassette. The *mibS* (SGR1269) mutant was created by replacing the +3 and +1291 region from the start of the gene by the apramycin resistance cassette. For the *gcoA* (SGR2079) mutant the +36 to +1291 region relative to the start of the gene was replaced, for the *gecA* (SGR6065) mutant the +36 to +928 region of the gene, and for the *geoA* (encoding geosmin synthase, SGR6839) mutant the +36 to +2011 region of the gene. In all cases, the apramycin resistance cassette was subsequently removed by expressing the Cre recombinase from the pUWLCre plasmid generating a marker-less deletion mutant. The double mutant lacking the genes encoding GeoA and MibS was created using the *geoA* mutant as a starter strain followed by deletion of *mibS* using the same strategy as described above. For the volatile terpene non-producer strain (VTN), the ∆*gecA* strain was used as a starter strain. The *geoA* gene was then knocked out as described above,

and after the removal of the apramycin resistance cassette a double knockout was created allowing the removal of a third gene encoding a terpene synthase (Δ*gecA/*Δ*geoA/*Δ*mibS*). After the creation of a triple deletion mutant, the removal of the apramycin resistance cassette from this strain was no longer possible due to the presence of multiple *loxP* sites throughout the genome. For this reason, the fourth gene encoding a terpene synthase (*gcoA*) was replaced using a kanamycin resistance cassette. This VTN strain has therefore two in-frame deletions (Δ*gecA/* Δ*geoA)*, an apramycin resistance cassette replacing the *mibS* gene and a kanamycin resistance cassette replacing the *gcoA* gene.

Changes in volatile profiles of mutants deleted for terpene synthase genes

To characterize the VC profile emitted by the *S. griseus* mutants deleted for one or more genes encoding terpene synthases, the strains were grown on SFM agar and the headspace collected between three and five days of growth. The VCs were identified and compared to those emitted by the parent *S. griseus* DSM40236 (Table 4).

Table 4. List of putative VCs identified in the headspace of *S. griseus* mutants deleted in terpene synthase genes.

	RT^	**Compound**	**RI[$]**	WT		Δ*mibS*		Δ*gcoA*		Δ*gecA*		Δ*geoA*		ΔgeoA/ Δ*mibS*	VTN
1	1.89	sulfur dioxide	577	+	∝	+		+	∝	+	∝	+	∝	+	+
2	2.11	acetone	587	+		+				+		+		+	+
3	2.17	2-methyl-3-methylene-1-pentene	590	+		+		+		+		+			
4	2.25	methyl acetate	594			+									
5	2.32	carbon disulfide	597			+								+	+
6	2.56	3-methylpentane	609	+				+		+		+			
7	2.96	methylcyclopentane	629	+		+		+				+	∝		
8	3.33	benzene	646					+							
9	4.80	dimethyl disulfide	718	+		+		+		+		+		+	+
10	6.04	cyclopentanone	783	+		+		+		+	∝	+	∝	+	+
11	7.99	s-methylthiobutyrate	846			+	∝								
12	10.94	alpha-thujene	921	+						+	∝	+	∝		
13	11.23	alpha pinene	931	+		+	∝	+		+		+			
14	11.90	camphene	947	+				+		+		+			
15	12.41	benzaldehyde	959	+	∝	+	∝	+	∝	+	∝	+	∝		
16	12.64	dimethyl trisulfide	964	+		+		+		+		+		+	+
17	12.90	sabinene	971	+				+		+		+			

18	13.08	tp-like	975	+		+	∝	+		+		+			
19	13.48	3-octanone	985			+									
20	13.50	2-methyl-2-bornene	985	+				+		+		+			
21	13.61	beta-pinene	988	+	∝					+					
22	13.65	beta-myrcene	989	+		+	∝	+				+			
23	14.87	2-methylenebornane	1017	+				+		+		+			
24	15.33	limonene	1028	+				+		+		+			
25	16,17	tp-like (3-caren-10-al?)	1047											+	
26	16.60	gamma terpinene	1057	+				+		+		+			
27	16.73	tp-like	1061	+	∝			+		+	∝	+	∝		
28	17.75	terpinolene	1084	+	∝			+	∝	+	∝	+	∝		
29	18.61	6-nonenal	1105	+	∝	+	∝								
30	19.21	tp-like	1120	+	∝			+	∝	+	∝	+	∝		
31	19.40	tp-like	1124	+				+		+		+			
32	20.32	camphor	1146	+	∝			+	∝	+		+	∝		
33	21.84	tp-like	1184	+				+	∝						
34	22.01	2-methylisoborneol	1187	+				+		+		+			
35	22.17	tp-like	1191									+			
36	22.72	decanal	1206	+	∝			+	∝						
37	22.99	dimethyl tetrasulfide	1212			+									
38	23.58	tp-like	1228									+	∝		
39	23.62	tp-like	1230	+				+		+					

