Natural product antibiotics: cues for modulating bacterial biofilm formation

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Abstract

Cell-cell communication enables bacteria to coordinate their behavior through the production, recognition, and response to chemical signals produced by their microbial neighbors. An important example of coordinated behavior in bacteria is biofilm formation, where individual cells organize into highly complex, matrix-encased communities that differentiate into distinct cell types and divide labor among individual cells. Bacteria rely on environmental cues to influence biofilm development, including chemical cues produced by other microbes. A multitude of recent studies have demonstrated that natural-product antibiotics at subinhibitory concentrations can impact biofilm formation in neighboring microbes, supporting the hypothesis that these compounds may have evolved as signaling molecules that mediate cell-cell interactions. In this review we discuss the role of antibiotics in modulating biofilm formation and interspecies communication in bacteria.

Keywords

Biofilm formation; cell-cell communication; bacterial interactions; natural products; specialized metabolites

Antibiotics as signaling molecules

Antibiotics have long been instrumental in fighting infectious disease. The majority of antibiotics used in clinical settings are derived from small molecule natural products [1] many of which are produced as 'secondary' or specialized metabolites by microorganisms [2]. Despite their extensive use as therapeutics, the function of these molecules in the natural environment remains poorly characterized; this disconnect is surprising because such

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knowledge might accelerate the discovery of additional therapeutic molecules produced by microbes. Although debate remains about the function of these metabolites in their natural environments, the antibiotic concentrations used for killing pathogens in clinical settings are likely higher than those present in nature, where the population of the producing organisms, molecule concentration, and diffusion rate may all fluctuate with environmental variabilities and microbial community composition. In addition, an emerging body of research has highlighted the importance of microbial metabolites as signaling molecules able to modulate gene expression in bacterial populations and affect important physiological functions and cellular processes such as metabolite production, motility, pigmentation, and biofilm formation [3–5]. This suggests that these molecules may not have evolved exclusively for killing, but rather as a means of communication between microorganisms that mediate interspecies and intraspecies interactions. Significant challenges remain for validating this premise, including a need to expand our understanding of the (non-antibiotic) mechanisms of action of these metabolites and to devise experimental capacities that will enable us to monitor their activities at realistic concentrations and spatial scales. Nevertheless, uncovering the potential non-killing biological functions of these compounds is crucial not only for understanding chemical communication within microbial communities but also for ensuring the judicious clinical application of antibiotics, since subinhibitory concentrations could otherwise potentially impact pathogens in a manner that increases their fitness in the host. Here we review recent reports of natural product antibiotics that specifically impact the densely packed, matrix-embedded microbial communities called biofilms, and discuss the potential roles of these natural products in bacterial cell-cell communication (see Table 1 for an overview).

Biofilm formation

Biofilms are microbial aggregates encased in a self-produced extracellular polymer matrix that can be either surface-attached or free-floating. These highly complex microbial accretions are widely distributed in the natural environment as well as in industrial and medical settings, and are thought to have been a major mode of eubacterial survival for billions of years [6]. Gene expression and metabolic activity is profoundly different between biofilm cells and their planktonic counterparts [7]. Additionally, there is cellular and spatial heterogeneity within biofilms that arises as cells undergo differentiation in response to local conditions and are exposed to different developmental signals [8]. Emergent properties arise in biofilms due to complex social interactions and as the structural and chemical properties of the biofilm matrix develop [8]. Finally, biofilms are highly resistant to environmental stresses, predators, detergents, and antibiotic treatment [6]. These protective effects have been demonstrated to be greatly enhanced when multiple species are present within the biofilm [9], suggesting that multi-species interactions among biofilm cells are beneficial to their fitness. Many natural-product antibiotics affect biofilm formation, suggesting that specialized metabolite production by neighboring microorganisms may act as important environmental signals that regulate biofilm formation and shape multispecies interactions in these communities. A multitude of assays have been used to detect the impacts of these specialized metabolites on biofilm formation, some of which are depicted in Figure 1. We begin by focusing on two bacteria (*Bacillus subtilis* and *Pseudomonas aeruginosa*) that are

extremely well-characterized in terms of their ability to form biofilms, and thus have also been the focus of many of the studies examining how antibiotics impact the development of biofilms in these species.

