Volatile compounds from Actinobacteria as mediators of microbial interactions

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CHAPTER 6

Streptomyces volatiles as an air defense system against protist predators

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ABSTRACT

Microbial interactions in the soil are intricate and the majority of those interactions is yet unexplored. Protists are major bacterial predators that sense and select their prey through the metabolites that bacteria produce. Here, we show that *Streptomyces* volatile compounds (VCs) can act as long-distance defense molecules against their protist predators. Our data show that a mutant of *Streptomyces griseus* deleted for all genes for terpenes was far less capable of inhibiting protists than its parent strain. The well-known terpenes geosmin and 2-methylenebornane (produced by many *Streptomyces* strains) had a differential effect on protists, suggesting a novel function of these terpenes in bacteria-protist interactions. Taken together, our work revealed that this potential extends to long-distance interactions and volatile compounds such as geosmin and 2-methylenebornane playing important role as anti-predators.

INTRODUCTION

Research on soil microbiomes has grown exponentially during the last ten years, revealing the tremendous diversity of microbial communities including bacteria, fungi, protist and archaea. At the same time, our knowledge on the chemical interactions in the soil, which shape microbial communities, is still rudimentary.

Actinobacteria are a large bacterial taxon that are abundant in the soil (Janssen 2006), with streptomycetes representing about 95% of the Actinomycetales strains isolated from soil (Barka et al 2016). Besides bacteria and fungi, protists are present in high abundance and diversity in soil (Fierer and Jackson 2006, Geisen et al 2015). Bacteria are a common food source for protists and it is known that protist sense bacteria by their morphological differences and metabolites (Jousset 2012).

The low molecular weight and high vapour pressure of volatile compounds (VCs) allow them to diffuse through the pores in soil and

reach long distances, and often play a role in interspecies communication (Audrain et al 2015, Effmert et al 2012, Kai et al 2009, Schmidt et al 2015a). VCs belong to many different chemical classes, including alkanes, alkenes, alcohols, esters, ketones, terpenoids, sulfur-containing compounds and a range of small inorganic compounds. Of these, terpenes are the largest class of natural products (Gershenzon and Dudareva 2007, Tyc et al 2017b). They are produced by almost all living organisms and have been suggested as a "*lingua franca*" between inter- and intra-species interactions (Schmidt et al 2017, Schulz-Bohm et al 2017b). Recent findings show that bacterial VCs alter protist activity and help protists localize their prey (Schulz-Bohm et al 2017a).

Streptomycetes are mostly recognized for their biotechnological potential as the main producers of the antibiotics used in the clinic (Barka et al 2016, Hopwood 2007). Besides soluble secondary metabolites, *Streptomyces* are bountiful producers of VCs, with blends of up to 200 compounds identified from one strain (Schöller et al 2002). Terpenes produced by streptomycetes include the well-known geosmin and 2-MIB, which lend streptomycetes their characteristic earthy smell. The biological function of these compounds and their role in microbial interactions has so far remained elusive. In this work, we have analysed the potential of VCs emitted by *Streptomyces* as deterrents of protist activity and show that geosmin and 2-methylenebornene inhibit protist proliferation and induce cyst formation.

MATERIALS AND METHODS

Bacteria and protist strains, media and culture conditions.

Bacterial strains used in this study were *Streptomyces griseus* DSM40236 a mutant deleted for all four terpene synthases (See Chapter 5), called VTN (volatile terpene non-producer). Monoxenic protist cultures of *Acanthamoeba* sp and *Tetramitus* sp from the Amoebozoa and Excavata eukaryotic supergroups respectively were obtained from enrichment cultivation. Briefly, 0.1 g of well-mixed sandy soil (Millingerwaard, The

Netherlands) was added to a 10 cm Petri Dish filled with sterile water. Three days after inoculation, individual protists were manually transferred to 6 cm petri dishes filled with 0.15% wheat grass (WG) medium (Geisen et al 2014). The resulting protist cultures were routinely checked for potential contamination and stored at room temperature.

