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Review

The Ecological Role of Volatile and Soluble Secondary Metabolites Produced by Soil Bacteria

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The rich diversity of secondary metabolites produced by soil bacteria has been appreciated for over a century, and advances in chemical analysis and genome sequencing continue to greatly advance our understanding of this biochemical complexity. However, we are just at the beginning of understanding the physicochemical properties of bacterial metabolites, the factors that govern their production and ecological roles. Interspecific interactions and competitor sensing are among the main biotic factors affecting the production of bacterial secondary metabolites. Many soil bacteria produce both volatile and soluble compounds. In contrast to soluble compounds, volatile organic compounds can diffuse easily through air- and gas-filled pores in the soil and likely play an important role in long-distance microbial interactions. In this review we provide an overview of the most important soluble and volatile classes of secondary metabolites produced by soil bacteria, their ecological roles, and their possible synergistic effects.

Trends

Bacteria produce a vast array of secondary metabolites, both soluble and volatile, which have diverse and important ecological functions.

It has become increasingly clear that secondary metabolite production often is triggered by intra- and interspecific interactions between soil bacteria.

Secondary metabolites may be used as agents of warfare or as infochemicals, with volatile compounds potentially acting over a greater spatial scale than soluble compounds.

New analytical techniques are transforming the study of secondary metabolite chemistry, physiology, and ecology.

Introduction to Soil Bacteria

Soil is a highly complex, heterogeneous, and nutrient-limited environment consisting of an organic matrix with liquid and gaseous pores possessing the highest microbial diversity on earth. The **rhizosphere** (see Glossary), defined as the narrow region of soil attached to plant roots and influenced by plant root exudates, is a hotspot of microbial interactions and activities [1,2]. Molecular phylogenetic analyses reveal that the soil and rhizosphere can contain thousands of unique bacterial species per gram [3]; however, to date, only a small fraction of these bacteria has been cultured and studied for their ability to produce bioactive secondary metabolites. Culture-based studies have revealed that even a single bacterial strain can produce a vast array of secondary metabolites encoded by cryptic gene clusters that are not transcribed under *in vitro* conditions. Secondary metabolites are not directly involved in the growth, development, or reproduction of the producing bacteria, yet they may play important ecological roles in the interactions with other organisms. Bacterial secondary metabolites originate from a few clearly defined compound classes via their corresponding biosynthetic pathways, but for each compound class a variation of building blocks, enzymatic mechanisms, and tailoring steps can lead to an extremely diverse array of chemical structures. This review provides a short description of the main classes of soluble and volatile secondary metabolites produced by soil bacteria, and the abiotic and biotic environmental factors affecting the production of these secondary metabolites. We highlight the importance of interspecific interactions and competitor sensing for the production

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of both volatile and soluble secondary metabolites, their ecological roles, and the possible synergism between volatile and soluble compounds, a new and virtually unexplored area.

Main Classes of Secondary Metabolites Produced by Soil Bacteria

Soil bacteria produce a large amount of secondary metabolites which have many different physiochemical and biological properties. In this article volatile organic compounds and soluble metabolites will be distinguished. On the one hand, volatile organic compounds are small molecules (<300 Da) belonging to different chemical classes that can evaporate and diffuse easily through air- and water-filled pores [4,5]. These physiochemical properties make volatiles ideal candidate metabolites for cooperation and competition between soil microorganisms that do not live directly adjacent to each other. On the other hand, soluble secondary metabolites have a higher polarity which makes them soluble in water. They act for shorter distances, but usually exhibit stronger biological activities as toxins or antibiotics, as a consequence of their high degree of functionalization. Here, we first summarize the main classes of secondary metabolites produced by soil bacteria, including both soluble and volatile compounds (Figure 1; also see Table S1 in the supplemental information online), and give a brief introduction to their biosynthetic background.

Soluble Compounds

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria. They exhibit activity against other microbes, either from the same species (narrow spectrum) or across genera (broad spectrum) [6]. It has been hypothesized that the production of bacteriocins is a strategy for controlling competing bacteria in the hunt for nutrients and space in an environmental niche. Therefore, it is not surprising that it has been estimated that more than 99% of bacteria produce at least one bacteriocin [7] which may help them to influence the surrounding population dynamics, both at the population and community level. Many bacteriocins are commonly produced by rhizosphere and soil bacteria and are important for plant protection. For example, *Pseudomonas putida* BW11M1, isolated from banana roots, produces putidacin which inhibits the plant pathogen *P. putida* GR12-2R3 [8]. Other examples are bacteriocin Bac 14B from *Bacillus subtilis* 14B that is effective against the causative agent of crown gall disease, *Agrobacter tumefaciens* [9], and Bac GM17 from *Bacillus clausii* GM17 that possesses both antibacterial and antifungal activity [10].

Nonribosomal Peptides are synthesized by large nonribosomal peptide synthetases (NRPSs) with a modular organisation, these enzymes exhibit one module per chain extension with one amino acid. The biosynthesis proceeds via a thiotemplate process, with the growing peptide chain bound to a phosphopantetheinylated peptidyl carrier protein (PCP) during chain assembly. Each chain extension requires the activity of an adenylation (A) domain for selection, activation, and uploading of an amino acid, and of a condensation (C) domain for the formation of the peptide bond. Optional domains can catalyse *inter alia* oxidative modifications or epimerisations, and product release is performed by a terminal thioesterase (TE) domain to yield a free acid or a cyclised product such as a lactam. Two important classes of secondary metabolites made by NRPSs include siderophores and lipopeptides.

