

## REVIEW ARTICLE

# Role of bacterial volatile compounds in bacterial biology

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**One sentence summary:** The present review describes how airborne volatile compounds produced by bacteria can influence bacterial physiology and behavior, which constitutes an unexplored aspect of bacterial interactions.

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## ABSTRACT

Bacterial interactions with neighboring microorganisms via production of small metabolites enable bacteria to respond and adapt to environmental changes. The study of intercellular interactions primarily focused on soluble metabolites, but bacteria also produce and release into their headspace a wide variety of volatile secondary metabolites, the ecological roles of which have generally been overlooked. However, bacterial volatile compounds are known to contribute to interkingdom interactions (plant, fungi and nematodes), and recent studies also identified their at-a-distance influence on bacterial behavior. The present review describes the biological roles of bacterial volatile compounds in inter- and intraspecies bacterial interactions, a new and yet unexplored research area, with potential clinical and industrial applications.

**Key words:** bacterial volatile compounds; antibiotic resistance; biofilm; pathogenesis

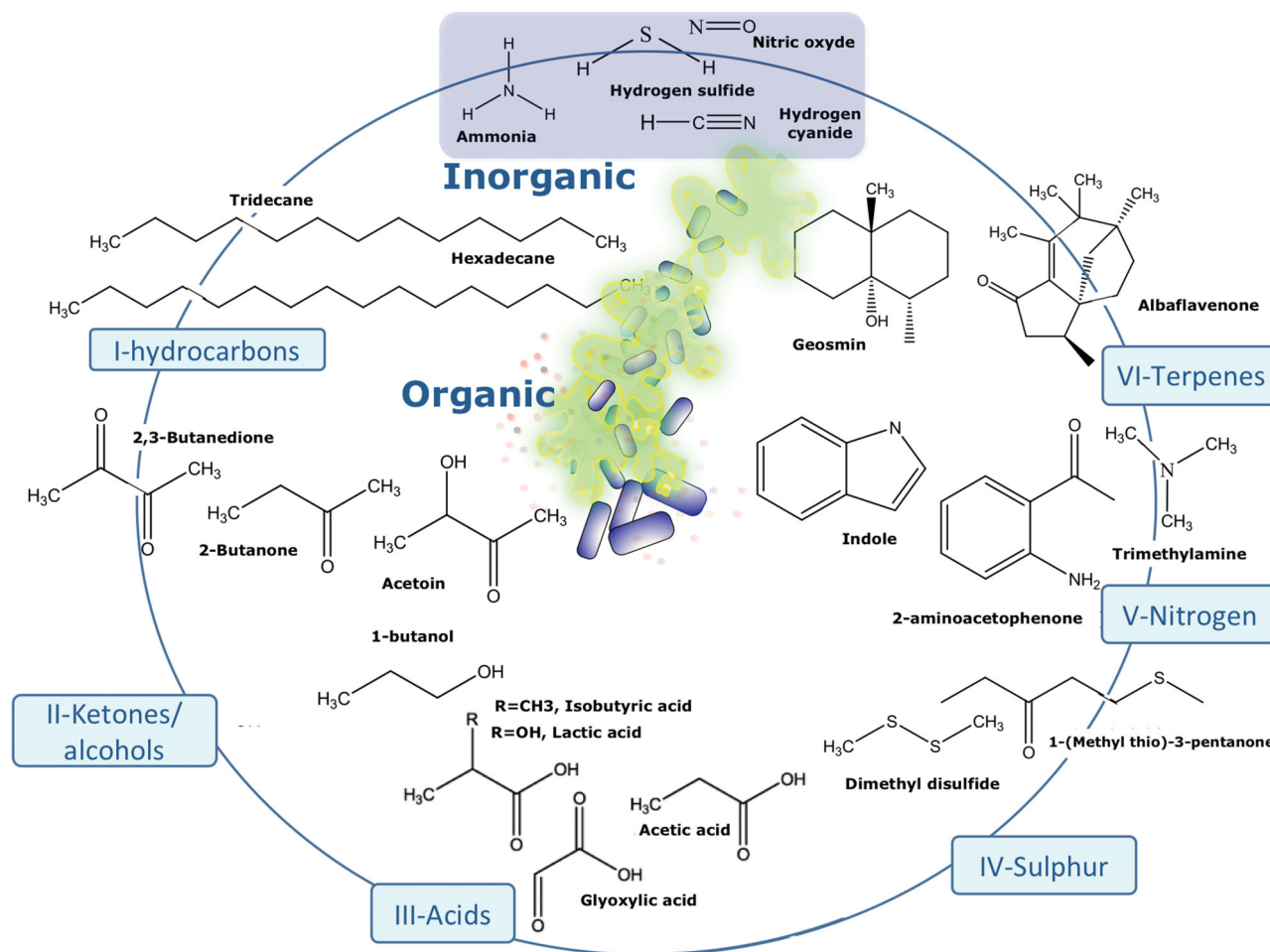
## INTRODUCTION

Many bacterial species coexist in dynamic communities and produce a wide diversity of secondary metabolites as cues potentially involved in competition and cooperation, enabling them to adapt to biotic and abiotic stresses (Hughes and Sperandio 2008; Surette and Davies 2008). Among them, bacteria release molecules of low molecular weight (<300 Da) and high vapor pressure (0.01 kPa at 20°C) that can readily evaporate and diffuse through heterogeneous mixtures of solids, liquids and gasses (Schulz and Dickschat 2007). While more than 1000 bacterial volatile compounds (BVC) have thus far been described, the diversity of bacterial environmental niches suggests that this could be a gross underestimation of the diversity of volatile or-

ganic and inorganic compounds produced by bacteria (Schulz and Dickschat 2007; Kai et al., 2009; Lemfack et al., 2014). BVC are generally produced by catabolic pathways, including glycolysis, proteolysis and lipolysis, and belong to different chemical classes (Fig. 1) (Schulz and Dickschat 2007; Penuelas et al., 2014). While detection and quantification of BVC as attractive or repellent odors and aromas are of great interest in food and cosmetic bioprocesses, BVC also contribute to bacterial ability to interact with their own environments. Indeed, several BVC were shown to influence growth, differentiation, stress resistance and/or behavior in fungi, plants or invertebrates (Kai et al., 2009; Kai and Piechulla 2010; Effmert et al., 2012; Wenke et al., 2012; Davis et al., 2013). Beyond such interactions with a wide range of eukaryote

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**Figure 1.** Chemical classes of volatile compounds released by bacteria. Synthetic representation of volatile compounds able to influence at-a-distance bacterial behavior and discussed in the review. The structure of biologically active organic volatile compounds are regrouped in six chemical classes, including hydrocarbons (I), ketones/alcohols (II), acids (III), sulfur compounds (IV), nitrogen-containing compounds (V) and terpenes (VI). Inorganic compounds are grouped in the gray box in the upper part of the diagram.

organisms, recent studies have revealed the role of BVC in bacterial interactions in various environments including soil, animal and plant microbiota, and biofilms. The aim of this review is to present our current knowledge of the impact of volatile molecules released by bacteria upon bacterial behavior, including their effect on the host during infectious processes, and potential applications in clinic or industry.