Exposure of protists to *Streptomyces* volatiles

To examine the effect of *S. griseus* VCs on the activity of two different protists a 3.5 cm diameter petri dish within a two-compartment petri dish (Greiner bio-one B.V., the Netherlands) was used (Figure 1). One compartment was filled with 12 mL of SFM (Soy Flour Mannitol) agar (Kieser et al 2000) and *Streptomyces* were inoculated using 1 x 10^6 spores/mL and incubated at 30°C for three days. Protists were washed with sterile phosphate-buffer (10 mM KH_2PO_4, pH 6.5) containing three different antibiotics (5 mg ml^{-1} ampicillin, 0.4 mg ml^{-1} rifampicin, and 0.5 mg ml^{-1} kanamycin) to inhibit the growth of associated and co-transferred bacteria. A volume of 100 µL of protists suspension was mixed with 3 mL of 10 mM KH_2PO_4, pH 6.5 in the 3 cm plate-within the empty compartment of the two-division petri-dish. Five replicates were used for each treatment and incubated at 20°C. Trophozoites and cysts (active and inactive stage of the protists) were counted after one, three and seven days of protists exposure to the VCs of the *Streptomyces* strains. Non-inoculated SFM agar was used as control treatment. VCs effect was assessed as the comparison of the number of protists in active stage (trophozoites) vs the number of inactive protists (cysts). We quantified protists microscopically using an inverted Leica DMIL microscope (Germany) with a Leica C Plan L20x/0.30 or L40x/0.50 PH2 objective.

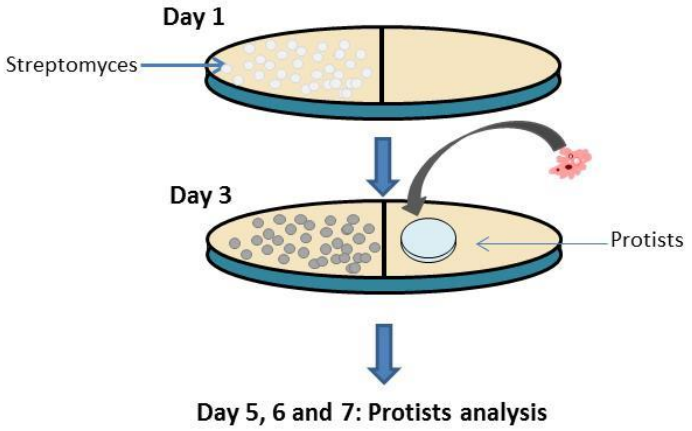

Figure 1. Experimental setup to evaluate the effect of *Streptomyces* VCs on protists.

Exposure of protists to geosmin

To study the effect of one of the most widely distributed terpenes amongst *Streptomyces* strains we tested geosmin as a pure compound (20 µg/mL). Stock solutions of pure geosmin (Sigma Aldrich, the Netherlands) were prepared in 50% Methanol and stored at -20 °C upon usage. The same setup described before was used but instead of SFM with *Streptomyces* growth, a volume of 10 µL geosmin (0.02µg) was spotted on a 5.5 mm diameter filter disc (Whatman™ filter paper, 6 µm pore size) on one compartment right after protist inoculation. A volume of 10 µl 50 % v/v methanol was spotted as control.

Exposure of protists to 2-methylenebornane and dimethyl disulfide

The effect on growth and behavior of pure 2-methylenebornane and dimethyl disulfide was tested on the protist *Tetramitus* sp. The assays were performed in 96 well plates (Greiner bio-one B.V, the Netherlands). Protists were grown with three antibiotics (5 mg ml^{-1} ampicillin, 0.4 mg ml^{-1} rifampicin, and 0.5 mg ml^{-1} kanamycin) to inhibit the growth of