Small molecule natural products as a cue for biofilm formation

Bacillus subtilis

B. subtilis is a Gram-positive soil-dwelling bacterium that has been well-studied for its ability to form highly structured biofilms (Figure 2A). Within a *B. subtilis* biofilm, subpopulations of matrix-producing, surfactin-producing, sporulating, and motile cells, among other cell types, coexist in discrete regions of the structure [10]. The *B. subtilis* biofilm matrix is comprised of exopolysaccharides (EPS) and two major matrix proteins: TasA, which provides structural integrity [11] and BslA, which confers hydrophobicity to the biofilm structure [12]. These matrix components are encoded by the *epsA-O* operon, the *tapA* operon, and the *bslA* gene, respectively. The master transcriptional regulator Spo0A controls over 100 genes, depending on its concentration and phosphorylation state, including these three critical structural components of the biofilm matrix, [13,14]. Intermediate levels of phosphorylated Spo0A (Spo0A~P) induce matrix gene expression, while higher levels induce sporulation [13,15]. Levels of Spo0A~P are primarily controlled by the activity of five sensor histidine kinases (KinA, KinB, KinC, KinD, and KinE), which phosphorylate Spo0A both indirectly and directly [16,17]. However, with a few notable exceptions [15] the environmental cues that control the kinase or phosphorylation activities of these kinases are poorly defined.

One of the first studies that indicated that the secretion of small molecules by soil bacteria can influence biofilm development in *B. subtilis* screened purified compounds for their ability to induce pellicles (floating biofilms that form at the liquid-air interface) [18]. This approach identified nystatin and surfactin as biofilm inducers in *B. subtilis* [18]. Nystatin is a polyene polyketide produced by *Streptomyces noursei* and is well-known for its antifungal activity: it forms pores in cell membranes that allow cation efflux. Surfactin is a lipopeptide produced by *B. subtilis* itself. By characterizing the effect of a variety of structurally and/or functionally related molecules, this study specifically found that cation (potassium) leakage, not simply membrane disruption, was responsible for the induction of biofilm formation in *B. subtilis* [18]. The authors also showed that *kinC* was required for *B. subtilis* to respond to surfactin, suggesting that KinC may be responsible for sensing potassium leakage and mediating biofilm matrix gene expression [18]. Many of the other biofilm-inducing compounds identified in this report were, like nystatin and surfactin, microbially produced metabolites that have characterized antibiotic activity (e.g. gramicidin and valinomycin). Because the concentrations of nystatin and surfactin used did not lead to a decrease in growth rate, this phenomenon does not appear to be a stress response per se, but rather a mechanism *B. subtilis* might use to sense the presence of specific microbial neighbors by sensing a change in the state of their cell membranes [18].

In a complementary approach, another study used microbial coculture rather than purified compounds to identify soil microbes secreting compounds that stimulated biofilm formation in *B. subtilis* [19]. This interspecies-interaction screen identified the common soil microbes

Bacillus cereus, Bacillus thuringiensis, Bacillus mycoides, Bacillus megaterium, Bacillus luciferensis, and *Pseudomonas monteilli* all as inducers of biofilm matrix gene expression in *B. subtilis* [19]. The results from this work demonstrate that *B. subtilis* responds to the secreted molecules produced by these and other soil bacteria using mechanisms that depend on the phylogenetic relatedness of the interaction partner [19]. A follow-up publication used matrix-assisted laser desorption/ionization time-of-flight imaging mass spectrometry (MALDI-TOF IMS) to identify the thiocillins as biofilm-inducing metabolites produced by *B. cereus* [20]. Thiocillins are ribosomally encoded, post-translationally modified peptide antibiotics that interfere with the 50S ribosome. In addition to the thiopeptides, this study demonstrated that a range of structurally and functionally diverse thiazolyl peptides (thiostrepton, nosiheptide, berninamycin), as well as two other strains of *Bacillus* species whose genomes contained putative thiazolyl biosynthetic gene clusters all induced biofilm matrix production in *B. subtilis* [20]. These results suggest that thiazolyl peptide-induction of biofilm formation may be a widespread signaling mechanism among soil microbes. Indeed, putative thiazolyl-like biosynthesis genes were present in roughly 5 % of the sequenced *Bacillus* genomes and 40 % of the *Streptomyces* genomes available at the time this work was published [20]. Although all of these thiazolyl peptides affect *B. subtilis* biofilm formation under the conditions examined, it remains unknown whether or how these metabolites may influence biofilm formation in the producing species themselves. In addition, although the thiocillins were identified as a biofilm-inducing metabolite secreted by *B. cereus*, none of the other soil microbes identified in the initial coculture screen appear capable of producing thiazolyl peptides, suggesting that a range of additional metabolites able to alter biofilm formation in *B. subtilis* await discovery.