## NATURE AND BIOSYNTHESIS OF BVC

Bacteria produce and emit highly diverse inorganic and organic volatile compounds. In this section, we will present different chemical subclasses and focus on biologically active BVC (Fig. 1). For more details on the nature and biosynthesis of BVC, see Schulz and Dickschat (2007).

### Organic compounds

Bacterial volatile compounds of organic origins include several chemical classes such as fatty acid derivatives (hydrocarbons, ketones, alcohols), acids, sulfur and nitrogen-containing compounds and terpenes.

### Hydrocarbons

Linear-chained hydrocarbons likely derived from products of the fatty acid biosynthetic pathway via two routes either the ‘elongation–decarboxylation’ or the ‘head-to-head condensation’ pathways (Ladygina, Dedyukhina and Vainshtein 2006). While short-chain alkanes (from decane to tetradecane) are occasionally found in microbes, longer hydrocarbons such as hexadecane are particularly abundant in cyanobacteria, which are also known for their ability to synthesize branched hydrocarbons (Tellez, Schrader and Kobaisy 2001; Ladygina, Dedyukhina and Vainshtein 2006).

### Ketones/alcohols

Methyl ketones are produced via decarboxylation of fatty acids. Acetoin (3-hydroxy-2-butanone) and its oxidized form 2,3-butanedione are derived from pyruvate fermentation under anaerobic conditions (Ryu et al., 2003). Acetolactate synthase catalyzes the condensation of two pyruvate molecules into acetolactate, which is decarboxylated to form acetoin, and a further oxidative step leads to 2,3-butanedione formation. The specific environmental and cell-life-cycle conditions that regulate acetoin and 2,3-butanedione syntheses are still unclear.

Long-chain aliphatic alcohols (i.e. 1-octanol, 1-decanol and 1-dodecanol) are commonly associated with Enterobacteriaceae; they are produced through  $\beta$ - or  $\alpha$ -oxidation of fatty acid derivatives, and thus, their concentration are markedly increased from cultures supplemented with fatty acids (Hamilton-Kemp *et al.*, 2005). In contrast, Proteobacteria and Firmicutes produce short-chain alcohols (i.e. 2,3-butanediol) under low-oxygen conditions (i.e. sputum of cystic fibrosis patients or bacteria grown in the rhizosphere) (Farag, Zhang and Ryu 2013; Whiteson *et al.*, 2014) to provide an alternative electron sink for the regeneration of NAD<sup>+</sup> when aerobic respiration is limited from pyruvate (Ramos *et al.*, 2000; Xiao and Xu 2007). *In vitro* production of 2,3-butanediol is also favored in the presence of sucrose as major nutrient in growth media (Ryu *et al.*, 2004). Microbial-derived short-chain-branched alcohols such as 3-methyl-1-butanol and 2-methyl-1-butanol are produced by enzymatic conversion of branched chain amino acids i.e. leucine and isoleucine via the Ehrlich pathway (Marilley and Casey 2004).

### Acids

Organic acids are less abundant compared to ketones and alcohols in bacteria (Schulz and Dickschat 2007). Nevertheless, several short-chain fatty acids have been described as released from bacteria such as acetic, propionic or butyric acids (Schulz and Dickschat 2007). These are saturated aliphatic organic acids and represent major by-products of anaerobic metabolism; indeed, they are formed during bacterial fermentation of carbohydrates and are especially abundant in human gut. Glyoxylic acid is produced via the glyoxylate shunt, an anaplerotic pathway of the tricarboxylic acid cycle that allows cells to utilize simple carbon compounds in case of glucose limitation (Lorenz and Fink 2002). However, glyoxylic acid is a hub metabolite that could be derived from several other metabolic pathways including ethylene glycol or amino acids metabolism (i.e. glycine, serine, threonine, arginine and proline metabolism) (Muckschel *et al.*, 2012).

### Sulfur compounds

Sulfur compounds are well known to contribute to fermented foods aroma, including cheese and wine. The biogenesis of methionine-derived volatiles, such as dimethyl sulfide and 1-(methyl thio)-3-pentanone, is often mediated by bacteria. The pathway for the formation of most volatile sulfur compounds involves cleavage of 3-dimethylsulfoniopropionate, generated by higher plants and marine algae from L-methionine (Stefels 2000).

### Nitrogen-containing compounds

Trimethylamine (TMA) is a tertiary volatile amine that contributes to the odor of spoiling fish and is produced upon biogenic reduction of trimethylamine oxide (TMAO). TMAO can be used as an alternate electron acceptor in anaerobic conditions but the bacterial conversion from TMAO to TMA occurred in aerobic and anaerobic conditions. Both compounds (TMA and TMAO) are especially abundant in fish but also in animal and human intestines.

2-amino-acetophenone (2-AA) is an aromatic compound responsible for the grape-like odor of *Pseudomonas aeruginosa* cultures and could be used to detect *P. aeruginosa* infections (Que *et al.*, 2013). The synthesis of 2-AA, a non-4-hydroxy-2-alkylquinoline volatile molecule, is controlled by MvFR (multiple virulence factor regulator) via the regulation of the *pqs*ABCD operon and requires *pqsA* and *pqsD* genes (Kesarwani *et al.*, 2011; Que *et al.*, 2013); it is the first quorum sensing (QS)-regulated volatile molecule identified. 2-AA is also produced

by *Burkholderia thailandensis* or the myxobacterium *Chondromyces crocatus*, accompanied in this latter case by the related 2-aminobenzaldehyde and methyl anthranilate. 2-AA might also serve as the biosynthetic precursor for the production of 4-methylquinoline, produced concurrently in the marine species *Streptomyces caviscabies* and in *Myxococcus xanthus* (Dickschat *et al.*, 2004).

Indole production has been described in 85 bacterial species, including 14 Gram-positive bacteria (Lee and Lee 2010). Its biosynthetic pathway has been well characterized in *Escherichia coli*, where it depends on tryptophanase encoded by *tnaA* that can reversibly convert tryptophan into indole, pyruvate and ammonia. Indole biosynthesis is regulated by several environmental factors such as presence of extracellular tryptophan, cell population density, catabolite repression, temperature or pH (Lee and Lee 2010).

