Volatile compounds from Actinobacteria as mediators of microbial interactions

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op
Dinsdag 24 September 2019
klokke 15:00 uur

door

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geboren te Cuautla Morelos, Mexico
26 mei, 1986

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This work was supported by grant No. 313599 from CONACyT to Mariana Avalos Garcia.

Tell me and I will listen,
Teach me and I will remember,
Involve me, and I will learn.

From a collection of Chinese writings attributed to Xungzi.

Contents

CHAPTER 1

General introduction and thesis outline

Smell is the most chemically focused of our senses. It is the result of small molecules traveling through the air, passing up through the nasal cavity and reaching the olfactory epithelium where they come into contact with the odour receptors. These molecules are volatile compounds (VCs). It is easy for us to smell them because the compounds have a low vapour pressure and a low molecular weight which means that they can readily evaporate and come into the air we breathe. VCs have diverse origins. They are released from plants, microorganisms, food and even organic and inorganic materials such as compost and plastics. Examples of VCs are the perfume of flowers like the molecule geraniol responsible for the smell of roses. Dimethyl trisulfide is that pungent odour we smell when opening the wrapping of a good Limburger cheese and let's not forget geosmin, the scent that fills the air after the first drops of rain have fallen.

Many VOCs are produced by bacteria, whereby members of the genus *Streptomyces* in the family of Actinobacteria are a major source (Dickschat et al., 2007; Yang et al., 2018). Streptomycetes are soil-dwelling bacteria with a complex mycelial life cycle, that can reproduce via sporulation (Barka *et al*., 2016). Actinobacteria are prolific producers of natural products, including two-thirds of all known antibiotics, the majority of which are produced by *Streptomyces* (Kieser et al., 2000). Some examples of these antibiotics are streptomycin (Distler et al., 1987), vancomycin (Levine, 2006), the more recently discovered daptomycin (Miao, 2005), as well as the β-lactam inhibitor clavulanic acid that, in combination with antibiotics like penicillin, overcomes the antibiotic resistance generated by the secretion of beta-lactamases (Paradkar, 2013). The antibiotic-producing potential of these bacteria includes the 'soluble' secondary metabolites and the smaller volatile compounds, which have not yet been extensively explored. Despite the recent interest in VCs, only a few compounds have been found to have antibiotic activity, namely the sesquiterpenes albaflavenone and pentalenolactone produced by *Streptomyces coelicolor* and *Streptomyces avermitilis* respectively (Zhao et al., 2008; Tetzlaff et al., 2006). The physicochemical properties of volatile compounds make them ideal molecules to participate in microbial communication and interactions. For this reason, the aim of this work is to analyse the potential of volatile compounds as antibiotics as well as to obtain a deeper understanding of the function of these molecules.

Over the last decade, research on microbial volatiles and their biological activity, including potential biotechnological applications has gained attention. **Chapter 2** presents a review of the potential of microbial volatiles as antimicrobials and as modulators of antibiotic resistance. The review highlights the bioactivity of volatiles against pathogenic bacteria and describes the modes of action behind antibiotic volatiles and as modulators of antibiotic resistance.

To identify *Streptomyces* with volatile antibiotic activity, we performed a screening using our actinomycetes collection from the Himalaya and Qinling mountains as well as soil from The Netherlands (Zhu et al., 2014). In **Chapter 3** we show that *Streptomyces* produce an abundance of VCs both organic and inorganic. These VCs show antibiotic activity inhibiting the growth of *B. subtilis* and *E. coli*. The antibiotic effect was strain specific since none of the *Streptomyces* strains that were able to inhibit *E. coli* inhibited the growth of *B. subtilis* and vice versa. Surprisingly, *Streptomyces* can produce high concentrations of volatile ammonia, which has antibiotic activity. This inorganic molecule can be produced by the bacteria at low cost and diffuse over long distances, thereby accumulating in the agar, with an inhibitory effect as the result. The high concentrations of ammonia that accumulated altered the pH of the growth media and influenced the activity of common antibiotics and the behaviour of neighbouring streptomycetes. This shows the importance of VCs in air-borne interactions, not only via a direct effect but also via collateral effects such as pH change. In **Chapter 3** we also analysed the headspace from different *Streptomyces* strains and observed that it is dominated by the terpenes 2-methylisoborneol and its dehydrogenation molecules 2-methylenebornane and 2-methyl-2-bornane.

E. coli is a Gram-negative bacterium commonly found in the intestine of animals, including humans (Gorbach, 1996). Pathogenic strains can cause diarrhea, urinary tract infections, respiratory illness and pneumonia, among others (CDC, 2018). Gram-negative bacteria have an outer membrane that protects the cell by limiting the entrance of toxic substances therefore reducing the efficacy of antibiotics (Zgurskaya et al., 2015). Pathogenic *E. coli* strains can also rapidly develop resistance to

antibiotics threatening the life of infected people (Collingnon, 2009). In **Chapter 4** we analyse the response of *E. coli* to ammonia. The target bacteria respond to the high concentrations of ammonia by down-regulating the porin master regulatory two-component system OmpR-EnvZ. The concomitant reduced expression of the outer membrane porins OmpC and OmpF, limits the entrance of ammonia into the cell. In confirmation of the toxic influence of ammonia, *E. coli* also reduces its own ammonia production.

In **Chapter 5** we show that apart from 2-methylisoborneol and its derivatives, the headspace of *Streptomyces griseus* consists mostly of terpenes. 36 out of the 46 VOCs identified belong to the terpene class, including the well-known sesquiterpene geosmin. To study the importance of such molecules, we constructed several mutants lacking one or more genes responsible for the production of the volatile terpene compounds, including a quadruple mutant that was unable to produce any volatile terpenes. In this chapter, the evident and not-so evident phenotypical changes that arose in the different mutants are presented. A first approach towards understanding the biological and ecological role of such compounds in *Streptomyces* is also presented.

In **Chapter 6** we concentrated on the role of VOCs in ecological interactions. The effect of VOCs emitted by *Streptomyces* in its interaction with protists are discussed. When protists were grown in the presence of VOCs emitted by *Streptomyces,* an inhibition of the activity of protists was observed. The inhibitory effect was confirmed when the pure compounds dimethyl disulfide and 2-methylenebornane were used. Bacterial VOCs can be detected by protists and possibly used as food source as previously suggested (Schulz-Bohm et al., 2017). Here we observed a similar behaviour, indicating that some protists can use VOCs emitted by *Streptomyces* as nutrients. At the same time, VOCs released by *Streptomyces* acted as a defence mechanism against protist predators. The overall findings obtained in this work are integrated in the general discussion of **Chapter 7** with a summary of the most important observations and future perspectives.

CHAPTER 2

Healthy scents: microbial volatiles as new frontier in antibiotic research?

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Current Opinion in Microbiology 2018, 45:84–91|doi:

10.1016/j.mib.2018.02.011

ABSTRACT

Microorganisms represent a large and still resourceful pool for the discovery of novel compounds to combat antibiotic resistance in human and animal pathogens. The ability of microorganisms to produce structurally diverse volatile compounds has been known for decades, yet their biological functions and antimicrobial activities have only recently attracted attention. Various studies revealed that microbial volatiles can act as infochemicals in long-distance cross-kingdom communication as well as antimicrobials in competition and predation. Here, we review recent insights into the natural functions and modes of action of microbial volatiles and discuss their potential as a new class of antimicrobials and modulators of antibiotic resistance.

INTRODUCTION

The problem of antimicrobial resistance

The discovery and use of antibiotics to treat infectious diseases has dramatically affected human life span. Nevertheless, the increasing use of antibiotics has led to a rapid acquisition of antibiotic resistance by pathogenic microorganisms (Davies 1994, Wright 2011) as was already predicted by Alexander Fleming shortly after he discovered penicillin (Fleming 1929). The threat of the increased frequency of antibiotic resistance is further augmented by the reduced interest and efforts of the pharmaceutical industry to discover and develop novel antibiotics (Cooper and Shlaes 2011, Payne et al 2007, Wright 2015). Therefore, scientists are taking the lead in finding new strategies to identify new antibiotics to turn the tide of antibiotic-resistance (Kolter and van Wezel 2016). In particular, we need to expand the chemical space of bioactive molecules with different modes of action and for which resistance development is less likely to occur. To date, the attention of industrial screening efforts has been almost exclusively directed at canonical antibiotic classes such as polyketides (PKS), nonribosomal (NRPS) and ribosomal (RiPP) peptide antibiotics, β-lactams and aminoglycosides. However, there is a major and

highly diverse class of natural products that has been largely ignored by the pharmaceutical industry, namely the volatile compounds. Research on microbial volatiles is an emerging field with immense potential for both human, animal and plant health (Kai et al 2009, Kanchiswamy et al 2015, Luhachack and Nudler 2014, Weisskopf 2013). Here, we provide a brief and up-to-date overview of recent studies concerning the natural functions of microbial volatiles with a specific focus on volatiles that have antimicrobial activity or that act as modulators of antimicrobial resistance.

Chemical diversity and natural functions of microbial volatile compounds (MVCs)

Bacteria and fungi release a plethora of organic and inorganic volatile compounds, small molecules with low molecular weight and high vapour pressure. These physicochemical properties enable MVCs to diffuse more easily, allowing dispersal over longer distances than other microbial metabolites. A decade ago, the excellent review by Schulz and Dickschat (Schulz and Dickschat 2007) on microbial volatiles marked the rise of this emerging and exciting research field of natural product chemistry. Since then, numerous structurally diverse MVCs produced by marine and terrestrial microorganisms have been described (Piechulla et al 2017, Schulz et al 2010). MVCs belong to diverse chemical classes, including alkanes, alkenes, alcohols, esters, ketones, terpenoids, sulfur-containing compounds and a range of small inorganic compounds. Moreover, within these classes there appears to be an enormous chemical diversity of MVCs that remains to be discovered such as the terpenes sodorifen (von Reuß et al 2010) and pristinol (Klapschinski et al 2016). MVCs may be unique to a single phylogenetic group or even species, which also allows the use of MVCs for chemotaxonomic purposes (Cordovez et al 2015) and for the selective detection of pathogens in both indoor and outdoor environments (Bos et al 2013). For example, VCs produced by *M. tuberculosis* help to detect the pulmonary infection and asses the treatment (Zetola et al 2017) (Figure 1).

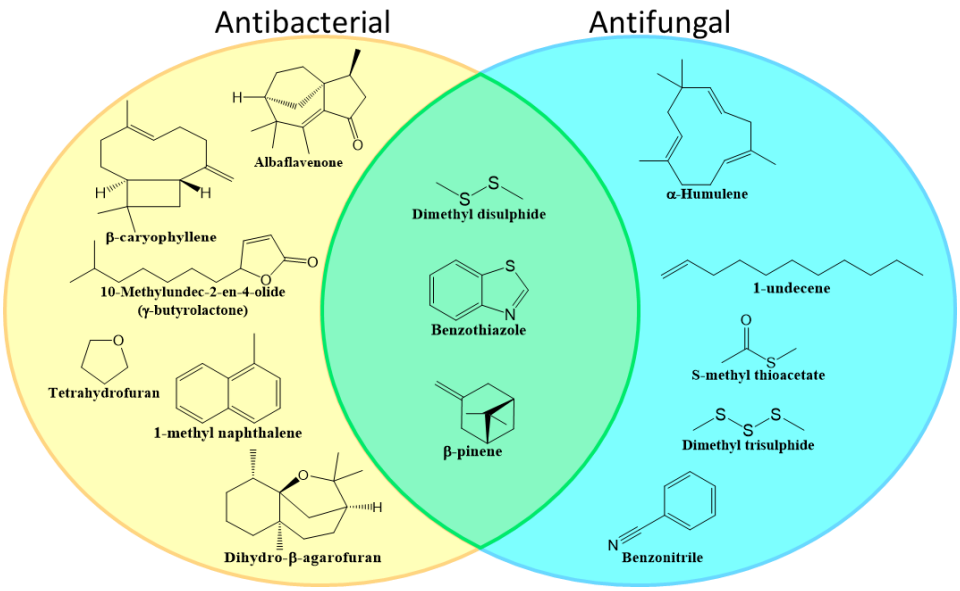

Figure 1. Examples of MVCs with antimicrobial activity

MVCs play important ecological roles in intra- and inter-kingdom interactions (Garbeva et al 2014, Schulz-Bohm et al 2017b). Activities reported for MVCs include modulation of growth, motility, virulence and biofilm formation as well as production of specialized metabolites (e.g. toxins), antibiotic resistance and spore germination in competing microorganisms (i.e. bacteria, fungi) (Chitarra et al 2005, Cugini et al 2007, Kim et al 2013, Lemfack et al 2016, Que et al 2013, Schmidt et al 2015b). For example, some *Streptomyces* species surpass obstacles by so-called 'explorer cells', whereby they colonize new areas in the face of competition induced by the biogenic volatile trimethylamine (Jones et al 2017). The skin-borne *Staphyloccoccus schleiferi* produces schleiferons A and B that modulate the skin microbiome possibly by inhibiting the growth of Gram-positive bacteria and by interfering with prodiginines production (Lemfack et al 2016). Plants also respond to and utilize MVCs leading to growth promotion or inhibition, induced systemic resistance or alteration of the plant metabolome (Kai et al 2010, Kanchiswamy et al 2015, Park et al 2015). Recent studies further pointed to other intriguing ecological roles of MVCs in cross-kingdom interactions. For example,

ammonia produced by bacteria promotes the symbiosis between a fungus and a beetle by regulating the consumption sequence of the carbon sources pinitol and glucose (Zhou et al 2017). Other studies indicated that volatiles from *Bacillus subtilis Pseudomonas fluorescens*, *Serratia odorifera*, and *Xanthomonas campestris* act as infochemicals disclosing a food source to bacterial predators, whereby the nematode *Caenorhabditis elegans* responded by crossing a 3-cm plastic barrier presumably to feed on the bacteria (Kai et al 2009). By contrast, MVCs like acetaldehyde, cyclohexene and dimethyl disulfide, were reported to reduce the motility of nematodes (Gu et al 2007). Recent studies in our labs further revealed that terpenes from *Collimonas* may act as a defense mechanism against protozoan predation (Schulz-Bohm et al 2017b). Also, the terpene geosmin produced by *Streptomyces* and other bacteria has been proposed to be multifunctional as a signaling molecule involved in sporulation of the producing strain (Schöller et al 2002) and as a deterrent in food for *Drosophila* flies (Stensmyr et al 2012). For more comprehensive overviews of other natural roles of MVCs in intra- and interspecific interactions and cross-kingdom communication, we refer to several recent reviews (Audrain et al 2015, Effmert et al 2012, Junker and Tholl 2013, Kai et al 2009, Kai et al 2016, Piechulla et al 2017, Schmidt et al 2015a).

Microbial volatile compounds as antimicrobials

MVCs can have significant inhibitory effects on the growth or development of other microorganisms (Figure 2). The activity spectrum of MVCs appears to be as diverse as their chemistry. Most of the studies to date have focused on antifungal activities of MVCs and only a few have reported their antibacterial properties. Examples include: the hormone-like γ-butyrolactones with broad spectrum activity against bacteria, fungi and yeast (Schulz et al 2010); furfuryl isovalerate that inhibits growth of Gram-positive and Gram-negative bacteria, and acts as a quorum quencher in Gram-negative bacteria (Schulz et al 2010); pyrazines (2,5-bis (1-methylethyl)-pyrazine), produced by *Paenibacillu*s in interaction with *Burkholderia* (Tyc et al 2017a), with activity against human pathogenic

bacteria like *Escherichia coli*, *Staphylooccus aureus* and the yeast *Candida albicans*. Interestingly, only few studies to date have looked into the antibacterial activities of MVCs produced by actinobacteria (Figure 2), the most prolific producers of known antibiotics, anticancer, antifungal, immunosuppressant and herbicidal compounds (Barka et al 2016, Berdy 2012). Preliminary experiments conducted in our labs suggest that the role of actinobacterial MVCs as antibiotics has been grossly underestimated. Our experiments indicated that approximately 15% of all actinobacteria species tested (N=200) can inhibit the growth of *Bacillus subtilis* or *Escherichia coli* in an experimental setup where the actinobacteria were physically separated from the target (see **Chapter 3**). Intriguingly, those actinobacterial strains that produce volatiles that inhibit the growth of the Gram-positive *B. subtilis* did not inhibit growth of the Gram-negative *E.coli* and *vice versa*, suggesting that actinobacterial MVCs exhibit different modes of action.

The antimicrobial activity of MVCs can be further enhanced via synergy with 'soluble' antibiotics. For example, pre-treating antibiotic-resistant bacteria with the terpene eugenol lowered the minimal inhibitory concentration (MIC) such that they became antibiotic sensitive again (Hemaiswarya and Doble 2009). Similarly, phenylpropanoids such as β- cinnamic and ferulic acid, conferred sensitivity to amikazin, erythromycin and vancomycin (Hemaiswarya and Doble 2009). A mixture of the monoterpenes γ-terpinene, 1S-α-pinene, β-pinene and β-myrcene produced by *Collimonas pratensis* inhibited growth of *S. aureus* and *E. coli* (Song et al 2015). Additionally, essential oil components such as limonene, sabinene, α-pinene, thymol and carvacrol have been shown to have potential as enhancers of anti-tuberculosis drugs like ethambutol, rifampicin and isoniazid (Sieniawska et al 2017).