associated and co-transferred bacteria. The protist culture was washed twice and re-suspended in 10 mL sterile phosphate-buffer (10 mM KH_2PO_4, pH 6.5). A volume of 35 µl of the protist solution was added to each well in the 96 well plate. Stock solutions of 2-methylenebornane ($C_{11}H_{18}$) were prepared in 50% MeOH (Merck, Germany). Dimethyl disulfide ($CH_3S_2CH_3$) was obtained as pure compound from Sigma-Aldrich (Sigma Aldrich, The Netherlands). To test the compounds a volume of 5 µl of 2-methylenebornane ($C_{11}H_{18}$) and dimethyl disulfide ($CH_3S_2CH_3$) were added into each well, resulting in a final concentration of 12 % (v/v). As controls 5 µl of 50% Methanol or water were applied. The 96 well plates were incubated for 1 week at room temperature. For the analysis, cysts and trophozoites (inactive and active stages of the protists) were counted under an inverted Leica DMIL microscope (Germany) with a Leica C Plan L20x/0.30 or L40x/0.50 PH2 objective. All treatments were performed in triplicates.

Statistical analysis

For the effect of VCs emitted by *S. griseus*, *S. griseus* mutant VTN and pure geosmin, the experiments were performed in quintuplet. The data was analyzed with IBM SPSS Statistics 24 (IBM, Somers, NY, USA). The results were analyzed using one-way ANOVA with post-hoc TUKEY (HSD- test) between the treatments. Results were considered significantly different when $p \leq 0.05$. The effect of pure 2-methylenebornane and dimethyl disulfide on protist growth and development was analyzed using a Generalized Linear Model (Two-Way ANOVA) followed by a post-hoc TUKEY (HSD) test (Treatment*Phenotype) when at least one of the model terms was significant ($P \leq 0.05$).

RESULTS

As an initial test of the effect of bacterial VCs on protists, we analysed the response of two phylogenetically different soil protists (*Acanthamoeba* and *Tetramitus*) to VCs emitted by streptomycetes. For this, we compared the responses of *Streptomyces griseus* DSM40236 and its mutant strain VTN, which fails to produce terpenes due to the deletion of all genes for terpene synthases (Chapter 5). VCs emitted by *S. griseus* and *S. griseus* VTN significantly affected the activity of protists after 3 days of exposure. The number of active protists exposed to VCs released by *S. griseus* and its terpene non-producer were lower compared to the control. Significant differences were also observed between treatments, where the number of active protists was lower when exposed to VCs emitted by *S. griseus* compared to *S. griseus* VTN (Figure 2, Table 1). This indicates that terpenes play a major role in the defense of *S. griseus* against *Acanthamoeba*. After 7 days of exposure of *Acanthamoeba* to *Streptomyces* VCs, there was no clear difference between the parent *S. griseus* and mutant VTN in terms of the effect on the protists. After 7 days no active protists were seen in the non-exposed control, while some active protists were still seen when exposed to VCs emitted from the *S. griseus* strains. This suggests that VCs are used to sustain the activity of the protists.

Table 1. Summary of the statistically significantly differences in the analysis of the *Streptomyces* VCs effect on *Acanthamoeba* using One-Way ANOVA with post-hoc Tukey HSD Test.

	(I)Treatment #	(J) Treatment #	(I-J) Mean difference	Std. Error	Sig.
Day 1	No significant differences				
Day 3	*S. griseus* active	VTN active	-98.8	20.08	0.001
	S. griseus active	Ctrl active	-185.4	20.08	0.000
	S. geiseus VTN active	Ctrl active	-86.6	20.08	0.003
Day 7	*S. griseus* active	Control active	349.8	80.1	0.003
	S. griseus inactive	Control inactive	-616.0	80.1	0.000
	S. griseus VTN active	Control active	362.6	80.1	0.002
	S. griseus VTN inactive	Control inactive	-393.4	80.1	0.001

#active indicates that the comparison was made using the count of the active forms of the protists; inactive indicates that the comparison was made using the count of inactive forms of the protists.