Siderophores are low-molecular-weight, high-affinity, iron-chelating compounds produced by microorganisms under iron-limited conditions and function in the solubilization, transport, and storage of iron [11,12]. Siderophore production can act as an antagonistic mechanism by scavenging limited iron from the soil environment, thereby reducing the amount of available iron for other organisms. Well studied siderophores are pyoverdines from *Pseudomonas*, bacillibactin from *Bacillus*, desferrioxamine from *Streptomyces*, and ornibactin from *Burkholderia*.

Lipopeptides (LPs) are compounds composed of a lipid tail with a linear or cyclic oligopeptide [13]. The structural diversity of the LPs is due to differences in length, and composition of the fatty

Glossary

GC–MS: gas chromatography–mass spectrometry (GC–MS) is an analytical method that combines the features and separation capabilities of gas-chromatography (GC) with the mass analysis capabilities of mass spectrometry to identify different substances of a volatile nature within a test sample.
GC-Q-TOF–MS: gas chromatography combined with time-of-flight mass spectrometry (Q-TOF–MS) is a method of mass spectrometry in which an ion's mass-to-charge ratio is determined via time of flight measurements using quadrupole mass filter technologies. This is one of the most sensitive technique used to date for accurate analysis of complex volatile mixtures in ultra-low concentrations.
ISR: induced systemic resistance (ISR) is an important mechanism by which selected plant growth-promoting bacteria and fungi prime the whole plant body for enhanced defense against a broad range of pathogens and insect herbivores.
LAESI–MS: laser ablation electrospray ionization mass spectrometry (LAESI–MS) is an ambient ionization method for mass spectrometry analysis. This method combines laser ablation with a secondary electrospray ionization (ESI) process.
LC–MS: liquid chromatography–mass spectrometry (LC–MS) is an analytical method that combines the separation capabilities of liquid chromatography of an HPLC with the mass analysis capabilities of a mass spectrometer (MS).
LESA: liquid extraction surface analysis (LESA) is an automated chip-based nano-electrospray source for high-resolution mass spectrometry analysis.
MALDI–IMS: matrix-assisted laser desorption/ionization imaging mass spectrometry is an imaging possibility that combines the matrix-assisted laser desorption ionization method with mass spectrometry imaging. With this technique the sample is moved in 2D while the mass spectrum is recorded and the image of the present molecules (m/z) is created. With MALDI–MS it is possible to directly measure molecules *in situ* ranging from small metabolites to large metabolites such as proteins.

acid tail, as well as the number, type, and configuration of the amino acids in the peptide moiety, and these compounds exhibit surfactant, antimicrobial, antipredation, and cytotoxic properties [14,15].

Polyketides are a large class of secondary metabolites produced by many bacteria, such as *Actinobacteria* [16], *Pseudomonas* [17], *Myxococcus* [18], *Bacillus* [19], and *Burkholderia* [20]. They are synthesized by polyketide synthases (PKSs) which are a family of multidomain enzymes or enzyme complexes. The general mechanism of polyketide biosynthesis shares great similarities with that of fatty acid biosynthesis, but allows for a much greater variability of building blocks and (reductive) modifications during chain extensions, thus leading not only to simple, usually unbranched and fully reduced, alkyl chains, but highly functionalized molecules.

Polyketide biosynthesis is also a thiotemplate-based process in which the growing acyl chain is bound to an acyl carrier protein (ACP). Acyl transferase (AT) domains select the starter and elongation units and upload them to the ACPs. In each chain extension the ketosynthase (KS) catalyses the condensation of the next extension unit with the so far assembled acyl chain, while a ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domain are required for optional reductive modifications. The terminal TE domain is responsible for product release, usually under formation of a free acid or lactone [21]. PKSs are currently classified into three major types [22]. Type I PKSs consist of large, multidomain proteins that produce polyketides with one module (set of domains) per successive condensation reaction (modular PKSs) or by iterative usage of one and the same set of domains (iterative PKSs) in polyketide chain assembly [23]. The antibiotic erythromycin is the paradigm of a type I PKSs which plays an important role in the treatment of infectious diseases [22]. Type II PKSs are composed of individual proteins that are used in the formation of aromatic polyketides [24]. Actinorhodin and doxorubicin (cancer chemotherapy drugs) and tetracyclines (antibiotics) are among the products synthesized by the type II PKS biosynthesis pathways [24]. Type III PKSs are involved in the synthesis of polyhydroxy phenols in bacteria [25], such as 2,4-diacetylphloroglucinol (2,4-DAPG) produced by *Pseudomonas* species [26].

PKS–NRPS Hybrid Compounds. The striking structural and functional similarities between PKSs and NRPSs allow for the formation of clusters that contain elements of both classes [27]. These hybrid NRPS–PKS clusters can provide more diversity for potential secondary metabolites produced by microorganisms [28]. Hybrid NRPS–PKS metabolites have been isolated from numerous Gram-positive and Gram-negative soil bacteria. Important examples are the immunosuppressant rapamycin from *Streptomyces hygroscopicus* [29], rhizoxin from the fungal endosymbiont *Burkholderia rhizoxinica* [30], that also occurs in other soil microorganisms [31,32], and epothilone from the myxobacterium *Sorangium cellulosum* that is used in cancer therapy [33].