40	23.77	tp-like	1234	+				+		+					
41	24.30	tp-like	1247									+			
42	27.84	tp-like	1347					+	∝						
43	28.81	copaene	1376	+		+	∝					+			
44	29.25	beta elemene	1389	+	∝										
45	29.85	alpha gurjunene	1407	+		+	∝	+	∝			+			
46	30.00	geosmin	1412	+				+		+					
47	30.03	tp-like	1413			+	∝					+	∝		
48	30.20	tp-like	1418							+	∝				
49	30.37	tp-like	1423	+		+		+				+			
50	30.57	tp-like	1430									+			
51	30.61	tp-like	1431	+		+		+				+			
52	30.71	tp-like	1434	+											
53	30.79	calarene	1437			+	∝	+	∝						
54	30.97	aromadendrene	1442			+	∝	+	∝			+	∝		
55	31.4	tp-like	1456	+				+		+					
56	31.53	tp-like	1460			+	∝					+	∝		
57	31.77	tp-like	1467			+	∝								
58	32.00	germacreneD	1474	+		+	∝	+	∝	+	∝	+	∝		
59	32.40	tp-like	1487			+									
60	32.74	alpha muurulene	1498	+		+		+		+	∝	+			
61	33.00	germacrene isomer	1507	+	∝										

62	33.34	delta cadinene	1518	+		+				+	∝	+			
63	33.44	calamenene isomer	1521	+						+	∝	+			
64	33.55	calamene isomer	1525			+	∝								
65	33.78	cubenene	1532					+				+			
66	33.89	4-Isopropyl-1,6-dimethyl-1,2,3,4,4a,7-hexahydronaphthalene	1536	+	∝	+	∝	+	∝	+	∝				
67	34.02	alpha calacorene	1541	+	∝			+	∝	+	∝	+	∝		
68	34.66	beta calacorene	1562			+	∝								
69	36.46	cubenol	1629	+		+		+				+			

^RT indicates the retention time from each compound

$RI refers to the experimental retention index.

∝ refers to VCs found in very small amounts.

The parental strain produced many terpene compounds indicating that each terpene synthase produces multiple terpenes and terpene isomers. 2-methylisoborneol (2-MIB) and its dehydrogenation products 2-methylenebornane and 2-methyl-2-bornene, (RT: 22.18, 14.95 and 13.5 min respectively) min were identified in all strains except those lacking *mibS*. A terpene-like molecule at 30.61 min RT and cubenol at 36.46 min RT were identified in all strains except in the *gecA* mutant. Caryolan-1-ol could not be found under these growth conditions in any of the *S. griseus* strains, and we therefore hypothesize that it is not produced when grown on SFM agar plates. Finally, geosmin was identified as a peak corresponding to an RT of 30.02 min (Figure 3, Table 4). The compound was found in all strains except for strains lacking either *geoA* or *mibS*. Geosmin has a characteristic ion peak at m/z 112, which was not found in the extracted ion chromatogram of the headspace of Δ*geoA* as expected. Interestingly, geosmin was also missing in the *mibS* mutant (Figure 4). The failure of *mibS* mutants to produce geosmin was seen previously in our laboratory, when the genes SCO7700-7701 were deleted in *Streptomyces coelicolor* M145 (GPvW, unpublished).