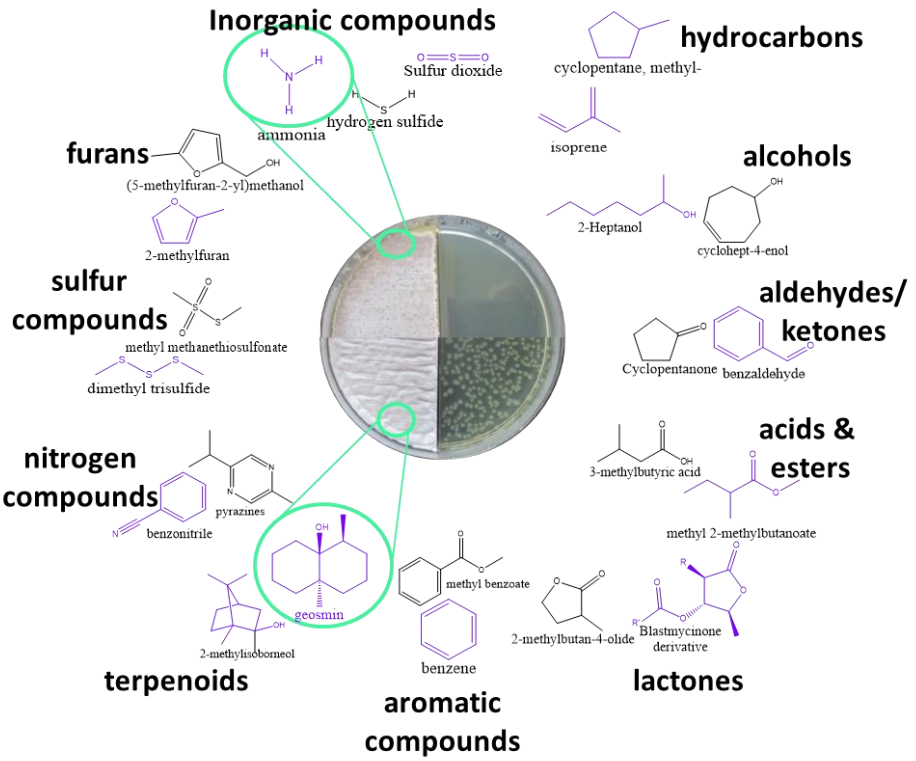

Figure 2. Chemical classes of volatile compounds released by bacteria. Highlighted in purple are the VCs identified in *Streptomyces* strains. Zoomed compounds in green correspond to the widespread terpene geosmin (non-active as antibiotic) and ammonia that when produced in high concentrations by some *Streptomyces* strains inhibits *E. coli* growth.

Modes of action of MVCs

Despite the observations that many MVCs have antimicrobial activity, only few studies provided insight into their modes of action. Pentalenolactone is an example with a specific intracellular target; it impedes glycolysis by inhibiting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tetzlaff et al 2006). Another example comes from *Muscodor albus*, a fungus that produces an antimicrobial blend of volatiles (Strobel et al 2001) that appears to operate by causing DNA damage; *E. coli* cells became

particularly sensitive to these MVCs when they lack the enzymes (e.g. RecA) for DNA repair (Alpha et al 2015). Another mode of action proposed is the modification of membrane fluidity/permeability by MVCs, allowing their entry into the cell or causing increased leakage of intracellular materials (Trombetta et al 2005). This mode of action might be widespread among MVCs from different microbial families. For example, VCs from yeasts such as 3-methyl-1-butanol disrupt the fungal membrane by increasing the peroxidation levels of membrane lipids, thereby causing a non-selective permeability of the plasma membrane (Dalilla Carvalho Rezende 2015). Other modes of action comprise the interference of MVCs with cell-to-cell communication or quorum sensing. The aforementioned Schleiferon A and B inhibit quorum sensing, thereby reducing the expression of prodigiosin and bioluminescence in Gram-negative bacteria (Lemfack et al 2016). Another well-studied example is the diffusible signal factor DSF (cis-11-methyl-2-dodecenoic acid) which modulates the virulence factors in *Xanthomonas campestris* (Barber et al 1997, Wang et al 2004). In *B. subtilis,* DSF-family signals significantly decreased the transcription of drug efflux systems and biofilm formation (Deng et al 2014). Homologs occur in many bacteria. One example is *Stenotrophomonas maltophilia*, a Gram-negative bacterium that is found ubiquitously in different environments, including nosocomial infections where it affects biofilm formation in *Pseudomonas aeruginosa*, a lung pathogen associated with cystic fibrosis (Berg et al 2005, Graff and Burns 2002).

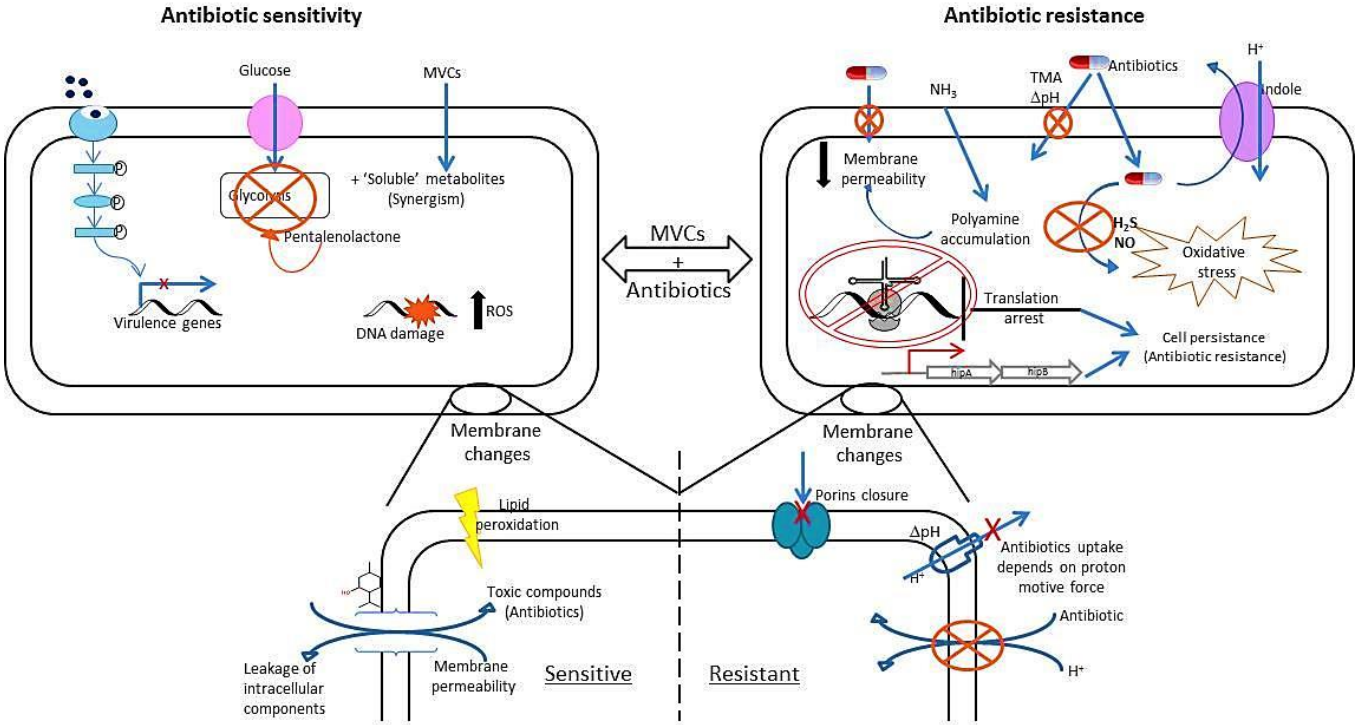

Figure 3. MVCs modes of action as antimicrobials and modulators of antibiotic resistance

MVCs as modulators of antibiotic resistance

An important activity of MVCs in the light of this review is their ability to modulate antibiotic resistance. Recently, Groenhagen et al. (Groenhagen et al 2013) demonstrated that the volatiles 1-methylthio-3-pentanone and o-aminoacetophenone from *Burkholderia ambifaria* increased resistance of *E. coli* to aminoglycoside antibiotics like gentamicin and kanamycin. Trimethylamine (TMA) modified resistance to tetracycline by increasing the pH and lowering the transport of tetracycline inside the cell due to changes in transmembrane pH and proton motive force (Letoffe et al 2014). Slow-growing cells present in a normal growing population, also referred to as persister cells or persisters, evolve tolerance to antibiotics and other environmental stresses. Recently, a link was established between persistence and a toxin-antitoxin system. High persistence (*hip*) mutants exhibited significant survival after treatment with cell wall inhibitors. The mutations were mapped to a toxin-antitoxin locus (*hipBA*). In this example, the toxin HipA inactivates glutamyl-tRNA synthetase by phosphorylation, thereby inhibiting cell growth (Germain et al 2013, Kaspy et al 2013). The anti-toxin HipB is a transcriptional repressor that neutralizes HipA and regulates *hipBA* expression (Page and Peti 2016, Schumacher et al 2009). Microarray data of *E. coli* exposed to VCs emitted by *B. subtilis* showed induction of the expression of *hipA* and *hipB*, thereby inducing resistance (Kim et al 2013, Schumacher et al 2015). Specifically, 2, 3-butanedione and glyoxylic acid produced by *B. subtilis* enhance resistance to ampicillin and tetracycline in *E. coli* through *hipBA* (Kim et al 2013). 2-Aminoacetophenone (2-AA) produced by *P. aeruginosa* regulates quorum sensing and also stimulates persister cell formation. The long-range effect of this volatile also influenced persister cell formation in pathogenic bacteria belonging to a different genus that do not produce 2-AA, like *Acinetobacter baumanii* (Que et al 2013). Interestingly, microorganisms may also 'eavesdrop' the signalling molecules produced by other microorganisms, thereby taking advantage of their effect. As an example, *Pseudomonas putida* recognizes indole produced by *E. coli*, which induced the *Pseudomonas* TtgGHI antibiotic efflux pump allowing its growth in the presence of ampicillin (Molina-Santiago et al 2014).

Besides organic MVCs, it is becoming clear that small inorganic MVCs (such as hydrogen cyanide (HCN)) can have a major effect on antibiotic resistance. We propose that this is a general phenomenon and to exemplify our proposition we discuss three molecules here, hydrogen sulphide (H_2S), nitric oxide (NO) and ammonia (NH_3). H_2S and NO have overlapping activities and play an important role in the protection against oxidative stress and against antibiotics. Interference with H_2S production by *Bacillus anthracis, P. aeruginosa, S. aureus*, and *E. coli* rendered these human pathogenic bacteria sensitive to a range of different antibiotics, which could be reversed to resistance by adding exogenous H_2S (Shatalin et al 2011). Interestingly, H_2S provided protection against many classes of antibiotics targeting DNA, RNA, cell wall or protein biosynthesis (Shatalin et al 2011). These modulating activities have also been attributed to nitric oxide (NO), mainly due to the pioneering work of Gusarov and Nudler (Gusarov and Nudler 2005, Gusarov et al 2009, Shatalin et al 2008, van Sorge et al 2013). NO is produced from arginine by nitric oxide synthases (bNOS) that are present in many Gram-positive bacteria. NO was first recognized as being critical for the survival of bacteria such as *Bacillus anthracis* against oxidative stress and survival in macrophages (Gusarov and Nudler 2005, Shatalin et al 2008). However, NO also directly protected bacteria against a broad spectrum of antibiotics (Gusarov et al 2009, van Sorge et al 2013). In nature, this trait likely evolved to allow NO-producers to share their habitat with other antibiotic-producing species (Gusarov et al 2009). *B. subtilis* cells producing NO are able to grow in the presence of *P. aeruginosa* producing the toxin PYO (Gusarov et al 2009). Similarly, *B. subtilis* and *S. aureus* grow in the presence of cefuroxime only when producing NO, while *nos* null mutants cannot (Gusarov et al 2009). NO-mediated resistance is achieved through direct chemical modification of toxic compounds (Gusarov et al 2009). The role of NO may even go a lot further, as it was shown that the lifespan of the NO non-producing *C. elegans* is expanded significantly when it feeds on NO-producing bacilli, offering a striking new example of symbiosis mediated by a volatile compound (Gusarov et al 2013).

Ammonia (NH_3) generated by the catabolism of aspartate, promotes intracellular accumulation of polyamines modifying *E. coli* membrane permeability thereby increasing resistance to tetracycline

(Bernier et al 2011). In contrast, exposure to ammonia decreased resistance to kanamycin. This effect could be explained by a higher expression of the polyamine-induced protein OppA, a periplasmic binding protein involved in uptake of aminoglucosides (Kashiwagi et al 1992). We have recently established that *E. coli* cells become more resistant to NH_3 by reducing the expression of the regulatory system (*ompR*/*envZ*) of the major outer membrane porins OmpF & OmpC. These results suggest that porins represent a major point of entry for ammonia (see **Chapter 4**).

Outlook and perspectives

Microorganisms are rich sources of VCs. However, their functions and role as antimicrobial are yet poorly understood. Some MVCs are produced by many different bacterial genera, while others are unique at the species level providing useful information for microbial chemotaxonomy and detection. Furthermore, some MVCs are only induced during interspecific interactions, suggesting a major role in communication and/or competition.

For most bioactive MVCs the genes involved in biosynthesis and regulation are yet unknown. There is indication that the production of some 'soluble' and volatile compounds is encoded by the same genes, for example blastmycinones and butenolides are derived from the antimycin biosynthetic pathway (Riclea et al 2012). Salinisporamide A, an anticancer compound presently undergoing clinical trials is synthesized using previously unknown volatile cyclohexene derivatives as intermediates (Groenhagen et al 2016). Such evidence calls for integrative bioinformatics and systems biology approaches to unravel their biosynthesis and study synergism between soluble and volatile compounds. This should shed more light on how closely related these seemingly different 'worlds' of natural products are.

Examples reviewed here bring attention to the fact that MVCs can serve as a self-defence mechanism for the producer or the community. We firmly believe that the near complete omission of volatile compounds

from drug discovery efforts needs to be reconsidered especially at a time where new antibiotic treatments are so desperately needed to counteract antimicrobial resistance. One argument that is often heard is that volatile compounds need to be solubilized before they can be applied. This may be true for topical or IV application, but we would like to point out that some of the diseases that are most difficult to treat such as the lung diseases tuberculosis and cystic fibrosis may be targeted by MVCs. Inhaling MVCs should be considered as a possible therapy in addition to regular antibiotic regimes, taking advantage of their direct and modulating effects described in this review. The information gathered can also be used in the design and development of novel chemical structures and therapies such as the example given by Abed, N. et al., (2015) (Abed et al 2015), where they use the natural terpenes farnesyl and geranyl to design a nano-device that takes advantage of the formation of an environmentally sensitive bond between the terpenes and penicillin helping the delivery of the antibiotics directly into cells.

'Small-talk' molecules like the terpene 3-carene produced under poor-nutrient growth conditions (Schmidt et al 2015b) or the pyrazines produced by *Paenibacillus* during its interaction with *Burkholderia* (Tyc et al 2017a) play an important role as infochemicals as they are produced specifically when needed while the smaller inorganic molecules like NH_3 or NO produced in high amounts make a 'loud noise' that is easily perceived by different organisms.

Improving our understanding of the natural roles of volatile compounds in the microbial environment (i.e. learning from nature) would greatly help in the search for novel bioactive molecules for drug development or as biomarkers for clinical and industrial purposes.

Acknowledgements

This work was supported by a Conacyt grant 313599 from the Mexican government to MA, by grant 14221 from the Netherlands Organization for Scientific Research (NWO) to GPvW and by the NWO, VIDI personal grant 864.11.015 to PG. This is publication 6496 of the NIOO-KNAW.

CHAPTER 3

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

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Submitted for publication

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.

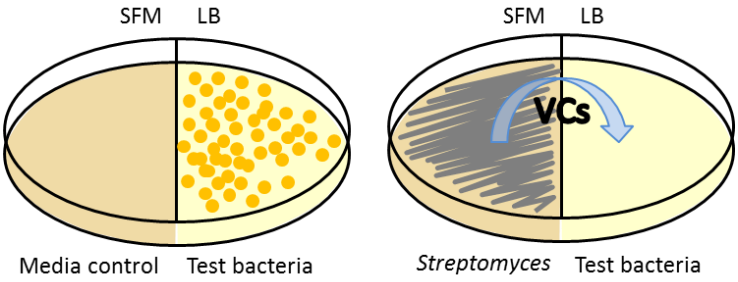


Figure 1. Experimental setup pf the two division petri dishes used for volatile antibiotic assays.

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.

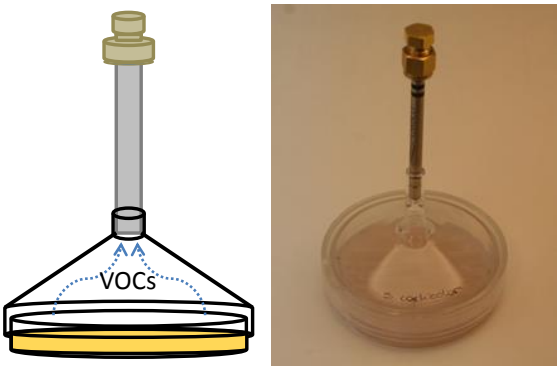


Figure 2. Left: Illustration of the system for the collection of VOCs. Right: Picture of the real glass petri connected to a Tenax® column and inoculated with *S. coelicolor*.

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).