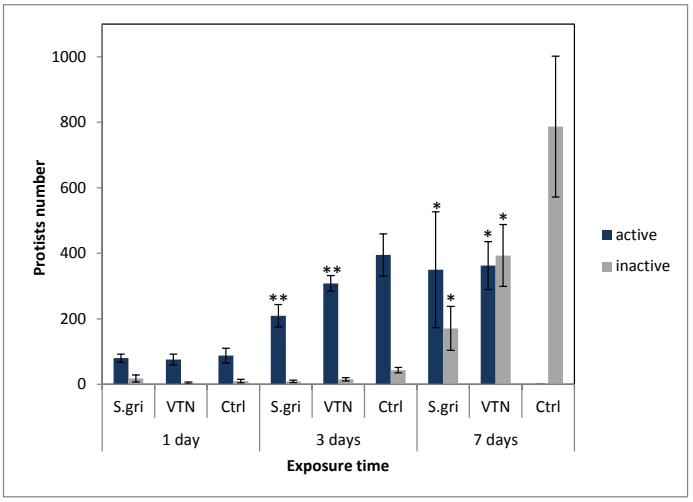

Figure 2. Abundance of active (blue) and inactive (gray) forms of *Acanthamoeba* when exposed to VCs from *S. griseus* and *S. griseus* VTN. * indicates significant difference compared to control (only culture media); ** indicates significant differences between *S. griseus* parent strain and its mutant VTN as well as against the control.

Tetramitus did not show significant differences in the number of active protists when exposed to VCs from either *S. griseus* or its mutant VTN

(Figure 3, Table 2). From day 3, the number of active protists was at least four times lower when exposed to VCs from either *S. griseus* or *S. griseus* VTN compared to the control (p <0.001), showing that VCs induce cyst formation of *Tetramitus* and therefore limits their proliferation. After three days, the number of inactive protists was higher when exposed to VCs produced by the mutant than to VCs from the wild-type strain. This suggests that *Tetramitus* is less sensitive to terpenes, but instead may be sensitive to other compounds, such as DMDS or DMTS, which are produced in much higher amounts by the mutant (see Chapter 5). From day 3, the number of inactive protists was more than fifty times higher as compared to day 1 under all conditions tested.

Table 2. Summary of the statistically significant differences in the analysis of the *Streptomyces* VCs effects on *Tetramitus* using One-Way ANOVA with post-hoc Tukey HSD tests.

	(I)Treatment #	(J) Treatment #	(I-J) Mean difference	Std. Error	Sig.
Day 1	*S. griseus* active	Control active	-22.8	6.8	0.029
Day 3	*S. griseus* active	Control active	-245.0	43.2	0.000
	S. griseus inactive	VTN inactive	-232.4	43.2	0.000
	S. griseus inactive	Control inactive	-204.4	43.2	0.001
	S. griseus VTN active	Control active	-266.0	43.2	0.000
Day 7	*S.griseus* active	Control active	-245.0	72.2	0.026
	S. griseus inactive	Control inactive	-315.0	72.2	0.003
	S. griseus VTN active	Control active	-271.6	72.2	0.011

[#]active indicates that the comparison was made using the count of the active forms of the protists; inactive indicates that the comparison was made using the count of inactive forms of the protists.

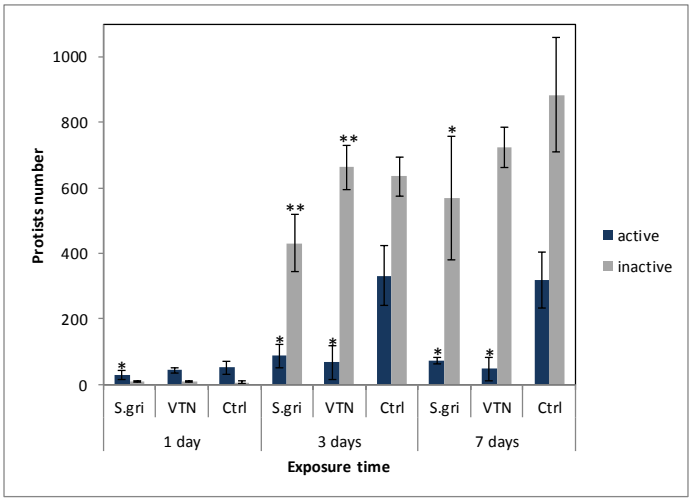

Figure 3. Abundance of active (blue) and inactive (gray) forms of *Tetramitus* when exposed to VCs from *S. griseus* and *S. griseus* volatile terpene non-producer (VTN). * indicates significant difference compared to control (only culture media). ** indicates significant differences between *S. griseus* parent strain and the mutant VTN as well as against the control.