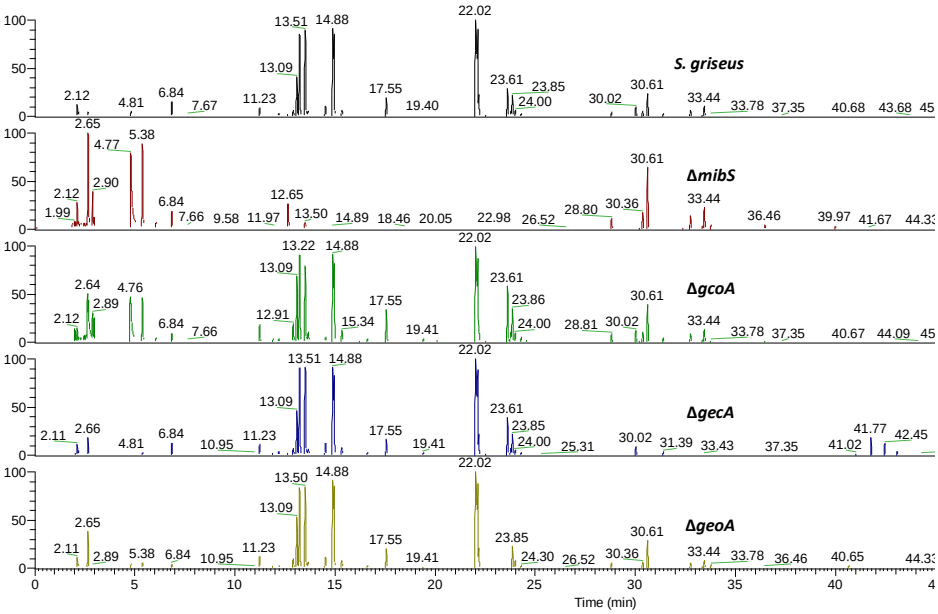

Figure 3. GC-chromatogram from the headspace of *S. griseus* strains grown for 3 days on SFM agar. *S. griseus* DSM40236 (black), Δ*mibS* (red), Δ*gcoA* (green), Δ*gecA* (blue) and Δ*geoA* (light green).

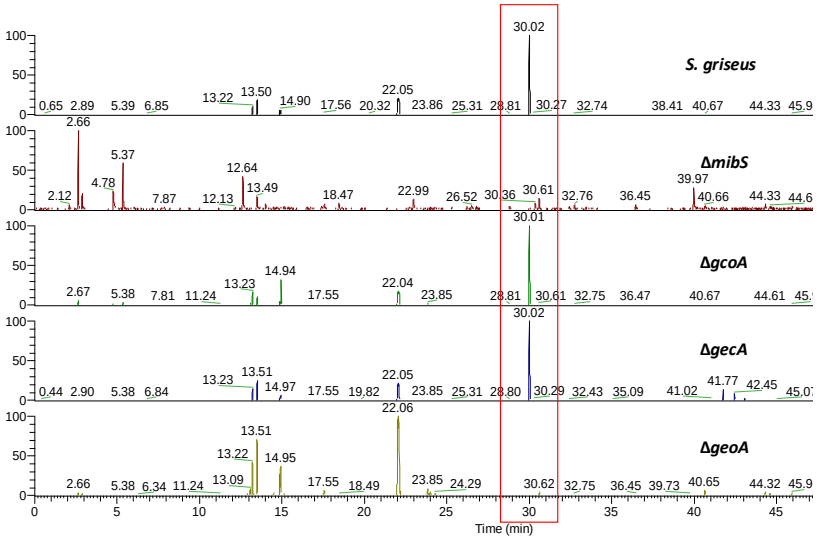

Figure 4. Extracted ion chromatogram of geosmin m/z 112 in *S. griseus* DSM40236 and the non-terpene producing single mutants.

***S. griseus* Δ*mibS* shows increased accumulation of sulfur compounds.**

The comparison between the mutants of *S. griseus* unable to produce 2-MIB and the parent *S. griseus* showed that the mutants that cannot produce MibS had an increased production of dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and dimethyl tetrasulfide (DMTES) (Table 4; Figure 5).

The production of sulfides was increased in all the mutants that do not produce 2-MIB, however, the production was higher in the *mibS* single mutant, followed by the double (Δ*geoA*/Δ*mibS*) and the quadruple (VTN) mutant.

