Volatile compounds from Actinobacteria as mediators of microbial interactions

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CHAPTER 3

Streptomyces low-cost volatile ammonia as antibiotic and modulator of antibiotic sensitivity.

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ABSTRACT

Research on volatile compounds has recently received a lot of interest. Volatile compounds represent an untapped reservoir of molecules with yet undescribed biological functions. Here we focus on the potential of streptomycetes to kill bacteria over long distances via air-borne antibiosis. Soil-inhabiting streptomycetes are nature's largest supplier of canonical antibiotics but also produce chemically diverse VCs. Our research shows that the low-cost volatile ammonia is a key player in killing Gram- and Gram+ opponents. Addition of the ammonia precursor glycine allowed non-producing streptomycetes to kill *E. coli* and *B. subtilis* cells, while inactivation of the glycine cleavage system annihilated air-borne antibiosis. We further show that ammonia enhances the activity of the costlier canonical antibiotics, suggesting that streptomycetes adopt a low-cost strategy to sensitize competitors for antibiosis over longer distances.

INTRODUCTION

Volatile compounds (VC) are small molecules with high vapor pressure and low molecular weight that easily diffuse through air, water or soil (Schmidt et al 2015a, Schulz and Dickschat 2007). VCs have a broad activity-spectrum, acting as infochemicals, growth-promoting or inhibiting agents, modulators of quorum sensing and drug resistance or as a carbon-release valve, influencing their neighbor's behavior and phenotypes such as stress response, colony morphology, biofilm, virulence and pigment production (Audrain et al 2015, Kim et al 2013, Nijland and Burgess 2010, Que et al 2013). Actinobacteria are one of the largest bacterial phyla present in soil (Barka et al 2016, Cordovez et al 2015) and producers of a wide range of VCs (Schöller et al 2002). They are well known for their capability of producing bioactive secondary metabolites, whereby streptomycetes alone produce half of all known antibiotics in the clinic (Hopwood 2007). The role of VCs in bacterial competition is virtually unknown, although antimicrobial activity has been reported for the sesquiterpene albaflavenone produced by *Streptomyces albidoflavus* (Gürtler et al 1994). There is also some experimental evidence that

suggests that VCs may affect membrane integrity (Fadli et al 2014, Yung et al 2016), which in turn may make the cells more susceptible to other cell-damaging compounds, such as antibiotics. The lack of information makes it hard to mimic the biological effect and more so to pinpoint the responsible molecules of such activity. The natural role of antibiotics may lie in cell to cell communication (Davies 2006). However, antibiotics may well act as weapons, and bioactivity is influenced by social and competitive interactions between strains (Abrudan et al 2015). Evidence has been provided of VCs acting as enhancers of antibiosis, with some studies reporting on plant VCs acting as potentiators of antibiotics (Andrade-Ochoa et al 2015, Gallucci et al 2009, Sieniawska et al 2017). Thus, VCs may help to potentiate the bioactivity of antibiotics in the soil and the plant microbiome.

This chapter shows how volatile ammonia released in high concentrations by *Streptomyces* inhibits the growth of *E. coli* and *B. subtilis* and can also modify the surrounding environment modulating growth and antibiotic production from neighboring actinomycetes.

MATERIALS AND METHODS

Strains Media and culture conditions.

Strains used in this study were obtained from an actinomycete collection previously collected from soil samples from the Qinling and Himalaya mountains (China) and The Netherlands (Zhu et al 2014). The strains were grown on SFM agar media (Soy Flour Mannitol) to prepare spore stocks and as inoculum for the experiments performed. *Escherichia coli* strain AS19-RlmA$^-$ (Liu and Douthwaite 2002a) and *B. subtilis* were used as test microorganisms. Strains used are summarized in Table 1.

Volatile antimicrobial assays were performed using a petri dish with two compartments (Figure 1), one filled with SFM media for *Streptomyces* growth and the second one with LB +/- TES buffer 50-100mM as indicated. *Streptomyces* strains were streaked on the SFM side

and allowed to grow for 5 days after which, *E. coli* or *B. subtilis* were inoculated on the LB side using a concentration of 10^4 and 10^3 cfu/mL respectively.

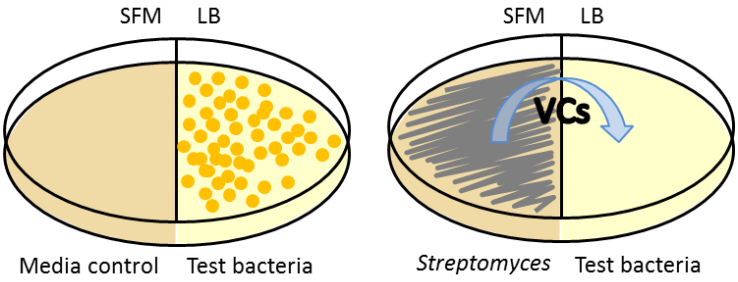


Figure 1. Experimental setup pf the two division petri dishes used for volatile antibiotic assays.

Table 1. Bacterial strains used in this study

Strains	Description	Reference
E. coli AS19-*rlmA*$^-$	Hypersusceptible, KAN^R resistant	(Liu and Douthwaite 2002a)
E. coli BREL606	Parent strain of *E. coli* AS19	*E. coli* Genetic stock center
B. subtilis 168	Wild-type strain	(Barbe et al 2009)
S. coelicolor A3(2) M145	SCP1$^-$SCP2$^-$	(Bentley et al 2002)
S. lividans 1326	Wild-type strain	(Hopwood et al 1983)
S. griseus DSM40236	Wild-type strain	Krainsky, 1914 (Liu et al 2005)
S. venezuelae ATCC 15439	Wild-type strain	(Song et al 2016)
Streptomyces sp. MBT11 Streptomyces sp. MBT21	*Streptomyces* isolates	(Zhu et al 2014)
UTR-T	*S. griseus* IFO13350 with deletion of 5'-untranslated region of *gcvTH*	(Tezuka and Ohnishi 2014)
GAL61	M145 Δ*gcvP*(::*aac(3)IV*)	(Zhang 2015)
Δ*mibS*	*Streptomyces sp.* MBT11 Δ*mibS*	This work
Δ*cyc*	*Streptomyces sp.* MBT11 Δ*cyc*	This work
Δ*cy*cΔ*mibS*	*Streptomyces sp.* MBT11 Δ*cyc*Δ*mibS*	This work

Δ*arcAD*	*Streptomyces sp.* MBT11 Δ*arcAD*	This work
Δ*cyc*Δ*mibS* Δ*arcAD*	*Streptomyces sp.* MBT11 Δ*cyc*Δ*mibS* Δ*arcAD*(::*aac(3)IV*)	This work

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

VCs collection and analysis.

Streptomyces volatile compounds were collected from monocultures grown on SFM agar using a glass petri dish designed for headspace volatile trapping (Garbeva et al 2014) Figure 2). 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.

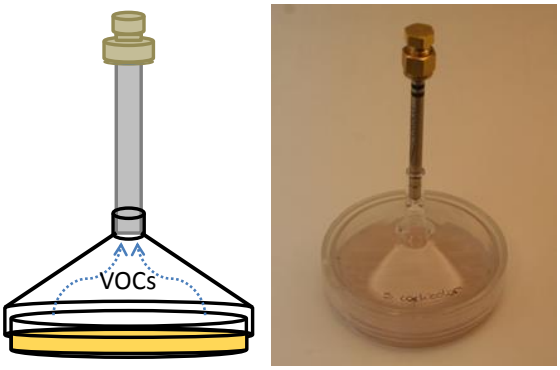


Figure 2. Left: Illustration of the system for the collection of VOCs. Right: Picture of the real glass petri connected to a Tenax® column and inoculated with *S. coelicolor*.

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 VOCs were detected by the MS operating at 70 eV in EI mode. Mass-spectra's 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's 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 2012, Xia et al 2015)).

