Volatile compounds from Actinobacteria as mediators of microbial interactions

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CHAPTER 2

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

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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).

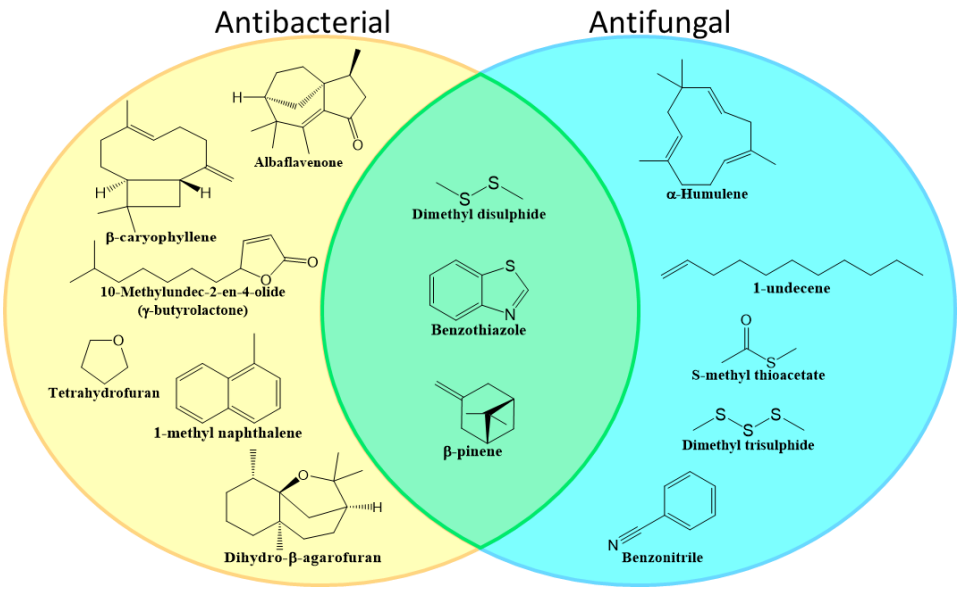

Figure 1. Examples of MVCs with antimicrobial activity

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).