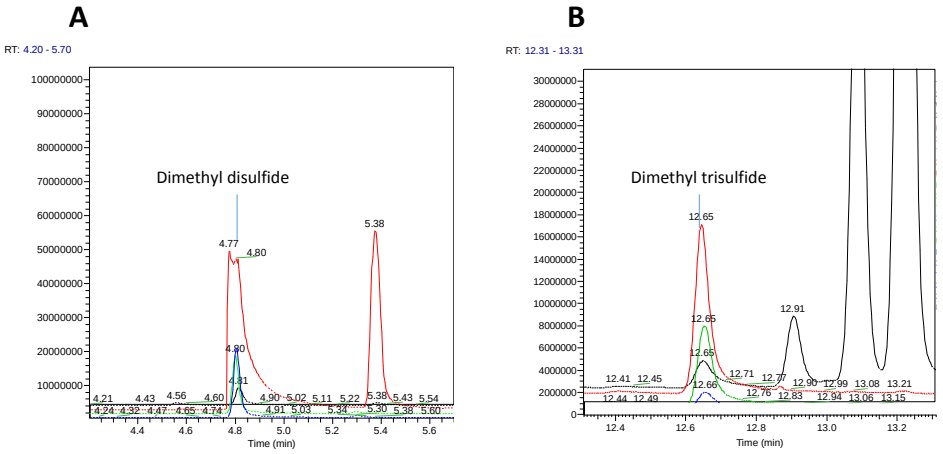

Figure 5. GC-Chromatogram showing increased production of dimethyl disulfide (A) and dimethyl trisulfide (B) in mutants of *S. griseus* lacking *mibS*(red), a double mutant lacking both *mibS* and *geoA* (green) and the terpene non-producer with all four terpene synthase genes deleted (blue). Peak abundance is given relative to wild-type *S. griseus* DSM40236 (black).

Morphological analysis of the mutants deleted for terpene synthase genes of *S. griseus* DSM40236

The mutants and their parental strain were compared for differences in their morphology. No significant changes in colony morphology were observed when the mutants were grown on SFM agar plates (Figure 6) or when grown in liquid TSBS (data not shown). The strains were also checked for their ability to sporulate in submerged cultures (van Dissel et al 2014). Spores were inoculated in TSBS media and the cultures grown for 24 h, followed by nutritional shift down by washing the cells and re-suspending them in minimal media + mannitol and glycerol 1% (Girard et al 2013); no apparent changes were seen when the strains were grown under these conditions (data not shown). Nevertheless, phenotypic differences were observed when cultures were grown under a high osmolyte concentration (sucrose 20% + 25 mM $MgCl_2$). *S. griseus* formed large mycelial clumps or pellets, while the single mutants produced an uncharacterized yellow pigment that was not seen in cultures of the parental strain or in those of the double or quadruple mutants (Figure 7). These multiple mutants in fact formed smaller pellets and seem to fragment more as seen by the smaller pieces of broken mycelia dispersed through the field of view.

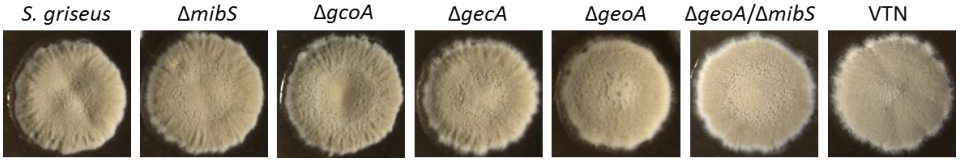

Figure 6. Colony morphology of *S. griseus* and the terpene non-producer mutants grown on SFM agar after 4 days of growth at 30°C. No major differences were observed in colony morphology between the mutants and the wild type.