AntiSMASH

Antibiotic gene clusters were found by analysing the whole genome sequence using the free web tool antiSMASH 3.0 (http://antismash.secondarymetabolites.org; (Weber et al 2015).

Gene disruption of terpene synthases in *Streptomyces* sp. MBT11

A 1.47 kbp DNA fragment upstream of the gene encoding a terpene synthase (*cyc*) containing the -1421 to +52 and a 1.42 kbp DNA fragment downstream of the *cyc* gene containing a flanking region from +1014 to +2442 were amplified by PCR. These fragments were cloned by means of EcoRI/XbaI and XbaI/HindIII respectively into pSET151, a vector suitable for DNA transfer from *E. coli* to *Streptomyces* by conjugation (Bierman et al 1992). The engineered XbaI site was used to insert the apramycin resistance cassette *aac(3)IV* flanked by loxP sites. LoxP recognition sites allow the removal of the apramycin resistance cassette after introducing the plasmid puWLCre expressing the Cre recombinase (Fedoryshyn et al 2008, Khodakaramian et al 2006). The same method was used to delete the gene encoding the 2-methylisoborneol synthase (*mibS*) and the arginine deiminase + transporter (*arcAD*). The double and triple knock-out mutants were made sequentially using as background the Δ*cyc* and Δ*cyc*Δ*mibS mutants* respectively. Plasmids and constructs used are detailed in Table 2 and primers in Table 3.

Table 2. Plasmids and constructs used in this study

Plasmid	Description	Reference
pSET151	*E. coli/Streptomyces* Conjugable non-replicating vector	Bierman *et al*., 1992
pWHM3	*E. coli/Streptomyces* shuttle vector, multi-copy and very unstable in *Streptomyces*	Vara *et al*., 1989
pMAG1	pSET151 containing flanking regions -1478/+12 upstream and +1389/+2785 downstream of the 2-MIB terpene cyclase gene with apra-lox inserted in between	This work
pMAG2	pSET151 containing flanking regions -1421/+52 upstream and +1014/+2442 downstream of the cyc2_3 terpene cyclase gene with apra-lox inserted in between	This work
pMAG3	pSET151 containing flanking regions -1417/+78 upstream and +2636/+4059 downstream of the arginine deiminase arcA and arginine/ornithine antiporter arcD gene apra-lox inserted in between	This work

Table 3. Oligonucleotides used in this study

Name	5'-3' sequence
2-MIB-LF+2785-EcoRI	GTCA**GAATTC**GGTGCCCGAGTCGAACCAGG
2-MIB-LR+1389-XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**TACAGC CTGCCCGATTTCTGG
2-MIB-RF+12-XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**GGGTTC GGGCATCCGTGACTC
2-MIB-RR-1478-HindIII	GTCA**AAGCTT**AAGCAGTCCAGCCTCAGTACC
CD5cyc2_3-LF-1421-EcoRI	GTCA**GAATTC**TGATCATGCCGATCACCCTGG
CD5cyc2_3-LR+52-XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**GCAGTG GATAGGGCATCCACA
CD5cyc2_3-RF+1014-XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**GACCTC TCCGTACCGGAGCAG
CD5cyc2_3RR+2442-HindIII	GTCA**AAGCTT**ATGGGCCTCTACGAGGAACTGC
arcA_2+arcD-LF-1417-EcoRI	GTCA**GAATTC**CTGGATCCGCTGCTTGAAGTC
arcA_2+arcD-LR+78-XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**CAGGG TGACGAGCGTCAGTTTG
arcA_2+arcD-RF+2636-XbaI	GTCAGAAGTTATCCATCACC**TCTAGA**TTCACG TACGACCGCAACACC
arcA_2+arcD-RR+4059-HindIII	GTCA**AAGCTT**CGACGAACGCACACGAGAAAG

Antibiotic assays

To assess the effect of VCs produced by Streptomyces strains on the activity of common antibiotics, two division Petri-dishes were used. Streptomycetes were inoculated on the right compartment using SFM media and grown for 4 days allowing the VCs to accumulate on the LB side of the plate. *E. coli* and *B. subtilis* were grown up to an O.D of 0.5 and 100 μL of this culture were streaked into the LB. A whatman disk filter was placed on top and 10 μL of the antibiotic were spotted on it. Plates were incubated overnight at 37 °C. Halos sizes were scored the next day.

pH change, ammonia determination and toxicity.

pH change in LB agar media was determined by the addition of phenol red indicator (0.002%) into the agar. Pictures were taken after 0, 3 and 5 days of incubation under the presence of the *Streptomyces* growth. For NH_3 test, *Streptomyces* were grown for 5 days in SFM using the two-compartment petri-dish, the other half of the plate was left empty. After 5 days, a Quantofix® ammonium test trip was put into the empty compartment and 10 µL of water were spotted onto the test strip. After 2 minutes incubation, 10 µL of the Quantofix® NH_4^+-1 solution (NaOH solution) were spotted on the test stripe to develop the reaction. Pictures were recorded to obtain a qualitative measurement of ammonia production from each strain.

Quantification of ammonia accumulation inside the LB agar was determined by extracting the liquid from the LB agar by centrifugation. For this, centrifuge tube filters were used (spin-X® 0.22 um cellulose acetate, Corning Inc. USA), 1 cm^2 of agar was put inside the filter tube and centrifuge at 13,000 rpm for 20 minutes. The eluate (~200 µL) was used to quantify the ammonium concentration in comparison to a standard curve. The standard curve was made with LB agar containing 0-50mM concentrations of ammonia. Ammonia solution (25% in H_2O, J.T. Baker 6051) was used as source of ammonia. The liquid was extracted from the agar the same way as described before and used together with the Quantofix® ammonium kit to obtain a semi-quantitative measure of ammonia accumulation inside the agar.

To determine the toxicity of ammonia, *E. coli* and *B. subtilis* were incubated in the automated Bioscreen C (Lab systems Helsinki, Finland) in the presence of increasing concentrations of ammonia. Each dilution was prepared in LB containing an inoculum of 10^5 cfu/mL + different volumes of ammonia solution (J.T. Baker) to give the following final concentrations: 1, 5,10,15,16,17,18,20, 25, 30, 40 and 50 mM. The final working volume in each well of the honeycomb was 100µL. Cultures were incubated at 37°C overnight with continuous shaking. O.D. measurements (wideband) were taken every 30 minutes for 20 hours. The data and growth curves were calculated from triplicates.

HCN determination.

To detect hydrogen cyanide in the headspace of *Streptomyces* growth we used a method adapted from Castric and Castric (Castric and Castric 1983). For this, whatman paper was soaked in suspension containing 5 mg ml^{-1} of copper(II) ethyl acetoacetate (Sigma-Aldrich, USA) and 4,4'-methylenebis-(N,N-dimethylaniline) (Sigma-Aldrich) dissolved in chloroform and allowed to dry protected from light. The filter paper was placed next to *Streptomyces* pre-grown for 2 days. *Pseudomonas donghuensis* P482 was used as positive control. Strains were incubated at 30°C until a blue coloration in the filter paper appeared.

RESULTS

Screening for strains with volatile antibiotic activity

Streptomycetes release volatile compounds (VCs) that inhibit the growth of filamentous fungi (Wang et al 2013a, Weisskopf 2013). To establish if also antibacterial VCs are produced, streptomycetes were grown on one half of an agar plate that was physically separated by a polystyrene barrier from the other half, where indicator strains were grown. This allows passage of air-borne VCs, but not of soluble antibiotics. As indicator strains we used *Bacillus subtilis* and *Escherichia coli* strain AS19-RlmA$^-$ (referred to as *E. coli* ASD19 from now on) (Avalos et al 2018a). The latter has known antibiotic sensitivity (Liu and Douthwaite 2002a). A collection of actinomycetes from the Qinling mountains, the Himalaya Mountains (Zhu et al 2014) and soil from the Netherlands was tested for the production of volatile organic compounds with antibiotic activity. Interestingly, *E. coli* ASD19 failed to grow adjacent to *Streptomyces* sp. MBT11 or *S. venezuelae*, while it grew normally next to *S. coelicolor*, *S. lividans* and *S. griseus* (Figure 3A). From 180 actinobacteria tested from our collection, seven strains produced VCs that inhibited growth of *B. subtilis* and 16 that killed *E. coli*. Surprisingly, strains with activity against *B. subtilis* did not kill *E. coli* and *vice versa*, indicating that the activity

could be due to a diversity of molecules that specifically target Gram-positive or Gram-negative bacteria (Figure 3B).