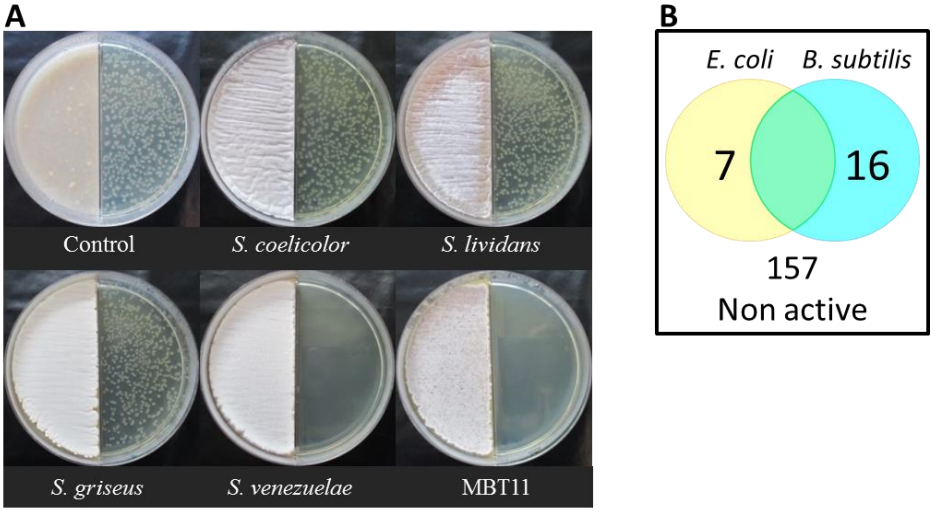


Figure 3. A. Bioactivity of VCs released by selected *Streptomyces* strains against *E. coli* strain ASD19. **B.** Venn Diagram showing target-strain specificity of the bioactivity from *Streptomyces* VCs.

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.

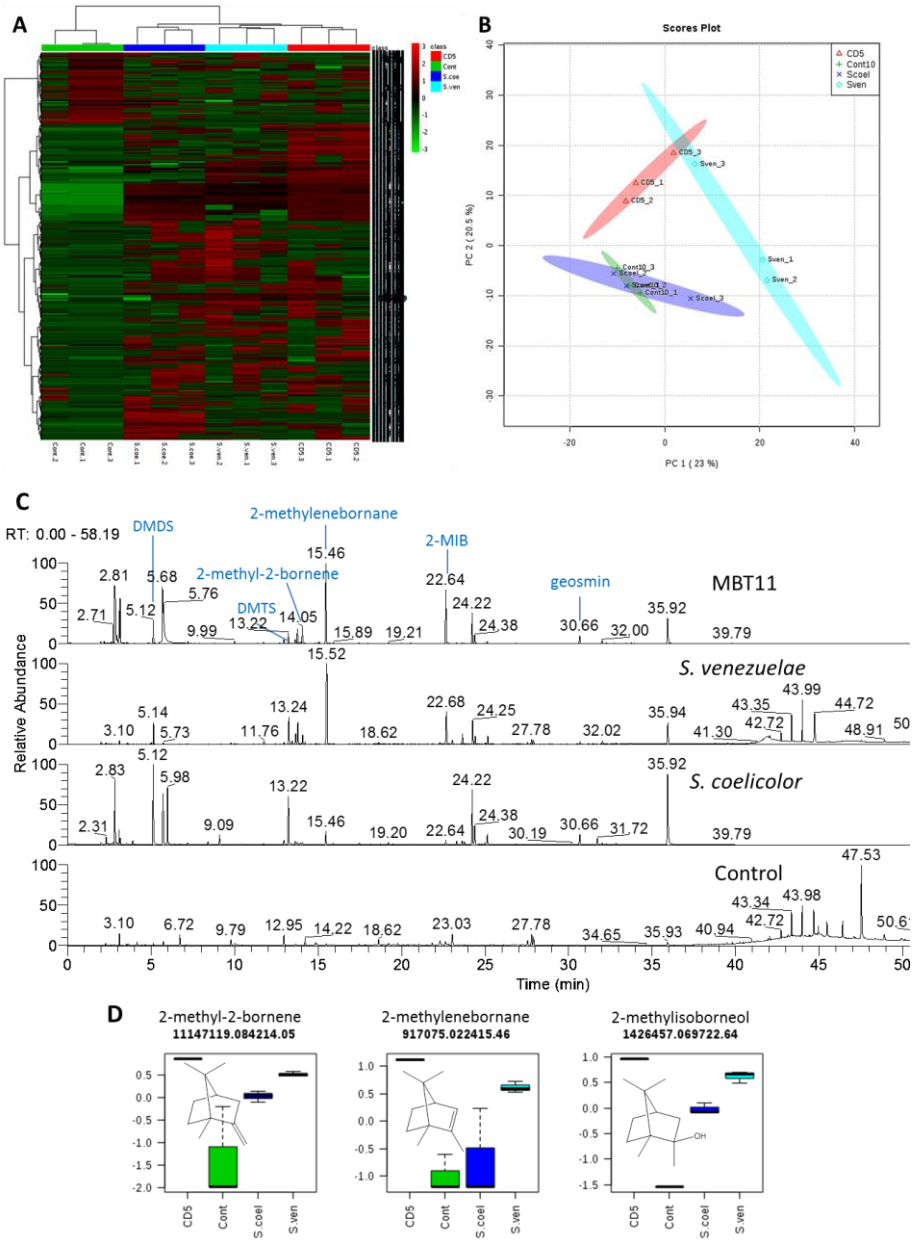


Figure 4. A. Heatmap and clustering, **B.** PCA-2D plot of the VCs produced by *Streptomyces* sp. MBT11 (red), *S. venezuelae* (turquoise), *S. coelicolor* (purple) and SFM media as control (green). **C.** GC-chromatogram of VCs from bioactive strains compared to non -bioactive and SFM media control.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

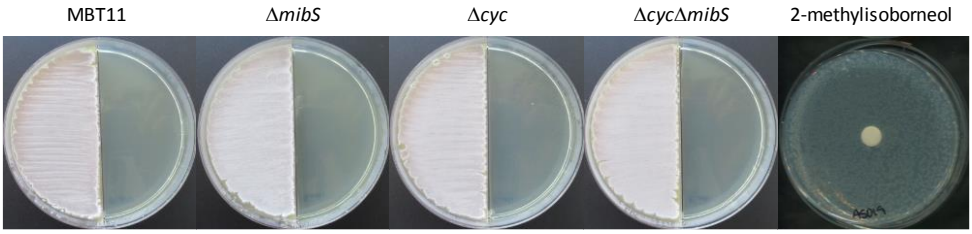


Figure 5. Bioactivity of *Streptomyces* sp. MBT11 and the mutants impaired in terpene production against *E. coli* ASD19. Far right: bioactivity of pure terpene 2-methylisoborneol.

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).

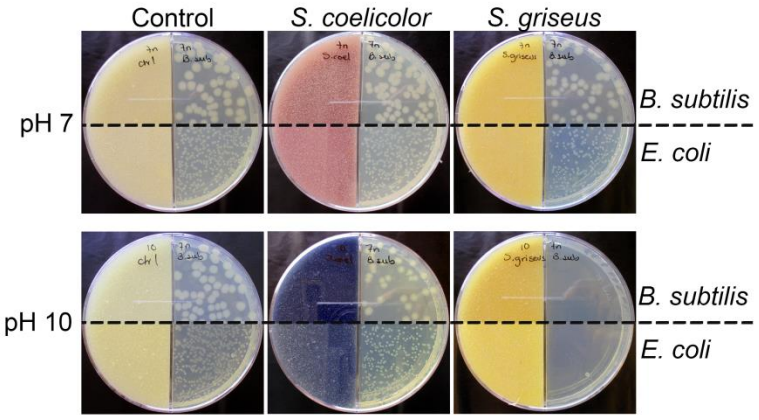


Figure 6. Volatile antibiotic activity of different *Streptomyces* strains grown at pH 7 and pH 10 (using a glycine buffer) against *E. coli* strain ASD19.

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).

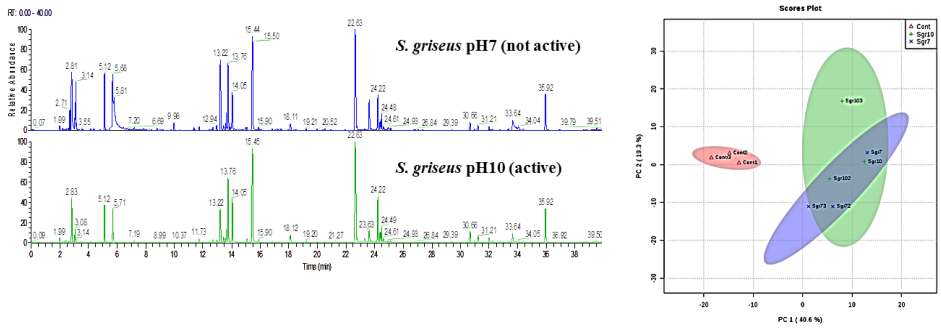


Figure 7. *S. griseus* chromatograms and metabolomic analysis at pH7 and pH10. No difference is seen; however, terpene compounds are the main VCs in both conditions.

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.

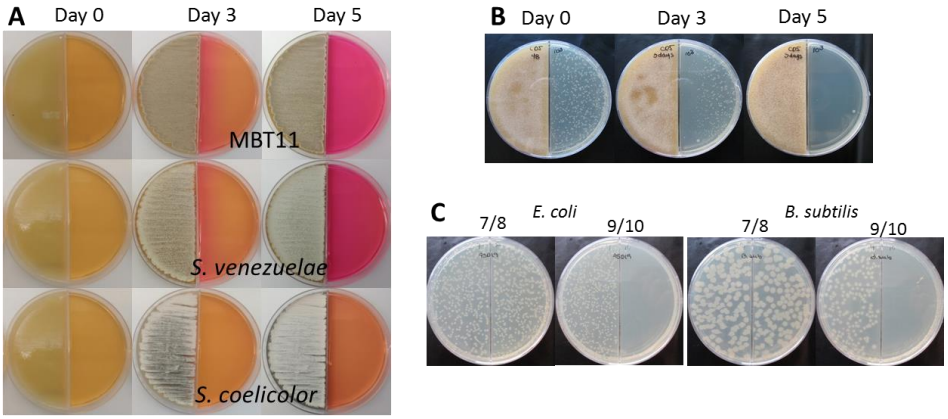


Figure 8. A. pH change illustrated by the color change of the indicator (Phenol red 0.002%). LB medium alkalization after 3 and 5 days of growth of *Streptromyces* sp. MBT11 and *S. venezuelae,* no alkalization was seen from *S. coelicolor* volatiles. **B.** *Streptomyces* sp. MBT11 Antimicrobial VCs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

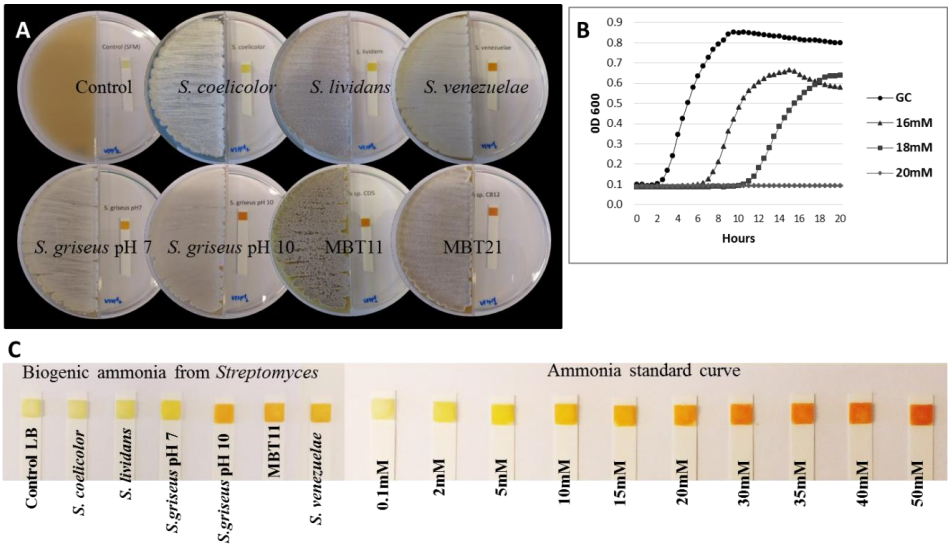


Figure 9. A. NH_3 emission. Test strips on the right compartment show the production of NH_3 by *Streptomyces* strains. *S. coelicolor* and *S. lividans* (~10mg/L); *S. griseus, S. venezuelae*, *Streptomyces* sp. MBT11 and *Streptomyces* sp. MBT21 (~100mg/L); Control: SFM media (0mg/L). Concentrations 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.

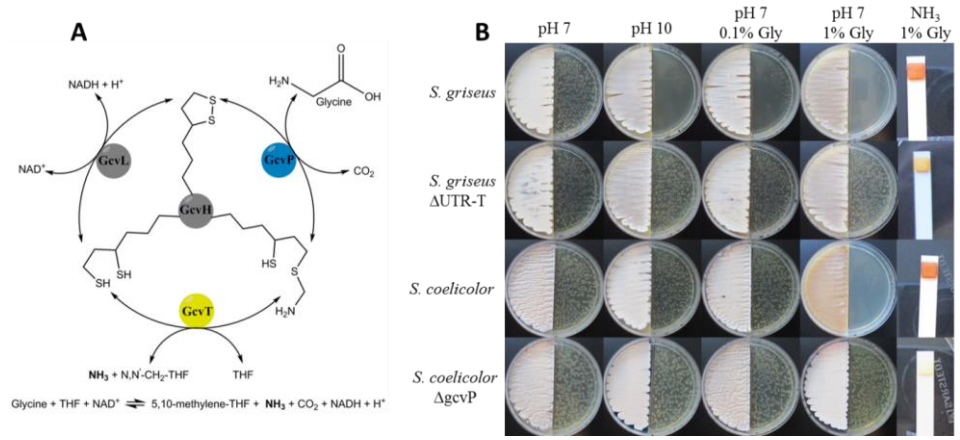


Figure 10. A. Glycine cleavage system schematic representation. **B.** Volatile activity and ammonia production from *S. griseus* WT, *S. griseus* glycine cleavage system mutant *gcvT* (ΔUTR-T), *S. coelicolor* WT and *S. coelicolor* glycine cleavage system mutant Δ*gcvP*(::*aac(3)IV*).

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	+

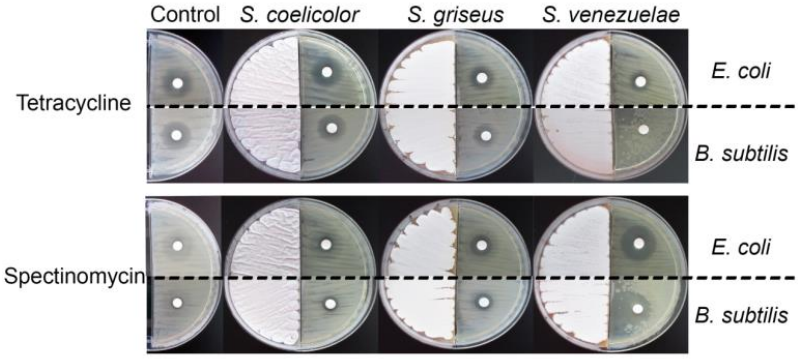


Figure 11. Table indicating the changes in antibiotic sensitivity under the presence of *Streptomyces* VCs. (-) means decrease in halo size, (+) means increase in the halo size, NC: no changes and NA: not active. Pictures are an example of the changes in halo size.

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.

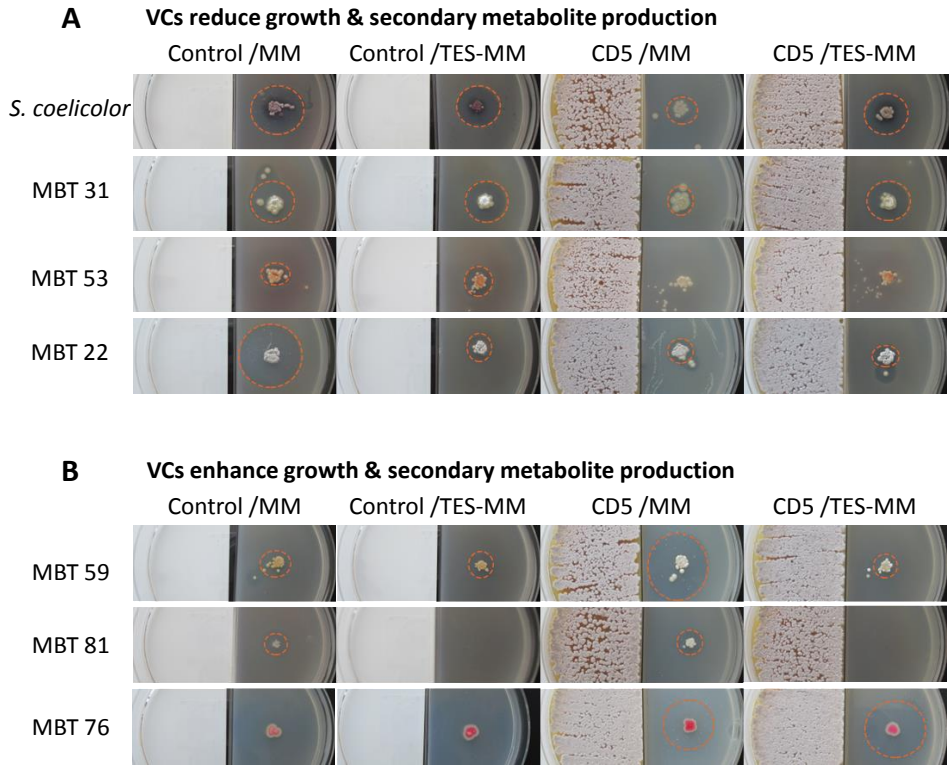


Figure 12. VCs from *Streptomyces* sp. MBT11 modify the development and secondary metabolite production of neighboring streptomycetes.