Volatile Compounds

Terpenes are derived from the terpene-building units dimethylallyl pyrophosphate and isopentenyl pyrophosphate, which can arise either from the mevalonate pathway or from the deoxyxylulose phosphate pathway [34]. These compounds are best known as plant metabolites, but recent studies have revealed that terpenes are produced throughout the tree of life, including prokaryotes [35–38], fungi [39], and social amoebae [40]. Although terpenes of bacterial origin have been known for over a century, their biological and ecological roles are still unknown. Even for geosmin, the most well-known terpene emitted by soil bacteria, no biological function has been reported so far.

An odoriferous *Streptomyces albidoflavus* isolate from corn seeds was shown to produce a novel sesquiterpene, named albaflavenone, with antibacterial properties [41]. More recently,

MALDI–TOF: matrix-assisted laser desorption/ionization (MALDI) is an ionization technique used in mass spectrometric analysis. It is based on embedding samples in a special matrix from which they are desorbed by laser light. This technique allows the analysis of biomolecules and organic molecules such as polymers. It is similar in character to electrospray ionization (ESI).
Metabolome: the metabolome is a collection of all the metabolites in a biological cell, which are the end products of cellular processes.
nanoDESI: nanospray desorption electrospray ionization technique is a novel ambient mass spectrometry imaging technique in which a solvent is used for localized extraction of molecules from a sample followed by nano-electrospray ionization and introduction into a mass spectrometer (MS).
PGPR: plant-growth-promoting rhizobacteria (PGPR) are root-colonizing bacteria that form symbiotic relationships with plants, promote plant growth, and protect plants from pathogen attack by a range of mechanisms.
Rhizosphere: the rhizosphere is the narrow zone surrounding, and influenced by, plant roots and root exudates.

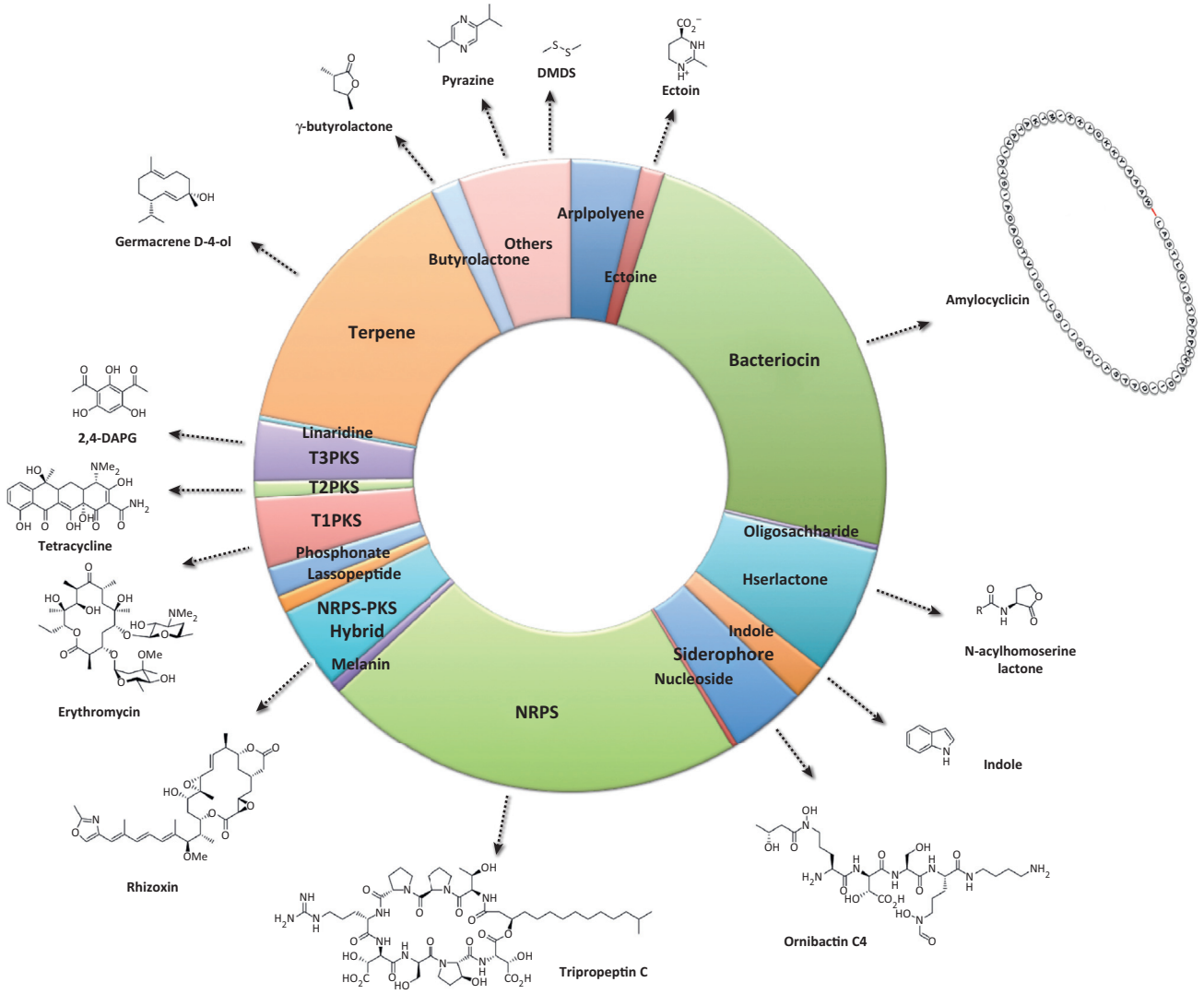

Figure 1. Most Abundant Secondary Metabolite Categories Based on the AntiSMASH *In Silico* Analysis of 30 Different Whole-Genome Sequences of Soil Bacteria. 2,4-DAPG, 2,4-diacetylphloroglucinol; DMDS, dimethyl disulfide; NRPS, nonribosomal peptide synthetase.