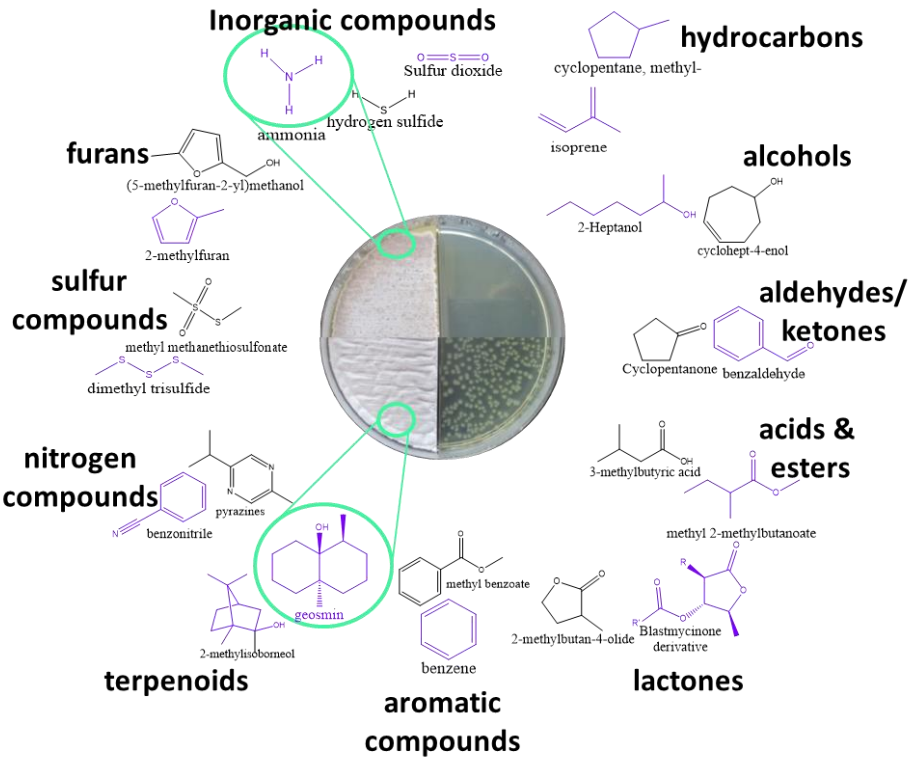

Figure 2. Chemical classes of volatile compounds released by bacteria. Highlighted in purple are the VCs identified in *Streptomyces* strains. Zoomed compounds in green correspond to the widespread terpene geosmin (non-active as antibiotic) and ammonia that when produced in high concentrations by some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

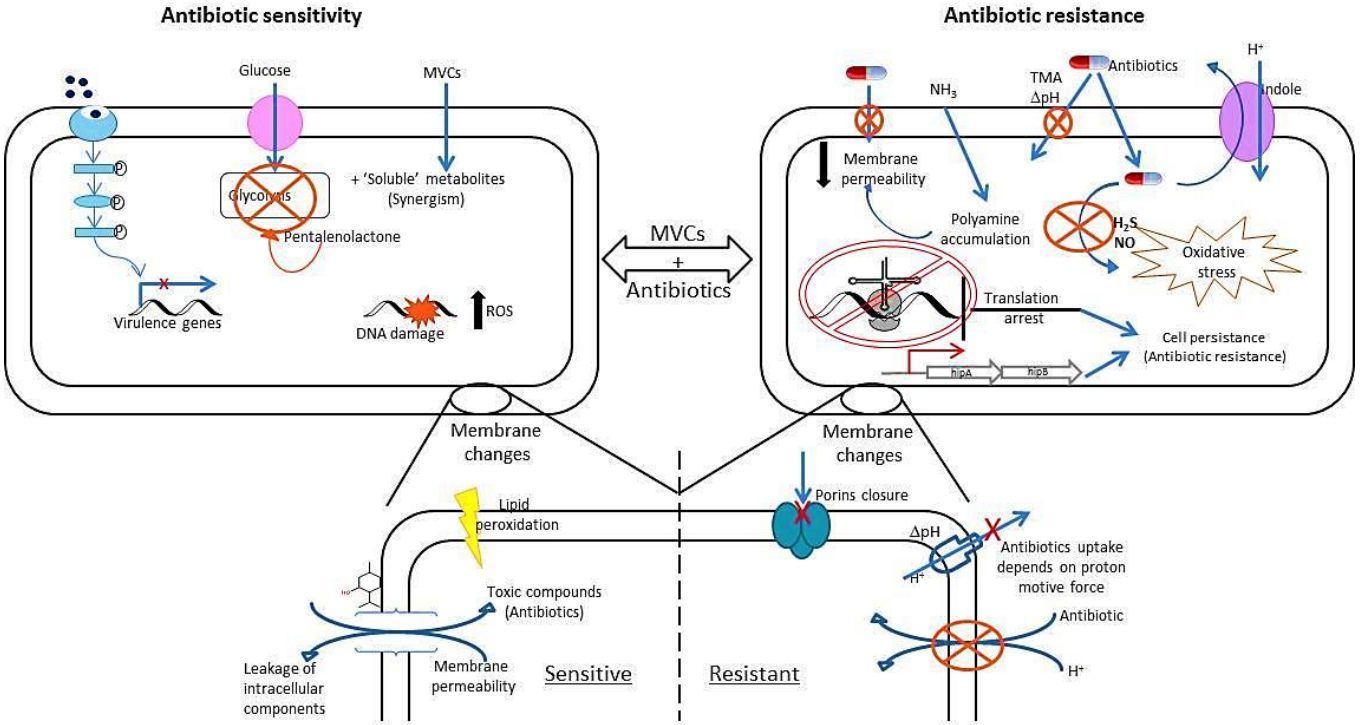

Figure 3. MVCs modes of action as antimicrobials and modulators of antibiotic resistance

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.

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