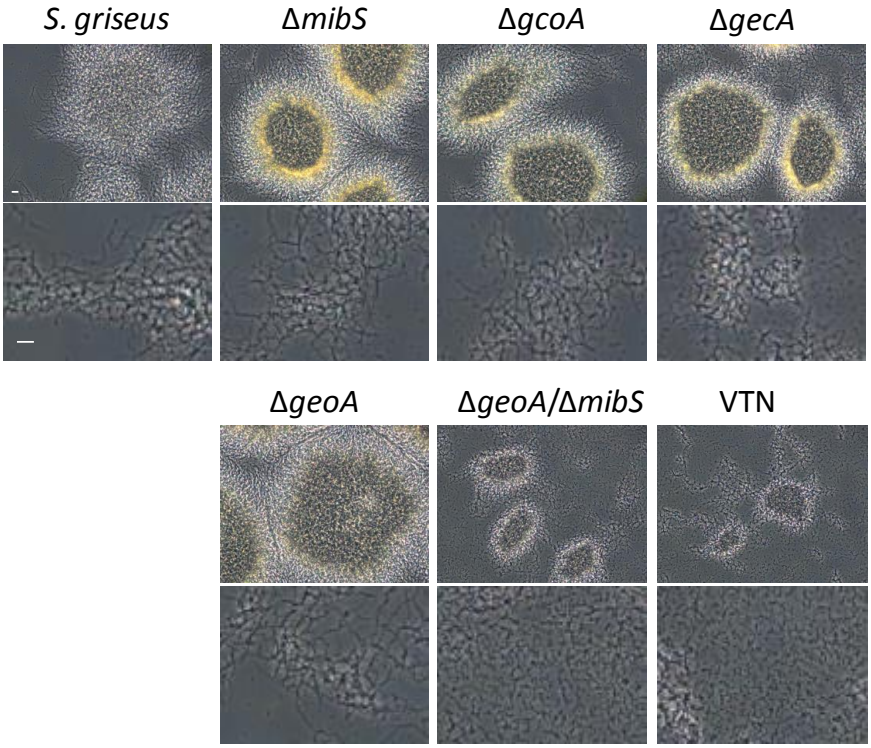

Figure 7. Phase-contrast pictures (10x) of liquid-grown cultures of *S. griseus* and the mutants unable to produce volatile terpene compounds in a high osmolarity medium (20% sucrose + 25 mM $MgCl_2$). Both morphologies: pellets (top) and open mycelia (bottom) were seen for each strain. Bar 20 µm.

Biological role of VCs from *S. griseus* in long-distance bacterial-fungal interactions

S. griseus and *Fusarium culmorum* produce complex blends of VCs that are dominated by terpenes ((Schmidt et al 2017); this work). Both are soil microorganisms making it an interesting setup to study the role of these compounds in air-borne interactions.

The double plate within a plate system was used to assay phenotypical responses of *F. culmorum* when exposed to volatiles from *S. griseus* and its mutants lacking one or more terpene synthases. Growth and pigment production were the main phenotypes that changed under the presence

of the different VCs from *S. griseus* strains. The radial growth of *F. culmorum* was significantly inhibited when grown next to *S. griseus* Δ*mibS* (Figure 8, 10). Despite the growth inhibition, the fungal hyphal micro-morphology did not change (data not shown). However, pigmentation of *F. culmorum* was slightly altered in response to the VCs produced by the different *Streptomyces* strains. The major difference was observed when *F. culmorum* was exposed to the VCs of Δ*mibS* (Figure 9), as it produced a red pigment. When exposed to VCs from *S. griseus* and the other terpene synthase mutants, *F. culmorum* produced a yellow to light-orange pigment. As shown above, Δ*mibS* has an increased production of sulfur compounds (DMDS and DMTS) suggesting that the inhibitory activity could be due to the higher concentration of dimethyl disulphide and dimethyl trisulfide. Both showed an antifungal effect against *F. culmorum* when tested as pure compounds, indicated by the reduced diameter of the fungal growth (Figure 8B). DMTS had a stronger inhibitory effect than DMDS, but the mixture of both compounds resulted in the largest inhibition zone (Figure 8B). Sulfide compounds have antifungal activity against different *Fusarium* strains, especially when used in high concentrations (Gilardi et al 2017, Wang et al 2013b), and are involved in the induction of suppression against the plant fungal pathogen *Rhizoctonia solani* in soil (Carrion et al 2018). Nevertheless, our results were not consistent in all the mutants missing the enzyme responsible of the production of 2-MIB. The differences in growth and pigment production were less apparent in the double mutant (Δ*geoA*/Δ*mibS*) and the quadruple mutant (VTN). These results indicate that even though dimethyl disulfide and dimethyl trisulfide play an important role in the inhibition of the fungal growth, the mix of volatiles exerts the overall effect. The *mibS* mutant produced an increased amount of sulfur compounds and still produced most of the terpenes, which could have a synergistic effect and hence a stronger inhibitory effect compared to the double and quadruple mutants. Figure 8A shows that the overall effect (growth inhibition and pigment production) is stronger in the *mibS* mutant followed by the double mutant but it is lost in the quadruple mutant. These results support the idea that a combination of the sulfides with the terpenes inhibits the growth of *F. culmorum* and induces a change in pigment production.

