Universiteit
Leiden
The Netherlands

Volatile compounds from Actinobacteria as mediators of microbial interactions

Avalos Garcia, M.

Citation
Avalos Garcia, M. (2019, September 24). *Volatile compounds from Actinobacteria as mediators of microbial interactions*. Retrieved from https://hdl.handle.net/1887/78556

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/78556

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/78556 holds various files of this Leiden University dissertation.

Author: Avalos Garcia, M.
Title: Volatile compounds from Actinobacteria as mediators of microbial interactions
Issue Date: 2019-09-24

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.