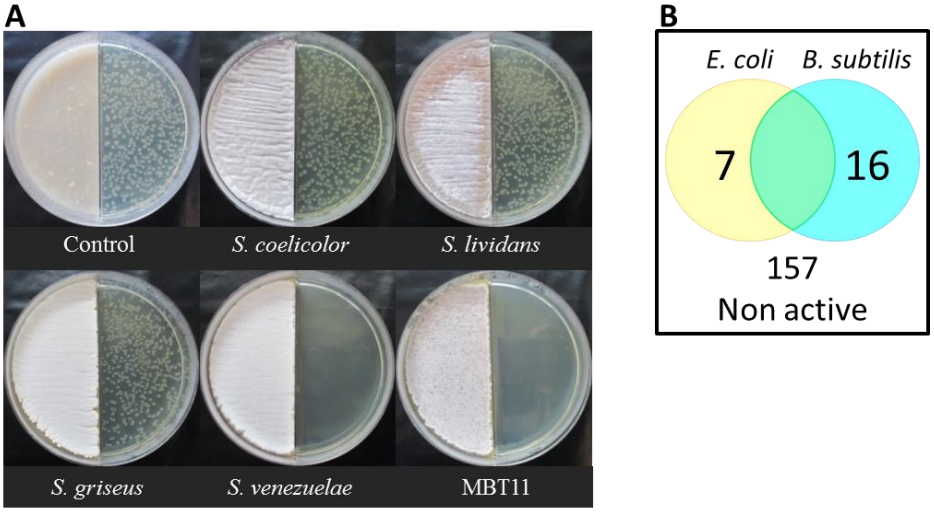


Figure 3. A. Bioactivity of VCs released by selected *Streptomyces* strains against *E. coli* strain ASD19. **B.** Venn Diagram showing target-strain specificity of the bioactivity from *Streptomyces* VCs.

Streptomyces sp. MBT11 showed a stronger activity, inhibiting up to 10^5 cfu/mL of *E. coli* ASD19 cells. Previous 16S rDNA sequencing and phylogenetic analysis showed it is close related to *Streptomyces venezuelae* (Zhu et al 2014). For this reason, these two strains were selected to perform a whole VC analysis and comparison against VCs from *S. coelicolor*. The VC profile from MBT11 and *S. venezuelae* was very similar as seen in the chromatogram (Figure 4A). The compounds identified are listed in Table 4.

Table 4. List of VOCs emitted by *Streptomyces* isolates.

RT	Compound	RI exp	RI lit
1,99	sulfur dioxide	488	
2,31	Isoprene	522	520
2,45	carbon disulfide	539	549
2,59	2-pentene	556	551
2,65	ammonium acetate	559	
2,71	3-methylpentane	563	571
2,96	2-methylfuran	587	585
3,07	trifluorobenzene∅	601	
3,14	methylcyclopentane	638	638
3,55	benzene∅	657	
4,15	s-methyl ethanethioate	687	688
5,12	dimethyl disulfide	734	739
5,68	1H-Inden-4-ol	760	
5,98	methyl 2-methylbutanoate	767	767
9,08	methyl 2-methylpentanoate*	853	853
11,71	alpha-pinene	923	927
11,73	alpha-thujene	924	933
12,95	benzaldehyde	953	959
13,22	dimethyl trisulfide	960	968
13,63	Sabinene	971	978
14,05	2-methyl-2-bornene*	983	981
14,16	2,2,4,4-tetramethylpentane	989	
14,89	3-carene*	1007	1011
15,47	2-methylenebornane*	1017	1017
15,9	Limonene	1026	1028
22,64	2-methylisoborneol*	1186	1180
24,22	tp-like*	1231	-
24,38	tp-like*	1235	-
24,98	tp-like	1251	-
25,13	tp-like	1255	-
30,66	geosmin	1415	1403
31,18	beta-copaene	1430	1433
31,99	7-Isopropenyl-1-methyl-4-methylenedecahydroazulene	1457	

32,82	germacrene-D	1482	1488
33,64	hexathiane	1510	1499
34,03	calamene	1524	1522

Putative identified compounds according to NIST/NIOO library. RT indicates the retention time from each compound; RI exp refers to the experimental retention index. RI lit: Retention indices found in literature. * indicates compounds different between *S. coelicolor, Streptomyces* sp. MBT11 and *S. venezuelae*. tp: terpene. ∅compounds are most likely contaminants however they were not found in the control (uninoculated media).

In order to find the compounds that were produced in a significantly higher amount in the bioactive strains, a metabolomics analysis was performed using MetaboAnalyst 3.0 (Xia et al 2015). The PLS-DA plot shows a clear separation between strains (Figure 4B), the major differences between *Streptomyces* sp. MBT11 and *S. coelicolor* were terpenes, more specifically, 2-methylisoborneol and the related terpenes 2-methylenebornane and 2-methyl-2-bornene. Most streptomycetes can produce 2-methylisoborneol, however, the metabolomic analysis showed that these terpenes were produced in a significantly different amount in *Streptomyces* sp. MBT11 and *S. venezuelae* compared to *S. coelicolor* (Figure 4C, D).

To identify candidate biosynthetic genes responsible for terpene production in *Streptomyces* sp. MBT11 and *S. venezuelae*, the antiSMASH algorithm was used (Weber et al 2015). Table 5 shows a high similarity between *Streptomyces* sp. MBT11 and *S. venezuelae*, from the 31 putative biosynthetic gene clusters (BGCs) identified in *Streptomyces* sp. MBT11, 16 are present in *S. venezuelae.* From the six BGCs containing terpene synthases/cyclases, identified in *Streptomyces* sp. MBT11, five were shared with *S. venezuelae* (Table 6), from which only three could possibly be responsible for the production of volatile terpenes.