Streptomycetes are prolific terpene producers, of which geosmin is produced by almost all streptomycetes (Figure 4). We evaluated if geosmin has a specific role in below-ground interactions such as repellent or attractant on both *Acanthamoeba* and *Tetramitus* when exposed to pure geosmin.

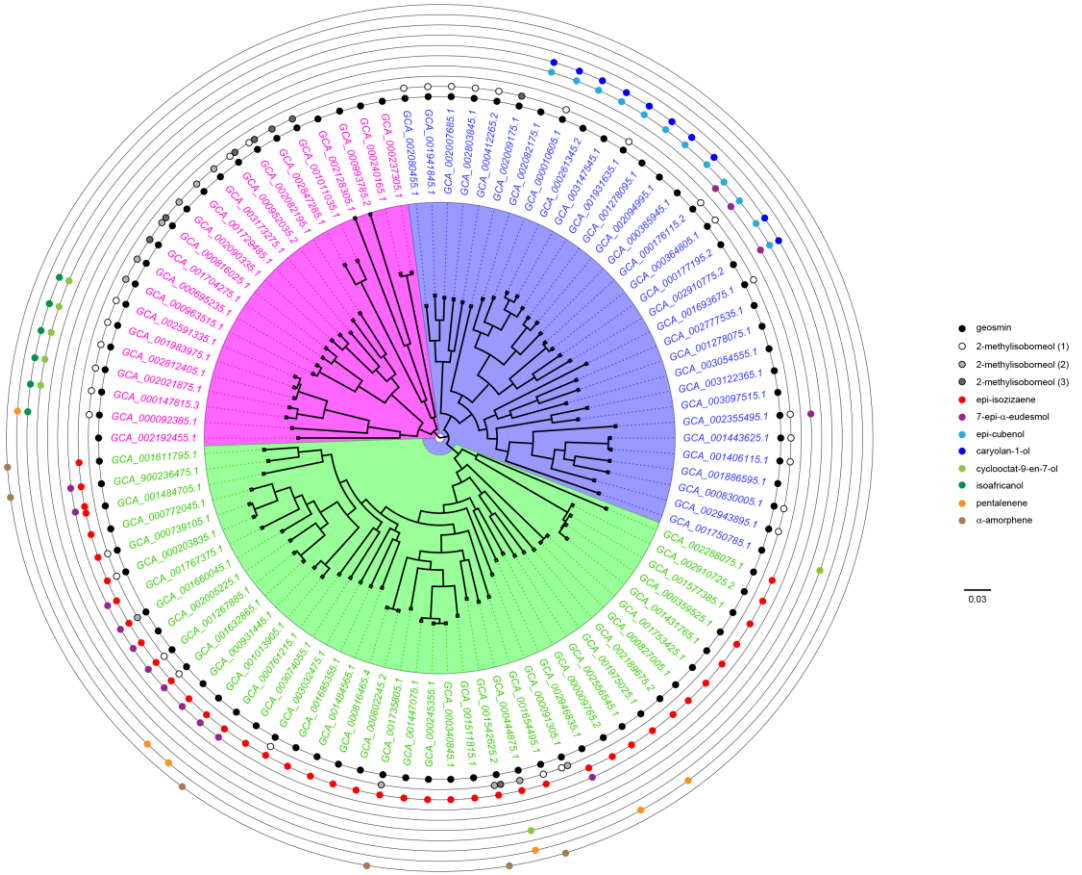

Figure 4. Phylogenetic tree of terpene synthases found in *Streptomyces* with their whole genome available.