### Terpenes

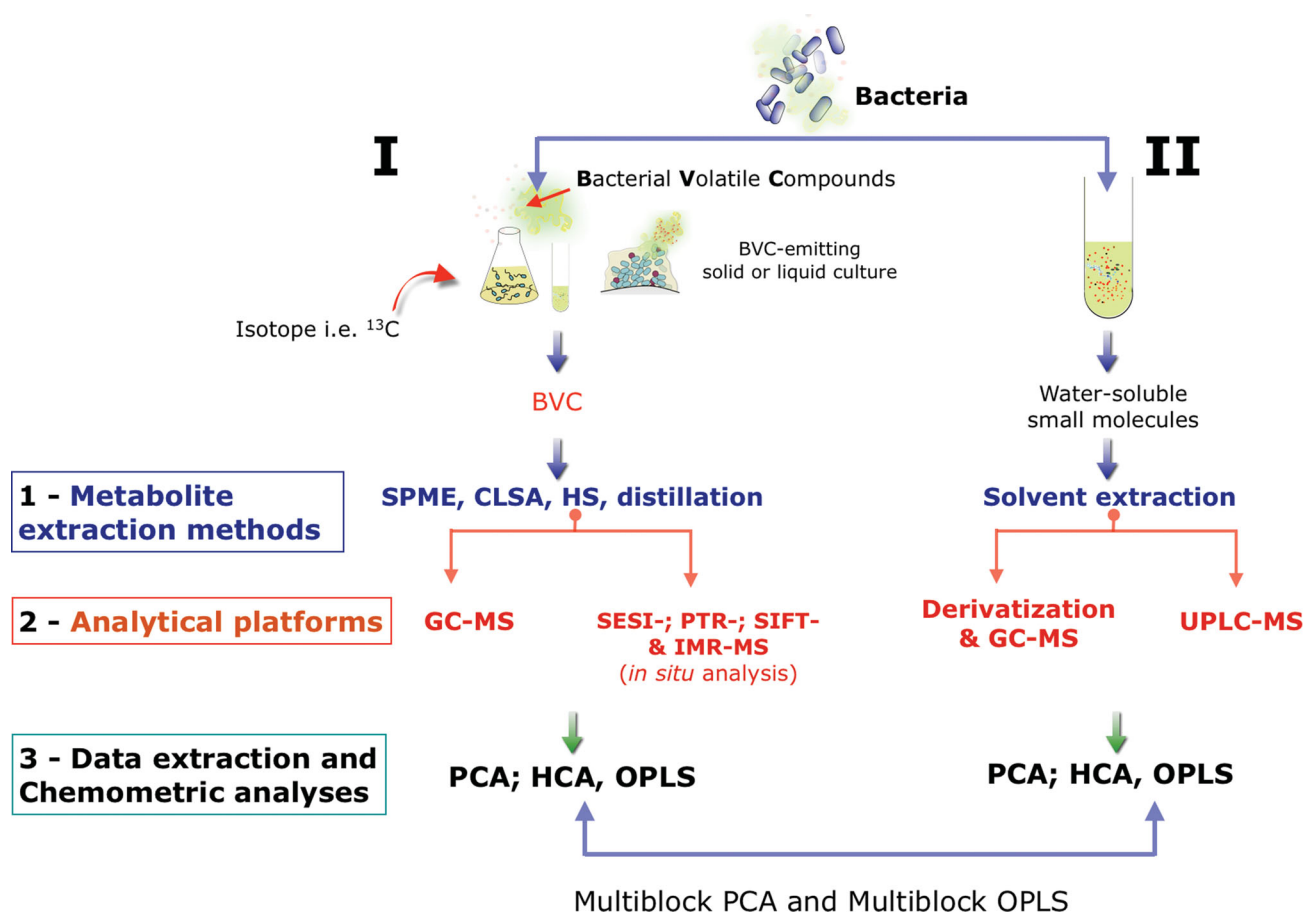
In nature, terpenes are derived from the terpene building units dimethylallyl pyrophosphate and isopentenyl pyrophosphate, which can arise either from mevalonate pathway or deoxyxylulose phosphate pathway. Only monoterpenes (C<sub>10</sub>), sesquiterpenes (C<sub>15</sub>) and their derivatives or degradation products have been reported from bacterial volatile blends (Schulz and Dickschat 2007). In particular, the earthy odorant geosmin and antibiotic albaflavone are both terpenoid compounds produced by bacteria; while geosmin, a degraded sesquiterpene, is produced in several bacterial species, albaflavone was first isolated from *Streptomyces albidoflavus* and is exclusively found in *Streptomyces* (Schulz and Dickschat 2007).

### Inorganic compounds

Bacteria also emit inorganic volatile compounds such as nitric oxide (NO), hydrogen sulfide (H<sub>2</sub>S), ammonia or hydrogen cyanide (HCN). While most H<sub>2</sub>S-producing bacteria generate this gas through degradation of cysteine (by orthologs of mammalian enzymes), NO is produced mostly from L-arginine by nitric oxide synthases, homologs to mammalian enzymes (Mattila and Thomas 2014). Ammonia is produced from the metabolism of peptide and amino acid, especially via L-aspartate catabolism (Bernier *et al.*, 2011); indeed, the conversion of aspartate to fumarate by an aspartate ammonia lyase released ammonia as a by-product in *E. coli* K12 (Bernier *et al.*, 2011). Finally, HCN has been detected as emitted from a few bacterial species, and its production is for now restricted to some species of *Pseudomonas*, *Chromobacterium* and *Rhizobium*. HCN biosynthesis is catalyzed by HCN synthase, encoded by *hcnABC* genes, which forms HCN and CO<sub>2</sub> from glycine. The HCN production usually occurs at the end of the exponential phase and under low oxygen concentration (involvement of the anaerobic regulator ANR); moreover, regulation of cyanogenesis through QS appears to be strain-specific since QS regulators are required in *P. aeruginosa* PAO1 or *Chromobacterium violaceum* CVO but not in *Pseudomonas fluorescens* 2P24 (Blom *et al.*, 2011).

## METHODS TO EXTRACT AND ANALYZE BACTERIAL VOLATILE COMPOUNDS

Analysis of BVC is an arduous task due to the wide range of volatiles abundance and the complexity of mixtures and/or matrices in which they are usually present and from which they are released. The standard approach for robust and reproducible



**Figure 2.** A workflow showing key steps for the analysis of BVC (I) and water soluble primary metabolites serving as volatile precursors (II). Bacteria were inoculated into a liquid or solid culture medium, (i.e. a broth or agar). (1) Volatiles extraction using either SPME, CLSA, headspace (HS) or distillation in parallel to solvent extraction of water soluble low molecular weight metabolites. (2) BVC chemical analysis using either offline GC-MS or the *in situ* analysis, multi-capillary column- ion-molecule reaction (IMR-MS), SIFT-MS, SESI-MS and proton-transfer reaction MS (PTR-MS); for analysis of water soluble metabolites, an ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) or GC-MS following derivatization is adopted. (3) Volatile and non-volatile metabolite data are extracted and analyzed using chemometric analyses including PCA, hierarchical cluster analysis (HCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA); for combining metabolites, data derived from the two different platforms I and II, multiblock (PCA) and multiblock (OPLS) should be used.