The most notable aspect of this work was the discovery that matrix induction by thiocillin is structurally separable from its antibiotic activity – in other words, different parts of the molecule appear to be responsible for its antimicrobial and signalling activities [20]. This was ascertainable because – unlike most antibiotics, which are synthesized using matabolite-specific enzymatic machinery – thiocillin is ribosomally encoded. Therefore, structural variants were easily generated through standard molecular genetics approaches. This allowed the identification of a variant (T4V) that had no antibiotic activity against *B. subtilis*, yet still induced biofilm matrix gene expression and enhanced colony wrinkling (a phenotype correlated with biofilm matrix production) [20]. This result is important because it provides direct evidence that, at least in the case of thiocillin, biofilm formation in response to antibiotics is not solely a consequence of stress-induced killing, as has frequently been proposed. Instead, this paper establishes that microbial 'antibiotics' possess biological activities independent of their killing activity and thus may have evolved as signaling molecules specifically to alter bacterial physiology. Future work will be required to determine how broadly true this finding might be for antibiotics in general. Notably, the identification of a biofilm-inducing, antibiotic-null thiocillin variant (T4V) also provides a unique tool for future studies focused on ascertaining whether the biofilm-enhancing or killing activity (or both) of this metabolite are pertinent at microbially relevant spatial scales and concentrations.

In addition to these antibiotics that enhance biofilm formation in *B. subtilis*, some bacterial natural products have been identified that inhibit biofilm formation in this bacterium.

Pseudomonas protogens is a Gram-negative bacterium found in soil and associated with plants, and thus inhabits the same environments as *B. subtilis*. *P. protegens* produces 2,4-diacetylphloroglucinol (DAPG), a broad-spectrum antibiotic whose biosynthesis genes are conserved across many pseudomonads [21], and which has been shown to stimulate biofilm formation in *Azospirillum brasilense* at subinhibitory concentrations [22]. In contrast, when applied at subinhibitory concentrations to *B. subtilis*, DAPG was recently shown to inhibit biofilm formation (and sporulation) [21]. One intriguing detail that emerged from this study is that DAPG (a biofilm-inhibitor) appears to be overproduced on the medium MSgg (a *B. subtilis*-biofilm-inducing medium); this suggests that in the same environmental conditions where *B. subtilis* is forming biofilms, *P. protegens* produces large amounts of its biofilm-inhibiting compound DAPG. This raises interesting questions about how these two bacteria may interact and coexist in nature.

These findings highlight a challenge with the effort to understand the biological activities of microbial metabolites: DAPG has been shown in different *in vitro* contexts to kill fungi, to kill bacteria, to affect the hatching of nematodes, and to modulate *B. subtilis* biofilm formation. A similarly broad range of roles have been attributed to molecules such as surfactin, and to a variety of other microbal metabolites as well. How then are we to know which (if any) of these activities are relevant to the producing organisms in the natural environment? In some ways it may be irrelevant: these studies have advanced our understanding of diverse biological processes and provided potential chemical tools to modulate interspecies interactions to our benefit. But for those driven by a desire to discern the native functions of microbial metabolites, such reports serve as a vital starting point, providing critical reagents (signaling mutants, purified compounds, etc.) to test the hypothesis of whether the observed *in vitro* results align with their *in vivo* or *in situ* activity. This question will be facilitated by the development of experimental systems that allow us to examine chemically mediated interactions at the microscale: we predict that the behavior of bacteria at the single cell (or tens-of-cells level) will differ dramatically compared to the responses of the massive populations of cells typically examined in *in vitro* laboratory assays.

Pseudomonas aeruginosa

P. aeruginosa is a Gram-negative model bacterium whose biofilm formation has been extensively studied (Figure 2B). This opportunistic pathogen is capable of colonizing plants and humans and is of considerable medical importance as a colonizer of the lungs of cystic fibrosis patients. The ability of *P. aeruginosa* to form biofilms is thought to be a major contributor to its ability to cause disease in cystic fibrosis patients, as biofilm cells exhibit increased tolerance to antibiotics and resistance to phagocytosis [23]. Biofilm formation in *P. aeruginosa* requires flagellar motility (which facilitates localization to the liquid-surface interface) as well as type IV pili-mediated twitching motility (which mediates bacterial movement along surfaces) [24]. *P. aeruginosa* produces three matrix polysaccharides (alginate, Pel, and Psl) that contribute to the stability and structure of the biofilm [25,26]. In addition, extracellular DNA is important for biofilm structure in *P. aeruginosa* [27,28]. Cyclic di-guanosine monophosphate (c-di-GMP) is a second messenger molecule that controls polysaccharide production in *P. aeruginosa* [29,30] and promotes cell surface

adhesion [31]. Levels of c-di-GMP are modulated in response to environmental signals by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), which produce and degrade c-di-GMP respectively [32]. Quorum sensing (QS) is also integrated into the biofilm regulatory network in *P. aeruginosa* [33]. The QS system in *P. aeruginosa* is comprised of two hierarchical systems, the autoinducer synthetases/regulatory protein pairs LasI/LasR and RhlI/RhlR. LasR binds to the promoter region of the *psl* operon [34] and a lack of *lasI* causes a biofilm defect [35]. Both *lasI* and *rhlI* enhance Pel polysaccharide biosynthesis in *P. aeruginosa* [36].