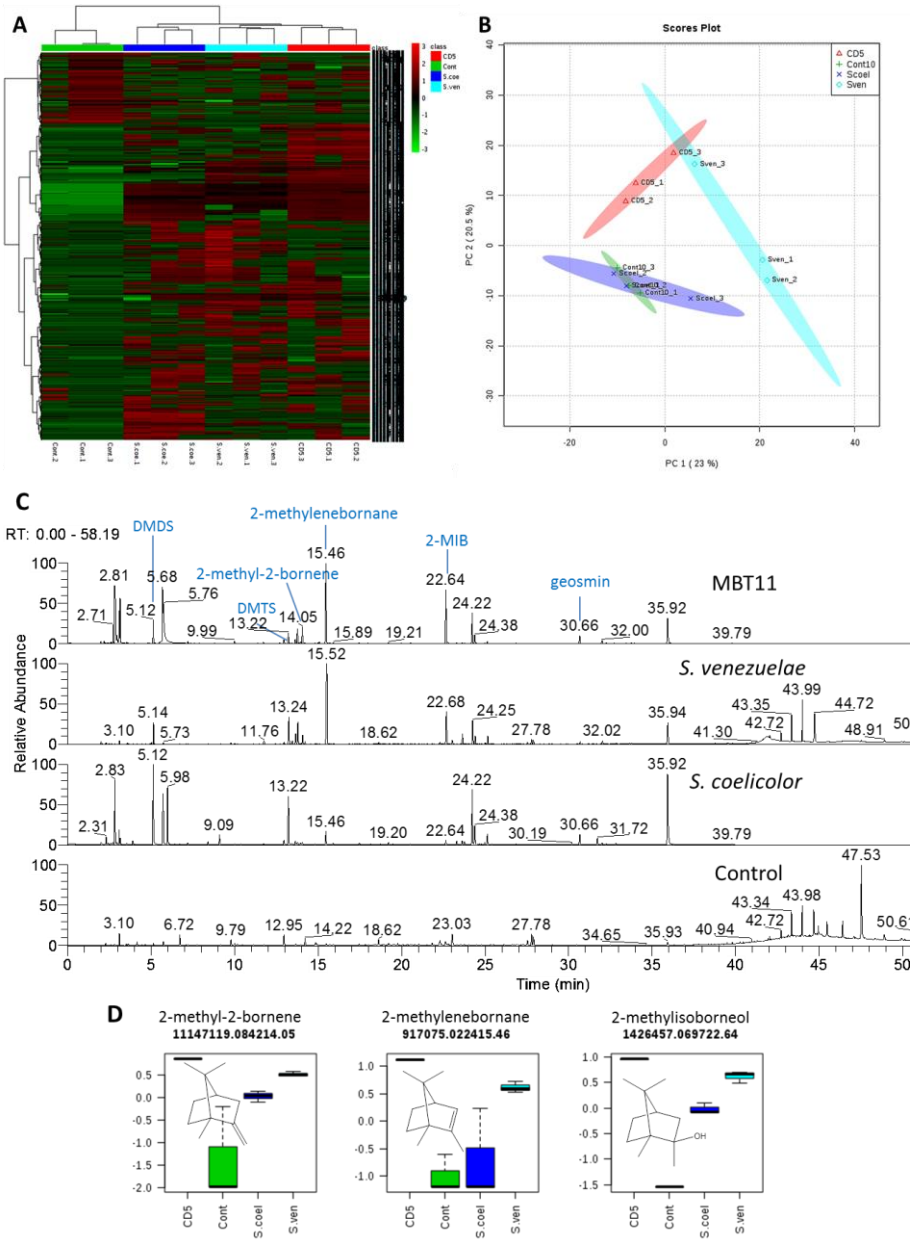


Figure 4. A. Heatmap and clustering, **B.** PCA-2D plot of the VCs produced by *Streptomyces* sp. MBT11 (red), *S. venezuelae* (turquoise), *S. coelicolor* (purple) and SFM media as control (green). **C.** GC-chromatogram of VCs from bioactive strains compared to non -bioactive and SFM media control. **D.** Plots showing 2-MIB and related compounds as statistically differently produced by *Streptomyces* sp. MBT11 compared *to S. coelicolor.*

Table 5. Secondary metabolites analysis of *Streptomyces* sp. MBT11 using antiSMASH.

Cluster	Type	Putative compound	% identity
1	Lantipeptide	SAL-2242	100
2	Bacteriocin	-	-
3	Terpene	Hopene	69
4	T2pKs	Alnumycin	62
5	T1pks-Nrps	Lipomycin	27
6	Butyrolactone	-	-
7	Bacteriocin	-	-
8	Nrps	Thiolutin	36
9	Nrps	Friulimicin	24
10	Siderophore	-	-
11	Siderophore	-	-
12	Thiopeptide	-	-
13	Butyrolactone	-	-
14	Melanin	-	-
15	Linaridin	Cypemycin	100
16	Other	Lankamycin	16
17	T1pks	-	-
18	Terpene	phytoene	-
19	Ectoine	Ectoine	100
20	Terpene	geosmin	100
21	Lantipeptide-terpene	-	-
22	Other	-	-
23	Siderophore	Desferroxamine B	100
24	Terpene	Isorenieratene	100
25	Lantipeptide	Venezuelin	100
26	Nrps	-	-
27	Nrps	Stenothricin	18
28	T2-pks-T1-pks-Nrps	Spore pigment	83
29	Thiopeptide-Terpene	2-methylisoborneol	100
30	Nrps	Scabichelin	70
31	Melanin	Melanin	28

Table 6. Putative Terpene cyclases/synthases identified from the genome of *Streptomyces* sp. MBT11.

Cluster	Function	*S. venezuelae* homologue	Volatile detected
Cluster 3	Squalene-hopene cyclase / phytoene synthase	SVEN6451	Non-volatile
Cluster 18	Phytoene synthase	SVEN7424	Non-volatile
Cluster 20	Geosmin synthase	SVEN0269	Yes
Cluster 21	Terpene cyclase	SVEN0552	N/I
Cluster 24	Lycopene cyclase / phytoene synthase	-	Non-volatile
Cluster 29	2-MIB synthase	SVEN7112	Yes ++

++ indicates high amount. N/I means not identified.

To study the role of the main volatile terpenes released by *Streptomyces* sp. MBT11, the gene encoding for the 2-methyisoborneol terpene cyclase (*mibS*) was deleted and the strain tested for its antimicrobial volatile activity with the two-division petri dish, however the strain remained active indicating that 2-methylisoborneol was not responsible for the antibiotic activity. From the list of detected VOCs (Table 4) a few terpene-like compounds could not be identified with the NIST library and our in-house NIOO-KNAW library. The genome of *Streptomyces* sp. MBT11 encodes an unknown terpene cyclase (*cyc*) most likely responsible for the production of the terpene-like molecules. The gene encoding for the unknown terpene cyclase (*cyc*) was deleted and the strain tested for its antibiotic activity. Unfortunately, all the terpene cyclase mutants including a double mutant (Δ*cyc*Δ*mibS*) conserved their antibiotic activity (Figure 5). Pure 2-methylisoborneol was also tested, but this compound failed to inhibit growth of ASD19 (Figure 5 far right). These results suggest that a different molecule other than terpenes or a combination of molecules is responsible for the antibiotic activity.

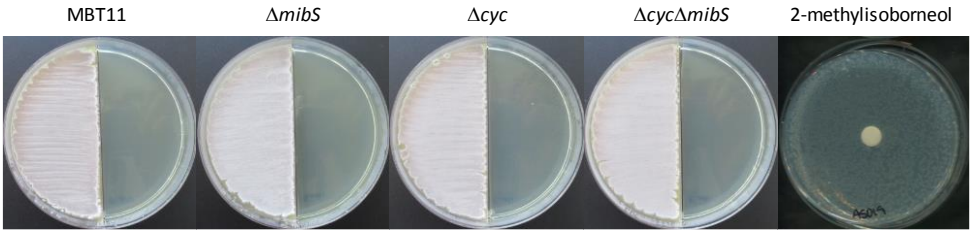


Figure 5. Bioactivity of *Streptomyces* sp. MBT11 and the mutants impaired in terpene production against *E. coli* ASD19. Far right: bioactivity of pure terpene 2-methylisoborneol.

The growth inhibition of *E. coli* cells indicates the presence of an antibacterial substance. To establish whether this compound was indeed volatile, the same experimental setup described before was used and the side with the *Streptomyces* growth was removed before inoculating *E. coli* on the other side. The antibacterial effect was observed regardless the presence or absence of *Streptomyces* growth (data not shown), indicating that the compounds released by *Streptomyces* strains were accumulated inside the agar on the other side of the plastic division.

We then wanted to assess whether the production of antimicrobial volatile compounds (AMVCs) could be elicited by varying the growth conditions. We previously showed that growth at pH 10 or on *N*-acetylglucosamine, starch or yeast extract pleiotropically enhanced the production of antibiotics in many *Streptomyces* species (Zhu et al 2014). Interestingly, in contrast to a neutral pH, when *S. griseus* was grown at pH 10 by addition of a glycine/NaOH buffer (25mM glycine; (Mohan 2006)), it produced VCs that completely inhibited growth of *E. coli* and *B. subtilis* (Figure 6). Volatile antibiotic production by *Streptomyces* species MBT11 was also enhanced by growth at pH 10. In contrast, *S. coelicolor* failed to produce AMVCs under any of the conditions tested (Table 7).