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

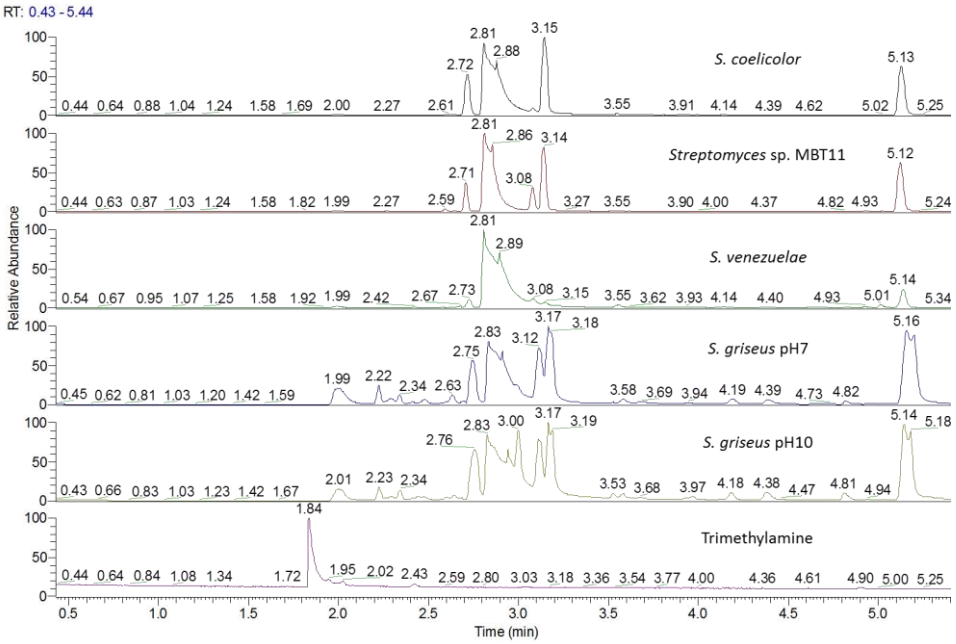
production of a small molecule such as ammonia is a strategy to enhance the activity of antibiotics that require many high-energy precursors (ATP, NADPH, acetyl-CoA etc.) for their synthesis and are therefore costlier to produce, such as polyketides, non-ribosomal peptides or β-lactams. This is applicable 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.

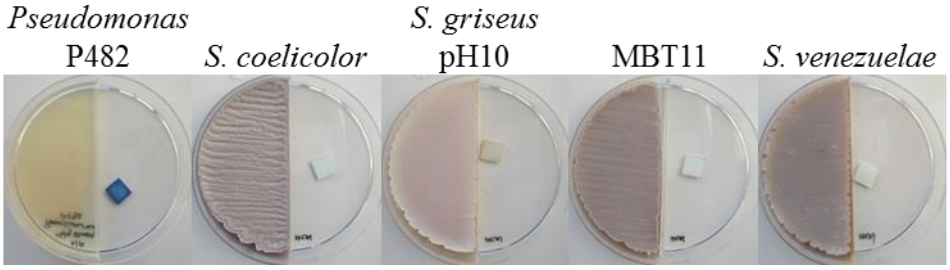
Acknowledgements

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.

CHAPTER 4

Escherichia coli mediates resistance to volatiles from *Streptomyces* in an OmpR-dependent manner.

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Genome announcements 2018, 6:18| doi: 10.1128/genomeA.00385-18

Submitted for publication in combination with Chapter 3

ABSTRACT

The rise of antibiotic resistance in pathogens is a worldwide health problem. *Escherichia coli* is a common bacterium found in human gut and as other bacteria that can rapidly acquire antibiotic resistance and become a life threat. Previously, a chemically mutagenized *Escherichia coli* strain AS19 was isolated based on its enhanced sensitivity to different antibiotics, in particular to actinomycin. The strain was later mutated to study how rRNA modifications confer antibiotic resistance. In this work we use the modified strain *E. coli* AS19-RlmA$^-$ to study the antibiotic effect of *Streptomyces* volatile compounds (VCs). Spontaneous ammonia-resistant *E. coli* derivatives had mutations in the porin master regulator OmpR, resulting in reduced membrane permeability for VCs and enhanced survival. Here we present the genomic and transcriptomic differences of the variant *E. coli* AS19-RlmA$^-$ in response to *Streptomyces* VCs.

INTRODUCTION

Escherichia coli is a Gram-negative bacterium commonly found in the human intestine as well as in other animals (Gorbach 1996). It has an outer membrane that acts as a barrier which limits the number of antibiotics that are effective (Zgurskaya et al 2015). Like other bacteria, *E. coli* pathogenic strains can rapidly acquire antibiotic resistance and become a life threat (Collignon 2009). The emergence of antibiotic resistance is a worldwide problem that reduces or inhibits the efficacy of antibiotics, putting the life of millions of people at risk. Obtaining insights into the genetics of antibiotic resistance is therefore of utmost importance.

E. coli strain AS19 is an actinomycin-sensitive strain that was selected to study antibiotic sensitivity and how this might be linked to cell permeability (Avalos et al 2018a). The strain was obtained by chemical mutagenesis of *E. coli* strain B with N-methyl-N'-nitroso-N-nitroguanidine (Sekiguchi and Iida 1967). AS19 has been used further in studies of

bacteriophage infection (Iida and Sekiguchi 1971) and antibiotic resistance/sensitivity (Liu and Douthwaite 2002b). In a more recent study, the derivative *E. coli* AS19-RlmA$^-$ (referred to as *E. coli* ASD19 from now on) was developed to examine how rRNA modifications affect susceptibility to MLS_B and ketolide antibiotics (Liu and Douthwaite 2002a). This latter strain harbors a kanamycin resistance cassette disrupting the rRNA methyltransferase gene *rlmA* (formerly *rrmA*).

The fact that *E. coli* AS19 has a higher permeability towards actinomycin (Sekiguchi and Iida 1967) as well as a general higher sensitivity towards a majority of antibiotics appeals to its use as a test microorganism for antibiotics that might be present in lower concentrations. The gaseous nature of volatile compounds (VCs) makes the accumulation of such molecules difficult but allows them to disperse over longer distances or areas. This trait enables these compounds to act as infochemicals in long distance communication. The sensitivity of *E. coli* ASD19 towards antimicrobial VCs (AMVCs) as shown in Chapter 3, will serve as a starting point to study how bacteria respond and protect themselves against these molecules.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Streptomyces sp. MBT11 (Zhu et al 2014) and *Streptomyces venezuelae* (Song et al 2016) were used as antimicrobial volatile producers. *E. coli* BREL606 (Daegelen et al 2009) and its hypersusceptible variant *E. coli* AS19-RlmA$^-$ (Liu and Douthwaite 2002a) referred to as *E. coli* ASD19 from now on were used as test strains. Volatile antimicrobial assays were performed using a petri dish with two compartments, one filled with SFM agar (Soya Flour Mannitol) for *Streptomyces* growth (Kieser et al 2000) and the other half with LB agar for *E. coli* growth (Sambrook 1989). *Streptomyces* sp. MBT11 and *S. venezuelae* were streaked on the SFM half and allowed to grow for 5 days at 30°C. After that, *E. coli* strains were inoculated on the LB side using a concentration of 10^4 cfu/mL. A resistant

E. coli ASD19 mutant (ARM9) insensitive to antimicrobial volatile compounds (AMVCs) was obtained spontaneously after prolonged growth under *Streptomyces* sp. MBT11 VCs.

Minimal inhibitory concentration (MIC)

Two-fold serial dilutions were made with LB in 96-well plates. Wells were inoculated in triplicates with a final concentration of 10^5 cfu/mL (3µL of a dilution made from a fresh culture grown to an O.D. = 1.0 and diluted 100 times to give a concentration of 10^7 cfu/mL (100 µL culture O.D. = 1 + 9.9 mL LB)). The final volume in each well was 150 µL. Plates were incubated overnight at 37°C and OD_{600} was measured after 20 h of growth. The MIC was assigned as the lowest concentration where the O.D was not higher than the LB not inoculated.

Whole genome sequencing

Genomic DNA from *E. coli* was isolated as described elsewhere (Sambrook 1989). Briefly, *E. coli* cells were harvested from an overnight culture and re-suspended in lysis buffer (TE, SDS 10%, Proteinase K), incubated for 1h at 37°C. classical extraction with phenol-chloroform was performed and the aqueous layer was precipitated with absolute ethanol. The DNA pellet was washed with 70% Ethanol, dried and solubilized in TE to perform RNA digestion with RNase 50 µg/mL (RNase A, Thermo Fischer). Degraded RNA was removed by phenol/chloroform extraction followed by ethanol precipitation. DNA was re-suspended in nuclease-free water.

Genome sequencing of *E. coli* ASD19 and its mutant ARM9 was performed using Illumina HiSEQ and PacBio RS at Baseclear BV, Leiden (The Netherlands). Paired-end sequence reads were generated using the Illumina HiSeq 2000 system and mapping the individual reads against the reference genome of *E. coli* B str. REL606. The contigs were placed into superscaffolds based on the alignment of the PacBio CLC reads. Alignment

was performed with BLASR (Chaisson and Tesler 2012). Genome annotation was performed using the Baseclear annotation pipeline based on the Prokaryotic Genome Annotation System (http://vicbioinformatics.com). Variant detection was performed using the CLC genomics workbench version 6.5 (QIAGEN Bioinformatics). The initial list of variants was filtered using the Phred quality score and false positives were reduced by setting the minimum variant frequency to 70% and the minimum number of reads that should cover a position was set to 10. Relevant mutations were confirmed by PCR analysis. The genome has been submitted to the NCBI; the accession number is CP027430.

RNA sequencing

For RNA extraction, *E. coli* cells were grown to an OD_{600} of 0.5, RNA Protect Bacteria Reagent (Qiagen Cat No. 76506) was added according to manufacturer instructions. Cells were pelleted and re-suspended in boiling 2% SDS + 16mM EDTA followed by extraction with Phenol:chloroform:Isoamyl alcohol (25:24:1) pH 6.6. (VWR Prolabo 436734C). Aqueous phase was precipitated with 3M sodium acetate pH 5.2 and pure ethanol, washed with 70% ethanol and re-suspended in RNAse free water. DNA was removed using 5 units of DNAse I (Fischer Scientific, The Netherlands) with further purification using again phenol:chloroform:isoamyl alcohol and precipitation with sodium acetate and ethanol. The final pellet was dissolved in RNase free water.

RNA sequencing and analysis was performed by Baseclear BV (Leiden, The Netherlands). For this, the RNA quality was determined using a Bioanalyzer. Ribosomal RNA was subsequently removed with a Ribo-Zero kit (Epicenter) and the remaining RNA used as input for the Illumina TruSeq RNA-seq library preparation. Once fragmented, it was converted into double strand cDNA, the fragments (on average 100-200 bp) were ligated with DNA adapters at both ends and amplified via PCR. The resulting library was then sequenced using Illumina Sequencing. The FASTQ sequence reads were generated using the Illumina Casava pipeline version 1.8.3. Initial quality assessment was based on data passing the

Illumina Chastity filtering. Subsequently, reads containing adapters and/or PhiX control signals were removed using an in-house filtering protocol. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0.

For the RNA-Seq analysis the quality of the FASTQ sequences was enhanced by trimming off low-quality bases using the "Trim sequences" option present in CLC Genomics Workbench Version 6.0.4. The quality-filtered sequence reads were used for further analysis with CLC Genomics Workbench. First an alignment against the reference and calculation of the transcript levels was performed using the "RNA-Seq" option. Subsequent comparison of transcript levels between strains and statistical analysis was done with the "Expression analysis" option, calculating so-called RPKM values. These are defined as the Reads Per Kilobase per Million mapped reads (Mortazavi et al 2008) and seeks to normalize for the difference in number of mapped reads between samples as well as the transcript length. It is given by dividing the total number of exon reads by the number of mapped reads (in Millions) times the exon length (in kilobases).

The RNAseq data has been submitted to the GEO (Gene Expression Omnibus) from NCBI (National Biotechnology Center Information). The GEO accession number is GSE111370.

Volatile suppressor mutant. *ompR* and *envZ* complementation.

E. coli strain ASD19 suppressor mutant ARM9 was complemented by inserting the *ompR* or *envZ* genes in pCA24N from the ASKA collection (Kitagawa et al 2005). Cells of suppressor mutant ARM9 containing the plasmid were inoculated in LB + chloramphenicol (25µg/mL) with or without IPTG 0.1 mM for induction of the gene expression.

RESULTS

Escherichia coli ASD19 sensitive to soluble and volatile antibiotics

Escherichia coli strain AS19 is an actinomycin-sensitive strain that is also sensitive to other antibiotics like tylosin, erythromycin and streptomycin (Table 1).

Table 1. Minimum inhibitory concentrations (MIC, μg/mL) of *E. coli* Brel606 and *E. coli* ASD19.

	Brel606	**ASD19**
Antibiotic	μg/mL	
Ampicillin	3.9	1
Erythromycin	80	1.25
Kanamycin	8	>500
Polymyxin	0.06	0.25
Spectinomycin	31.25	31.25
Streptomycin	>500	4
Tetracycline	<1	<1
Tylosin	500	15.6

Both *E. coli* strains were tested for its sensitivity against antibacterial volatile compounds released by streptomycetes. *Escherichia coli* ASD19 was chosen as the indicator strain. As shown in Chapter 3, growth of these strains is completely inhibited when exposed to VCs from *Streptomyces* sp. MBT11.

E. coli resistance to volatile ammonia from *Streptomyces* sp. MBT11

After 48 h of incubation adjacent to *Streptomyces* MBT11, a few colonies appeared, that had likely undergone suppressor mutations. These colonies were streaked again to test if they had indeed become resistant to the AMVCs produced by *Streptomyces* sp. MBT11. Four colonies that showed different levels of resistance as indicated by the number of colonies and its size were further analyzed (Figure 1A). Of these,

suppressor mutant ARM9 was selected for its higher resistance (Figure 1A, far right). As shown in Chapter 3, *Streptomyces* sp. MBT11 produces high concentrations of ammonia. It is logical to assume that ammonia is the main AMVC and that ARM9 had sustained one or more suppressor mutations to enable ammonia resistance. This notion is supported by the fact that ARM9 was also resistant against AMVCs from *S. venezuelae* (Figure 1B), which is also known to produce high concentrations of ammonia.

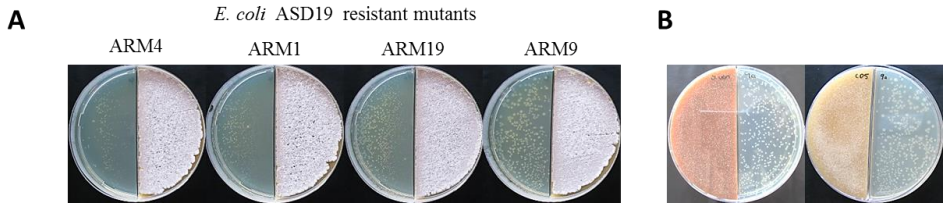

Figure 1. Colonies from *E. coli* strain ASD19 with different resistance patterns to VCs from *Streptomyces* sp. MBT11. **B.** Strain ARM9 is completely resistant to VCs from *S. venezuelae* (left) and *Streptomyces* sp. MBT11.

Strain ASD19 and ARM9 were grown under different concentrations of ammonia. The suppressor mutant ARM9 was more resistant to ammonia than its parent strain, with MICs of 25 mM and 20 mM, respectively (Figure 2). This is a small but very significant difference, as we previously showed that 20 mM is the tipping point between ammonia resistance and sensitivity.

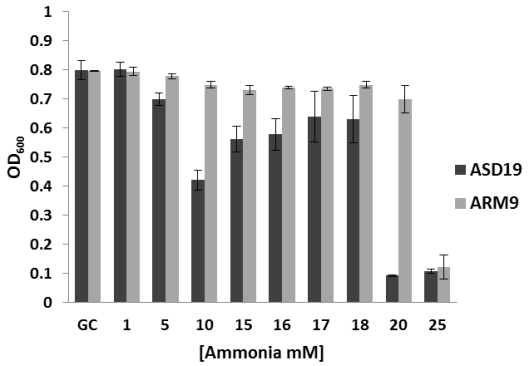

Figure 2. Growth of *E. coli* strain ASD19 (black) and *E. coli* strain ASD19 suppressor mutant ARM9 (gray) under the presence of different concentrations of ammonia.

OmpR is key to ammonia toxicity

It is known that under low availability of nitrogen, the AmtB transporter facilitates the intake of ammonium inside the cell (Conroy et al 2007, Wirén and Merrick 2004). Our conditions include high concentrations of ammonia; therefore we hypothesised that a mechanism other than the AmtB channel would be involved in the resistance towards ammonia. To identify the nature of the mutation(s) sustained by ARM9, its genome sequence was compared to that of its parent *E. coli* ASD19 (Supplementary Table S1). In total 658 mutations were found by SNP analysis, of which 198 gave rise to amino acid changes, insertions or deletions. However, one change immediately stood out, namely the introduction of two insertion elements (insA_31 and insB_31) in-between the -35 and -10 consensus sequences of the promoter for *ompR*-envZ, which encode the two-component system (TCS) consisting of response regulator OmpR and sensory kinase EnvZ (Figure 3). This TCS is involved in osmoregulation in response to environmental signals (Nikaido 2003) and regulates the expression of outer membrane porins OmpF and OmpC. Importantly, these are known to be involved in antibiotic resistance regulated by osmotic pressure and pH (Fernandez and Hancock 2012), and to reduce the responsiveness of *E. coli* cells to VCs (Kim et al 2013).