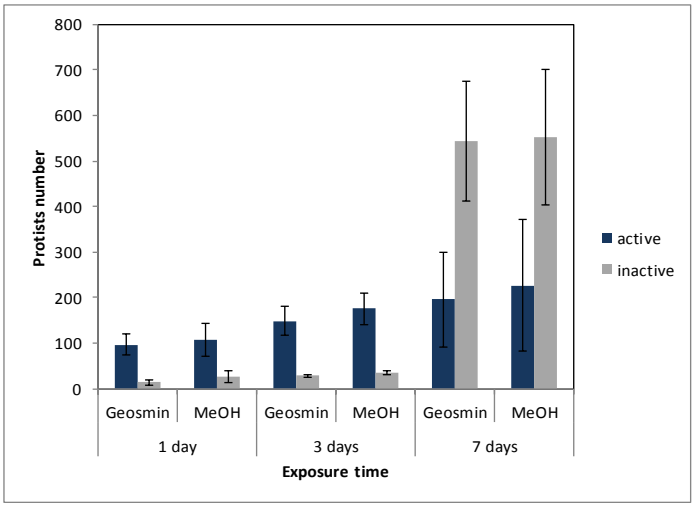

Figure 5. Abundance of active (blue) and inactive (gray) forms of *Acanthamoeba* when exposed to pure geosmin (0.02µg).

No significant differences were observed when *Acanthamoeba* was exposed to pure geosmin as compared to the control methanol (Figure 5). From this result we can infer that geosmin by itself is not responsible for the inhibition of *Acanthamoeba* by *S. griseus*.

For *Tetramitus* we observed one significant difference within the number of inactive protists after seven days exposure to geosmin compared to the methanol control (Figure 6, Table 3).

Table 3. Summary of the statistically significant differences in the analysis of the effect of geosmin on *Tetramitus* using One-Way ANOVA with post-hoc Tukey HSD tests.

	(I)Treatment	(J) Treatment	(I-J) Mean difference	Std. Error	Sig.
Day7	Geosmin inactive	MeOH inactive	-715.4	223.4	0.026

[#]inactive indicates that the comparison was made using the count of inactive forms of the protists.

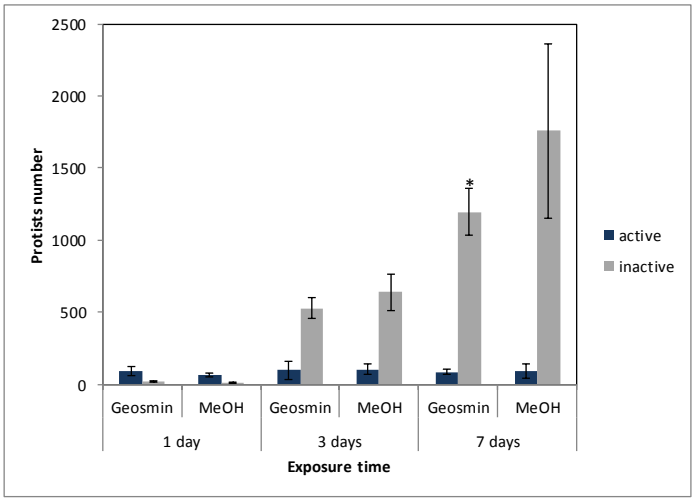

Figure 6. Abundance of active (blue) and inactive (gray) protists when exposed to pure geosmin (0.02µg) * indicates significant difference compared to control (only culture media).

The data presented in Chapter 5 showed that a suite of related terpenes is produced at high level by *S. griseus*, which are produced by the enzyme 2-methylisoborneol terpene synthase MibS (SGR1269). These are 2-methylisoborneol (2-MIB), 2-methyl-2-bornene and 2-methylenebornane. Furthermore, when the gene for 2-MIB synthase was deleted, it resulted in strong upregulation of sulfides, particularly dimethyldisulfide (DMDS).