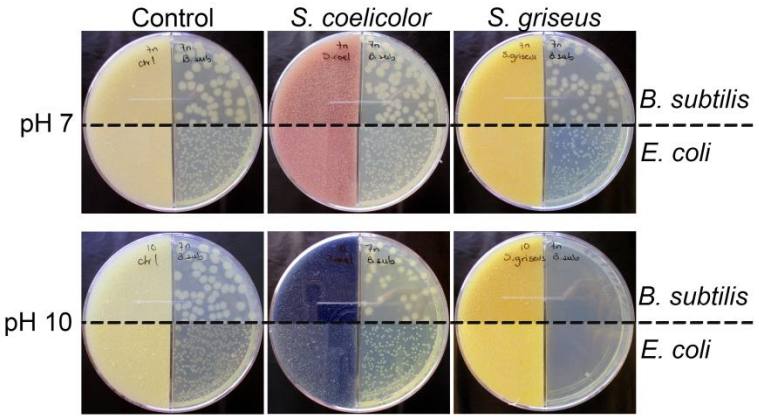


Figure 6. Volatile antibiotic activity of different *Streptomyces* strains grown at pH 7 and pH 10 (using a glycine buffer) against *E. coli* strain ASD19.

Table 7. Volatile antimicrobial activity from *Streptomyces* under different growth conditions.

	0.8% peptone		**1% starch**		**MM**		**pH10**	
	E. coli	*B.sub*	*E. coli*	*B.sub*	*E. coli*	*B.sub*	*E. coli*	*B. sub*
S. coelicolor	+	+	+	+	+	+	+	+
S. lividans	+	-	+	+	+	+	+	+
MBT11	-	-/+	-	+	+	+	-	+/-
S. venezuelae	-	-/+	+	+	+	+	-	+/-
S. griseus	-/+	-	+	+	+	+	-	-

growth (+) or inhibition (-) of test microorganism. (+/-) means poor growth as very small colonies.

The induction of AMVCs by *S. griseus* when grown at pH 10 offered an ideal system to elucidate the nature of the bioactive molecules by statistical methods. For this, GC-MS-based metabolomics was performed to compare the VC profiles of *S. coelicolor*, *Streptomyces* sp. MBT11, *S. venezuelae* and *S. griseus* grown at pH 7 and pH 10. However, no volatile organic compounds (VOCs) correlated statistically to the bioactivity, nor did we see any significant difference between the metabolome profiles of *S. griseus* grown at pH 7 and pH 10 (Figure 7).

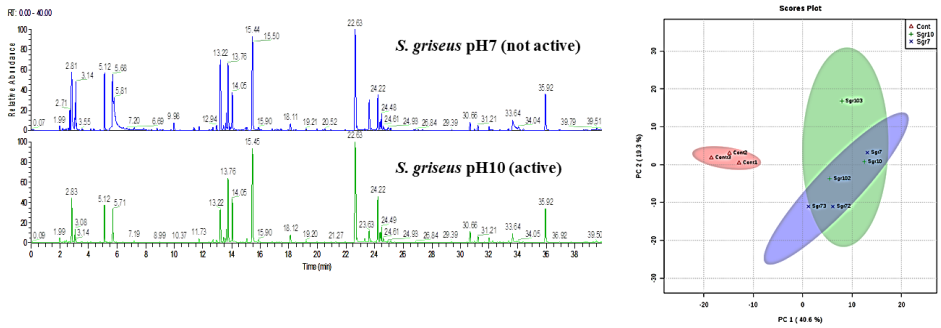


Figure 7. *S. griseus* chromatograms and metabolomic analysis at pH7 and pH10. No difference is seen; however, terpene compounds are the main VCs in both conditions.

Bioactive VCs increase the pH of the surrounding media.

In order to find the active compounds, we needed to understand their nature, therefore we aimed to examine changes in the receiving media. There are evidences that VCs can induce a pH change (Chitarra et al 2005, Jones et al 2017, Letoffe et al 2014). To determine if the pH had changed due to accumulation of the VCs, we used bromophenol blue and phenol red as indicators. The indicator changed from pale orange to a bright pink when alkalization of the surrounding media took place. Interestingly, a gradual change in the pH was seen, as shown in figure 8A; after 3 days, only the area close to the *Streptomyces* growth exhibited alkalization, but after 5 days, the whole agar section had turned pink. This result also confirmed the accumulation of volatile compounds inside the agar. Such accumulation of compounds was also seen when an antibacterial VCs production curve was made. After 3 days *E. coli* was only inhibited in the area close to the *Streptomyces* growth, but after 5 days, the growth of *E. coli* was fully inhibited (Figure 8B). *S. coelicolor* growth did not show either alkalization or acidification. The pH increase of the LB media was measured using indicator strips. The color change of the strips indicated an increment to a pH around 8.5. However, the pH itself was not the cause of the inhibition, since the *E. coli* cells grew apparently normal on media adjusted to pH 9 (Figure 8C). Also, we previously showed that even

at pH 10 growth is normal, and antibiotic susceptibility is similar to growth at pH 7.

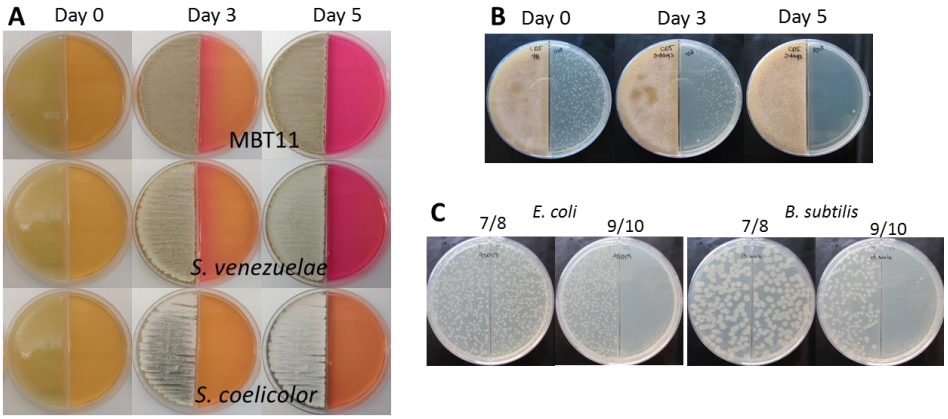


Figure 8. A. pH change illustrated by the color change of the indicator (Phenol red 0.002%). LB medium alkalization after 3 and 5 days of growth of *Streptromyces* sp. MBT11 and *S. venezuelae,* no alkalization was seen from *S. coelicolor* volatiles. **B.** *Streptomyces* sp. MBT11 Antimicrobial VCs production curve against *E. coli* strain ASD19. **C.** *E. coli* strain ASD19 and *B. subtilis* growth under different pH.

Ammonia is a key factor for the toxicity of VCs

Likely candidates for the pH increase were ammonia and trimethylamine (TMA) (Bernier et al 2011, Čepl JJ 2010, Jones et al 2017, Letoffe et al 2014). TMA production by *Streptomyces* sp. MBT11 and *S. venezuelae* was assessed and compared to a standard solution of TMA (sigma Aldrich). Under our growth conditions, TMA was not detected in any of the headspace of the *Streptomyces* strains (Supplementary figure 2). We then assessed the concentration of ammonia using the Quantofix® Ammonium detection kit from Macherey-Nagel. Interestingly, all strains that have antimicrobial activity produce a higher concentration of ammonia according to the color scale concentration provided by the kit (Figure 9A).

Ammonia toxicity was tested against *E. coli* ASD19 by growth in LB media supplemented with increasing concentrations of ammonia. Figure 9B shows that *E. coli* ASD19 is sensitive to ammonia above 15 mM as seen by the extension of the lag phase and the diminished O.D. Full inhibition of *E. coli* ASD19 growth was seen upon a concentration of 20 mM of ammonia.