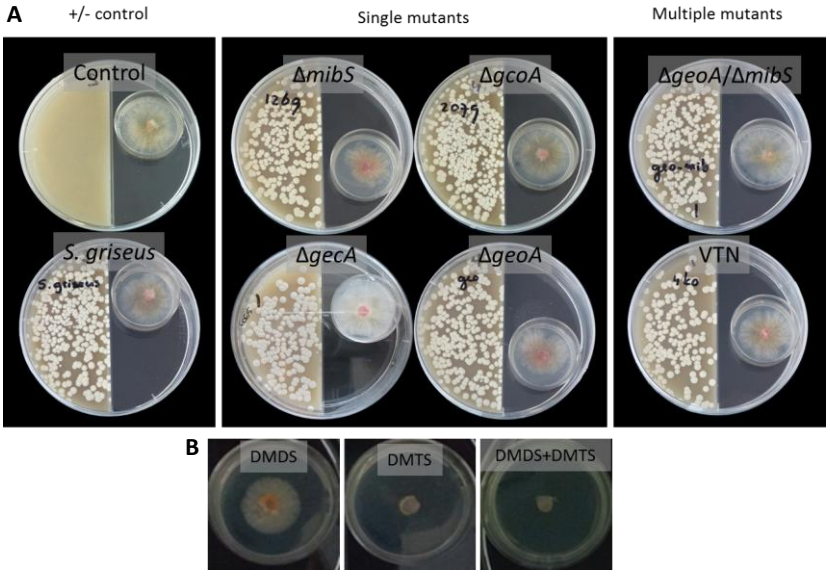

Figure 8. A. Volatile antifungal activity of *S. griseus* DSM40236 and the mutants unable to produce volatile terpene compounds. Left: control: SFM media and wild-type *S. griseus* DSM40236. Centre: *S. griseus* single mutants lacking one terpene synthase. Right: *S. griseus* double mutant Δ*geoA*/Δ*mibS* (top) and *S. griseus* quadruple mutant VTN unable to produce any volatile terpene compounds (bottom). **B.** Volatile antifungal effect of 1µg of each pure compound: dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) and in a mixture 1:1 (2µg total).

Figure 9. Stereo micrographs of *F. culmorum* showing hyphal macro-morphology and pigment production when exposed to the VCs produced by *S. griseus* and its terpene non-producer mutants. Left: control: SFM media and wild-type *S. griseus* DSM40236. Centre: *S. griseus* single mutants lacking one terpene synthase. Right: *S. griseus* double mutant Δ*geoA*/Δ*mibS* (top) and *S. griseus* quadruple mutant VTN unable to produce any VCs (bottom). Scale bar 2 mm.

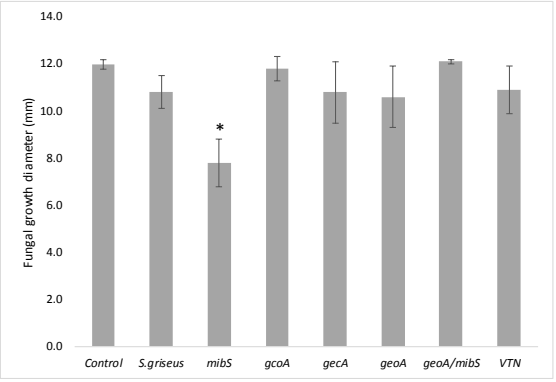

Figure. 10. Antifungal effect measured by the diameter of fungal colonies under exposure of VCs emitted by *S. griseus* and its terpene non-producer mutants.
* indicates significantly reduced growth of *F. culmorum* next to the *mibS* mutant as compared to the growth next to the parental strains *S. griseus* DSM40236.

DISCUSSION

Streptomycetes are abundant in soil and coexist in microbial communities of bacteria and fungi. They are well-known producers of terpene compounds, but little is known about the biological role of these molecules. In this study we constructed several mutants of *S. griseus* unable to synthesize one or more terpene synthases. Biochemical changes were observed as the diversity of VCs in the headspace of the mutants were significantly changed. When no terpenes were synthesized, the whole volatile profile changed and very few VCs were emitted. Interestingly, from the few compounds identified, dimethyl disulfide and dimethyl trisulfide remained, and more importantly, these compounds were upregulated in all the mutants lacking the gene for MibS.