BVC analysis therefore includes three key aspects: BVC extraction, analysis and identification (Fig. 2).

### Extraction of BVC

A variety of systems have been developed to capture volatiles released from bacteria, and the number of detectable volatiles generally increases with the diversity and sensitivity of the extraction methods used, and the different trapping materials (Wenke et al., 2012). While the closed-loop stripping apparatus (CLSA) is widely used to pre-concentrate volatiles from aqueous samples (Meruva, Penn and Farthing 2004), the solid-phase microextraction (SPME) is a rapid sample preparation method allowing to combine sampling, extraction, concentration and introduction in an analytical instrument into a single solvent-free step (Goupry et al., 2000; Marilley and Casey 2004). However, fiber coatings in SPME limit the sensitivity of the analysis by preferentially absorbing or excluding particular molecules based on polarity or size. Since use of SPME only provides discontinuous measurement, methods such as static diffusive passive sampling ought to be considered for epidemiological day- or week-long studies (Matysik, Herbarth and Mueller 2009). Finally, direct and real-time gas sampling of bacterial headspace

can be performed using secondary electrospray ionization-mass spectrometry (SESI-MS), which has several advantages, such as a sensitive detection limit and the potential for high-throughput sample analysis (Zhu and Hill 2013). SESI-MS has been used for detection of human breath vapor (Martinez-Lozano et al., 2009) and clinically relevant pathogens (Zhu et al., 2010).

### Analysis of BVC

Standard approach to analyze BVC profiles relies on gas chromatography coupled with mass spectrometry (GC-MS), characterized by a powerful separation capacity and highly sensitive detection performance; indeed, recent GC-MS software improves the detection limit thanks to adjacent peak deconvolution and background subtraction (Farg et al., 2006; Farg and Wessjohann 2012). Optimal GC-MS analysis includes the use of the time-of-flight (TOF)-MS detector, especially when it is coupled with 2D column gas chromatography (GC×GC). The high degree of separation afforded by the two columns and the high sensitivity of TOF-MS are suitable solutions for analysis of trace volatile components (Meruva, Penn and Farthing 2004; Salvador et al., 2013). Nevertheless, the GC-MS-based method often requires pre-concentration and does not allow rapid *in situ*

analysis. Selected-ion flow-tube mass spectrometry (SIFT-MS), ion-mobility spectrometer (IMS) and electronic noses (eNoses) are therefore often preferred for real-time analysis of volatiles (Lirk et al., 2003; Dolch et al., 2012a). Indeed, SIFT-MS and IMS are compatible with *in-situ* real-time measurement of BVC (Bos, Sterk and Schultz 2013), whereas eNoses rely on pattern recognition (Rock, Barsan and Weimar 2008). IMS and SIFT-MS are very promising analytical methods, with short measurement times performed in ambient conditions and high sensitivity (Tiebe et al., 2009; Kunze et al., 2013). For instance, the dynamics of bacterial species growing in mixed populations was determined *in situ* via SIFT-MS monitoring of volatile compounds present in the community headspace (Sovova et al., 2013), suggesting potential applications for metabolic engineering, bioprocess control and health care. However, detection of volatiles from clinical samples and/or tissues has been only rarely reported and was mainly performed in a targeted manner on a selected volatile or group of volatiles (Whiteson et al., 2014).

### Identification of BVC

Identification of compounds present in a volatile sample can be realized by comparing mass spectra with spectra from different databases like the Wiley or NIST libraries. However, these libraries tend to mislead the inexperienced user, since the closest hit within the library might uncritically be taken as a positive identification. A database of microbial volatiles, called mVOC, is now available online at (<http://bioinformatics.charite.de/mvoc>). This database lists almost 1000 microbial volatile compounds and contains information about their emitting organisms, the biosynthetic pathways and their biological effects (Lemfack et al., 2014). This new database provides different possibilities for the user to search for a volatile compound (PubChem-ID, name, molecular formula, species, etc.). In case of unknown volatiles, an isolation step is required and followed by spectral analysis using MS and nuclear magnetic resonance (NMR) spectroscopy.

Multivariate statistical methods, broadly classified into two categories—unsupervised and supervised—are required to estimate potential differences between BVC released from various species, especially when comparing several samples. Use of supervised methods such as partial least squares (PLS) or PLS discriminant analysis allows comparing samples analyzed with different machines and not in the same time. However, extreme care should be taken in setting up these models, since such analyses often tend to over fit data, leading to odd results (Farag 2014). Finally, a correlation-based multiblock hierarchical principal component analysis (MB-PCA) and MB-PLS enable the comparison of data sets derived from different analytical approaches i.e. NMR versus MS (Fig. 2).

### Challenges in chemical characterization of BVC

Detected BVC profiles are often quite complex and it is therefore important to run blank controls to ascertain which signals correspond to volatile compounds released by bacteria or by the culture medium itself (Tait et al., 2014). Fortunately, most modern GC-MS commercial data processing and software are able to subtract background signals due to media from bacterial data files, significantly simplifying the chromatogram.

Another major challenge in BVC analysis lies in frequent result discrepancies due to genomic variation between bacterial species subtypes, altering the metabolic pathway leading to volatile compounds (Tait et al., 2014). In addition to medium

composition, variations in metabolite availability according to growth phase, in oxygen concentration or initial inoculum influence the production and emission of BVC (Bos, Sterk and Schultz 2013; Broekaert et al., 2013; Kunze et al., 2013). Analysis of *in vitro* and *in vivo* BVC profiles revealed that the host response can also affect BVC emission (Bos, Sterk and Schultz 2013). For instance, comparison of *in vitro* and *in vivo* volatile profiles of several lung pathogens showed that only 25–34% of peaks are shared between the two volatile fingerprints detected using SESI-MS (Zhu et al., 2013).