To prove that these concentrations can be produced by the *Streptomyces* strains, the LB agar on the right side of the plate was extracted by centrifugation after five days of incubation under the presence of *Streptomyces* sp. MBT11 VCs. A standard curve prepared in LB agar with different concentrations of ammonia was extracted the same way. The ammonia present in the solution extracted from the agar was determined using the same Quantofix Ammonium kit. As seen in figure 9C, the strains that lack the antibiotic effect (*S. coelicolor* and *S. lividans*) produce less ammonia, around 2 mM while the growth inhibiting *S. venezuelae*, *Streptomyces* sp. MBT11, a close-related strain *Streptomyces* sp. MBT21 and *S. griseus* (the latter only with added Gly/NaOH buffer pH 10) had accumulated between 15-30 mM ammonia, proving that the concentrations tested before are biologically relevant (Figure 9C).

A different molecule found in the headspace of several bacteria including rhizospheric *Streptomyces* isolates is hydrogen cyanide (HCN) (Anwar et al 2016). This molecule also has an inhibitory effect on the growth of various organisms including fungi and bacteria (Blumer and Haas 2000, Ossowicki et al 2017, Popova et al 2014). For this reason, the *Streptomyces* strains were tested for hydrogen cyanide production by growing them next to a whatman filter paper soaked with the appropriate indicator (copper(II) ethyl acetoacetate + 4,4-methylenebis-N,N-dimethylaniline).

Pseudomonas donghuensis P482 (Ossowicki et al 2017) was used as positive control giving a dark blue coloration after 24 h incubation at 30°C. None of the *Streptomyces* strains gave a positive reaction showing that the toxicity of the VCs from

Streptomyces against *E. coli* is not due to the presence of toxic HCN. (Supplementary Figure 2).

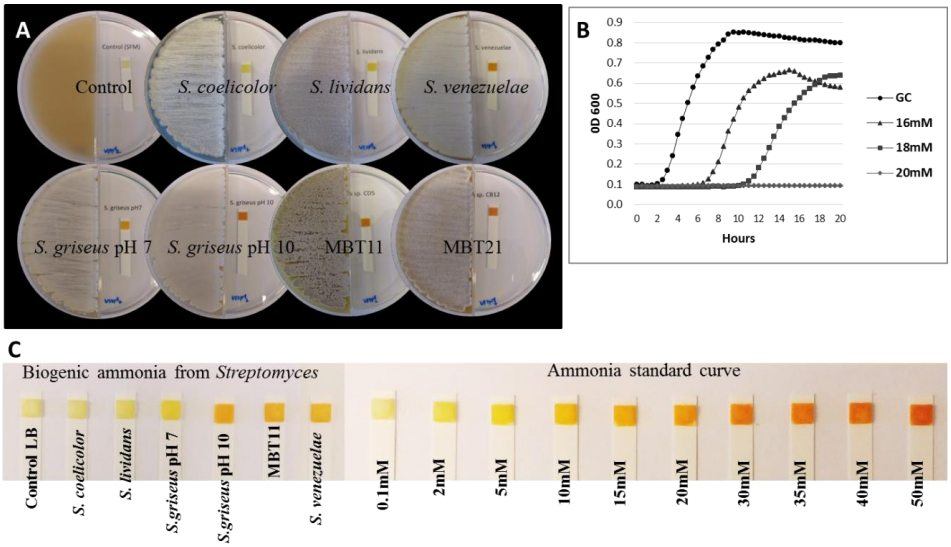


Figure 9. A. NH_3 emission. Test strips on the right compartment show the production of NH_3 by *Streptomyces* strains. *S. coelicolor* and *S. lividans* (~10mg/L); *S. griseus, S. venezuelae*, *Streptomyces* sp. MBT11 and *Streptomyces* sp. MBT21 (~100mg/L); Control: SFM media (0mg/L). Concentrations are estimated according to the color chart indicator from the Quantofix® ammonium detection Kit. **B.** Ammonia toxicity. *E. coli* strain ASD19 growth under increasing concentrations of ammonia. **C.** Ammonia quantification from LB agar extracts exposed to *Streptomyces* VCs (left). Ammonia standard curve from LB agar extract (right).

Ammonia is produced by the glycine cleavage system

We then wondered if ammonia was generated from amino acid metabolism, since the pH was set at the receiver side using a glycine/NaOH buffer. A major pathway for the catabolism of glycine is the glycine cleavage system (GCV) involving the conversion of glycine into CO_2, ammonia and a methylene group which is accepted by tetrahydrofolate (THF) to form N_5, N_{10}-methylene-THF (Kikuchi et al 2008, Tezuka and Ohnishi 2014). Indeed, when glycine alone was added to the

Streptomyces side of the plate at concentrations as low as 0.1% (w/v), *S. griseus* fully inhibited the growth of *B. subtilis* and *E.coli*, at neutral pH (Figure 10B top). We then tested the direct involvement of the GCV system (Tezuka and Ohnishi 2014), which consists of three enzymes (GcvL, GcvP, GcvT) and a carrier protein: GcvH (Figure 10A). *gcvP* and *gcvT* mutants in *S. griseus* used are deleted in the 5' UTR therefore lacking a glycine riboswitch that controls the expression of the *gcv* genes while the *gcvP* mutant in *S. coelicolor* has an apramycin resistance cassette replacing the *gcvP* gene. Since GcvT is the enzyme that generates ammonia, we tested the AMVC-producing capacity of an *S. griseus* mutant lacking the 5'UTR of *gcvT* (Tezuka and Ohnishi 2014). Excitingly, the mutant had completely lost volatile bioactivity, and this went hand in hand with strong reduction of ammonia production (Figure 10B). At high concentrations of glycine (1% w/v) also *S. coelicolor* inhibited the indicator cells due to the production of large amounts of ammonia, and this was also lost in *gcvP* mutant ((Zhang 2015); Figure 10B). This is conclusive evidence that the glycine cleavage system is the main responsible for the ammonia production in *S. griseus*. *S. venezuelae* and *Streptomyces* sp. MBT11 already produced high concentrations of ammonia in the absence of added glycine, suggesting a difference in ammonia-related metabolism. Many enzymes are responsible for the biosynthesis of ammonia in bacteria, including deaminases, deiminases, pyridoxamine phosphate oxidases and ammonia lyases. A gene for an arginine deiminase SVEN_7018 (*arcA* in MBT11) and for a putative transporter SVEN_7109 (*arcD* in MBT11) were found immediately adjacent to the genes for 2-MIB biosynthesis on the genomes of *Streptomyces* sp. MBT11 and *S. venezuelae*, and absent in the other streptomycetes analysed. However, mutational analysis (*ΔarcAD*) showed that these genes could not explain the enhanced ammonia production in *Streptomyces* sp. MBT11 (data not shown). We are currently performing a larger scale phylogenomics and mutational analysis to identify the gene(s) that are responsible for the overproduction of ammonia in these strains.

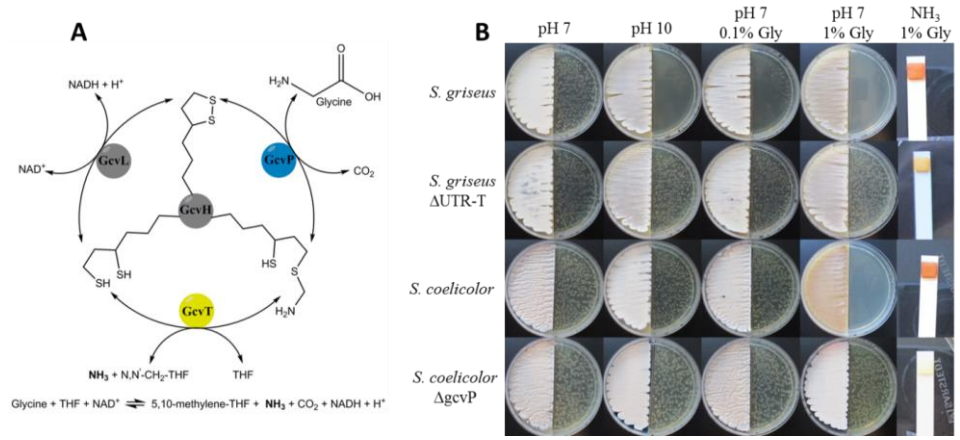


Figure 10. A. Glycine cleavage system schematic representation. **B.** Volatile activity and ammonia production from *S. griseus* WT, *S. griseus* glycine cleavage system mutant *gcvT* (ΔUTR-T), *S. coelicolor* WT and *S. coelicolor* glycine cleavage system mutant Δ*gcvP*(::*aac(3)IV*).