2-MIB is a methylated sesquiterpene synthesized by the addition of a methyl group by a S-adenosyl methionine (SAM) methyltransferase on the C2 position of geranyl diphosphate and its subsequent cyclization by MibS. Many heterotrophic bacteria including soil bacteria produce sulfur compounds like methanetiol (MeSH) and dimethyl sulfide (DMS) from inorganic sulfide by the action of S-adenosylmethionine:thiol methyltransferases (Drotar et al 1987). Sulfur compounds can also be derived from methionine or cysteine by direct lysis, thereby releasing

ammonia, 2-oxobutyrate and methanethiol (MeSH). The enzymes that catalyse these reactions are methionine γ-lyase (EC 4.4.1.11) and cystathionine γ-lyase (EC 4.4.1.1). Both enzymes are present in *S. griseus*, supporting the idea that the production of MeSH in these bacteria is possible via these pathways. Besides this, polysulfides have been widely found in the headspace of many *Streptomyces* strains (Schöller et al 2002), however, their biosynthesis is not completely understood yet. DMDS and DMTS have been suggested to originate spontaneously by autoxidation from hydrogen sulfide (H_2S) and MeSH mediated by ascorbate and transition-metal ions (Chin and Lindsay 1994). We suggest that further methylation of MeSH could be done by SAM-methyltransferase generating dimethyl disulphide, dimethyl trisulfide and even dimethyl tetrasulfide in the mutant lacking the gene for MibS (Figure 11).

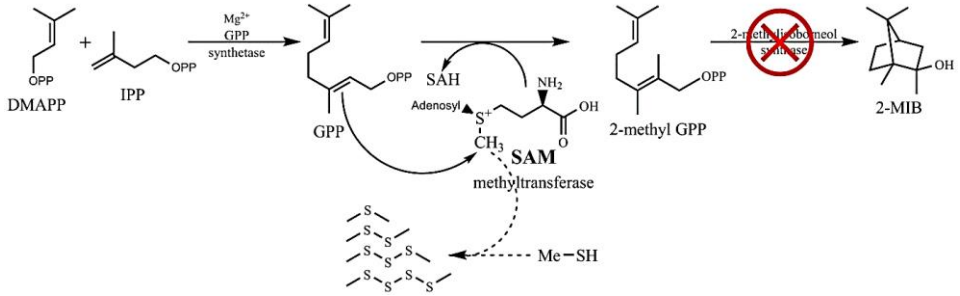

Figure 11. Proposed mechanism for the generation of dimethylated sulfides from the mutants lacking the gene for MibS.

The up regulation of sulfide compounds when the major terpenes are down regulated hints to the idea that *S. griseus* has several strategies to overcome competitors. However, the reason why this pathway is preferred rather than the up-regulation of the other terpene synthases is still unknown.

The deletion of the gene for MibS also affected the production of geosmin as this compound was also absent in the headspace of the mutant. Geosmin and 2-methylisoborneol are synthesized independently from each other, by completely different terpene synthases and both biosynthetic pathways have been thoroughly studied (Gust et al 2003,

Jiang et al 2007, Komatsu et al 2008, Wang and Cane 2008) without any suggestion of a linked biosynthesis. Therefore, we lack enough information to speculate if the synthesis of the terpenes is somehow related. Complementation studies and a larger screen of *Streptomyces* mutants lacking the gene encoding MibS are undergoing to answer this question.

Terpenes produced by streptomycetes have a role in morphological differentiation and pellet formation when grown in liquid culture under high osmolarity regarded as a stressful condition. In plants, terpenes are known to act as protectants under stress conditions. The down-regulation of terpene biosynthesis induced an increase in the jasmonic acid response in orange making it more resistant to fungal pathogens (Rodríguez et al 2014). Additionally, an increase in isoprenoids alleviated the effects of oxidative stress in plants (Vickers et al 2009). Based on these observations, we speculate that the production of terpenes in *Streptomyces* may also provide plant protection under stress conditions.

S. griseus produces grixazone, a yellow antibiotic, under phosphate limitation in an A-factor dependent manner (Higashi et al 2007, Ohnishi et al 2004). The media used to grow the *S. griseus* strains does not contain added phosphate, enabling grixazone production; however, this was only seen in the single mutants, but not in the parental strain or its double or quadruple mutants. A more likely explanation could be the expression of the 'cryptic' carotenoid (*crt*) gene cluster (Lee et al 2001). Carotenoids are also terpenoids which means they share the same precursors (isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)). A balance in terpenoid production has been observed in citrus fruit where a decreased carotenoid production was accompanied by an increase in volatile terpenoids (Liu et al 2015). However, this is unlikely the case as the pigment production is only seen in *S. griseus* single mutants. The double knockout (Δ*geoA*/Δ*mibS*) that does not produce the major terpenes, nor the quadruple mutant that is unable to produce any terpenes did not produce the yellow pigment and also exhibited major morphological differences relative to the parental strain. Presumably, the terpenes may act as signals that regulate the production of other secondary metabolites and of morphological differentiation.