P. aeruginosa has been isolated from numerous environments including plants, animals, soil, natural and man-made aquatic environments, and clinical settings [37–40]; thus there is great potential for interspecies and interkingdom interactions with this organism. Subinhibitory concentrations of tobramycin, an aminoglycoside produced by the soil bacterium *Streptomyces tenebrarius*, was found to induce biofilm formation in *P. aeruginosa* [41]. Other aminoglycosides (streptomycin and gentamicin, produced by the soil bacteria *Streptomyces griseus* and *Micromonospora purpurea*, respectively) induce biofilm formation in *P. aeruginosa* as well [41]. Aminoglycosides are protein synthesis inhibitors that are a clinically important for treatment of chronic heart, lung, and urinary tract infections. The fact that these antibiotics provide a signal to *P. aeruginosa* to form highly recalcitrant biofilms is therefore of significant clinical relevance, since subinhibitory concentrations of these compounds may stimulate a bacterial response that is counter to their desired clinical effect.

The induction of biofilm formation by tobramycin requires a functional *arr* (aminoglycoside response regulator) gene, which encodes an inner membrane PDE that degrades c-di-GMP [41]. Tobramycin-inducible biofilm formation is inhibited by the addition of exogenous GTP (a c-di-GMP inhibitor), suggesting that tobramycin impacts biofilm formation through the Arr phosphodiesterase via a still undefined mechanism [41]. Analysis of the *P. aeruginosa* transcriptome upon exposure to antibiotic treatment (at subinhibitory concentrations) revealed that tobramycin differentially regulates 40 genes (out of 555 genes represented in the microarray used), including those involved in transcriptional regulation, secondary metabolite regulation, outer membrane composition, secretion, chemotaxis, and motility [42]. This study also demonstrated that subinhibitory concentration of tobramycin and tetracycline (a protein synthesis inhibitor produced by multiple streptomycete species) both stimulated biofilm gene expression, but otherwise altered expression in unique subsets of genes [42]. Thus, *P. aeruginosa* may have multiple sensing systems that integrate to influence biofilm formation in response to subinhibitory concentrations of antibiotics. Also notable is the fact that these antibiotics cause major changes to the transcriptome of *P. aeruginosa* without measurably altering its growth rate or global protein synthesis [41], further suggesting that these antibiotics may have specific biological functions beyond just killing.

Another indication that antibiotics are molecules important for bacterial communication is that several antibiotics impact the QS pathway in *P. aeruginosa* (as well as in other bacteria as discussed below), which may be an important mechanism for controlling bacterial biofilm formation. Azithromycin (AZM) is a macrolide antibiotic derived from the soil-dwelling

bacterium *Saccharopolyspora erythraea* commonly used for treating chronic lung infections in cystic fibrosis patients. Subinhibitory concentrations of AZM transcriptionally repress both the *las* and *rhl* QS systems in *P. aeruginosa*, and inhibit the expression of the *N*-acylhomoserine lactone (AHL) synthesis enzymes upstream of *lasI* and *rhlI*, leading to lower concentrations of autoinducers being produced [43,44]. Transcriptome and proteome analysis of *P. aeruginosa* indicates that AZM influences mRNA message abundance of over 10 % of the QS regulon and almost 32 % of QS-dependent proteins, while also reducing the expression of multiple flagellar biosynthesis proteins required for biofilm surface attachment [45]. AZM also delays biofilm formation in *P. aeruginosa* in flow cell systems [46], and inhibits biofilm formation in static systems [47], presumably through its effect on QS pathways and by impairing motility.

Other natural products also inhibit biofilm formation in *P. aeruginosa* by impacting QS pathways. Halogenated furanones are natural compounds secreted by the red alga *Delisea pulchra*. Using a *lasB*-based AHL reporter assay, a halogenated furanone was found to reduce quorum sensing-controlled gene expression at concentrations that had no effect on growth [48]. Confocal imaging demonstrated that this antibiotic penetrates microcolonies within the *P. aeruginosa* biofilm and blocks cell signaling, which in turn impacts biofilm architecture and biomass [48]. Several other natural compounds from plants and animals have anti-QS properties in *P. aeruginosa* [4]. Thus, chemical communication mechanisms that influence biofilm formation in *P. aeruginosa* appear to be widespread in both the eubacterial and eukaryotic kingdoms.

Antibiotics impacting biofilm formation in other bacteria

Fewer studies have examined the impact of antibiotics on biofilm development in bacterial species other than the model microbes *B. subtilis* and *P. aeruginosa*. Nevertheless, such studies, some of which are summarized below, demonstrate that many microorganisms produce specialized metabolites that affect biofilm formation in other bacteria, and suggest that microbes possess a range of possible mechanisms for sensing and responding to subinhibitory concentrations of antibiotics. In many cases, however, there is insufficient data available to discern whether the tested antibiotics impact biofilm formation independently of their killing activity, or whether they are simply acting as antimicrobial and/or antibiofilm molecules, a distinction that should be more thoroughly explored by the field going forward.