Ammonia released by *Streptomyces* modulates antibiotic activity further away from the colony

Since ammonia is an AMVC that can reach far from the colony, the molecule may play a role in long-distance competition with other microbes in the soil. Ammonia may enhance the effect of canonical antimicrobials produced by the strain itself, or by other bacteria such as actinobacteria, *Burkholderia*, *Bacillus* and *Myxococcus* species in their neighborhood. The latter would be an interesting new concept, namely a form of piracy whereby weapons produced by other microbes are used to its own advantage, by potentiating them via ammonia. To test this, the streptomycetes were grown on the left side for four days to allow accumulation of compounds on the receiver side containing LB. After that, *B. subtilis* and *E. coli* BREL606 (more resistant to AMVCs) were plated next to *Streptomyces* strains and a filter disk placed on the agar containing different antibiotics. Interestingly, we noticed a significant increase in the sensitivity of *B. subtilis* and *E. coli* to macrolide, aminoglycoside and β-lactam antibiotics when ammonia-producing streptomycetes were grown

adjacent to the receiver cells (Figure 11). Conversely, a decrease was observed in the susceptibility of *E. coli* and *B. subtilis* to tetracycline.

Antibiotic	*E. coli*	*B. subtilis*
Tetracycline	-	-
Ampicillin	NC	+
Erythromycin	+	+
Kanamycin	+	NA
Tylosin	NA	+
Actinomycin	NA	+
Spectinomycin	+	+
Streptomycin	NA	+

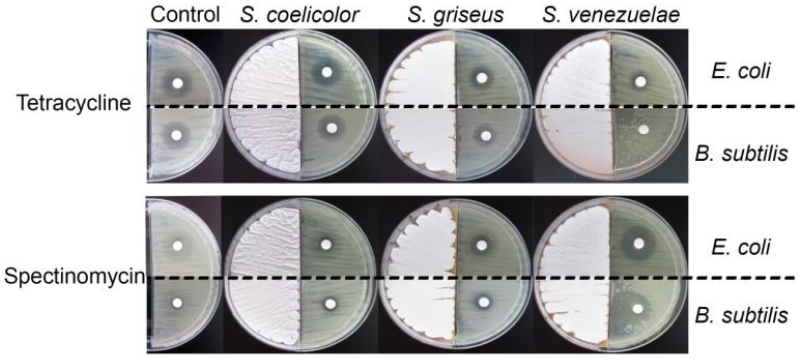


Figure 11. Table indicating the changes in antibiotic sensitivity under the presence of *Streptomyces* VCs. (-) means decrease in halo size, (+) means increase in the halo size, NC: no changes and NA: not active. Pictures are an example of the changes in halo size.

Streptomycetes are ubiquitous microorganisms present in almost every environment, in highly diverse communities. For this reason, it is important to know how they interact between each other. In soil they can operate as antibiotic producers, however there is little information about its ecological role or how they interact with other bacteria from the same genus with similar antibiotic production abilities. As an initial approach, several actinomycetes from the MBT strain collection were tested to analyze their response to VOCs produced by *Streptomyces* sp. MBT11.

Figure 12A shows some actinomycetes with a diminished antibiotic production in as well as a delayed development seen by a reduced amount of sporulation. The opposite is observed in Figure 12B where secondary metabolite production seems enhanced by means of an increased zone of inhibition or increase in pigment production as well as sporulation. Strain MBT11 produces a high concentration of ammonia which leads to an increase of the pH of the surrounding media. In most cases, the effect of the VCs produced by MBT11 on the growth of other actinomycetes was alleviated when the media was buffered with TES 50 mM. This suggests that the effect is indeed predominantly caused by the production of high levels of ammonia by MBT11.

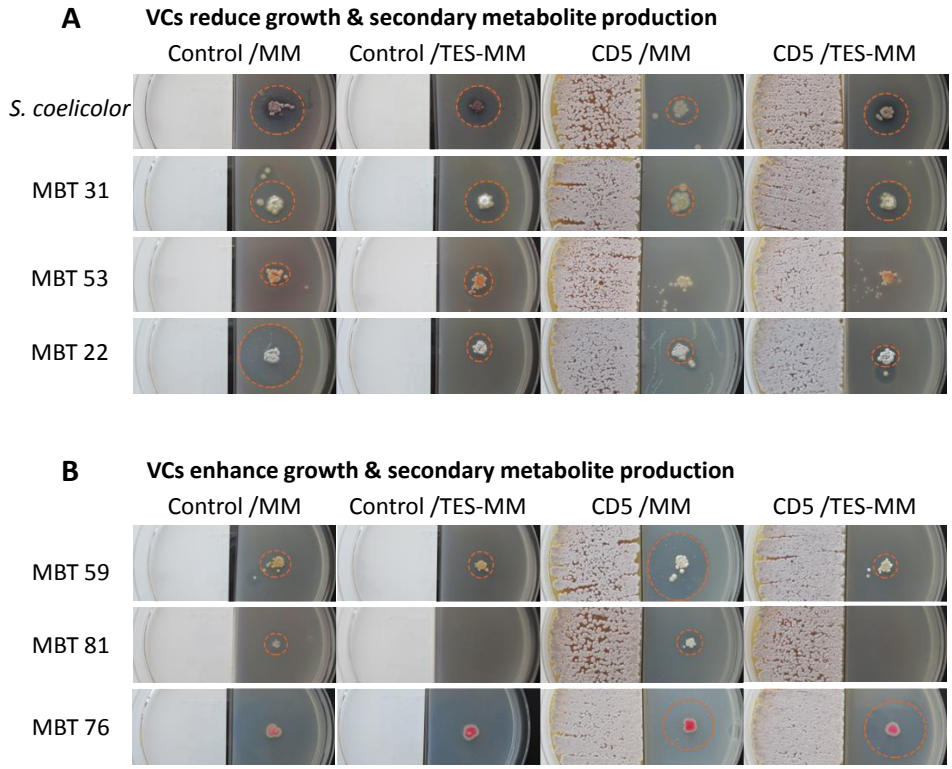


Figure 12. VCs from *Streptomyces* sp. MBT11 modify the development and secondary metabolite production of neighboring streptomycetes.

DISCUSSION

Streptomyces are soil bacteria recognized for their capability to produce antimicrobial compounds. Volatile compounds (either organic or inorganic) are a gaseous type of secondary metabolites that are also produced extensively by these bacteria. The potential of VCs as antimicrobial substances is starting to gain attention but still, very little is known about these molecules.

Our initial screening showed that the VCs bioactivity is strain specific, this trade has been reported before in bacteria (Groenhagen et al 2014, Kanchiswamy et al 2015, Ryu et al 2003). Species specific VCs have also been found in fungi which can be useful for the identification of exposure to dangerous fungal metabolites or spores indoor or in the workplace (Fischer et al 1999). The fact that bacteria can produce specific compounds points out to a specific role of such compounds. Our results show a complete separation between strains and volatile bioactivity. None of the strains tested had activity against both *B. subtilis* and *E. coli* suggesting a target-specific bioactivity. Strain-specific activity has been reported from the genus *Serratia* which produces sodorifen in response to the presence of *Fusarium culmorum* (Schmidt et al 2017).