## IMPACT OF BVC ON BACTERIAL GROWTH AND STRESS RESISTANCE

### Altering growth and differentiation

Following the demonstration that BVC can influence fungi growth and differentiation (Kai et al., 2009; Effmert et al., 2012), several groups investigated the impact of BVC produced by soil-associated bacteria on bacterial differentiation and growth. In *Streptomyces* spp., emission of headspace geosmin, a terpenoid compound frequently produced in this bacterial genus, correlates with sporulation in *S. albidoflavus* AMI 246 (Scholler et al., 2002), whereas albaflavenone, an antibiotic volatile sesquiterpene ketone, exhibits antibacterial activity against *Bacillus subtilis* (Gurtler et al., 1994). Similarly, emission of dimethyl disulfide from two rhizospheric bacteria, *P. fluorescens* and *Serratia plymuthica*, shows bacteriostatic effects against two plant bacterial pathogens *Agrobacterium tumefaciens* and *Agrobacterium vitis* (Dandurishvili et al., 2011). In addition to soil environments, animal intestines are also characterized by a mixture of solid, liquid and gas phases. Volatile short-chain fatty acids produced through fermentation by anaerobic intestinal bacteria such as *Veillonella* spp. inhibit growth of the enteropathogens *Salmonella enteritidis*, *Salmonella typhimurium*, *E. coli* and *P. aeruginosa*, suggesting a role for these BVC in control of enteropathogen colonization in the intestinal tract (Hinton and Hume 1995). Similarly, co-cultures of *Bacteroides fragilis*, a commensal member of the intestinal flora, and *Clostridium perfringens*, a pathogen causing food poisoning, result in inhibition of *C. perfringens* sporulation (Wrigley 2004). This effect can be mainly attributed to two short-chain volatile fatty acids, isobutyrate and succinate, produced by *B. fragilis*, inhibiting *C. perfringens* growth and sporulation when used at 100 mM. Although results were produced during experiments in which short-chain fatty acids were used in solution, these metabolites are also volatile, and are commonly detected in the headspace of many bacteria; it is speculated that these volatile compounds play a role in intestinal microbiota resistance to pathogen colonization (Fig. 3).

### Boosting antibiotic and stress resistance

BVC were also reported to modulate the bacterial response to different stresses, including exposure to antibiotics (Heal and Parsons 2002) (Fig. 3). Volatile ammonia released from a bacterial population of high density increases at-a-distance resistance to tetracycline and ampicillin, and decreases resistance to aminoglycosides, in several Gram-negative and Gram-positive bacteria (Bernier et al., 2011). In *E. coli*, upon import through the AmtB channel, ammonia metabolism induces an increase in polyamine synthesis involved in modulation of antibiotic resistance profiles (Bernier et al., 2011). TMA, another volatile compound produced by several Enterobacteriaceae, also modulates bacterial resistance to tetracycline and

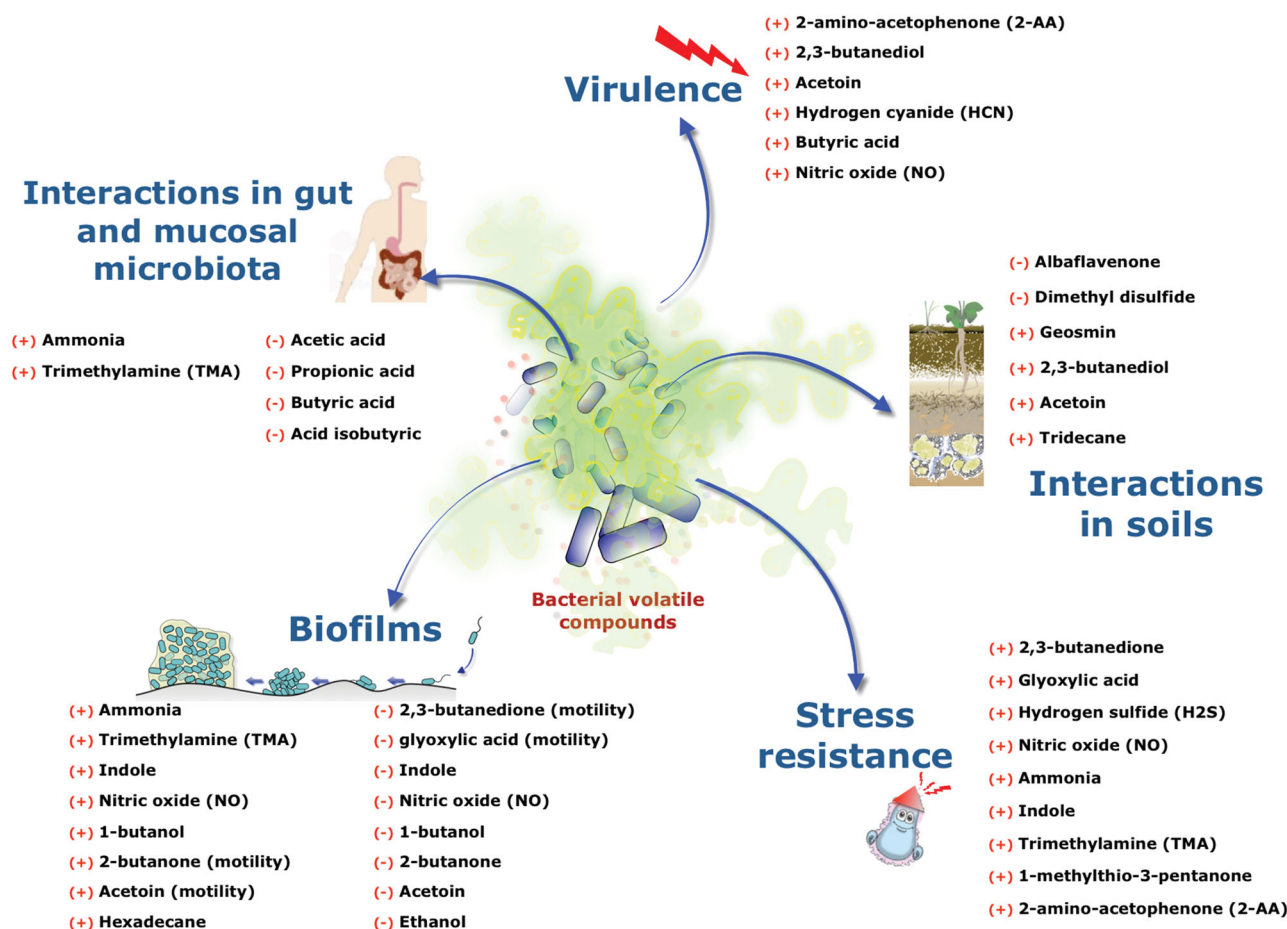


Figure 3. Phenotypic consequences of exposure to BVC in various environments. Volatile compounds released from bacteria are listed according to their positive and/or negative influence on different bacterial phenotypes in various environments. +, stimulation; -, inhibition.