Sensing and responding to subinhibitory concentrations of antibiotics

We begin by providing examples of publications that describe the effects of subinhibitory concentrations of antibiotics on biofilms. Subinhibitory concentrations of β-lactams have been shown to disperse established *Listeria monocytogenes* biofilms; to stimulate biofilm formation in *L. monocytogenes* [49]; to induce biofilm formation and extracellular DNA release in multiple strains of *S. aureus* [50]; and to cause *Klebsiella pneumoniae* biofilm cells to round, bleb, and dimple [51]. These studies demonstrate that functionally related β-lactam molecules have widely divergent impacts on different bacterial species. Similar conclusions can be drawn from studies examining the effects of biosurfactants on biofilms. Surfactin, as mentioned above, is a biosurfactant that activates the biofilm formation

pathway in *B. subtilis* [18]. Meanwhile, the putisolvin lipopeptides inhibit or break down *Pseudomonas* biofilms [52], while other biosurfactants inhibit adhesion and biofilm formation in *Acinetobacter baumannii*, *Escherichia coli*, and *Staphylococcus aureus* [53]. As a final example of this concept, *P. aeruginosa* grown on a polymer surface loaded with usnic acid (a specialized metabolite produced by lichens) showed enhanced biofilm structure (but no change in cell number compared to its growth on a non-usnic acid surface), while no such difference was observed with *S. aureus* grown on the same surfaces [54]. These data all emphasize that the same compound can have significantly different effects on different bacteria.

On the other hand, it is also the case that different molecules have unique impacts on the same organism: one study demonstrated that while two antibiotics (tetracycline and a streptogramin) at subinhibitory concentrations enhanced biofilm gene expression in *Staphylococcus epidermidis*, numerous others had no impact [55]. Taken together, these studies exemplify a significant challenge in the field: while it would be satisfying to be able to build a coherent framework for predicting the action of subinhibitory antibiotics on bacterial biofilms, the diversity of activities currently observed makes this impossible. Indeed, such a consistent framework may not exist. However, only by achieving a better understanding the molecular targets of the non-killing activities of antibiotics, as well as by constructing reproducible *in vitro* assay systems that allow direct comparisons to be made across different bacterial systems (and laboratories) will we be able to make a conclusion either way.

In an effort to identify the impact of subinihibitory antibiotics on non-stress-related, biofilm-relevant genes, some studies have used global analyses to examine the effects of subinhibitory antibiotics on the transcriptional responses of bacteria. For instance, subinhibitory concentrations of penicillin results in the differential regulation of 386 genes in *Streptococcus pneumoniae*, including those involved in competence, quorum sensing, cell envelope, capsular polysaccharide biosynthesis, fatty acid chain elongation, and polyamine transporters [56]. A similar study identified genes involved in twitching motility, flagellar assembly, biofilm formation, and quorum sensing that were differentially regulated in *P. aeruginosa* in response to subinhibitory concentrations of the human host defense peptide LL-37 [57]. The results from such studies emphasize the breadth of cellular functions that subinhibitory antibiotics affect, but in most cases they provide only tantalizing hints as to the potential mechanisms by which these non-killing functions are mediated.

One way to gain additional insight into this problem is to compare the transcriptional responses of related molecules within the same bacterium. For example, the biofilm-inhibiting peptides LL-37 and 1037 were observed to dysregulate more than 400 genes in *S. aureus*, but only 14 of them were similarly differentially regulated, nearly all of which were important in biofilm formation; these genes thus represent potential targets of action of these peptides [58]. In other cases, more directed studies have begun to elucidate the connections between antibiotic sensing and a change in biofilm physiology. In *Vibrio cholerae*, for instance, the two-component signal transduction system CarRS was shown to directly regulate the transcription of the *almEFG* operon, which both promotes polymyxin B resistance and also represses the major biofilm transcriptional regulators *vpsR* and *vpsT*

[59]. In spite of these examples, however, in most instances significant future work needs to be applied to follow-up on either transcriptional or biofilm analyses to identify the potential mechanisms by which subinhibitory antibiotics impact bacterial physiology. Only then will it be possible to more accurately assess whether these metabolites have distinct modes of action for their killing and biofilm-modulating activities (as suggested by the thiocillin results obtained in *B. subtilis* described above).