The activity of 2-methylenebornane and DMDS against *Tetramitus* was tested by adding them directly into a liquid culture of *Tetramitus*, achieving a soluble concentration of 12% v/v. The assay was performed in triplicate in a 96 well plate (see Materials and Methods section). When *Tetramitus* was exposed to either dimethyldisulfide (DMDS) or 2-methylenebornane (2-MB) we observed a drastic decrease in the total number of protists (around 30 times less protists) with no active protists in any of the treatments (Figure 7, Table 4). These results show that both compounds (DMDS and 2-MB) are active against protists

Table 4. Summary of the statistically significant differences in the analysis of effect of 2-methylenebornane and dimethyldisulfide (DMDS) on *Tetramitus* using Two-Way ANOVA with post-hoc Tukey HSD tests.

	(I)Treatment	(J) Treatment	(I-J) Mean difference	Std. Error	Sig.
Day7	2-methylenebornane	MeOH	-9.000	2.13	0.000
Day7	DMDS	water	-2.833	2.13	0.186

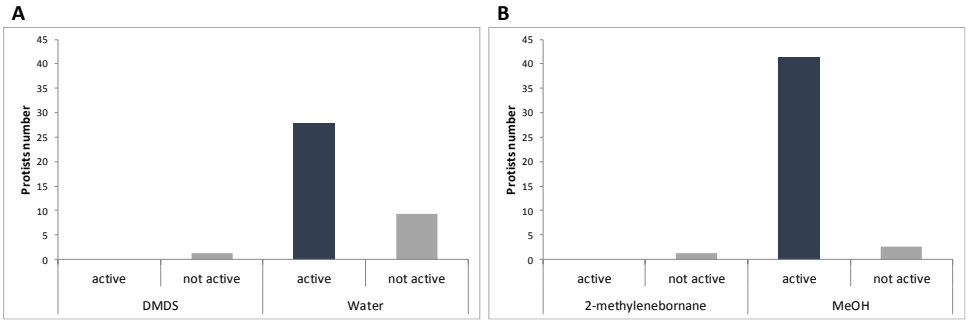

Figure 7. Abundance of active (blue) and inactive (gray) protists when exposed to pure (12% v/v) dimethyldisulfide (A) and 2-methylenebornane (B).

DISCUSSION

Streptomycetes are well known for their production of a wide range of natural products. An important class are the volatile terpenes, and in particular geosmin and 2-MIB. However, their function has so far remained largely elusive. A report has pinpointed the ability of fruit flies to identify suitable feeding and breeding sites by the detection of geosmin which alerts the insect of the presence of harmful microbes (Stensmyr et al 2012). In this work, we show that terpenes and other volatiles may play a role in controlling the activity of protists, which are known predators of streptomycetes. Both *Streptomyces* and protists are abundant in the soil and compete for resources. Not only streptomycetes but also social amoebae produce terpenes (Chen et al 2016, Kuzuyama 2017). Our results show that *Streptomyces* VCs inhibit the activity of protists and that the activity was particularly lower when exposed to the VCs from *S. griseus* as compared to those produced by its terpene non-producer VTN.

This is a clear indication that bacterial terpenes can act as deterrants of its protist predators.

After 7 days of incubation, no active protists were observed in the non-exposed control. However, *Acanthamoeba* remained active when exposed to the VCs from both *S. griseus* and its VTN mutant. *Acanthamoeba* can grow in axenic conditions (Weekers and Vogels 1994); still, active protists were only seen when *Acanthamoeba* was exposed to VCs from *S. griseus* and its mutant VTN, and we therefore hypothesize that *Acanthamoeba* used *Streptomyces* VCs as a source of nutrients. It was shown previously that fungi can use VCs as a nutrition source when grown on carbon-poor substrates (Cale et al 2016). In contrast, *Tetramitus* failed to grow in axenic cultures.

Tetramitus was inhibited with equal efficacy by wild-type *S. griseus* and its mutant VTN. The latter showed enhanced production of sulfur compounds like DMDS and DMTS. Sulfide compounds play an important role in microbial interactions; *e.g.* DMDS produced by *Bacillus cereus* induces systemic resistance in plants against necrotrophic pathogens (Huang et al 2012). These compounds are also produced by rhizobacterial isolates with antifungal activity against diverse fungi like *Rizochtonia solani* and *Alternaria alternata* (Carrion et al 2018, Groenhagen et al 2013, Li et al 2010). Interestingly, our results for the first time show that protists are sensitive to DMDS.