aminoglycosides in all tested bacteria, but increases resistance to chloramphenicol and decreases resistance to oxidative stress (Letoffe et al., 2014). The TMA mode of action was shown to rely on an increase of the pH of the culture medium, affecting proton motive force and membrane permeability (Letoffe et al., 2014). In TMAO-rich environments such as animal gut and tissues (Barrett and Kwan 1985; Bos, Sterk and Schultz 2013), TMA production and pH modifications may have profound ecological consequences, such as contribution to pH homeostasis and an alteration of concentration-dependent bacterial responses to antibiotic stress (Bernier and Surette 2013). Interestingly, at-a-distance medium alkalization (up to pH 8.5), leading to increased *Serratia marcescens* and *Serratia rubidaea* resistance to ampicillin, was also reported (Cepl et al., 2014). While Bernier et al. (2011) did not report an impact on pH medium after aerial exposure to ammonia, differences in experimental conditions could account for these divergences. Metabolic modification of the medium and/or a change in permeability of the target bacterial membrane could be complementary mechanisms involved in the increase of ampicillin resistance in different Gram-negative and Gram-positive bacteria upon exposure to volatile ammonia. As described for ammonia, volatile compounds released from *B. subtilis* also increase ampicillin and tetracycline resistance of *E. coli* (Kim, Lee and Ryu 2013). In the latter case, the authors revealed genes differentially expressed upon *E. coli* exposure to *B. subtilis* volatile compounds. Among them, *hipA*,

encoding an anti-toxin module previously described as mediating persistence, is upregulated and involved in modulation of volatile compound-induced antibiotic resistance (Kim, Lee and Ryu 2013). The volatile-compound-dependent phenotypes identified in this study are regulated by YpdB, the function of which is yet unknown, but it modulates expression of three known transcription factors in addition to *hipA* (Kim, Lee and Ryu 2013). Two volatile compounds produced by *B. subtilis* GB03, namely 2,3-butanedione and glyoxylic acid, were identified as being able to alter *E. coli* antibiotic-resistance profiles in a range of concentration compatible with physiologically relevant conditions (Kim, Lee and Ryu 2013). In *Burkholderia ambifaria*, resistance to aminoglycosides increases upon exposure to volatile compound blends emitted from three strains from various environments (clinical environment, rhizosphere of pea, roots of maize), including 1-methylthio-3-pentanone and 2-aminoacetophenone, whereas ampicillin and tetracycline resistance were not affected (Groenhagen et al., 2013).

Although H<sub>2</sub>S is produced by most bacteria, little is known about its physiological function in non-sulfur microorganisms. Recently, Shatalin and colleagues showed that this gas confers multidrug resistance upon different pathogens (*Bacillus anthracis*, *P. aeruginosa*, *Staphylococcus aureus* and *E. coli*) under aerobic conditions. H<sub>2</sub>S acts by mitigating oxidative stress induced by antibiotic treatment through a dual mechanism involving suppression of the DNA-damaging Fenton reaction and

stimulation of the main antioxidant enzymes (Shatalin *et al.*, 2011). Similarly, endogenous NO protects some Gram-positive bacteria against oxidative stress and antibiotic treatment, while NO and H<sub>2</sub>S gases show synergistic effects (Gusarov *et al.*, 2009).

Indole, a heteroaromatic compound, is able to modulate expression of several genes, including those encoding multidrug exporters. Indole also influences several phenotypes, such as drug resistance in non-indole-producing bacteria (*P. aeruginosa* and *S. enterica*) as well as in indole-producing bacteria. Indeed, while acid resistance is decreased in the presence of indole in *E. coli* (Lee, Jayaraman and Wood 2007), indole enhances drug resistance in *E. coli*, *P. aeruginosa* and *S. enterica* (Hirakawa *et al.*, 2005; Lee *et al.*, 2008; Nikaido, Yamaguchi and Nishino 2008; Lee *et al.*, 2009). However, impact of indole in such phenotypes has been investigated as a soluble compound added to culture medium and, until now, no experimental data have confirmed its role as an airborne signal affecting drug resistance. It should be noted that, although indole is detected in the headspace of several bacterial species (Effmert *et al.*, 2012), it has no impact on tetracycline resistance in recipient bacteria aerielly exposed to it (Letoffe *et al.*, 2014).

Finally, 2-AA, a volatile aromatic compound, was shown to enhance antibiotic tolerance through its influence on accumulation of persister cells (Que *et al.*, 2013). 2-AA is produced and released from *P. aeruginosa* and is responsible for the sweet grape-like odor used to detect *P. aeruginosa* in culture and burn wounds (Cox and Parker 1979). The biological role of 2-AA in virulence and, more recently, in antibiotic tolerance has been described (Kesarwani *et al.*, 2011; Bandyopadhaya *et al.*, 2012; Que *et al.*, 2013). The *P. aeruginosa* *mvfR* mutant impaired in 2-AA production produces less persistent bacteria (persisters) than the wild-type strain, whereas addition of exogenous 2-AA restores its persister formation to wild-type level. This promotion of persister formation occurs via 2-AA-mediated alteration of the translation capacity of the bacterial cell. 2-AA also increases accumulation of persisters in *B. thailandensis* and in the non-2-AA-producer *Acinetobacter baumannii*, two pathogens isolated along with *P. aeruginosa* during co-infection. The impact of 2-AA on producing and non-producing bacteria suggests that volatile 2-AA plays a role in the ability of Gram-negative bacteria to tolerate antibiotic treatment in polymicrobial infections. Since the synthesis, excretion and uptake of QS molecules is a common hallmark in bacteria, and translational machinery is highly conserved, it is speculated that modulation of the translational capacity of the bacterial cell via volatile QS molecules may be a general, far-reaching mechanism that promotes antibiotic tolerance among prokaryotes.

## ROLE OF BVC IN BACTERIAL BIOFILM FORMATION

Recent studies demonstrated the influence of volatile compounds on different stages of the development of bacterial biofilms, from bacterial motility to biofilm dispersal (Fig. 3). Several volatile compounds were shown to influence bacterial motility. Among 12 non-toxic volatile compounds tested, aerial exposure to 1-butanol or indole decreased *E. coli* and *P. aeruginosa* motility, respectively, whereas 2-butanone and acetoin increased *P. aeruginosa* motility (Letoffe *et al.*, 2014). Moreover, *E. coli* swarming, as well as that of *Burkholderia glumae*, *P. aeruginosa* and *Paenibacillus polymyxa*, is negatively affected upon exposure to volatile compounds emitted by *B. subtilis* (Kim, Lee and Ryu 2013). In particular, *E. coli* swarming was shown to be

decreased upon aerial exposure to glyoxylic acid; similarly, 2,3-butanedione also reduced *E. coli* motility (Kim, Lee and Ryu 2013). Interestingly, the reduced motility upon exposure to volatile compounds emitted by *B. subtilis* correlates with downregulation of 30 genes related to chemotaxis and motility in *E. coli* (Kim, Lee and Ryu 2013).

Several others BVC are able to influence bacterial ability to form biofilms. For instance, volatile ammonia induced biofilm formation in *Bacillus licheniformis*, *B. subtilis* and *S. aureus* (Nijland and Burgess 2010; Letoffe *et al.*, 2014). Although soluble indole was previously shown to increase biofilm formation in *Vibrio cholerae*, *P. fluorescens* and *P. aeruginosa*, other studies reported contradictory effects on *E. coli* biofilm formation (Martino *et al.*, 2002; Bansal *et al.*, 2007; Mueller *et al.*, 2009; Lee and Lee 2010). However, when used as an airborne volatile signal, it inhibits biofilm formation in both *E. coli* and *P. aeruginosa* while stimulating *S. aureus* biofilm formation (Letoffe *et al.*, 2014). Several other BVC (1-butanol, 2-butanone, acetoin, ammonia, ethanol, hexadecane, glyoxylic acid and TMA) slightly positively or negatively influence biofilm formation in one or several of the tested bacterial species, including *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis* (Letoffe *et al.*, 2014).