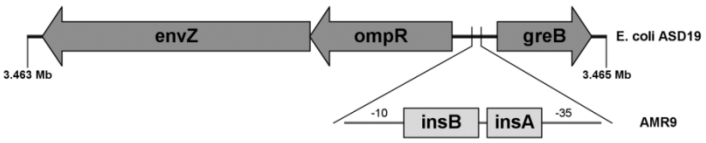

Figure 3. Diagram of the insertion sequences in the middle of the promotor region for *ompR/envZ* genes.

Reduced transcription of the *ompR-envZ* operon is the cause of ammonia resistance

Considering the location right in the middle of the promoter, we expected that the insertion elements (IS) in the *ompR-envZ* promoter reduced the transcription of these crucial TCS genes. To establish the transcriptional consequences of the IS insertion into the *ompR-envZ* promoter region, RNAseq was performed on *E. coli* ASD19 and its suppressor mutant ARM9 grown in LB media until mid-exponential phase (OD_{600} 0.5), and the global transcription profiles compared (see Supplementary Table S2 for the full dataset). Table 2 shows genes highly up/down regulated as a result of a clustering analysis using a cut-off value of a fold change +/- 2.0. These data confirm the downregulation of *ompR* and *envZ* genes and other related genetic elements like *omrA*, a small mRNA that negatively regulates *ompR* expression. Additionally, genes involved in amino-acid metabolism were down regulated, including the *astABCE* gene cluster involved in the ammonia-producing arginine catabolic pathway, *aspA* that is involved in the conversion of L-aspartate into fumarate and ammonia, and *tnaC* for catabolism of tryptophan, which again releases ammonia. Furthermore, the toxin-antitoxin system GhoT-GhoS (Wang et al 2012) appeared up-regulated. These toxin-antitoxin systems are known to be involved in the formation of persister cells (tolerant to antibiotics without undergoing genetic change) (Dorr et al 2010, Kim and Wood 2010). We lack evidence that this toxin-antitoxin system participates in the resistance to AMVCs, however we also observe down regulation of tRNAs which is linked to bacterial persistence by arrest of transcription (Que et al 2013).

Table 2. Clustering of the differentially down/up-regulated genes in *E. coli* ARM9 compared to *E. coli* ASD19.

Cluster	Differentially expressed genes	Function	Fold Change
		DOWN-REGULATED	
Membrane Function/ Transport	*envZ*	sensory histidine kinase in two-component regulatory system with OmpR	-16.43
	omrA	small regulatory RNA	-15.86
	yhdV	putative outer membrane protein	-15.86
	ompR	response regulator in two-component regulatory system with EnvZ	-14.84
	dacD	D-alanyl-D-alanine carboxypeptidase, penicillin-binding protein 6b	-6.47
	yqhH	outer membrane lipoprotein, Lpp paralog	-6.20
	ydiM	putative MFS transporter, membrane protein	-4.83
	yiaD	multicopy suppressor of bamB, outer membrane lipoprotein	-4.54
	yajR	putative transporter	-4.37
	yhfL	small lipoprotein	-3.22
Domain: EAL	*bluF*	anti-repressor for YcgE, blue light-responsive, FAD-binding, inactive c-di-GMP phosphodiesterase-like EAL domain protein	-2.55
	yhjH	cyclic-di-GMP phosphodiesterase, FlhDC-regulated	-2.13

	ycgG	putative membrane-anchored cyclic-di-GMP phosphodiesterase	-2.36
	yliE	putative membrane-anchored cyclic-di-GMP phosphodiesterase	-2.04
Aminoacyl-tRNA biosynthesis	*alaW*	tRNA-Ala	-2.02
	alaX	tRNA-Ala	-2.30
	argX	tRNA-Arg	-2.19
	asnV	tRNA-Asn	-2.76
	asnW	tRNA-Asn	-2.12
	glnV	tRNA-Gln	-2.18
	glnX	tRNA-Gln	-2.35
	hisR	tRNA-His	-2.03
	leuP	tRNA-Leu	-2.37
	leuT	tRNA-Leu	-3.34
	leuU	tRNA-Leu	-2.43
	pheV	tRNA-Phe	-3.56
	proK	tRNA-Pro	-2.24
	proL	tRNA-Pro	-2.94
	proM	tRNA-Pro	-2.76
	selC	tRNA-Sec	-2.07
	valV	tRNA-Val	-3.52
Aminoacid	*astA*	arginine succinyltransferase	-2.17

metabolism	*astB*	succinylarginine dihydrolase	-2.09
	astC	succinylornithine transaminase, PLP-dependent	-2.13
	astE	succinylglutamate desuccinylase	-2.14
	feaR	transcriptional activator for *tynA* and *feaB*	-2.02
	tnaC	tryptophanase leader peptide	-2.21
Fatty acid Oxidation	*fadA*	3-ketoacyl-CoA thiolase (thiolase I)	-2.20
	fadB	fatty acid oxidation complex, α component	-2.19
	fadH	2,4-dienoyl-CoA reductase, NADH and FMN-linked	-2.14
	prpB	2-methylisocitrate lyase	-2.07
		UP-REGULATED	
Prophage	*xisD*	pseudogene, exisionase in defective prophage DLP12	7.252
	ylcI	DUF3950 family protein, DLP12 prophage	5.318
Ribosome	*rrsC*	16S ribosomal RNA	3.93
	rrfB		3.32
	rrfC		2.18
	rrfD	5S ribosomal RNA	5.08
	rrfG		3.55
	rrfH		3.38
Pilus	*fimC*	periplasmic chaperone	2.42
	fimF	minor component of type 1 fimbriae	2.05
	ppdD	putative prepilin peptidase-dependent pilin	4.35
	ydeR	putative fimbrial-like adhesin protein	2.18

To confirm that indeed the reduced transcription of *ompR-envZ* is the major cause for the acquired ammonia resistance, *E. coli* mutant ARM9 was genetically complemented by the introduction of constructs from the ASKA collection (Kitagawa et al 2005) expressing either *ompR* or *envZ*. Introduction of constructs expressing either *ompR* or *envZ* restored ammonia sensitivity, while transformants harboring the empty plasmid continued to be resistant (Figure 4). This strongly suggests that the reduced expression of *ompR* and *envZ* is the main cause of the acquired ammonia resistance.

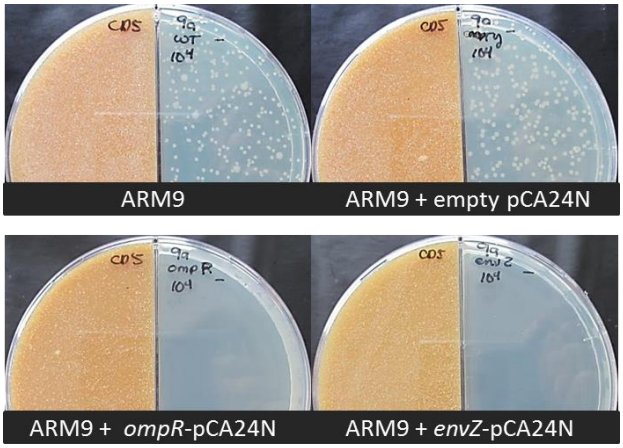

Figure 4. *E. coli* strain ASD19 suppressor mutant ARM9, complemented with empty pCA24N plasmid, *ompR*-pCA24N, *envZ*-pCA24N.

Taken together, these data show that *E. coli* responds to exposure to ammonia by reducing *ompR-envZ* transcription, down regulating the expression of OMPs to minimize the passage of small molecules, and by the reduction of ammonia biosynthesis. Both responses are aimed at defense against the accumulation of toxic levels of ammonia. A similar response was shown previously when an *ompR* mutant did not become as resistant to tetracycline as the wild-type *E. coli* when exposed to ammonia (Bernier et al 2011). Our results show that reducing the expression of OMPs is a defense mechanism against ammonia toxicity extending also earlier observations that *ompF* mutants show impaired response to VOCs that affect the motility of *E. coli* (Kim et al 2013).

DISCUSSION

In Chapter 3, it was shown that *Streptomyces* sp. MBT11 inhibits the growth of *E. coli* strain ASD19 via the production of large amounts of ammonia that accumulate over large distances. In this Chapter, this feature was exploited to study the molecular basis for ammonia resistance, by selecting for spontaneous suppressor mutants of *E. coli* strain ASD19 that have become resistant to volatile antibiosis caused by MBT11. Mutant ARM9 was selected, and genome sequencing revealed the insertion of a duplicated mobile element (insA_31 and insB_31) in the promoter region of the *ompR-envZ* operon. This resulted in the strongly reduced expression of the two-component system *ompR/envZ* that mediates the signal transduction in response to environmental osmolarity changes. This system is known to regulate the major outer membrane porins OmpC and OmpF. In *E. coli,* OmpC and OmpF are known to play a key role in antibiotic resistance (Fernandez and Hancock 2012) thereby reducing the number of entry channels. OmpC and OmpF are two of the main general porins that form a size-defined channel for the diffusion of hydrophilic molecules. The estimated amount of porins in a cell can reach up to 10^6 copies (Achouak et al 2001). These porins are regulated by environmental stimuli such as osmotic pressure and pH, therefore, when the regulator of such proteins is no longer expressed, the amount of porins present in the outer membrane will be much lower reducing the permeability to toxic compounds into the cell, thus increasing the resistance to antimicrobials (Jaffe et al 1982). Previously, an *ompF* mutant showed impaired response to VOCs affecting motility in *E. coli* (Kim et al 2013). Our results show that the suppression of the porin regulator 'OmpR' is responsible for the resistant phenotype, most likely because hydrophilic molecules such as ammonia and other AMVCs can no longer enter the cell in high amounts. Besides this, several studies show that resistance related to changes in the porins may only contribute partially to the overall resistance of a cell. For this reason, it is not uncommon to see that the effect of porin mutations is enhanced by additional mechanisms. For example, some strains of *Serratia marcescens* overproduce the beta-lactamase AmpC and also lack the porin OmpF (Suh et al 2010). *E. coli* clinical isolates lacking both *ompC* and *ompF* also showed an increased production of beta-lactamase (Beceiro et al 2011).

Our results show that the main effect is exerted by the down-regulation of *ompR-envZ*, supported by the loss of the resistance phenotype when the mutant ARM9 is complemented with the regulatory genes *ompR* and *envZ* confirming the idea that a mutant disturbing the *ompB* operon (*ompR*/*envZ*) is no longer affected by high concentrations of ammonia.

The fact that ammonia is a major player in the antimicrobial effect of VCs against *E. coli*, is reinforced by the down-regulation of genes related to amino acid metabolism with a collateral release of ammonia such as the arginine catabolic pathway (*astABCE*); *aspC* which converts L-aspartate into fumarate and ammonia and *tnaC*, the leader peptide of the tryptophanase operon involved in tryptophan degradation releasing ammonia. When cells are exposed to high amounts of toxic molecules that are in fact routine primary metabolites, such as ammonia, a negative feedback loop is a logical survival strategy, with the aim to reduce accumulation of the causing agent.

Ammonia is an important nitrogen source for most bacteria, however, our work shows that it becomes toxic when present in high amounts. Some strains of *Streptomyces* are capable of producing high concentrations of this volatile compound turning it into an antibiotic against bacteria like *E. coli.*

In conclusion, *E. coli* ASD19 is sensitive to high concentrations of ammonia and this work presents the genetic and transcriptomic basis of *E. coli* survival response against toxic concentrations of this volatile. Volatile compounds are implicated in diverse inter and intra-specific interactions all over different ecological niches, from soil to clinical environments. Therefore, the knowledge and understanding of the mechanisms behind such interactions is of great relevance.

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.

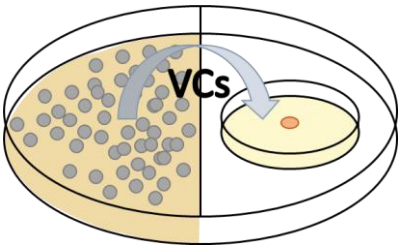

Figure 1. Schematic representation of the antifungal setup.

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.

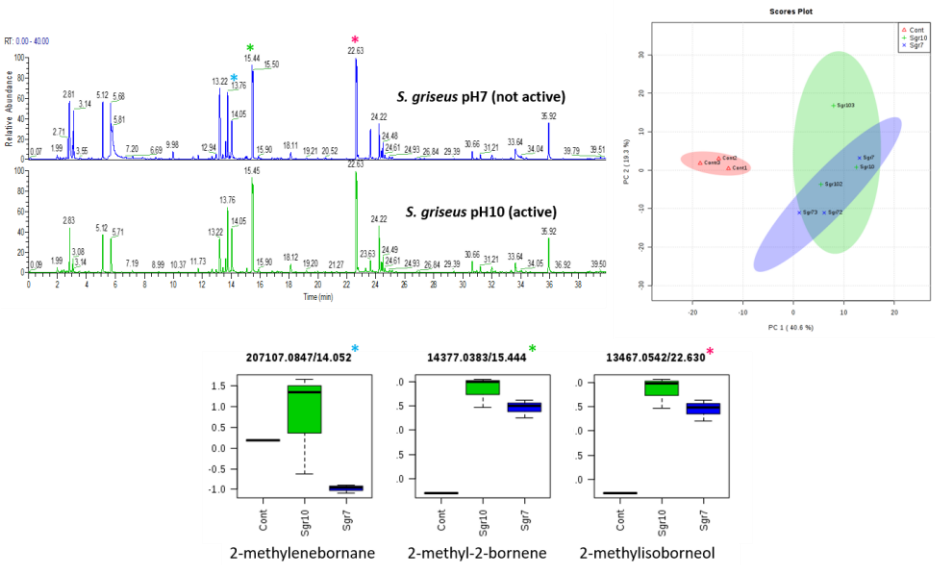

Figure 2. Chromatograms and metabolomic analysis of *S. griseus* grown on agar plates buffered to pH 7 and pH 10 using a glycine/NaOH buffer. Terpene compounds are the main VCs in both conditions.

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).

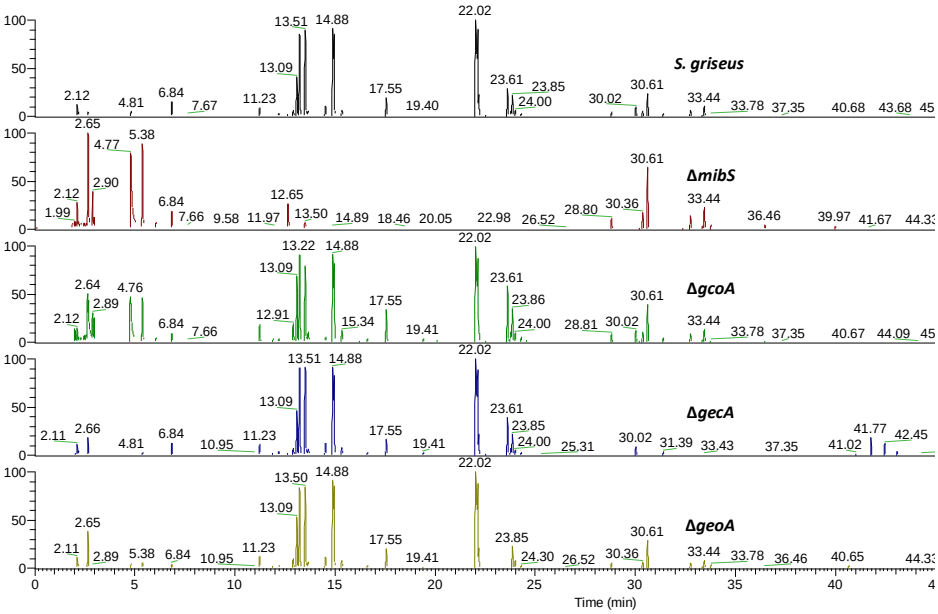

Figure 3. GC-chromatogram from the headspace of *S. griseus* strains grown for 3 days on SFM agar. *S. griseus* DSM40236 (black), Δ*mibS* (red), Δ*gcoA* (green), Δ*gecA* (blue) and Δ*geoA* (light green).

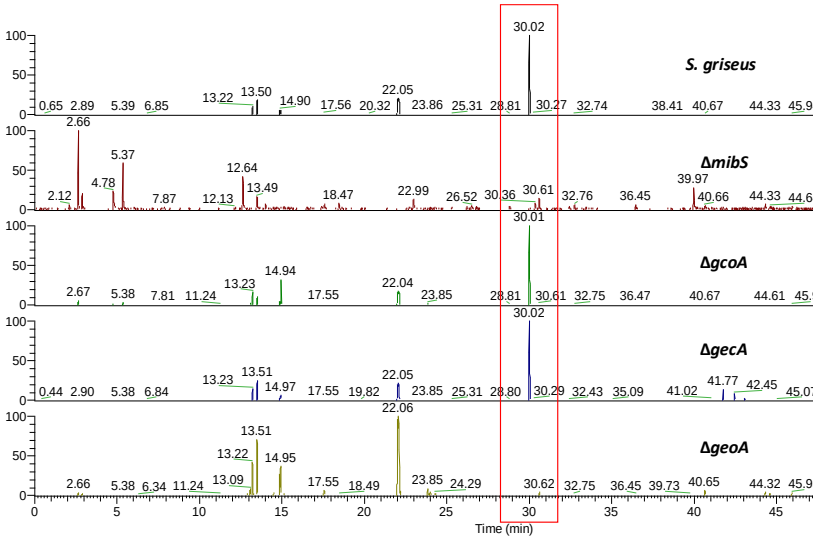

Figure 4. Extracted ion chromatogram of geosmin m/z 112 in *S. griseus* DSM40236 and the non-terpene producing single mutants.