Different terpenes can arise from a single terpene synthase and therefore may play a role in different pathways making it more complex to unveil their biological function. The presence of several terpene synthases and their high conservation amongst different *Streptomyces* strains points to an important role in the biology of these bacteria. The molecular promiscuity could be an evolutionary advantageous feature of the secondary metabolite's pathways (Fischbach and Clardy 2007). This idea goes hand in hand with the concept that they act synergistically and enhance each other's function. As a communication tool a mixture could be interpreted as a broader message that contains more information (Gershenzon and Dudareva 2007). Mixtures target a wider range of competitors, and because of the combined effect it is more difficult to develop resistance (Sieniawska et al 2017, Singh and Yeh 2017). In support of these theories, we see that the presence or absence of terpenes modifies the morphology and the chemical diversity of VCs in the headspace of *Streptomyces* itself, but it can also affect other microorganisms. *S. griseus* VCs mediate secondary metabolite production in *F. culmorum* as shown by the changes in pigment production.

Our work has shed light on the biological role of the VCs from *Streptomyces* particularly in the role of terpenes in inter and intraspecific interactions. It also suggests that there is a synergistic interaction between the mix of VCs released by *S. griseus*, as seen with the increased antifungal activity when compounds like dimethyl disulfide and dimethyl trisulfide are produced in higher concentrations together with terpenes.

In conclusion, *Streptomyces griseus* produces a chemically diverse blend of volatiles highly dominated by terpene compounds. These molecules are important in intra and interspecies communication changing the morphology and development of *Streptomyces* itself and modulating the release of other VCs when terpenes are absent. Our results demonstrate that the highly conserved terpene molecules can have several important roles in the biology of *Streptomyces*, and further studies are needed to unravel the function of these interesting small molecules.

Acknowledgements

We thank Jeroen S. Dickschat for the insightful discussions and suggestions and Hans Zweer for technical help with GC/Q-TOF analysis.

SUPPLEMENTARY MATERIAL

Table S1. Oligonucleotides used in this study

Name	5'-3' sequence#
SGR6839_LF-1409_ EcoRI	GTCA**GAATTC**CTGCCGAGAACCACAGTGCTC
SGR6839_LR+36_ XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**GACATA GAAGTCCGGCAGTGAG
SGR6839_RF+2011_ XbaI	GTCAGAAGTTATCGCGCATC**TCTAGA**GAGACC CTGTCGGGCTATGTG
SGR6839_RR+3461_ HindIII	GTCA**AAGCTT**TGAGCGTCTCCTTCGCCGAACAG
SGR1269_LF-1359_ EcoRI	GTCA**GAATTC**GCTTCCCTGGGTCGAGACCAA
SGR1269_LR-20_ XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**CATGCTG GACTCCTTGATGAGGT
SGR1269_RF+1291_ XbaI	GTCAGAAGTTATCGCGCATC**TCTAGA**TACAGCC TGCCCGACTTCTGGT
SGR1269_RR+2728_ HindIII	GTCA**AAGCTT**GTACCGGACTCCTCCAGCATGAC
SGR2079_LF-1196_ EcoRI	GTCA**GAATTC**GACGAGGGAGAAGGCCCCATCG
SGR2079_LR+36_ XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**CGGCATA TGAAACGCCGGTAAG
SGR2079_RF+979_ XbaI	GTCAGAAGTTATCGCGCATC**TCTAGA**GACTCGC TGTCCCGGCACTTC
SGR2079_RR+2467_ HindIII	GTCA**AAGCTT**CCGTACTGGCCGAGCTTCCAC
SGR6065_LF-1342_ EcoRI	GTCA**GAATTC**CTCCAGGACGGCGGAGAACTG
SGR6065_LR+36_ XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**GGTCCA GCTGTCCAGATGCC
SGR6065_RF+928_ XbaI	GTCAGAAGTTATCGCGCATC**TCTAGA**TATCTGG AGGAGACGGTGCTG
SGR6065_RR+2409_ HindIII	GTCA**AAGCTT**GGAACTGTGGCTCCAGGTCGA