Antibiotic signals that feed into quorum sensing networks

As described in the *P. aeruginosa* section above, another clue that antibiotic compounds may be important interspecies signals is the fact that they often impact QS systems, which in many bacteria then go on to impact biofilm formation. One furanone (structurally related both to bacterial acylhomoserine lactones (AHL) and the furanones that impact *P. aeruginosa* biofilms) was shown to enhance biofilm formation of *Staphylococcus epidermidis* and *Staphylococcus aureus* at subinhibitory concentrations in a manner that appears to be via the staphylococcal QS pathway [60]. Other studies have found that different furanones inhibit biofilm formation in *S. epidermidis* [61], *B. subtilis* [35–37] and *E. coli* [62], yet it remains unknown if QS pathways are impacted in these examples. In addition to furanones, subinhibitory concentrations of β-lactams have also been shown to act via QS pathways: penicillin stimulates biofilm formation in *Streptococcus pneumoniae* [56], while ampicillin induces biofilm formation in *Staphylococcus intermedieus* [63]. Finally, healthy vaginal lactobacilli produce bacteriocins that work as antibiofilm agents against the *Gardnerella vaginalis* [64,65] and reduce AI-2 production in this bacterial vaginosis-associated pathogen [66].

Conclusion: challenges and opportunities

Few studies have attempted to address the function of antibiotics in the natural environment, and many questions still remain about their potential roles there (see Outstanding Questions Box). However, subinhibitory concentrations of antibiotic compounds clearly affect biofilm formation in a multitude of bacterial species, and coculture experiments indicate that, at least *in vitro*, antibiotic production by one bacterium can affect biofilm gene expression and structure in other bacterial species. This indicates that natural-product antibiotics may play an important role as chemical signals that provide cues impacting biofilm development in nature.

Yet, as mentioned throughout this review, our knowledge of the mechanistic details of these effects and the identification of the (non-killing, biofilm-modulating) targets of subinhibitory antibiotics is still extremely limited-to-nonexistent. This makes it nearly impossible to know whether the observed effects are dependent or independent of the antimicrobial effect of the compound, a distinction critical for drawing conclusions about the potential function of these compounds in the natural environment. To address this challenge, we need experimental approaches that allow us to directly probe how metabolites affect microbial cells, ideally *in situ* and in real time. One such method is Attenuated Total Reflectance-Fourier Transform InfraRed (ATR-FTIR) spectroscopy, which was recently applied to gain insight into the molecular mechanism of action of an antimicrobial peptide (dermaseptin)

against *Pseudomonas fluorescens* biofilms [67]. This study revealed that dermaseptin led to a loss of membrane lipids and a rapid inhibition of nucleic acid biosynthesis in young *P. fluorescens* biofilms [67]. ATRFTIR thus can be used to probe and monitor, *in situ* and in real time, the biochemical changes induced by antibiotics in bacteria cells at the molecular scale during early biofilm formation. The further application of this and other novel spectroscopic methods may provide much-needed insights into how microbial metabolites impact biofilm formation in diverse bacteria.

In addition to obtaining a more mechanistic understanding of these interactions, future studies characterizing bacterial interactions mediated by specialized metabolites in non-model organisms and at small, microbially relevant spatial scales may provide important knowledge about the potential roles antibiotics play in nature. This information will be crucial to understanding how subinhibitory antibiotic concentrations can enhance potentially adaptive characteristics that increase fitness in bacterial pathogens. Furthermore, studies that address the role that antibiotics play in shaping microbial communities would provide important insights into how these compounds affect complex systems such as the human, plant, and soil microbiomes.

Acknowledgments

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Trends Box

- Many antibiotics are derived from natural products produced as specialized metabolites by microorganisms

- The function of antibiotics in the natural environment remains poorly characterized

- Bacteria produce a variety of natural-product antibiotics that can impact biofilm formation in other bacteria

- A single metabolite can have divergent effects on biofilm formation in different bacterial species

- The argument that the natural function of antibiotics may be signaling is strengthened by the finding that (at least for one natural product), different parts of the molecule are responsible for its biofilm-enhancing and killing activities.

- Specialized metabolites may act as important environmental cues that regulate biofilm formation and shape multispecies interactions in microbial communities

Outstanding Questions Box

- How do antibiotics affect biofilm development in non-model bacteria?

- What are the molecular mechanisms by which antibiotics impact biofilm formation?

- Are there commonalities in the mechanisms used to sense and respond to antibiotics among diverse bacterial species?

- Will expanding the breadth of data available (more compounds, bacterial species, conditions), allow us to formulate a coherent framework for predicting the activity of antibiotics on biofilm formation in different species?

- How do the effects of purified compounds on biofilm formation compare to those resulting from microbial coculture interactions?

- Do the activities of specialized metabolites observed in standard laboratory assays differ from those obtained at realistic (microbial) spatial scales from environmentally relevant (typically small) populations of cells?