Biofilm life cycle also includes active dispersal events allowing bacteria to revert to planktonic state. At low concentration, NO can trigger such biofilm dispersal in several Gram-negative and positive bacteria. Indeed, addition of low concentration of NO-releasing compounds induces biofilm dispersal of *P. aeruginosa*, *E. coli*, *V. cholerae*, *B. licheniformis*, *S. marcescens*, *Fusobacterium nucleatum* (oral anaerobic bacterium) (Barraud *et al.*, 2009a), *Shewanella woodyi* (Liu *et al.*, 2012), *S. enterica* (Marvasi *et al.*, 2014) and *Neisseria gonorrhoeae* (Potter *et al.*, 2009). A link between NO-induced dispersal and regulation of cyclic di-GMP (c-di-GMP) levels was established in *P. aeruginosa* and *S. woodyi* (Barraud *et al.*, 2009b; Liu *et al.*, 2012). In *P. aeruginosa*, presence of NO stimulates phosphodiesterase activity leading to degradation of c-di-GMP, especially responsible for the switching from biofilm to planktonic lifestyle; this process also requires the chemotaxis transducer BldA. While NO-mediated biofilm dispersal appears to be conserved in many bacterial species, some studies revealed a positive effect of NO on biofilm formation of *Shewanella oneidensis*, *Azospirillum brasilense* or *Vibrio harveyi* (Barraud *et al.*, 2014; Henares, Xu and Boon 2013).

Finally, given that sublethal concentrations of antibiotics increase or decrease biofilm formation in several bacterial species such as *P. aeruginosa* and *E. coli*, we speculate that exposure to BVC affecting antibiotic-resistance profiles could indirectly influence biofilm formation during antibiotic treatment (Bernier and Surette 2013).

## CONTRIBUTION OF BVC TO BACTERIAL PATHOGENESIS

### Promoting bacterial virulence

Although the contribution of volatile molecules produced by plants to plant resistance to pathogens and aggression has been extensively documented (Arimura *et al.*, 2000; Baldwin *et al.*, 2006), BVC can also play a critical role in completion of bacterial pathogenesis by affecting bacterial virulence (Fig. 2). The butanediol fermentation pathway, active in many enterobacterial plant pathogens including vegetable pathogens (*Pectobacterium* spp. and *Dickeya* spp.) and a tree pathogen (*Erwinia amylovora*), leads to production of 2,3-butanediol and acetoin, two widespread volatile end products of fermentation of glucose

(Lopez, Thoms and Rehbein 1975; Huang, Oppermann-Sanio and Steinbuchel 1999). This transformation is mediated by  $\alpha$ -acetolactate synthase encoded by the *budB* gene and is required for *Pectobacterium carotovorum* subsp. *carotovorum* full virulence (Marquez-Villavicencio Mdel et al., 2011a). Expression of the *budAB* operon is controlled by QS, pH and oxygen (Kovacikova, Lin and Skorupski 2005, 2010; Liu et al., 2008; Moons et al., 2011), and the *budAB* genes are among the most highly expressed genes in *P. carotovorum* during bacteria–plant interactions (Marquez-Villavicencio et al., 2011b). Recently, Venkataraman et al. (2014) confirmed that 2,3-butanediol also increased virulence factor production in *P. aeruginosa*; they showed that expression of LasI/R QS regulators was upregulated in the presence of 2,3-butanediol, resulting in an increase in phenazine production and biofilm formation. Two other volatile compounds, namely 2-AA and hydrogen cyanide produced by *P. aeruginosa*, may participate in its virulence. While production of hydrogen cyanide enables *P. aeruginosa* to rapidly paralyze and kill *Caenorhabditis elegans* (Gallagher and Manoil 2001), the QS-regulated volatile compound 2-AA attenuates acute infection by modulating the activity of the virulence regulator MvfR and also contributes to *P. aeruginosa* adaptation to chronic infections by promoting accumulation of *lasR* mutations and bacterial cell long-term survival (Kesarwani et al., 2011).

### Signals that alarm the host and modulate its response

While alteration of the production of virulence factors by BVC modulates bacterial pathogenicity, BVC also impact the course of infection by affecting host cell physiology (Fig. 3). For instance, short-chain fatty acids such as acetic, propionic or butyric acid are bacterial fermentation products relatively abundant in the colonic lumen that control enteropathogen colonization via reduction in bacterial growth or sporulation or via host immunomodulation (Wrigley 2004; Smith et al., 2013). Indeed, short-chain fatty acids can influence colonic homeostasis by regulating the size and function of the colonic regulatory T-cell pool that controls intestinal inflammation by limiting proliferation of effector CD4<sup>+</sup> T cells (Smith et al., 2013). Finally, volatile fatty acids, including butyric acid, produced by three periodontal pathogens (*Porphyromonas gingivalis*, *Prevotella loescheii* and *F. nucleatum*), impaired T- and B-cell proliferation responses and cytokine production and impacted periodontal disease (Kurita-Ochiai, Fukushima and Ochiai 1995). The butyric acid concentration reached 14.4 mM in subgingival plaque from periodontitis and suppressed more than 90% of the lymphocyte proliferation responses when used at 2.5 mM (Kurita-Ochiai, Fukushima and Ochiai 1995).

NO also seems to affect host cell functions (St John et al., 2013). Indeed, NO produced from *B. anthracis* nitric oxide synthase (baNOS) plays a role in *B. anthracis* virulence through different mechanisms according to the stage of the disease. At an early stage, it confers protection against the host-reactive species within macrophages and also contributes to macrophage death through depletion of macrophage bioenergetics via S-nitrosylation of mitochondrial proteins (Shatalin et al., 2008; Chung et al., 2013). Recent work demonstrated that baNOS-derived NO negatively affects host cell functions under hypoxic conditions occurring during later stages of the disease (hypoxic environment of the pre-mortal host) (St John et al., 2013). Indeed, it is involved in toxic effects against non-phagocytic cells through formation of peroxynitrite, which might be potentiated in the presence of serum albumin able to trap volatile NO (St John et al., 2013).