The total number of protists is the sum of active and inactive protists whereby new active protists are being formed while older active protists become cysts. Comparing the total number of *Tetramitus* exposed to VCs emitted by *S. griseus* in comparison to the total number of *Tetramitus* exposed to VCs emitted by the mutant VTN, we conclude that *S. griseus* inhibits proliferation of protists. Geosmin showed a species-specific response, and only had an effect on *Tetramitus.* The total number of *Tetramitus* protists was lower when exposed to geosmin, suggesting that this molecule inhibits proliferation of protists. Geosmin is produced only in small amounts by *S. griseus* under the growth conditions tested. The most abundant terpenes produced by *S. griseus* were 2-MIB and its derivatives 2-methylenebornane and 2-methyl-2-bornene. 2-

methylenebornane is emitted as one of the most abundant terpenes together with 2-MIB (Chapter 5). Indeed, 2-methylenebornane was shown to have an inhibitory effect against protists, considering the very few protists cells that were observed when exposed to the terpene.

In conclusion, our data show that *S. griseus* produces bioactive VCs that can act as weapons against protists. These VCs include geosmin and 2-methylenebornane as well as the sulfur-containing DMDS. The precise role of VCs in predator-prey interactions and their mode of action still need to be resolved.

Acknowledgements

We thank Lara Martín Sánchez and Kumar S. Singh for the collaboration doing the phylogenomic analysis of the terpene synthases from *Streptomyces*.

SUPPLEMENTARY INFORMATION

Terpene synthase gene knockouts in *Streptomyces*

S. griseus DSM40236 genome encodes 4 terpene synthases responsible of the production of volatile terpene compounds: SGR1269 (2-methylisoborneol synthase MibS), SGR2079 (caryolan-1-ol synthase), SGR6065 (epicubenol synthase) and SGR6839 (geosmin synthase). Mutants of these genes 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 S1 & S2. 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 S1 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. The VTN strain of *S. griseus* contains two in-frame deletions from position +36 to +928 relative to the start of SGR6065 and from +36 to +2011 relative to the start of SGR6839. An apramycin resistance cassette is replacing the SGR1269 gene and a kanamycin resistance cassette replacing the SGR2079 gene.

Table S1. Plasmids and constructs used in this study

Plasmids	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

Table S2. Oligonucleotides used in this study

Name	5'-3' sequence#
SGR6839_LF-1409_ EcoRI	GTCA**GAATTC**CTGCCGAGAACCACAGTGCTC
SGR6839_LR+36_ XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**GACATAGAAG TCCGGCAGTGAG
SGR6839_RF+2011_ XbaI	GTCAGAAGTTATCGCGCATC**TCTAGA**GAGACCCTGT CGGGCTATGTG
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**TACAGCCT GCCCGACTTCTGGT
SGR1269_RR+2728_ HindIII	GTCA**AAGCTT**GTACCGGACTCCTCCAGCATGAC
SGR2079_LF-1196_ EcoRI	GTCA**GAATTC**GACGAGGGAGAAGGCCCCATCG
SGR2079_LR+36_ XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**CGGCATAT GAAACGCCGGTAAG
SGR2079_RF+979_ XbaI	GTCAGAAGTTATCGCGCATC**TCTAGA**GACTCGCT GTCCCGGCACTTC
SGR2079_RR+2467_ HindIII	GTCA**AAGCTT**CCGTACTGGCCGAGCTTCCAC
SGR6065_LF-1342_ EcoRI	GTCA**GAATTC**CTCCAGGACGGCGGAGAACTG
SGR6065_LR+36_ XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**GGTCCAGC TGTCCAGATGCC
SGR6065_RF+928_ XbaI	GTCAGAAGTTATCGCGCATC**TCTAGA**TATCTGGAG GAGACGGTGCTG
SGR6065_RR+2409_ HindIII	GTCA**AAGCTT**GGAACTGTGGCTCCAGGTCGA