***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.

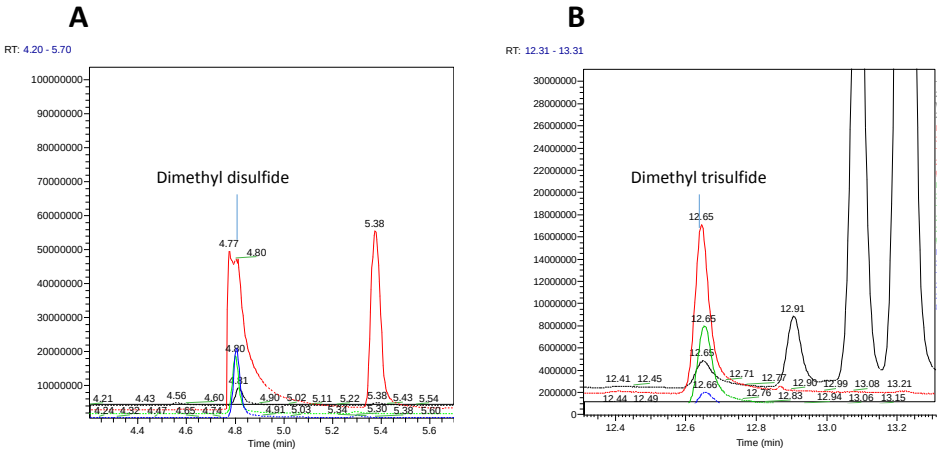

Figure 5. GC-Chromatogram showing increased production of dimethyl disulfide (A) and dimethyl trisulfide (B) in mutants of *S. griseus* lacking *mibS*(red), a double mutant lacking both *mibS* and *geoA* (green) and the terpene non-producer with all four terpene synthase genes deleted (blue). Peak abundance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

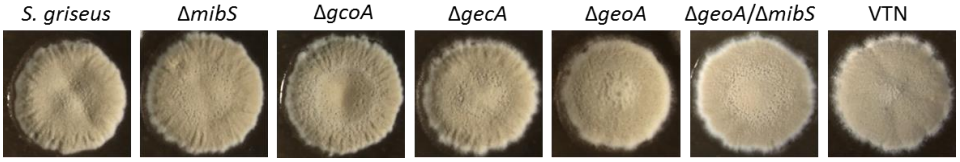

Figure 6. Colony morphology of *S. griseus* and the terpene non-producer mutants grown on SFM agar after 4 days of growth at 30°C. No major differences were observed in colony morphology between the mutants and the wild type.

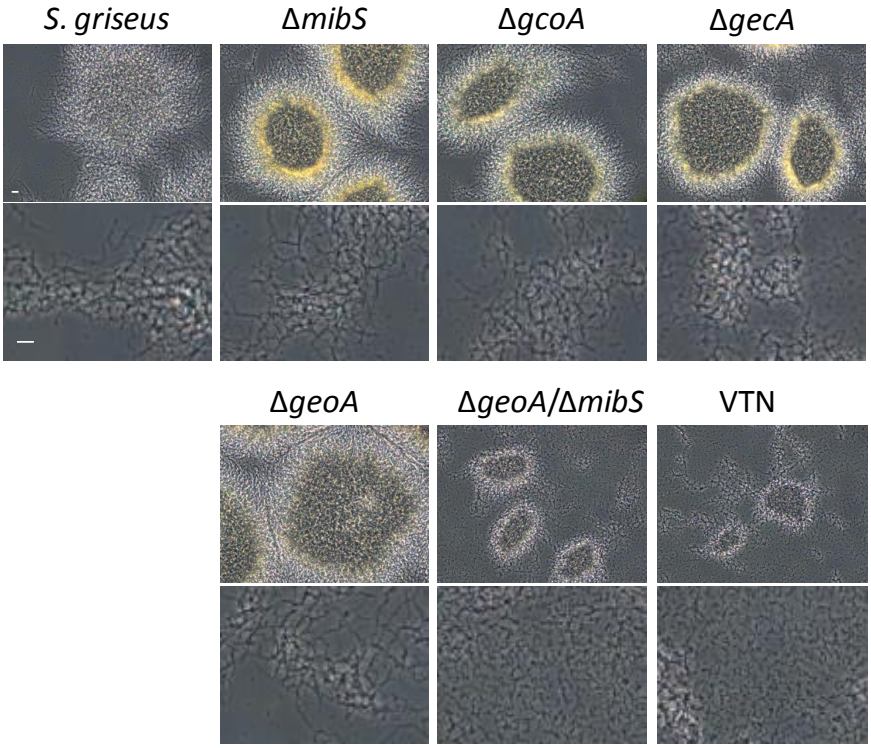

Figure 7. Phase-contrast pictures (10x) of liquid-grown cultures of *S. griseus* and the mutants unable to produce volatile terpene compounds in a high osmolarity medium (20% sucrose + 25 mM $MgCl_2$). Both morphologies: pellets (top) and open mycelia (bottom) were seen for each strain. Bar 20 µm.

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.

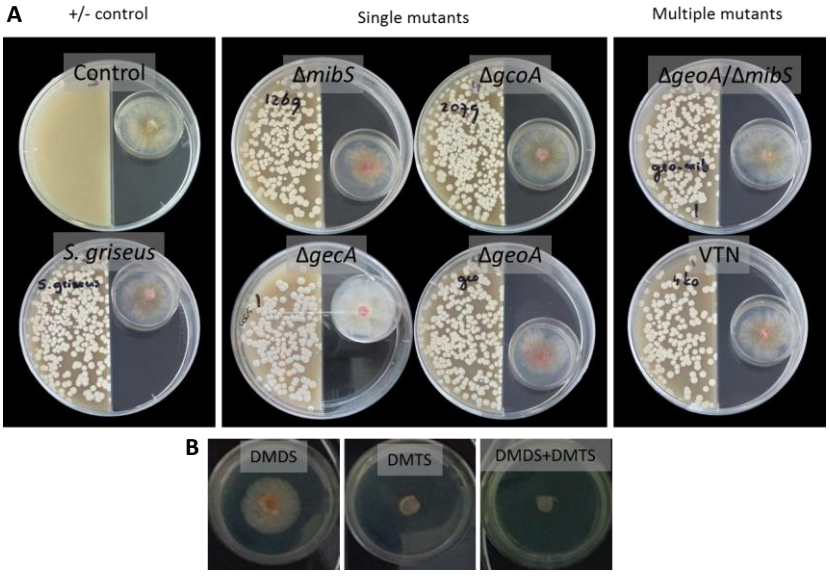

Figure 8. A. Volatile antifungal activity of *S. griseus* DSM40236 and the mutants unable to produce volatile terpene compounds. Left: control: SFM media and wild-type *S. griseus* DSM40236. Centre: *S. griseus* single mutants lacking one terpene synthase. Right: *S. griseus* double mutant Δ*geoA*/Δ*mibS* (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.

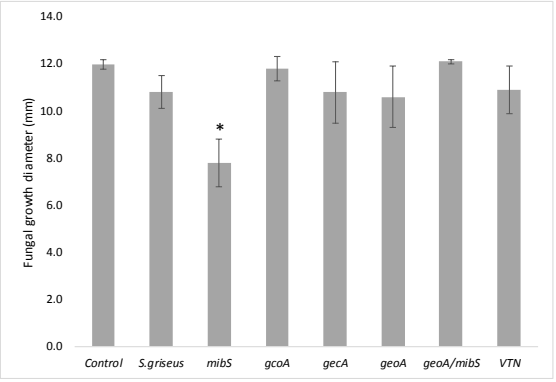

Figure. 10. Antifungal effect measured by the diameter of fungal colonies under exposure of VCs emitted by *S. griseus* and its terpene non-producer mutants.
* indicates significantly reduced growth of *F. culmorum* next to the *mibS* mutant as compared to the growth next to the parental strains *S.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

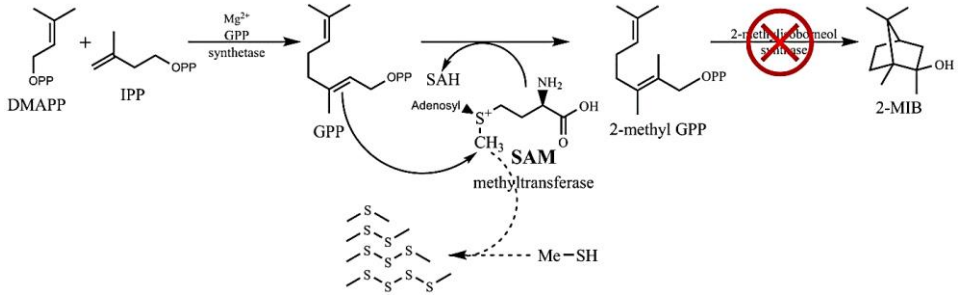

Figure 11. Proposed mechanism for the generation of dimethylated sulfides from the mutants lacking the gene for MibS.

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

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.

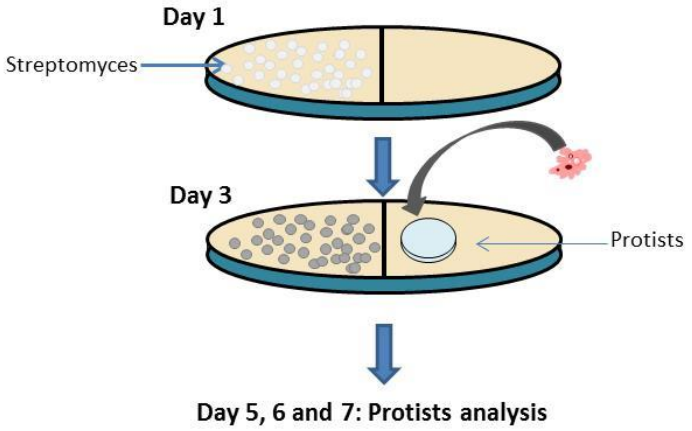

Figure 1. Experimental setup to evaluate the effect of *Streptomyces* VCs on protists.

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.

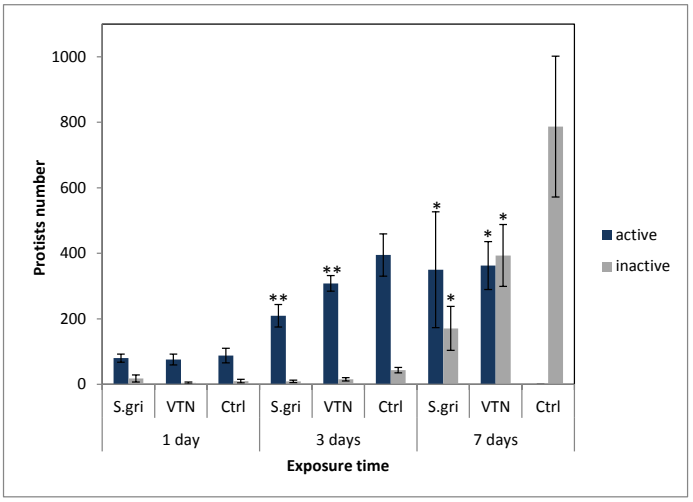

Figure 2. Abundance of active (blue) and inactive (gray) forms of *Acanthamoeba* when exposed to VCs from *S. griseus* and *S. griseus* VTN. * indicates significant difference compared to control (only culture media); ** indicates significant differences between *S. griseus* parent strain and its mutant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

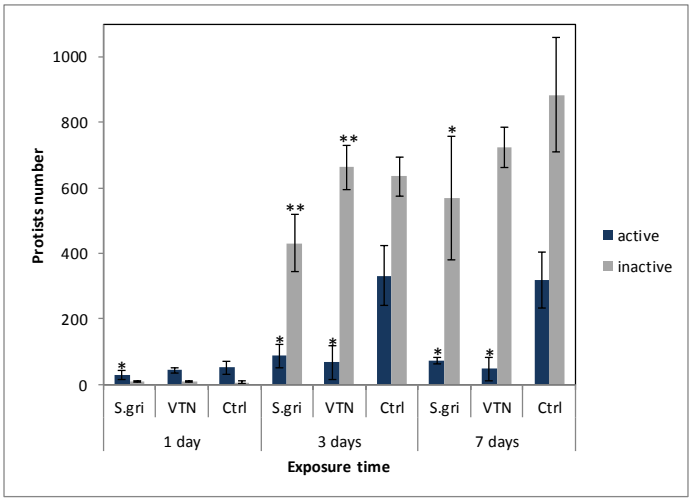

Figure 3. Abundance of active (blue) and inactive (gray) forms of *Tetramitus* when exposed to VCs from *S. griseus* and *S. griseus* volatile terpene non-producer (VTN). * indicates significant difference compared to control (only culture media). ** indicates significant differences between *S. griseus*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

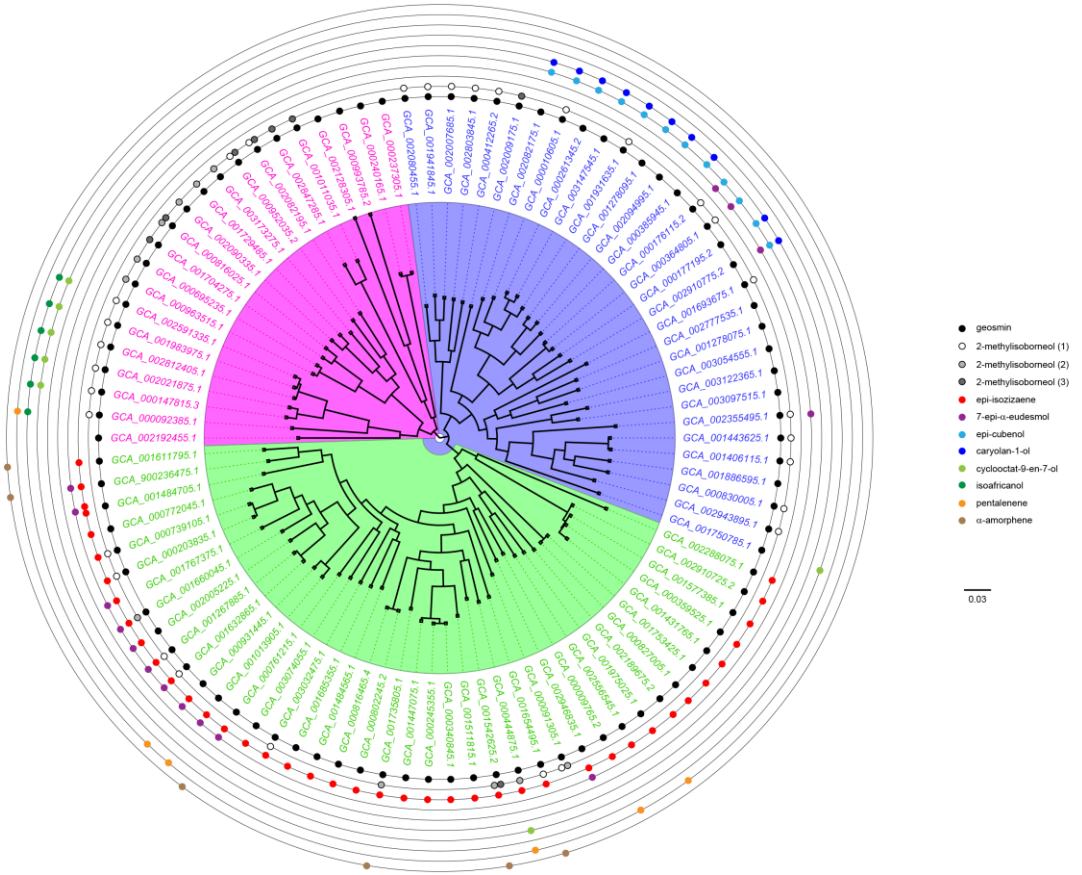

Figure 4. Phylogenetic tree of terpene synthases found in *Streptomyces* with their whole genome available.

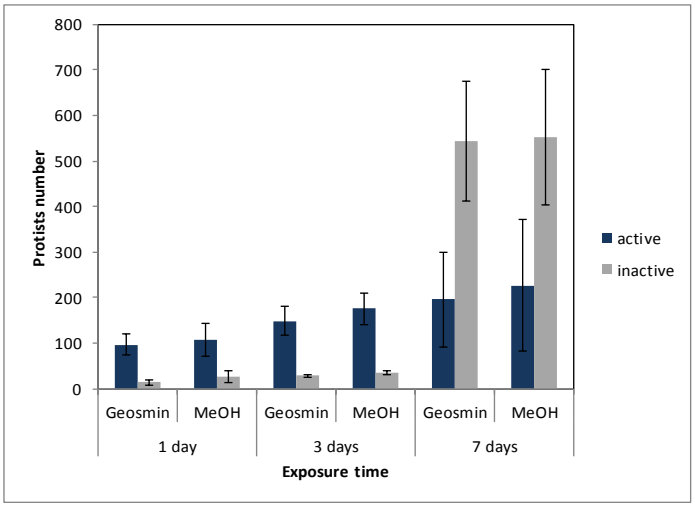

Figure 5. Abundance of active (blue) and inactive (gray) forms of *Acanthamoeba* when exposed to pure geosmin (0.02µg).

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.