albaflavenone was also isolated from other *Streptomyces* species and fungi [35,42,43]. While *Streptomyces* is certainly the best investigated genus in terms of bacterial terpene production, recent research also included other taxa of soil bacteria. A comparative genomics analysis of six *Collimonas* strains revealed that two *C. pratensis* strains harboured terpene synthase genes. After heterologous expression in *Escherichia coli* and biochemical characterization, it could be shown that these genes were responsible for the production of a mix of sesquiterpenes with Germacrene D-4-ol as major compound [44]. Four monoterpenes (γ-terpinene, ∝-pinene, β-pinene, and β-myrcene), detected in the headspace of *C. pratensis* strain Ter91, were tested individually and as a mixture for their antimicrobial activity. The β-pinene exhibited inhibition against *Staphylococcus aureus* and *Rhizoctonia solani*, and in addition the mixture of all four monoterpenes was shown to inhibit *E. coli* [44].

Nitrogen Compounds The most widespread nitrogen compounds occurring in many soil bacteria are pyrazines, while indole is an important signalling molecule. Other reported classes include pyrroles, thiazoles, pyridines, and aniline derivatives [45,46].

Pyrazines (1,4-diazabenzenes) are volatile organic compounds well known for their antimicrobial activities [47]. The production of pyrazines is widely distributed in plants, but so far only a few bacteria have been reported which synthesize pyrazines, including *Pseudomonas*, *Bacillus*, *Chondromyces* [48], and *Streptomyces* [47,49,50]. Two different biosynthetic pathways to pyrazines have been identified in bacteria. The pathway in *Corynebacterium glutamicum* requires activity of the acetolactate synthase and proceeds via acetolactate and its higher homologs [46]. In contrast, in myxobacteria, pyrazines arise from branched amino acids such as valine via reduction to valinal and dimerisation [51].

Indole is synthesized from tryptophan by tryptophanase by both Gram-positive and Gram-negative bacteria [52,53]. Indole and its derivatives can suppress bacterial pathogenesis of several antibiotic-resistant pathogens because of their ability to inhibit quorum sensing and virulence factor production [54]. In addition, indole is well known as a signaling molecule modulating spore formation, plasmid stability, cell division, antibiotic tolerance, and biofilm formation [55,56] and controls plant defense systems, growth, and root development [54,57]. Indole is known to be a stable compound within the bacteria producing it. However, many non-indole producing bacteria are able to modify or to degrade indole using diverse oxygenases, such as monooxygenases, dioxygenases, and P450 family members [58]. Indole derivatives are abundant in many microbial communities but very little is known about their biological roles and mechanisms of action.

Sulfur-Containing Volatiles Volatile sulfur compounds and alkyl sulfides have a large structural diversity ranging from relatively small compounds, such as dimethylsulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS), to more complex volatiles such as 2-methyltetrahydrothiophen-3-one that originates from homocysteine in bacteria [59]. Volatile microbial sulfur compounds play an important role in plant–microbe and interspecific microbe–microbe interactions [60–62]. DMDS was identified as a quorum-sensing-inhibiting compound [63] and has also been reported to stimulate bacterial growth and completely inhibit fungal growth [64].

Factors Affecting Secondary Metabolite Production in Soil Bacteria

The production of secondary metabolites by bacteria is influenced by a variety of environmental factors such as nutrients, temperature, pH, moisture, and light. Often, the production of secondary metabolites is triggered when bacterial growth is limited by the depletion of carbon, nitrogen, phosphate or other key nutrient sources. Nutrient composition and concentration affect complex mechanisms in global gene regulation, reflecting the range of conditions that trigger the production of different secondary metabolites in nature [65–67]. High concentrations of glucose, phosphate, or ammonium are often found to repress secondary metabolism [68] but this is not universal; for instance, high phosphate concentrations can also induce the production of specific secondary metabolites [69]. The triggers for secondary metabolite production are highly varied and are exploited in strategies of natural product discovery. The OSMAC (One Strain-Many Compounds) approach is based on cultivating a single bacterial strain under distinct conditions [70]. For example, the addition of low concentrations of rare earth elements such as scandium and/or lanthanum during cultivation enhanced several secondary metabolite-biosynthetic gene clusters in streptomycetes, or even activates otherwise silent biosynthetic gene clusters including that for actinorhodin biosynthesis in *Streptomyces lividans* [71].