2,3-butanediol has often been considered harmful to a wide variety of biological systems. While 2,3-butanediol produced by *V. cholerae* O1 El Tor shows a suppressive effect upon production of the proinflammatory cytokines IL-8 and TNF- $\alpha$  in epithelial cells, most likely through inhibition of NF- $\kappa$ B signaling (Bari, Song and Yoon 2011), non-2,3-butanediol-producing *P. aeruginosa* responds to 2,3-butanediol by stimulating production of phenazine pyocyanin (Venkataraman et al., 2011). Phenazines are well known for their antimicrobial activity and their toxic effects on eukaryotic cells. Indeed, pyocyanin produced by *P. aeruginosa* and detected in the sputum of infected cystic fibrosis patients has negative effects upon epithelial cells by affecting redox homeostasis (Price-Whelan, Dietrich and Newman 2006). By contrast, 2,3-butanediol emitted from specific bacterial strains of plant growth-promoting rhizobacteria has positive impact on plant biology since it is able to induce systemic resistance in plant *Arabidopsis thaliana* (Ryu et al., 2004). While the volatile tridecane, released from *P. polymyxa*, was shown to be more effective against the biotrophic pathogen *Pseudomonas syringae* pv. *tomato*, volatile 2,3-butanediol and acetoin from *B. subtilis* triggered a stronger induced systemic resistance (ISR) against *P. carotovorum* subsp. *carotovorum*. Moreover, direct application of 2,3-butanediol failed to elicit ISR against *P. syringae* pv. *tabaci*, but induced the ISR response to *P. carotovorum* subsp. *carotovorum* (Han et al., 2006). Previous transcriptome and proteome analysis of *A. thaliana* exposed to bacterial volatiles revealed that three major plant defense signaling pathways, including salicylic acid, jasmonic acid and ethylene, mediate these effects (Kwon et al., 2010; Zhang et al., 2010). Pretreatment of ethylene-insensitive plants with 2,3-butanediol did not elicit ISR, suggesting that ethylene is a major player in this interaction (for a more detailed overview of bacteria–plant interactions mediated by volatile compounds, see Farag 2014).

### Luring invertebrate hosts with bacterial pathogens' scent

Another facet of the role of BVC in bacterial pathogenesis is illustrated by attractive BVC emitted by *B. nematocida* to lure *C. elegans* nematodes. Once bacteria colonize the intestinal tract of their thus-attracted prey (a Trojan horse strategy), they secrete two proteases involved in pathogenesis, resulting in nematode death (Niu et al., 2010). Moreover, bacterial volatiles are used to attract a wide range of insects and could contribute to pathogenesis and dissemination (see Davis et al., 2013 for review). Several insect species such as sap beetles (*Carpophilus hueralis*), lygus bugs (*Lygus* sp.), cockroaches (*Nauphoeta cinerea*), Melanesian rhinoceros beetles (*Scapanes australis*), sorghum chafers (*Pachnoda interrupta*) and Mexican fruitflies (*Anastrepha ludens*) were found to be recruited by BVC (Buttery, Kamm and Ling 1984; Nout and Bartelt 1998; Moore and Moore 1999; Moore et al., 2002; Robacker and Lauzon 2002; Rochat et al., 2002; Bengtsson et al., 2009). However, the mode of action of such bacterial volatile-mediated insect attraction has only been described for fruit flies (Robacker and Flath 1995). 2,3-butanediol was also found to be effective in insect reproduction by increasing the time female cockroaches reach parturition, which increases their lifespan; however, it decreases the number of male offspring initially produced (Moore et al., 2001; Moore, Gowaty and Moore 2003).

### POTENTIAL APPLICATIONS

Recent advances in methods to detect and analyze bacterial-specific pattern of emission suggest that rapid and reliable



bacterial identification through BVC could be used as potential diagnostic tool in some clinical situations. Several studies reported that direct mass spectrometric methods such as SIFT-MS, IMS or SESI-MS allow *in vitro* detection of bacterial growth and differentiation of pathogenic bacteria after 5, 8 or 24 h of growth in synthetic media (Zhu et al., 2010; Thorn, Reynolds and Greenman 2011; Dolch et al., 2012a,b; Junger et al., 2012; Zhu and Hill 2013). Moreover, relevant *in vitro* studies were performed to detect and characterize volatile compound profiles from mixed cultures (Zhu et al., 2010) or monocultures realized in biological fluids such as human blood (Scotter et al., 2006).

The ability to identify bacteria by their volatile profiles offers new perspectives for rapid non-invasive tests able to identify bacterial infections *in situ*, particularly for diagnosis of lung infections via breath analysis (Zhu et al., 2013). For instance, analysis of BVC patterns enables distinguishing patients infected by *Mycobacterium tuberculosis* from healthy patients, whereas hydrogen cyanide could be considered a marker of *P. aeruginosa* in sputa from cystic fibrosis patients (Sethi, Nanda and Chakraborty 2013). Increasing our understanding of the nature and role of BVC in the context of bacterial infection could also lead to development of BVC-based biomarkers. For instance, p-menth-1-en-8-ol is considered a candidate biomarker for detection of *V. cholerae* from fecal samples, while three volatile compounds present in breath samples characterized patients infected by *Helicobacter pylori* (Sethi, Nanda and Chakraborty 2013).

BVC can also be used in greenhouse or controlled field settings in agriculture. For instance, direct application of volatile 2,3-butanedione reduced soft-rot symptoms of various vegetables by modulating QS-mediated virulence of the plant pathogen *P. carotovorum* subsp. *carotovorum* (Lee and Ryu, unpublished data). Hence, besides promising biomarker applications in clinic, BVC could also be used for plant disease control, growth promotion or abiotic stress resistance (Ryu et al., 2003; Farag, Zhang and Ryu 2013)

## CONCLUSIONS AND FUTURE PERSPECTIVES

This review reports the potential role of many BVC as airborne signals or environmental cues influencing stress responses, virulence and host responses (Fig. 2). In many instances, BVC enable bacteria to adapt to various environments such as microbial communities or the host, ultimately influencing bacterial competition and cooperation. Despite increasing reports, many aspects of BVC production, including metabolic pathways, regulation, perception and activity thresholds in recipient bacteria, are still poorly characterized. The combined analysis of metabolic and gene expression profiles will likely be an increasingly powerful approach to identifying candidate genes involved in the production and regulation of biologically active volatile compounds (Farag et al., 2009). Moreover, systematic use of  $^{13}\text{C}$ -mass isotopomers and  $^{13}\text{C}$ -labeling associated with MS analysis should lead to progress in characterization of biochemical pathways involved in BVC production. This could also be facilitated by simultaneous monitoring, directly in crude extracts without the need for fractionation, of a large number of metabolites by NMR spectroscopy and MS (Fan et al., 2009; Farag 2014). The question of how bacteria perceive and respond to single or multiple BVC could be addressed by using a large-scale mutagenesis screen based on BVC-dependent phenotypic responses, as well as by monitoring transcriptomic, proteomic and metabolomic changes in response to exposure to individual active BVC.

Investigations of the regulation of active BVC should also ultimately provide new insights into the dynamics of polymicrobial infections.

Finally, beyond fundamental advances in our knowledge of the role of BVC in bacterial biology as airborne signals, further elucidation of this poorly explored research area could lead to identification of biomarkers of clinical and industrial interest.

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