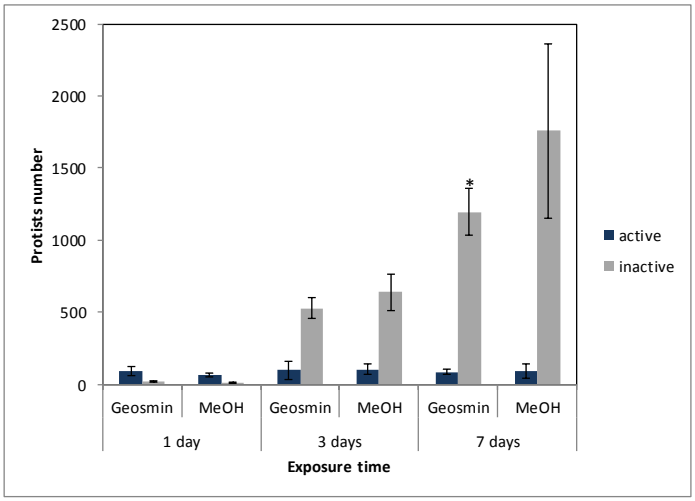

Figure 6. Abundance of active (blue) and inactive (gray) protists when exposed to pure geosmin (0.02µg) * indicates significant difference compared to control (only culture media).

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

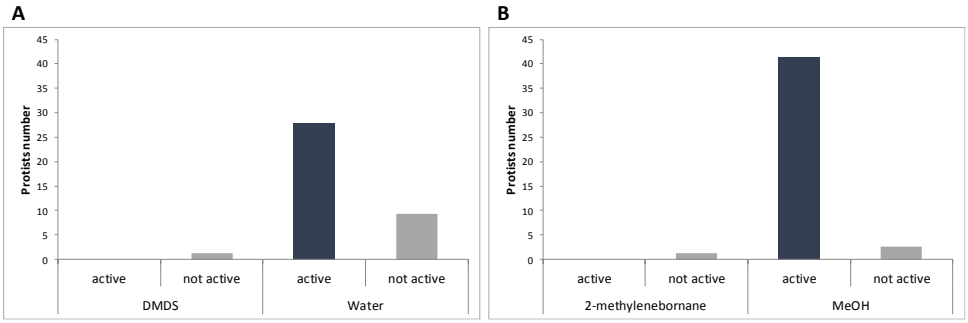

Figure 7. Abundance of active (blue) and inactive (gray) protists when exposed to pure (12% v/v) dimethyldisulfide (A) and 2-methylenebornane (B).

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

CHAPTER 7

General Discussion

Streptomyces as producers of volatile antibiotics

Microorganisms produce a wide range of natural products, many of which have application in the fields of human, animal and plant health. Actinobacteria are particularly prolific producers of such bioactive molecules including about two-thirds of the clinically used antibiotics; most of which are produced by members of the genus *Streptomyces* (Hopwood 2007). In recent decennia, the overuse of antibiotics has led to a rapid increase in antibiotic resistance and at the same time the development of new drugs has come to a standstill. In order to turn the tide, we need to find new molecules and develop novel strategies to find new drugs to treat clinical infections and overcome antibiotic resistance. The potential of actinobacterial secondary metabolism goes beyond the production of canonical 'soluble' antibiotics and extends to the production of smaller secondary metabolites (<300 Da). These so-called volatile compounds (VCs) are molecules with a high vapour pressure that allows them to easily diffuse through the air, soil and even water-filled pores. These properties enable VCs to function as communication tools in microbial interactions (Audrain et al 2015, Schulz-Bohm et al 2017b). This evidence underlines the importance of the roles bacterial VCs play in the biology and ecosystem of these bacteria. For example, bacteria can modulate plant growth by the production of sulfur compounds (Meldau et al 2013) or ammonia (Weise et al 2013). Furthermore, VCs participate in aerial warfare as antifungals, antibacterial compounds or modulators of antibiotic resistance (Avalos et al 2018b, Kim et al 2013, Schulz et al 2010, Wang et al 2013a). Our work shows that *Streptomyces* are capable of inhibiting bacterial growth by using small inorganic molecules such as ammonia (**Chapter 3**). The antibacterial activity observed in this study showed to be target-specific, suggesting that the various *Streptomyces* strains produce different compounds or different blends of compounds that target specific bacteria. Furthermore, specific bioactive molecules can be produced in response to the interacting partner, as seen for sodorifen produced by *Serratia* in the presence of volatiles from *Fusarium culmorum* (Schmidt et al 2017).

Along with *Streptomyces*, other bacteria have also shown to produce promising antibacterial VCs (**Chapter 2**). Schleiferon A and B are

synthesized by the skin-borne *Staphylococcus schleiferi* and affect the growth of Gram-positive bacteria and the quorum sensing system of Gram-negative bacteria. (Lemfack et al 2016). The lack of knowledge about the role of VCs in microbial interactions motivated us to explore and screen a collection of actinobacteria for their potential to produce volatile antibiotics. Besides a better understanding of the antibiotic potential of streptomycetes, these experiments also highlighted the role of VCs in microbial interactions. The high concentrations of ammonia released by streptomycetes modify the pH of the surroundings of the colony. The change in pH can influence the neighbourhood by increasing the adsorption of VCs (Serrano and Gallego 2006) or by modulating the microbial diversity favoring the growth of (certain classes of) bacteria (Bárcenas-Moreno et al 2011, Rousk et al 2009). A rise in pH enhances the activity of antibiotics, such as macrolides and aminoglycosides that work optimally at alkaline pH ((Yang et al 2014); **Chapter3**) and modulates development and antibiotic production in adjacent streptomycetes (**Chapter 3**). Altogether, these results point to a major role of ammonia in modulating the environment to favor growth and survival of the producer strain.

Chemical diversity of volatile compounds

Literature describes more than 1000 VCs emitted by fungi and bacteria (Effmert et al 2012, Lemfack et al 2014, Schmidt et al 2015a). Streptomycetes release complex blends of VCs with bouquets of more than 100 different compounds per strain (Groenhagen et al 2014, Schöller et al 2002). VCs belong to several classes including inorganic compounds, acids, alcohols, aromatic compounds, aldehydes, ketones, furans, lactones, nitrogen compounds, sulfur compounds and terpenoids (**Chapter 2**). So far, terpenes are the most studied group of volatile organic compounds (VOCs), and are in fact, the most widespread group of natural products, synthesized by almost every living organism. Terpenes are also the largest group of compounds with approximately 25,000 structures reported including volatile and non-volatile molecules (Gershenzon and Dudareva 2007). Consistently, in **Chapter 5** we found that the majority of VOCs emitted by *S. griseus* were terpenes (~80%).

Why do streptomycetes produce so many Terpenes?

Secondary metabolism is diversity oriented; if we consider that in extensive screenings molecules very rarely possess a potent biological activity, then microorganisms must exploit their ability to produce multiple molecules in order to strike a potent one. This theory suggests that evolution would favour the organisms that generate and keep diversity at a low cost (Firn and Jones 2000, Fischbach and Clardy 2007). Our results corroborate this, showing that the small and low-cost molecule ammonia acts as an antibiotic and in addition, can effectively influence the minimum inhibitory concentration of canonical antibiotics (**Chapter 3**). Besides ammonia, we observed a high diversity of terpene compounds produced by *S. griseus* (**Chapter 5**). Why streptomycetes produce so many different terpenes is yet unknown. One theory suggests that evolution has selected promiscuous terpene pathways because a gene encoding a terpene synthase that makes different products is more likely to produce novel natural products that meet a new selective need with only a few mutational steps. A shared pathway between several secondary metabolites also favours the low-cost theory by sharing the metabolic and genetic costs by using coexisting biosynthetic pathways (Fischbach and Clardy 2007). Terpenes are a good example of 'one pathway many products' as they all arise from the same building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) that condensate into more complex molecules that are cyclized, rearranged and modified in many different ways that create the chemical diversity known so far (Dickschat 2011, Oldfield and Lin 2012). In **Chapter 5**, a previously unknown link between different VC biosynthetic pathways was revealed. Surprisingly, when the biosynthesis of 2-methylisoborneol (2-MIB) - the main VC present in the headspace of *S. griseus* - was abolished, a completely different set of VCs was synthesized. These were the sulfur-containing dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and dimethyl tetrasulfide (DMTES). This is particularly surprising because the disruption of a pathway for terpene molecules elicits the biosynthesis of sulfur molecules, suggesting a connection between them. In the studies of 2-MIB biosynthesis, no such link had previously been observed. Immediately adjacent to the gene for the terpene synthase lies a gene for a SAM-methyltransferase, which is responsible for the

methylation of geranyl pyrophosphate (GPP); this enzyme may also methylate the sulfide precursor methanethiol (MeSH) in the absence of its original substrate GPP. SAM-dependent methyltransferases are functionally diverse; SAM methyltransferases methylate a wide range of substrates from nucleic acids and proteins to hormones and biosynthetic intermediates of secondary metabolites (Cooke et al 2009, Martin and McMillan 2002). The switch to the production of sulfide compounds instead of other terpene compounds when one or more terpene synthases are missing is the first report of a possible cross-link between the pathways for the biosynthesis of terpenes and sulfur-containing VCs.

Role of terpenes in *Streptomyces* biology

The headspace analysis of *S. griseus, S. venezuelae* and *Streptomyces* sp. MBT11 revealed that terpenes compounds are the most abundant molecules dominated by 2-MIB and its dehydrogenation products 2-methylenebornane and 2-methyl-2-bornene (**Chapter 3 & 5**). *S. griseus* is capable of producing around other 35 different terpenes (**Chapter 5**). The role of these molecules in *Streptomyces* biology has so far been elusive. VCs have been associated with the sporulation process since some non-sporulating strains were unable to produce 2-MIB or geosmin (Schöller et al 2002). This is not the case for *S. griseus*, as mutants lacking all of the terpene cyclase genes still developed abundant aerial mycelia and spores (**Chapter 5**). Nevertheless, the lack of all the volatile terpenes and in particular the 2-MIB and geosmin led to altered morphology of *S. griseus* in liquid culture (**Chapter 5**), suggesting a possible role in the biology of *S. griseus* itself. The volatile character of the terpenes points more to a neighbourhood-associated role. The questions we need to address are: what is their function? and do they act as signals, cues or perhaps as defence mechanisms? The presence of a mixture of compounds can be a mechanism to enhance their activity (Ge et al 2010, Sieniawska et al 2017) and thus prevent the induction of resistance in their competitors (Pimentel and Bellotti 1976). In terms of communication, the release of a mixture may carry a more specialized message (Gershenzon and Dudareva 2007).

The response of target bacteria to antibiotic volatile compounds

The response of microorganisms to a specific VC or a mix of VCs is often strain-specific and dependent upon the interacting partner (Schmidt et al 2015b). In **Chapter 6**, a different response was observed from the two protists tested, *Acanthamoeba* and *Tetramitus*. After one week of exposure to VCs emitted by *Streptomyces*, *Acanthamoeba* remained active as long as it was exposed to the VCs suggesting a possible role of VCs as a nutrition source. *Tetramitus* on the other hand is inhibited by the VCs released by *S. griseus*; its activity was affected by the terpenes geosmin and particularly inhibited by 2-methylenebornane and DMDS. The data presented in **Chapter 5** show the response of the fungus *Fusarium culmorum*, which is also able to produce terpenes, to the VCs released by *S. griseus*. The growth of *F. culmorum* was inhibited and the inhibition was linked to the overproduction of DMDS and DMTS. Sulfides have antifungal activity, especially when used in high concentrations (Gilardi et al 2017, Wang et al 2013a). This apparently correlates to the higher production of sulfides by *S. griseus* mutants that lack the gene encoding 2-MIB synthase.

Microorganisms live in highly diverse environments and microbial interactions are important in determining the community composition. The release of antibiotic VCs from *Streptomyces* will inevitably cause a response in the target bacteria. *E. coli* is known for having multiple ways to endure antibiotics; including the control of the permeability of the outer membrane to modulate the influx and efflux of toxic compounds, as well as the acquisition of resistance through the incorporation of new genetic material or as a result of selective mutations (van Hoek et al 2011, Woodford and Ellington 2007). In **Chapter 4**, we demonstrated that *E. coli* can combine these strategies to overcome the toxicity of ammonia. Insertion elements were found introduced in the promoter region of the *ompB* operon (*ompR* and *envZ*) that leads to the down-regulation of the main outer membrane porins OmpC and OmpF. This resulted in reduced membrane permeability and therefore conferred ammonia resistance. Interestingly, the level of resistance was just high enough to allow the cells to survive the toxic concentrations of ammonia released by *Streptomyces.* After all, OmpR and EnvZ are master regulators, and their

reduced expression affects *E. coli* cells in many ways. In addition, RNAseq experiments on *E. coli* cells exposed to ammonia showed that genes for amino acid metabolism like arginine, aspartate and tryptophan were also down-regulated, most likely in an attempt to help *E. coli* minimize its own intracellular ammonia concentrations.

Ecological implications on the role of volatiles produced by *Streptomyces* species

The aim of the work described in this PhD thesis was to identify volatiles with novel antimicrobial activity. The work did not deliver any novel VCs, but instead advanced our knowledge of the role of known VCs in microbe-microbe interactions and as antimicrobials (**Chapter 3, 5 & 6**).

The ability of *Streptomyces* to inhibit other organisms through the air was clearly demonstrated by the research described in this thesis. Streptomycetes produce VCs that travel long distances and accumulate and solubilize in surfaces containing water-filled pores like agar (**Chapter 3**). The accumulation of ammonia elevates the pH which could play an important role in the modulation of surrounding microbial communities (Bárcenas-Moreno et al 2011, Fierer and Jackson 2006, Rousk et al 2009). These changes also impacted microbial metabolism as well as the interactions between microorganisms. As a result, changes in the production of secondary metabolites were observed ((Schmidt and Spiteller 2017, Yang et al 2014, Zhu et al 2014); **Chapter 3**)). Developmental changes in response to VCs have also been studied. The basic VC trimethylamine triggers a previously unknown type of development called exploratory growth (Jones et al 2017). We showed that ammonia modifies colony morphology, as shown by a delayed sporulation process from strains subjected to high pH. Morphological changes were also observed in the mutants lacking one or more genes encoding terpene synthases, but particularly in a mutant unable to produce any terpenes. This behaviour suggests a regulatory role of the terpenes in *S. griseus* development.

Concluding remarks and future perspectives

Our data clearly demonstrate the ability of various actinobacteria from our strain collection to produce antibiotic volatiles that inhibited the growth of either *E. coli* or *B. subtilis*. This finding clearly supports the volatile antibiotic potential of these strains. The headspace of these strains should be analysed in order to identify the molecules responsible of the antibiotic effect.

The research on antimicrobial volatiles is a developing field and currently very little is known about the genes and the pathways involved in the biosynthesis and regulation of VCs. A few examples where the genes involved in VCs biosynthesis have been identified are those that also encode 'soluble' or 'non-volatile' antibiotics like blastmycinones and butenolides that derive from the antimycin biosynthetic pathway (Riclea et al 2012). Integrative bioinformatics and systems biology will help to elucidate their biosynthesis and unravel possible synergisms between pathways of secondary metabolites.

Microbial volatiles are chemically diverse, and this suggests that they have diverse biological functions. However, these functions are yet poorly understood. This thesis offers more clues on the role of VCs and in particular of terpenes in *Streptomyces* biology and ecology. VCs are widely used as infochemicals in interspecies communication. They can also have an important antagonistic activity. Our work further shows that VCs emitted by *Streptomyces* may act as antimicrobials, either against prokaryotes (bacteria) or eukaryotes (fungi, protists), which raises the question of whether VCs are used as infochemicals or as weapons. Whatever the answer is to this question, it appears that streptomycetes are well equipped for any encounter, whether with friends or foes.

In conclusion, the data presented in this thesis establish a basis for further study of the role of VCs released by streptomycetes. As the most prolific antibiotic producers, the knowledge of the biology and biochemistry of *Streptomyces* VCs could be translated into a possible biotechnological application in medicine, agriculture, food industry and even as alternatives against the increasing problem of antibiotic resistance.

Further studies are needed to reveal the biosynthesis of volatiles as well as their regulatory mechanisms and to understand their modes of action and synergistic activity with other volatile or non-volatile compounds to finally shed light on the applicability of VCs in the field of biotechnology and agriculture.

Inevitably, academic research will lead to new exciting questions. Some of the challenges and ideas that came from this PhD thesis work can be formulated as follows:

We have established that VCs have antimicrobial activity, now we need to understand how they act. In other words, do volatile compounds have a specific cellular target? For this, we need to perform mode of action studies in order to find the partner/receptor of these molecules.

Deletion of genes for terpene synthases led to the surprising and sharp increase in the production of sulfur-containing VCs. How are the pathways for VOCs and inorganic VCs connected? At present, virtually nothing is known of how VC pathways are controlled, and this is therefore a major goal to achieve. For this, the genes for sulfur compounds need to be identified, and the regulatory networks that govern the control of VCs uncovered.

The experiments performed in the frame of my thesis have been done under laboratory conditions. How well can these results be translated to bacterial communities, and ultimately to true environmental conditions? New methods should be developed to study these microorganisms in a more natural environment to fully understand their ecological role.

By addressing these questions, we will learn more about the fascinating world of the volatile small molecules; this will not only lead to better understanding of their role in nature but will also provide insights into their potential for medical and agricultural application.