While considerable research has been done on the effect of different nutrients and abiotic conditions on the production of secondary metabolites, research focusing on biotic interactions

such as interspecific competition has attracted research attention only in the last few years. In soil, and especially the rhizosphere, microbial communities are involved in complex and intimate interactions which have the potential to significantly affect the production of secondary metabolites. Recently, it could be demonstrated that soil bacteria growing under carbon limitation conditions can be specifically triggered to produce broad-spectrum antibiotics when challenged with other bacterial species [72]. Detailed transcriptomic analyses revealed that soil bacteria can distinguish among different bacterial competitors and fine-tune their competitive strategies [73]. The behavior and the transcriptional responses of the soil bacterium *Pseudomonas fluorescens* Pf0-1 was shown to elicit unique responses when confronted with evolutionary divergent bacterial species. In particular, the expression of genes involved in signal transduction and antibiotic production were strongly affected by the identity of the interacting strains.

The production of specialized metabolites by soil bacteria is the direct result of their interactions with other microorganisms in their immediate vicinity [74,75]. Genomes of soil and rhizosphere bacteria contain numerous cryptic gene clusters encoding enzymes involved in the production of secondary metabolites that are not expressed under typical laboratory conditions. While there have been numerous reports of 'waking up' the 'sleeping' gene clusters, many of them involving genetic intervention or nutrient challenges, the role of competing microorganisms has only been addressed in a few recent studies.

Using the newly developed techniques of **nanoDESI** and **MALDI–TOF** imaging mass spectrometry (Box 1 and Figure 2), Traxler *et al.* (2013) [74] were able to analyze the induction of secondary metabolite production by the model actinomycete *Streptomyces coelicolor* A3 (2) during interactions with other actinomycetes in great detail. Species interactions caused the production of many secondary metabolites that were not produced by the monoculture of *S. coelicolor* and the majority of the compounds were interaction-specific, occurring in only one of five pairwise interactions. In *Streptomyces lividans*, a very close relative of *S. coelicolor*, the production of red pigments (a mixture of prodiginines and actinorhodins) was induced due to interactions with mycolic-acid-producing bacteria, including *Tsukamurella pulmonis*, *Rhodococcus erythropolis*, and *Corynebacterium glutamicum* [76]. The interactions with different bacteria, such as *Bacillus*, *Myxococcus*, and *Serratia*, can induce the production of actinorhodin in *S. coelicolor* through unknown mechanisms [77–79]. The induction factor for the production of streptoaminals, a series of structurally related antimicrobial spiroaminals, by coculturing of *Streptomyces nigrescens* with *Tsukamurella pulmonis*, is also unknown, but this study showed impressively that coculturing can give access to previously unknown compounds [80].

The recently proposed 'competition sensing' hypothesis suggests that bacterial cells may be able to detect and respond to competitors through a lack of nutrients or via cellular damage [81]. The presence of neighboring bacterial colonies can alter the competitive behavior of many species of soil bacteria [75,82,83]. Interestingly, *Streptomyces* isolates from the same location are significantly more effective at inhibiting one another than are *Streptomyces* isolated from different soils, suggesting that these interactions are the result of local adaptation [84,85]. In addition, sympatric *Streptomyces* with similar carbon source utilization patterns tended to inhibit each other more intensely, consistent with competition sensing. The proportion of interactions either stimulating or inhibiting antibiotic production was substantial (35%) [85]. A high-throughput screening of 146 phylogenetically diverse soil bacteria revealed that interspecific interactions can have a major impact on antimicrobial compound production, both by inducing and suppressing antimicrobial compound production. From all screened isolates, 33% showed antimicrobial activity in monoculture, while 42% showed activity only during interaction with other species [75].

Most studies examining interactions between soil bacteria are focused on the production of soluble antimicrobial compounds; however, recent studies have revealed that such interactions

Box 1. Advances in the Analysis of Bacterial Secondary Metabolites

The development of "-omics" technologies during the last 15 years has enabled massive progress in the understanding of metabolic diversity, including analysis on the level of single cells [115]. Accordingly, metabolomics, the determination of a set of small molecules known as metabolites, has also flourished and enhanced our understanding of microbial chemistry [115–117]. The two principal methods in metabolomics that are used to detect and structurally elucidate metabolites are nuclear magnetic resonance (NMR) and mass spectrometry (MS). MS possesses high sensitivity and selectivity and can be easily combined with diverse separation methods such as GC or LC, and most metabolic studies are performed on these hybrid instruments [115]. These techniques provide information about the detected mass and fragmentation spectra, and the molecular formula of a metabolite can be determined from the accurate mass. Gas chromatography–mass spectrometry (GC–MS) is mainly used to analyse volatile compounds (boiling point 20–350 °C) and polar primary metabolites after derivatization to make them volatile. Liquid chromatography–mass spectrometry (LC–MS) is generally applied to analyse polar and semipolar, nonvolatile compounds. The recent technical developments in the field of mass spectrometry have led to improvement of volatile compound detection [111]. The **GC-Q-TOF–MS** is one of the most sensitive techniques used to date for accurate analysis of complex volatile mixtures in ultra-low concentrations.

In the last decade mass spectrometry imaging (IMS) has emerged as having great potential to visualize the **metabolome** at the cellular and subcellular levels [115]. Ambient ionization IMS is a technique in which ionization occurs at atmospheric pressure, meaning that samples are analysed in their native state. In ambient ionization the surface is sampled with minimum or no preparation, ionization occurs externally to the mass spectrometer, and only ions (not the entire sample) are introduced to the mass spectrometer. So far more than 30 different ambient ionization methods have been developed in the last 10 years [118].