Terpenes are the largest class of compounds known so far (Degenhardt et al 2009) nevertheless, many novel compounds belonging to this class are identified continuously. An example of this is observed in both *S. venezuelae* and MBT11. Both genomes encode a still unknown terpene synthase and their headspace is dominated by terpenes from which several could not be identified highlighting the lack of information on the role of the terpene and other volatile compounds in the biology of *Streptomyces* as well as other bacteria.

In this work, we show that some *Streptomyces* strains can inhibit the growth of *E. coli* from a distance by releasing high concentrations of the basic small volatile ammonia. The basic molecule is a side product of the amino acid metabolism, spiked when these molecules are present as a nutrient source. *S. venezuelae* and *Streptomyces* sp. MBT11 have several genes that encode for ammonia producing enzymes such as deaminases,

deiminases, pyridoxamine phosphate oxidases and ammonia lyases among others. An accumulated effect of the activity of the different ammonia producing enzymes could also lead to an increase production of ammonia. In *S. griseus* the increased ammonia production comes from the breakdown of glycine. Glycine is known to affect the development of the bacterial growth by interfering with the cell wall biosynthesis (Hammes et al 1973). The glycine cleavage system is responsible for the glycine detoxification in *Streptomyces* (Tezuka and Ohnishi 2014). The fact that ammonia is a release product from the amino acid metabolism is already known, however to the best of our knowledge this is the first report with scope to the high production and accumulation of the small low-cost volatile ammonia exerting a beneficial side effect. Glycine is the simplest material for protein synthesis and despite its toxicity is a material abundant in soil from plants exudates where streptomycetes are abundant (Cordovez et al 2015, Lesuffleur et al 2007, Phillips et al 2004).

Streptomyces VCs have a perceivable impact on the pH of their surroundings. Research has shown that richness and diversity of bacterial soil microbiomes is largely explained by the soil pH (Fierer and Jackson 2006) with acidic soils having the lowest diversity. Basic environments favor bacterial growth while acidic environments do it for fungi (Bárcenas-Moreno et al 2011, Rousk et al 2009). Studies have shown that VCs are more strongly adsorbed in alkaline soils, especially those containing a high organic carbon content (2.9%) (Serrano and Gallego 2006). The release of ammonia could help the solubility and diffusion of other types of secondary metabolites, while sensitizing competing bacteria. High concentrations of ammonia are achieved in densely populated environments, like the human intestine where ammonia concentrations range from 12 to 30 mM (Hughes et al 2000). It is also known that the urinary pH affects the effectivity of fluoroquinolones, aminoglycosides, and macrolides functioning optimally at alkaline pH (Yang et al 2014). Our data show that ammonia inhibits the growth of competitors and enhances the effectivity of certain antibiotics present at a distance. Nevertheless, in our work we see a strain specific response to the VOCs from another streptomycete as some of them seem to have an enhanced antibiotic production/effect while the opposite is true for other strains together with a reduced growth or development. We hypothesize that the

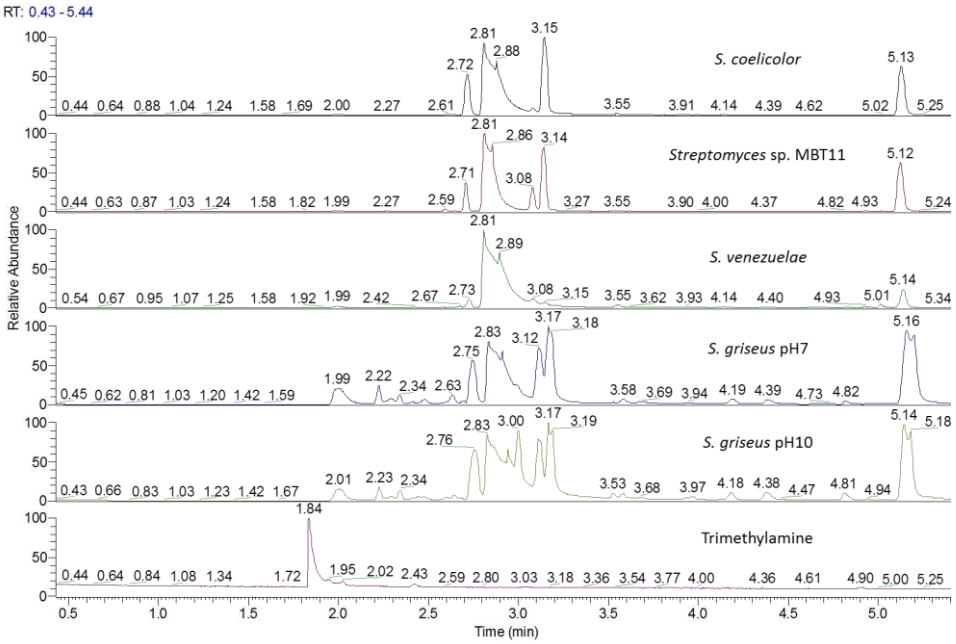
production of a small molecule such as ammonia is a strategy to enhance the activity of antibiotics that require many high-energy precursors (ATP, NADPH, acetyl-CoA etc.) for their synthesis and are therefore costlier to produce, such as polyketides, non-ribosomal peptides or β-lactams. This is applicable both to antibiotics produced by the organism itself, and to those produced by bacteria further away from the colony. The validity of the concept of "antibiotic piracy" requires further experimental testing. To reinforce the idea, ammonia released by *Streptomyces aburaviensis* has also shown to trigger droplet formation in different *Streptomyces* strains. Droplets are reservoir of nutrients, enzymes and secondary metabolites (Schmidt and Spiteller 2017).

In conclusion, our work offers an indication that several streptomycetes use ammonia as airborne weapon to change their surrounding environment, thereby making their own defense mechanism more effective.

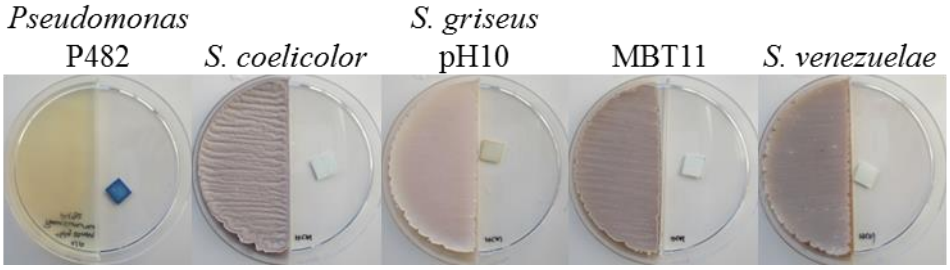
Acknowledgements

We thank Hans Zweer for technical help with GC/Q-TOF analysis and Lisanne Storm for the help with the volatile antimicrobial screening, Yasuo Ohnishi and Le Zhang for sharing the glycine cleavage system mutants from *S. griseus* and *S. coelicolor* respectively, and Stephen Douthwaite for providing *E. coli* AS19-RlmA^{-}.

SUPPLEMENTARY INFORMATION



Supplementary Figure 1. GC-chromatogram showing the absence of TMA in the headspace of *S. coelicolor* (black), *Streptomyces* sp. MBT11 (red), *S. venezuelae* (green), *S. griseus* grown at pH 7 (blue), *S. griseus* grown at pH 10 (yellow green). Chromatogram of a TMA standard (RT 1.8 min.) is shown below (purple).



Supplementary Figure 2. HCN determination from different *Streptomyces* strains. *Pseudomonas donghuensis* P482 was used as positive control. Blue coloration is developed from the oxidation product from HCN + copper (II) ethyl acetoacetate and 4,4'-methylenebis- (N, N-dimethylaniline). none of the *Streptomyces* strains gave a positive reaction showing that the toxicity of the VCs from *Streptomyces* against *E. coli* is not due to the presence of toxic HCN.