SUMMARY

NEDERLANDSE SAMENVATTING

RESÚMEN

Microorganisms live in a complex soil habitat participating in intricate and dynamic interactions. They compete for nutrients or space, therefore, the production of compounds with antibiotic activity results in a convenient trait to have. Molecules with antibiotic activity have a wide range of structures and these include volatile compounds (VCs); the latter have a low molecular weight and low vapour pressure that allows them to evaporate and diffuse through different surfaces like the soil pores, water and through the air. During this PhD work the diversity and functions of volatile compounds were studied. *Streptomyces* are one of the most abundant genus of bacteria in soil, and are the main producers of bioactive compounds, including volatile compounds. This work shows how even very small volatile molecules such as ammonia can act as antibiotics when produced in high amounts, by modifying the surrounding environment. Ammonia is derived from the metabolism of amino acids, in particular from glycine which can be regarded as an abundant molecule in soil as exudate from plants. The production of a bioactive compound would certainly trigger a response by the perceiving bacteria. We observed that *Escherichia coli* responds to the high concentrations of ammonia and other VCs by down-regulating its major outer membrane porins in order to reduce the amount of toxic compounds that enter the cell.

Streptomyces are excellent producers of terpenoid compounds dominating the headspace. Two of these molecules have been known for decades namely geosmin and 2-methylisoborneol, nevertheless their function remains unknown. The volatile character of such molecules could suggest that they participate in “long-distance” interactions. A role in communication has been suggested from plant terpenes; however, only a few examples are known in bacteria where terpenes play a role in interspecies communication.

The high-level production of terpenes under different growth conditions raised the question of their role as intra- and/or interspecies signals. The work in this thesis shows that these molecules are not vital for the growth and development of *Streptomyces*, but instead affect the development when grown under conditions of high osmolarity. The mutants unable to produce terpenes, in particular those lacking geosmin

and 2-methylisoborneol synthase were affected, and the typical mycelial pellet formation was altered, resulting in more open and fragmented mycelia.

VCs have also been suggested as waste products or as a carbon release valve. However, when the headspace of the mutant unable to produce any volatile terpene compound was analysed, almost no VCs were identified. The regulatory mechanism of these molecules is barely known, which makes it more important to continue the study of VCs in order to better understand their biosynthesis, regulatory mechanisms and more importantly, their function. This study sheds some light by showing strong upregulation of sulfide compounds when the major terpenes 2-methylisoborneol, 2-methylenebornane and 2-methyl-2-bornene were not produced anymore. This result suggests a possible link between these completely different and unrelated pathways, which had not been seen before. We hypothesize that the methyltransferase that is encoded by the gene cluster that is responsible for the production of the 2-methylisoborneol, may also participate in the methylation of sulfides, thus giving rise to dimethyl disulfide and dimethyl-trisulfide.

Several volatile compounds have been shown to have antimicrobial activity, and the upregulation of sulfides correlates with the higher antifungal activity shown by the mutant missing the 2-methylisoborneol synthase. The antifungal effect of dimethyl disulfide and dimethyl trisulfide is well known, thus confirming the results obtained in this work. The antimicrobial effect of VCs was also observed against protists known to be the main bacterial predators. VCs released by *Streptomyces* induced the formation of cysts in protists, particularly when a mix of VCs was present in the headspace. Geosmin, 2-methylenebornane and dimethyl disulfide were tested as pure compounds and showed an inhibitory effect in the activity of protists by the higher number of cysts. The effect correlated with in vivo anti-protist activity, and we believe that the effects of molecules produced in a blend could be cumulative. The anti-protist activity of VCs produced by bacteria is a novel concept and there is yet little evidence in the literature; still, it makes sense in the ecological context, considering that bacteria are the

main prey of protists. This idea could also be a very good reason for bacteria to produce VCs like terpenes in high amounts.

In conclusion, the work presented in this thesis shows the potential of VCs as antimicrobials. More effort needs to be put in understanding the role of VCs in microbial interactions and to elucidate the biosynthetic pathway and genetic control of this important class of natural products, which may well find their way towards clinical or agricultural application in the future.

Micro-organismen in de bodem ondergaan vele complexe interacties. Ze concurreren om voedingsstoffen en ruimte, wat soms resulteert in de productie van moleculen met antimicrobiële activiteit. We zijn inmiddels gewend aan de vele oplosbare antibiotica die geproduceerd worden door micro-organismen, waarvan penicilline ongetwijfeld de bekendste is. Naast antibiotica produceren ze nog veel meer bioactieve natuurstoffen. Wat veel minder bekend is, is dat hieronder ook vele vluchtige verbindingen (*volatile compounds* in het Engels, afgekort VC's) vallen. VC's hebben een laag molecuulgewicht en dampdruk, waardoor ze gemakkelijk verdampen en door bijvoorbeeld grondporiën, water en lucht kunnen diffunderen. Bacteriën die tot de zogenaamde streptomyceten behoren tot de meest voorkomende bodembacteriën en zijn tevens de belangrijkste producenten van bioactieve stoffen, waaronder ook vele vluchtige verbindingen.

In dit proefschrift worden de diversiteit en functies bestudeerd van door *Streptomyces* geproduceerde VC's. De hier beschreven experimenten laten zien dat zelfs zeer kleine VC's als antibiotica kunnen fungeren wanneer ze maar in voldoende grote hoeveelheden worden geproduceerd. Eén van deze moleculen is ammoniak. Ammoniak wordt geproduceerd door de afbraak van het aminozuur glycine, wat vaak wordt geassocieerd met de exudaten van planten en daarmee ook bodemomgevingen. Mutanten die verstoord zijn in de afbraak van glycine kunnen geen grote hoeveelheden ammonia meer produceren en dus ook geen andere bacteriën doden. Dit is niet alleen het geval wanneer streptomyceten gegroeid worden op voedingsbodems, maar hetzelfde zien we als we ze in grond groeien. Vele centimeters boven de grond worden bacteriële groei van *Escherichia coli* nog geremd. Dit geeft aan dat ammoniak in de natuur daadwerkelijk als antibioticum kan functioneren. Het ligt voor de hand dat de productie van dit soort bioactieve stoffen ook een reactie in de getroffen bacteriën teweegbrengt, als een vorm van resistentie. Onze resultaten laten inderdaad zien dat *E. coli* reageert op hoge concentraties ammoniak en andere VC's door het aantal porines in de buitenmembraan te reduceren en zo de import van toxische stoffen in de cel te verminderen.

Streptomyceten produceren ook een breed scala aan terpenen en terpenoïden. Hoewel twee van deze moleculen (met name geosmin en 2-methylisoborneol) al tientallen jaren bekend zijn, blijft hun functie onbekend. Het vluchtige karakter van deze moleculen wijst erop dat ze een rol spelen in het moduleren van interspecies interacties op grote afstand. Dit is bijvoorbeeld bekend van plantenterpenen. Desondanks zijn er slechts enkele voorbeelden van bacteriën die deze moleculen gebruiken voor communicatiedoeleinden. De productie van grote hoeveelheden terpenen onder verschillende groeicondities wijst op hun mogelijke rol als intra- en of interspecies communicatiesignalen. Het werk in dit proefschrift toont aan dat deze moleculen niet essentieel zijn voor de groei en ontwikkeling van *Streptomyces*. In plaats daarvan beïnvloeden ze de ontwikkeling onder omstandigheden van hoge osmolariteit. Mutanten die geen geosmin en 2-methylisoborneolsynthase meer kunnen produceren vertonen een andere morfologie in vloeibare cultures, waarbij de typische pellet structuur van de mycelia wordt verbroken, wat resulteert in mycelia met een open structuur die makkelijk kunnen fragmenteren.

VC's is ook een rol toegedicht als afvalproducten of als een manier om van een overmaat aan koolstof af te komen. Wanneer de gasfase in vloeibare cultures van mutanten werd geanalyseerd, zagen we echter nauwelijks nog VC's. Er is weinig of niets bekend van hoe de productie van VC's wordt gecontroleerd, en het is dan ook belangrijk om de studie van VC's voort te zetten met het doel hun biosynthese, regulatiemechanismen en hun biologische functie beter te begrijpen. De experimenten beschreven in dit proefschrift laten een zeer interessant fenomeen zien, namelijk dat er een sterke toename is van zwavelhoudende VC's als dimethyldisulfide (DMDS) en dimethyl-trisulfide (DMTS) in mutanten die geen 2-methylisoborneol of de gerelateerde 2-methyleenbornaan en 2-methyl-2-bornene meer kunnen produceren. Deze verrassende koppeling tussen de biosynthese van terpenen en van zwavelhoudende VC's wijst op een mogelijk direct verband tussen deze totaal verschillende en niet-gerelateerde biosyntheseroutes, die nog niet eerder waren geconstateerd. Een hypothese die nader moet worden onderzocht is of de methyltransferase welke gecodeerd wordt door het gencluster voor 2-methylisoborneol (2-MIB) ook een rol speelt bij de methylering van

sulfiden, wat nodig is voor de vorming van DMDS en DMTS. Als er geen 2-MIB meer wordt gevormd, zou het enzym zich in plaats daarvan kunnen richten op de productie van DMDS en DMTS. Van vele VC's is aangetoond dat ze antimicrobiële activiteit hebben. In dit kader correleert de stimulering van de productie van diverse sulfide VC's sterk aan de verhoogde antischimmelactiviteit van mutanten die geen 2-MIB kunnen produceren. Het antischimmeleffect van dimethyldisulfide en dimethyltrisulfide is algemeen bekend en dat is verdere validatie van de verkregen resultaten. We lieten daarnaast zien dat de VC's ook bioactiviteit hebben tegen protisten, de meest voorkomende predatoren van bacteriën in de bodem. Door *Streptomyces* geproduceerde VC's induceren de vorming van cysten in protisten, met name wanneer een mengsel van VC's aanwezig is in de gasfase boven de cultures. Geosmin, 2-methyleenbornaan en dimethyldisulfide werden ook getest als zuivere stoffen, geproduceerd door organische synthese, wat hun remmende effect op de proliferatie van protisten bevestigde. Deze observaties stroken met de in vivo anti-protistactiviteit, waardoor we kunnen veronderstellen dat deze moleculen cumulatief werken in termen van hun bioactiviteit. Hoewel de anti-protistactiviteit van bacteriële VC's een nieuw concept is, lijkt dit vanuit ecologisch perspectief logisch, aangezien bacteriën de belangrijkste prooi zijn van protisten. Dit zou tevens een extra reden zijn voor bacteriën om zo'n breed scala aan VC's te produceren.

Concluderend, het werk gepresenteerd in dit proefschrift toont het potentieel van VC's aan als antimicrobiële middelen. Meer onderzoek is noodzakelijk om de rol van VC's in microbiële interacties beter te begrijpen en om hun biosyntheseroute en genetische controle verder op te helderen. Deze belangrijke klasse van verbindingen zal in de toekomst wellicht haar weg gaan vinden naar klinische of agrarische toepassingen.

Los microorganismos viven en hábitats complejos como el suelo, en el cual participan en interacciones intrincadas y dinámicas. Compiten por nutrientes o espacio, por lo que la producción de compuestos antibióticos resulta en una habilidad conveniente. Las moléculas con actividad antibiótica tienen una amplia gama de estructuras incluyendo compuestos volátiles (VC por sus siglas en inglés). Los compuestos volátiles tienen un bajo peso molecular y una baja presión de vapor que les permite evaporarse y difundirse a través de diferentes superficies como los poros del suelo, el agua y el aire. Durante este trabajo de doctorado se estudió la diversidad y las funciones de dichos compuestos volátiles. *Streptomyces* es uno de los géneros de bacterias más abundantes en el suelo, y son los principales productores de compuestos bioactivos, incluidos los compuestos volátiles. Este trabajo muestra cómo incluso moléculas volátiles muy pequeñas, como el amoníaco, pueden actuar como antibióticos cuando se producen en grandes cantidades, modificando el entorno. El amoníaco se deriva del metabolismo de los aminoácidos, en este caso particular de la glicina, que puede considerarse como una molécula abundante en el suelo como exudado de las plantas. La producción de un compuesto bioactivo induce una respuesta por parte de las bacterias perceptoras. En este trabajo se observó cómo *Escherichia coli* responde a las altas concentraciones de amoníaco y otros VCs reduciendo la expresión de sus principales porinas de membrana externa para minimizar la cantidad de compuestos tóxicos que ingresan a la célula.

Los estreptomicetos son excelentes productores de compuestos terpenoides dominando su atmósfera. Dos de estas moléculas se conocen desde hace décadas: la geosmina y el 2-metilisoborneol, sin embargo, su función sigue siendo desconocida. El carácter volátil de tales moléculas podría sugerir que participan en interacciones a "larga distancia". Se conoce que los terpenos juegan un papel en la comunicación de las plantas; sin embargo, pocos ejemplos han sido descritos donde los terpenos producidos por bacterias juegan un rol en la comunicación entre especies.

La abundante producción de terpenos en diferentes condiciones de crecimiento sugiere que estas moléculas tienen un rol como señales intra

o inter especies. El trabajo en esta tesis muestra que estas moléculas no son vitales para el crecimiento y desarrollo de *Streptomyces*, pero afectan el desarrollo cuando se cultivan en condiciones de estrés como por ejemplo un medio de cultivo con elevada osmolaridad. Los mutantes incapaces de producir terpenos, se ven afectados morofológicamente, en particular, aquellos incapaces de producir geosmina y 2-metilisoborneol, desarrollan pellets con micelios más abiertos o fragmentados.

Los VCs también se han sugerido como productos de desecho o como una válvula de liberación de carbono. Sin embargo, al analizar la atmósfera del mutante incapaz de producir compuestos terpenoides volátiles, el número de VCs encontrados fue casi nulo. El mecanismo que regula la producción de estas moléculas es desconocido, por lo cual es de gran importancia continuar el estudio de los VCs para comprender mejor su biosíntesis y regulación así como su función. Este estudio muestra como la eliminación de los principales terpenos producidos por *Streptomyces*: 2-metilisoborneol, 2-metilenobornano y 2-metil-2-borneno se correlaciona con la sobreproducción de compuestos sulfurados. Este resultado sugiere un posible vínculo (hasta ahora desconocido) entre estas vías completamente diferentes y no relacionadas. Nuestra hipótesis sugiere que la metiltransferasa codificada por el gen adyacente a la metilisoborneol sintasa, responsable de la producción de 2-metilisoborneol, también puede participar en la metilación de sulfuros, dando lugar al dimetil disulfuro (DMDS) y dimetil-trisulfuro (DMTS).

La sobreproducción de DMDS y DMTS se correlaciona con una mayor actividad antifúngica mostrada por el mutante incapaz de producir 2-metilisoborneol. El efecto antifúngico del DMDS y el DMTS es conocido, lo cual confirma los resultados obtenidos en este trabajo.

El efecto antimicrobiano de los VCs también se observó contra los principales depredadores de bacterias, los protistas. Los VCs liberados por *Streptomyces* indujeron la formación de quistes en las especies de protistas estudiadas, particularmente frente a las cepas de *Streptomyces* capaces de emitir una veriedad de terpenos. La geosmina, el 2-metilenbornano y el disulfuro de dimetilo se probaron como compuestos puros y mostraron un efecto inhibitorio en la actividad de los protistas

induciendo la formación de quistes. El efecto concuerda con la actividad anti-protista in vivo, por lo cual se cree que los efectos de las moléculas producidas en una mezcla podrían ser acumulativos. La actividad anti-protista de los VCs producidos por bacterias es un concepto novedoso por lo cual existe poca evidencia en la literatura. Sin embargo, resulta lógico pensar que en un contexto ecológico las bacterias son la principal presa de los protistas por lo que la producción de compuestos volátiles antibióticos representaría una ventaja para la supervivencia de las bacterias.

En conclusión, el trabajo presentado en esta tesis muestra el potencial de los compuestos volátiles como antimicrobianos. Se necesita un mayor esfuerzo para comprender el papel de los VCs en las interacciones microbianas y para dilucidar la biosíntesis y el control genético de esta importante clase de productos naturales, con potencial para la aplicación clínica o agrícola en el futuro.

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CURRICULUM VITAE

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LIST OF PUBLICATIONS

CURRICULUM VITAE

Mariana Avalos Garcia was born in Cuautla, Morelos, Mexico in May 26th, 1986. She graduated from the College of Sciences and Humanities (Colegio de Ciencias y Humanidades) in Mexico City in 2004. From 2005-2009 she performed her undergraduate studies at the Faculty of Chemistry from the National Autonomous University of Mexico (UNAM) and in 2010 she obtained her Bachelor diploma in Pharmaceutical and Biological Chemistry. From 2010-2012 she carried out her MSc project "Characterization of secondary metabolites with antibiotic activity from *Streptomyces*" under the supervision of Prof. Dr. Sergio Sánchez Esquivel at the Biomedical Research Institute in Mexico City. In January 2013 she obtained her MSc degree with specialization in Biochemistry from the National Autonomous University of Mexico (UNAM). In 2013 she obtained a personal grant from the National Council of Science and Technology (CONACyT) to perform her PhD at Leiden University in The Netherlands under the supervision of Prof. Dr. Gilles van Wezel. The work done as a PhD student is presented in this thesis. Mariana is currently working as a postdoc researcher with Prof. Dr. Jos Raaijmakers studying the role of Oxalotrophic bacteria in disease suppressive soils at the Microbial Ecology department from the Netherlands Institute of Ecology (NIOO-KNAW).

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Sánchez S, Chávez A, Forero A, García-Huante Y, Romero A, Sánchez M, Rocha D, Sánchez B, **Avalos M**, Guzmán-Trampe S, Rodríguez-Sanoja R, Langley E, Ruiz B. (2010). Carbon source regulation of antibiotic production. *J Antibiot* (Tokyo). **63**:442-59

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Cover illustrations: Henk van Ruitenbeek – https://henkvanruitenbeek.nl/

Design: Mariana Avalos Garcia and Henk van Ruitenbeek

Printing: IPSKAMP printing / https://www.ipskampprinting.nl/