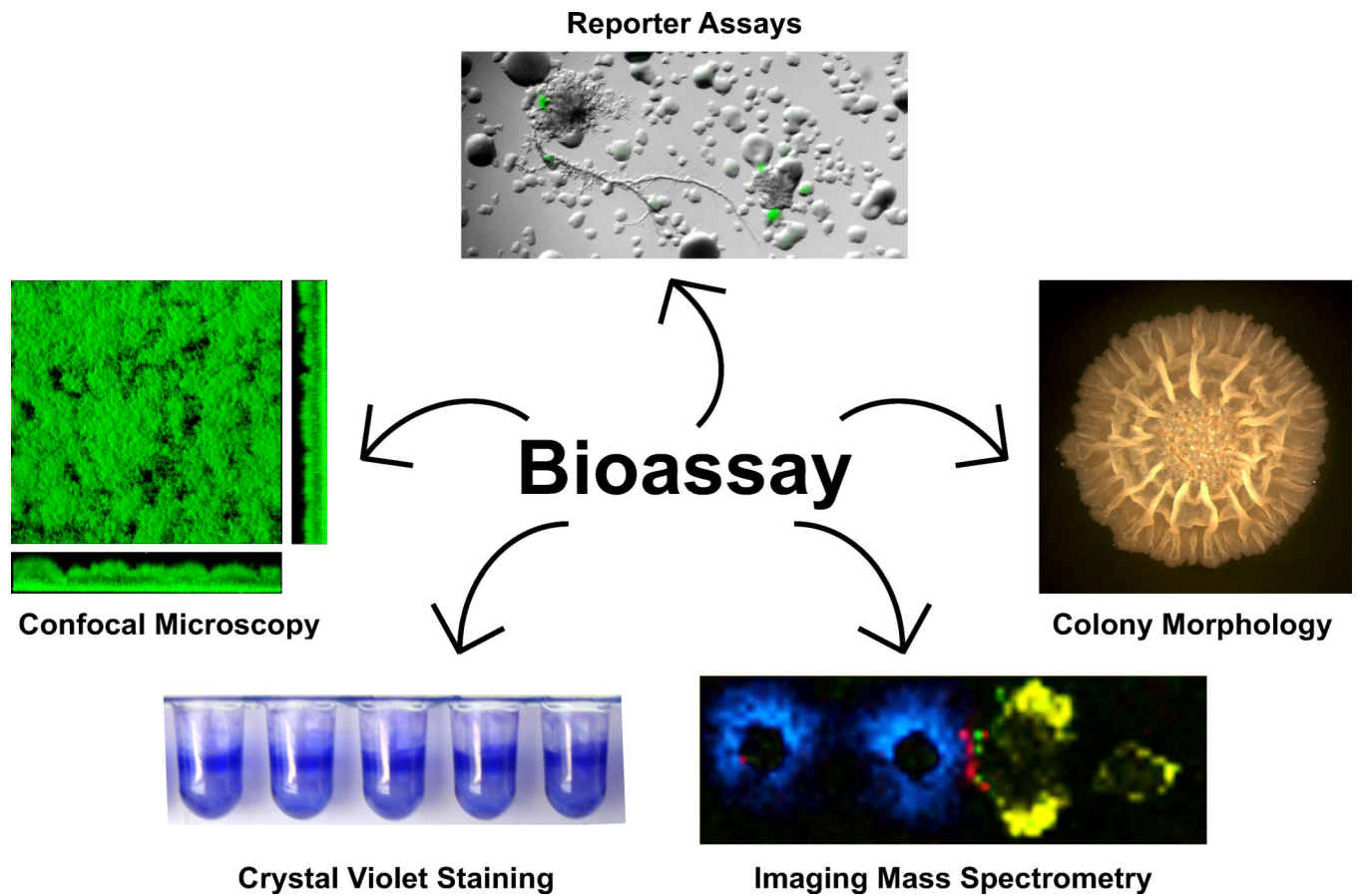- How do native environmental conditions impact antibiotic communication between bacteria?