The most widespread IMS scanning method is the matrix-assisted laser desorption/ionization method (MALDI) [119]. **MALDI–IMS** can be applied to characterise specialized metabolites produced by microbes in isolation or when interacting with other species. For example, MALDI–IMS analysis of *Streptomyces coelicolor* staged with other actinomycetes revealed the production of many interaction-specific metabolites not produced in monoculture [78]. MALDI–IMS has successfully been employed to observe antagonistic interactions between *Streptomyces* and *Bacillus* strains *in vitro* [77]. More recently, Kaltenpoth *et al.* combined MALDI–IMS with fluorescence *in situ* hybridization for simultaneous monitoring of antibiotic production and taxonomic identification using *Streptomyces* as model bacterium [120]. Using MALDI–IMS, the spatial distribution of extracellular metabolites produced by five *Lysobacter* strains was recently investigated [121]. The results revealed that the metabolomics data obtained by MALDI matched well with the gene clusters identified in the genomes of these five strains [121]. MALDI–IMS and live colony nanoDESI mass spectrometry were applied in a study by Song and coauthors to analyse the metabolites produced during bacteria–protozoa interactions [44]. In this way they were able to visualize the spatial distribution of the lipopeptide massetolide A during interactions of *Pseudomonas fluorescens* SS101 and *Naegleria americana*.

Laser ablation electrospray ionization mass spectrometry (**LAESI–MS**) was applied directly to a polymicrobial biofilm in order to visualize possible colocalization of *P. aeruginosa* and *S. aureus*. LAESI–MS was also used to analyse ions following LL-37 antimicrobial peptide treatment of the biofilm. This ambient ionization method holds promise for future biofilm studies and interspecific interactions [122].

A combination of liquid extraction surface analysis (**LESA**) automated chip-based nanoelectrospray ionization and high-resolution mass spectrometry proved to be a powerful tool for extracting and detecting thiazolyl peptide antibiotics from different actinobacteria [123]. LESA was found to be a suitable method for screening natural products produced by bacterial colonies on cultivation plates within the first 2 min following extraction and detecting antibiotics at high mass accuracy at low cost. This method can be applied as a high-throughput screening for dereplication of known antibiotics and rapid discovery of novel antibiotics.

Direct analysis in real-time high-resolution mass spectrometry (DART–HRMS) has proved itself as a versatile method for the analysis of solid, gaseous, or liquid samples. This method is able to detect small to medium-sized biomolecules (molecular mass range 50–1200 Da) and permits rapid qualitative and quantitative analysis [124].

The diversity of emerging mass-spectrometry imaging techniques are excellent for monitoring metabolic processes and for studying chemical communication in an ecological context. However, so far these methods have not been extended to *in situ* analyses of soil samples.

can significantly affect volatile bacterial emissions as well [62,86]. In contrast to soluble compounds, volatiles released during microbial interactions in the rhizosphere can have long-distance effects on the surrounding nonactive microbial community in nutrient-depleted bulk soils [60,86]. Recent research has revealed that microbial volatiles can play two major roles in the long-distance interactions in microbial communities: as infochemical molecules affecting the

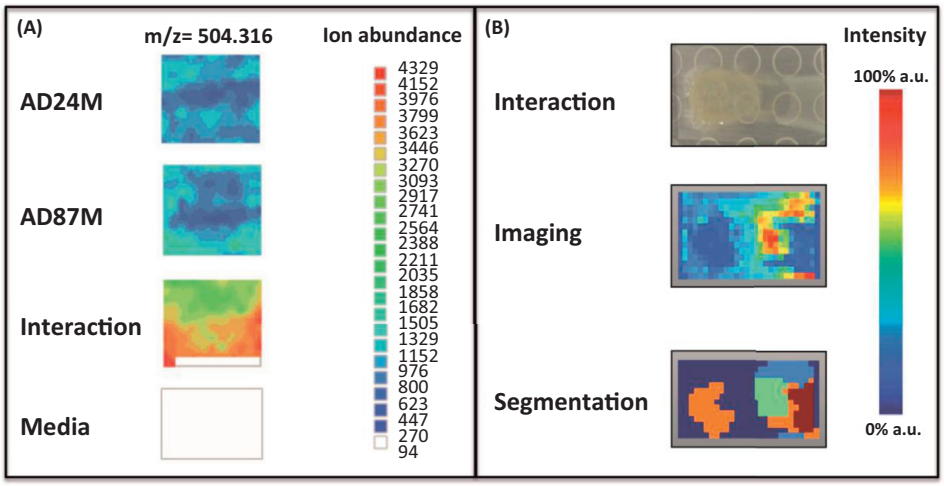

Figure 2. Results of the LAESI–MS (Laser Ablation Electrospray Ionization–Mass Spectrometry) Imaging and MALDI (Matrix-Assisted Laser Desorption/Ionization)–Imaging Mass Spectrometry (IMS). (A) Heat map targeting a Pederin-like compound with an *m/z* (mass-to-charge ratio) of 504.316 [M + H^+] showing specific accumulation of ions related to this compound in monocultures of *Paenibacillus* sp. AD87 (AD87 M) and the interaction of *Paenibacillus* sp. AD87 and *Burkholderia* sp. AD24, not in monocultures of *Burkholderia* sp. AD24 (AD24 M). (B) MALDI–IMS analysis of the *Pseudomonas fluorescens* SS101 (right inoculum)–protozoa *Naegleria americana* (left inoculum) interaction, including imaging of metabolite classes and spatial segmentation (analyzed with software SCiLS Lab version 2014b) of the MALDI–IMS data.

behaviour, population dynamics, and gene expression in the responding microorganism [60], and as antimicrobials providing an ecological advantage by suppressing or eliminating potential enemies [5,64,87]. Volatiles produced by *C. pratensis* can trigger the production of secondary antimicrobial metabolites in *P. fluorescens* Pf0-1 [60]. The production of soluble antibiotics triggered by volatiles in microbial interactions was also observed in *P. aeruginosa* during coculturing with *Enterobacter aerogenes*, producer of the volatile 2,3-butanediol [88]. For *Chromobacterium violaceum* and *P. aeruginosa*, several monoterpenes increased violacein and pyocyanin production [89].

Importantly, several studies have reported that volatiles can modify antibiotic bacterial resistance or tolerance. For example, exposure of *E. coli* to volatiles emitted by *Burkholderia ambifaria* increased its resistance to gentamicin and kanamycin [90], and exposure to the volatile compound trimethylamine (TMA) altered the antibiotic resistance profiles of several Gram-positive and Gram-negative bacteria, including important human pathogens [91]. The monoterpene α-pinene can act as a modulator of antibiotic resistance in *Campylobacter jejuni* [92].

Protists are major predators of bacteria in soils and can recognize prey quality when they are in direct contact through bacterial morphological differences and soluble compounds [93]. Recently, Schulz-Bohm *et al.* (2016) [94] revealed that volatiles can play an important role in species-specific bacterial–protist interactions and that terpenes are among the informative compounds that enable protists to sense suitable prey bacteria. Interestingly, the observed stimulation of protist activity by volatiles coincides with the direct trophic interaction assays suggesting that volatiles may serve as signals.

The Ecological Role of Bacterial Secondary Metabolites

It has been speculated that some secondary metabolites, especially volatile compounds produced by microorganisms, are metabolic spin offs from primary metabolism, sometimes

with coincidental activity. However, this viewpoint is not well supported since the large majority of secondary metabolites demonstrate biological activities with relevance for the producing organism in its ecological context. If secondary metabolites are merely spin offs from primary metabolism, how can one explain the great diversity of compounds produced by even single strains, regulated by different gene clusters and incurring costly investment for the cell?

Several studies have demonstrated that secondary metabolites produced by soil bacteria can serve as weapons in microbial warfare, providing an advantage to producer strains when competing against other microbial competitors in the same ecological niche [81,95]. A good example of this is the suppression of soil-borne plant pathogens by antimicrobial compounds from plant-beneficial soil bacteria [96]. This observation suggests that the ability to kill or inhibit the growth of microbial competitors evolutionarily favors antibiotic-producing microorganisms over antibiotic-susceptible ones. This is also supported by the observation that antibiotic resistance is widespread in soil bacteria. Secondary metabolites with antimicrobial activity can play a significant role in predation or protection against predators [97]. Knocking out genes involved in antibiotic production in the predatory soil bacterium *Myxococcus xanthus* results in a decreased growth rate on prey cells [98].

Antimicrobial compounds at subinhibitory concentrations might act as signaling molecules in inter- and intraspecies interactions, affecting a variety of cellular functions such as cellular development, biofilm formation, motility, virulence, and nutrient use [99–102]. A study on the model organism *Bacillus subtilis* revealed that bacillaene and surfactin play important roles as

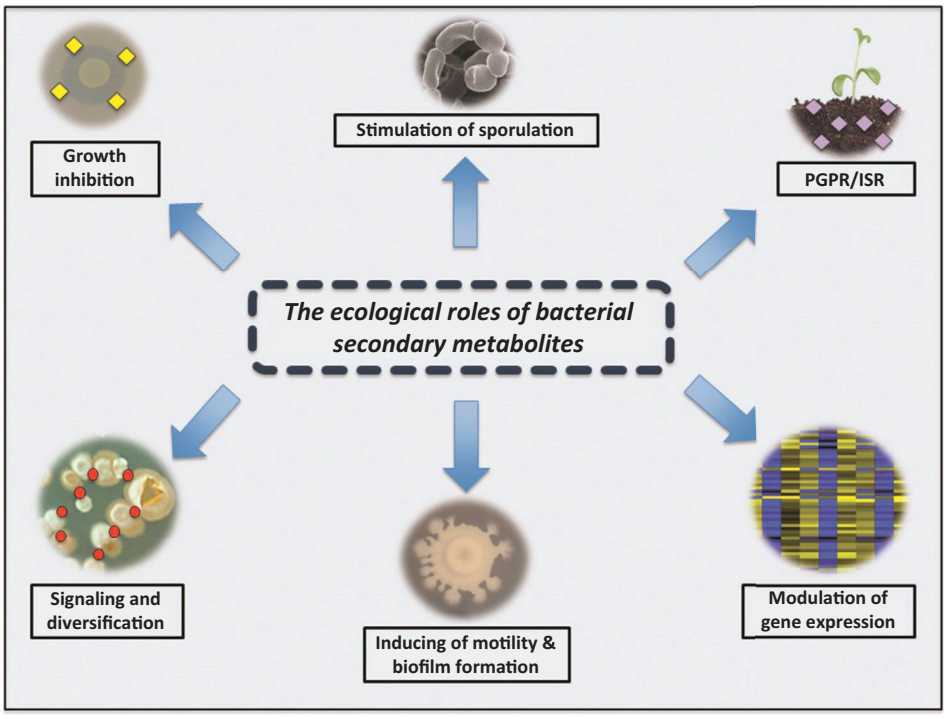

Figure 3. Schematic Representation of the Possible Different Ecological Roles of Bacterial Secondary Metabolites in Nature. Symbol abbreviations: ◇ = secondary metabolites with antimicrobial properties responsible for growth inhibition; ◇ = secondary metabolites with plant growth promoting properties; ⬭ = signalling molecules responsible for communication and diversification.

signaling molecules in cellular development, but also inhibited the development of aerial hyphae and spore formation in the phylogenetically distant species *Streptomyces coelicolor* [101].