Reporter Assays

Confocal Microscopy

Bioassay

Colony Morphology

Crystal Violet Staining

Imaging Mass Spectrometry

Figure 1.
Methods for detecting specialized metabolites that impact biofilm formation. Phenotypic assays that look for effects on biofilm formation include observing colony morphology, biofilm reporter activity, or biofilm architecture and attachment (using confocal microscopy and crystal violet staining) in the presence of purified compound or in co-culture. Imaging mass spectrometry can be used to identify specialized metabolites in respect to their spatial distribution.
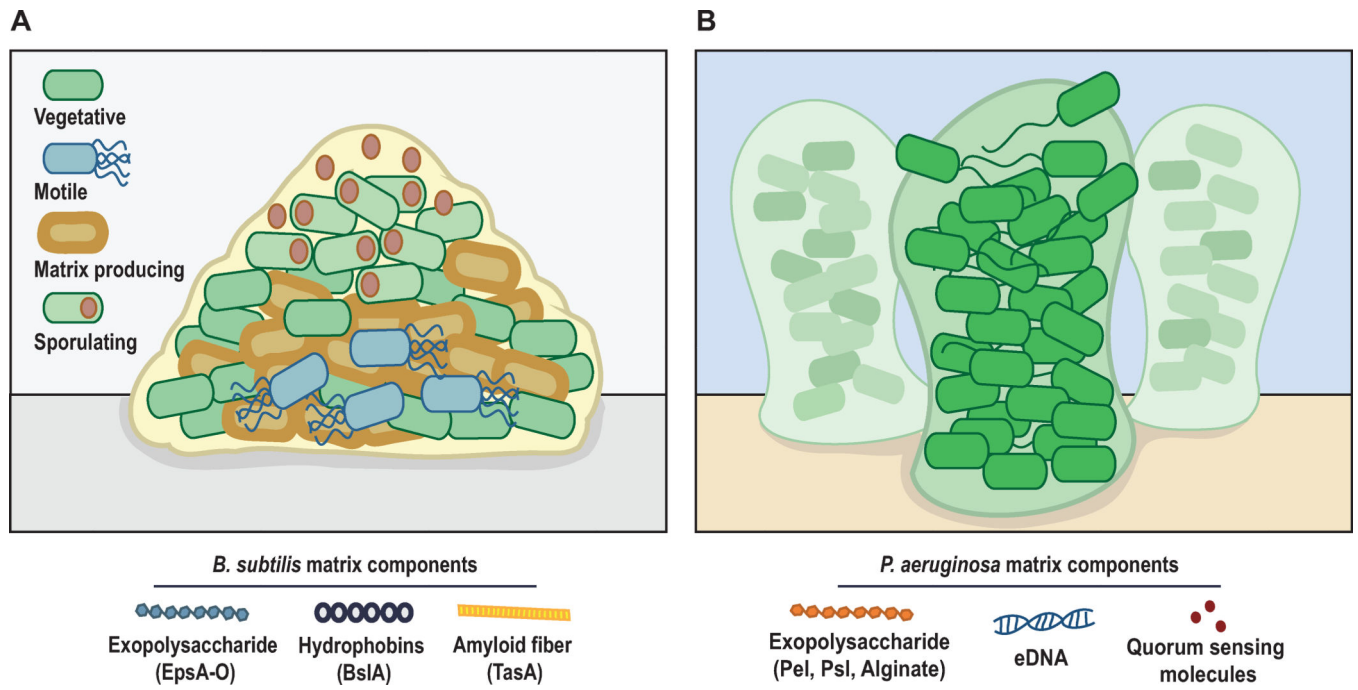
Figure 2.
Schematic representation of biofilm structure and composition in *B. subtilis* and *P. aeruginosa*. (A) *B. subtilis* and (B) *P. aeruginosa*.

Table 1

Antibiotics that impact biofilm formation that are mentioned in this paper.

Compound Source	Compound	Bacterial species effected	Reference
Amphibian skin	Dermaseptin S4 derivative	*Pseudomonas fluorescens*	67
Bacteria	2,4-diacetylphloroglucinol	*Bacillus subtilis*	21
Bacteria	Biosurfactants	*Acinetobacter baumannii* *Escherichia coli* *Staphylococcus aureus*	53
Bacteria	Biosurfactants putisolvins I and II	*Pseudomonas spp.*	52
Bacteria	Polymyxin B	*Vibrio cholerae*	59
Bacteria	Subtilosin	*Gardnerella vaginalis*	65, 64
Bacteria	Surfactin Nystatin	*B. subtilis*	18
Bacteria	Tetracycline	*Staphylococcus epidermidis*	55
Bacteria	Thiocillin Various thiazolyl peptides	*B. subtilis*	20
Bacteria	Tobramycin	*E. coli* *Pseudomonas aeruginosa*	41
Bacteria/semisynthetic	Carbapenem	*Klebsiella pneumoniae*	51
Bacteria/synthetic	Ampicillin Ciprofloxacin Tetracycline	*Streptococcus intermedius*	63
Human	Defence peptide LL-37	*S. aureus* *P. aeruginosa*	57
Lichen	Usnic Acid	*P. aeruginosa* *S. aureus*	54
Marine algae	Furanone	*E. coli* *P. aeruginosa* *S. aureus* *S. epidermidis*	48, 62, 60
Semisynthetic	Ampicillin Amoxicillin Cloxacillin Methicillin	*S. aureus*	50

Compound Source	Compound	Bacterial species effected	Reference
Semisynthetic	Azithromycin	*P. aeruginosa*	44–47
Synthetic	Peptide 1037	*P. aeruginosa* *Burkholderia cenocepacia* *Listeria monocytogenes*	58