Antibiotics have been reported to act as a source of nutrients promoting the growth of bacteria under nutrient-deprived conditions [103,104]; however, these findings are controversial as they could not be reproduced by another group [105]. Another potentially important ecological role of secondary metabolites with antimicrobial activity is that of stimulating sporulation [106].

Many bacteria are in mutualistic relationships with other organisms in which they protect or stimulate their host by providing secondary metabolites and receive nutrients in return. For example, a range of plant growth promoting rhizobacteria (**PGPR**) are able to drastically alter a plant's root system development and increase plant biomass by emitting complex blends of volatiles without actual physical contact [107]. Furthermore, increased resistance to pathogens can be conferred by exposure of plants to bacterial volatiles or by the induction of **ISR** (induced systemic resistance) and in addition the growth of pathogenic fungi can be reduced by exposure to microbial volatiles [108]. Another interesting timely topic is that of bacteria that are associated with soil-dwelling insects that produce numbers of secondary metabolites with diverse chemical structures, and many of these metabolites play important roles in protecting their host from infections; this topic has recently been reviewed [109] and will not be extensively discussed here.

There is so far no consensus on the ecological role and the evolutionary forces leading to the explanation for why antibiotics are produced. In summary, we can argue that antibiotics are bioactive secondary metabolites that have many functions in addition to being microbial warfare agents (Figure 3).

Concluding Remarks and Outlook

Although a considerable portion of bacterial secondary metabolites has been uncovered, very little is still known about why, when, and where soil bacteria produce secondary metabolites (see Outstanding Questions). One main contributing factor to this gap in knowledge is that most studies are focused on soil actinomycetes which, although a highly diverse and interesting group, represent only a small portion of phylogenetic and ecological microbial diversity. More generally, it is very hard to imitate the diverse and changing environmental conditions that are likely to drive secondary metabolite production in laboratory experiments. The successful isolation and structure elucidation of bacterial metabolites depends crucially on the extraction and purification protocol and is often hindered by the low concentration or chemical instability of highly active natural products. New developments in genomics, mass spectrometry and imaging offer solutions to these restrictions, allowing direct metabolite analysis, even at the level of single cells (Box 1). Notably, most studies are focused on either volatile or soluble compounds and conveniently ignore the fact that these compounds are usually produced simultaneously, sometimes by one and the same biosynthetic gene cluster (e.g., the fungicidal antimycins and the volatile blastomycinones [110]). This division of volatile and nonvolatile focused metabolomics is at least partly due to different techniques necessary to study them (**LC–MS** for soluble and **GC–MS** for volatile compounds). Furthermore, sample preparation is an important issue, because volatiles are easily lost in solvent evaporation steps after culture extraction and require specialized trapping techniques [111]. The newly available techniques may help to push the boundaries of detecting and identifying versatile metabolites *in situ* and to better understand the metabolic interactions occurring within complex multispecies communities (Box 1).

Bacteria are usually not directly inhibited by volatile compounds; however, volatiles can have synergistic effects with soluble antimicrobials. For example, hydrophilic antibiotics such as vancomycin and β-lactams, that have marginal inhibitory effects on Gram-negative bacteria, exhibit enhanced antibacterial activity when the exposed strains are pretreated with the volatile

Outstanding Questions

Do volatile metabolites usually function as signals rather than antimicrobials?

In which concentration range are volatile and soluble metabolites biologically active in the soil?

Do volatile and soluble metabolites have strong additive or synergistic effects?

What are the spatial and temporal scales of volatile- and soluble-metabolite-mediated interactions in soil?

Do volatiles play a more important role under dry soil conditions and soluble metabolites under wet soil conditions, and do bacteria switch expression from non-volatile to volatile metabolites under fluctuating moisture conditions?

phenylpropanoid eugenol [112]. Due to their lipophilic nature, volatiles may interfere with membrane structures, causing depolarization of the cell membrane and thus a higher sensitivity towards the more polar antibiotics.

Compared to soluble compounds that accumulate around the producing cells, volatiles can diffuse easily via air- and gas-filled pores in the soil and play a role in long-distance microbial interactions. It is plausible that volatile organic compounds are relatively more important in the interactions between soil bacteria when soil moisture levels decrease and more air-filled pores become available, but this has not yet received any experimental attention.

Bacterial volatile compounds offer a great potential for sustainable crop protection as environmentally friendly gaseous biofertilizers and alternatives to the deleterious pesticides [113,114]. For example, dimethyl disulfide, a volatile frequently emitted by bacteria, is used as a novel soil fumigant PALADIN® against nematodes and soil-borne pathogens. However, the research on the application of bacterial volatiles and the combination of volatiles and soluble compounds in agriculture is still in its infancy.

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