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Mechanisms of biofilm stimulation by subinhibitory concentrations of antimicrobials

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Biofilms are a typical mode of growth for most microorganisms and provide them with a variety of survival benefits. Biofilms can pose medical and industrial challenges due to their increased tolerance of antimicrobials and disinfectants. Exposure of bacteria to subinhibitory concentrations of those compounds can further exacerbate the problem, as they provoke physiological changes that lead to increased biofilm production and potential therapeutic failure. The protected niche of a biofilm provides conditions that promote selection for persisters and resistant mutants. In this review we discuss our current understanding of the mechanisms underlying biofilm stimulation in response to subinhibitory antimicrobials, and how we might exploit this 'anti-antibiotic' phenotype to treat biofilm-related infections and discover new compounds.

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Introduction

Most microbes live in surface-associated biofilms. This lifestyle affords benefits ranging from shared metabolism to protection from predation. The cues that influence biofilm development are complex and differ among species, but include both physical (surface topography, temperature, hydration, light) and chemical (nutrients and metabolites, quorum sensing molecules, antimicrobials) stimuli. The resulting biofilm is thus adapted to cope with the specific environment in which the microbes find themselves. In the case of antimicrobials and disinfectants, it is well established that microbes in biofilms can tolerate significantly higher concentrations than individual planktonic cells, leading to clearance failures in medical and industrial contexts. However, recent work has revealed that exposing bacteria to subinhibitory antimicrobials from many chemically distinct classes increases biofilm formation. This hormetic response could be viewed as a rapid and non-specific way to protect the population from impending chemical threats while a more targeted response to a particular molecule is developed. Here we review current hypotheses about the mechanisms by which subinhibitory antimicrobials modulate biofilm formation and propose ways in which we might inhibit this response to potentiate antibiotic action, or exploit it to identify antimicrobial activities present at subinhibitory concentrations in synthetic libraries or complex mixtures of natural products.

Biofilm stimulation is not species or antibiotic-class-specific

The dose-dependent stimulation of biofilm formation by antibiotics has been reported for multiple Gram positive and Gram negative species, and for multiple antimicrobial classes with distinct targets (Table 1). There are reports that natural products without antimicrobial activity can stimulate biofilm formation [1••], as can nutritional cues such as high iron concentrations [2]. We will not cover non-antibiotic chemical stimuli that influence biofilm formation here, but for excellent coverage of this topic we refer readers to a recent review by Townsley and Shank [3••]. Molecules of a given antimicrobial class may stimulate biofilm formation to different extents [4], making the potential mechanisms of stimulation unclear. Below we outline select examples of clinically relevant pathogens that respond to various subinhibitory antimicrobials with increased biofilm formation.

Pseudomonas aeruginosa

P. aeruginosa is an opportunistic pathogen that infects individuals whose defences are compromised by injury, immunosuppression, the presence of medical devices, or by cystic fibrosis (CF)-related impairment of mucociliary clearance. Its intrinsic resistance to many antimicrobials is further enhanced by growth in a biofilm. To treat exacerbations of chronic lung infections, CF patients use an inhaled form of the aminoglycoside tobramycin to achieve therapeutic concentrations without the systemic toxicity associated with this antibiotic class. The discovery that subinhibitory concentrations of tobramycin could increase the amount of biofilm formed by *P. aeruginosa* in

Table 1

Potential mechanisms of biofilm stimulation by subinhibitory antibiotics

Proposed mechanism	Species	Antibiotics	References
Increased biofilm by 'eDNA seeding'	*Enterococcus faecalis, Haemophilus influenzae*	Ampicillin, Ceftriaxone, Cefuroxime, Oxacillin, Fosfomycin, Amoxicillin/ Clavulanic Acid, Penicillin G	[12,27]
Induction of phage elements	*Pseudomonas aeruginosa, Staphylococcus aureus*	Ciprofloxacin, Methicillin, Ampicillin, Amoxocillin, Cloxacillin	[11,33••]
Regulatory responses	*Pseudomonas aeruginosa, Bacillus subtilis, Acinetobacter baumannii, Haemophilus influenzae*	Tobramycin, Ciprofloxacin, Tetracycline, Thiocillins, Imipenem, Bacitracin, β-defensin-3, Amoxicillin, Ampicillin, Cefuroxime, Rifampicin	[1••,5,9••,45–48]

a dose-dependent manner [5] was greeted with alarm, as it implied that limited diffusion of the antibiotic into deeper regions of the lung had the potential to perversely worsen infection [6]. The proposed mechanism underlying the development of increased biofilm in response to tobramycin exposure — expression of a putative cyclic-di-GMP phosphodiesterase, Arr — was later discounted. Although the biofilm stimulation response to aminoglycosides has proven highly reproducible, many strains of *P. aeruginosa* lack the *arr* gene [7].

The ability of subinhibitory antibiotics to increase *P. aeruginosa* biofilm production is not limited to aminoglycosides [8]. Other stimulatory classes include fluoroquinolones, beta-lactams, and tetracyclines. Diverse molecules including pyocins produced by competitor strains or ethanol produced by yeast also enhance biofilm formation [9••,10], suggestive of a generic response to potentially harmful chemical signals.

Staphylococcus aureus

S. aureus is a versatile pathogen capable of colonizing almost any bodily site. Methicillin resistant *S. aureus* (MRSA) strains, originally a problem in hospital settings, have since spread to the community. They are a frequent cause of intractable medical device and soft tissue infections, both associated with biofilm formation. Two well-studied strains — USA300 and USA500 — form little biofilm in the absence of methicillin, but exposure to subinhibitory concentrations of methicillin led to a dramatic increase in biofilm formation (Table 1) [11]. This increase was dependent on autolysis activity linked to *atl*, indicating a genetic mechanism that drives cell lysis to release common goods like eDNA that may increase biofilm formation.

Enterococcus species

E. faecalis and *E. faecium* are found as commensals in the gastrointestinal tract but can cause hospital-acquired urinary tract infections, endocarditis, and endodontic infections. *E. faecium* in particular has been associated with high levels of resistance to vancomycin, known as 'vancomycin-resistant enterococci' or VRE strains. Indwelling medical equipment such as catheters are a common site of infection. Recently, peptidoglycan synthesis inhibitors (ampicillin, oxacillin, ceftriaxone, and fosfomycin) were found to induce biofilm formation in *E. faecalis* V583, a clinical isolate, as well as in *E. faecalis* OG1RF, a common laboratory strain (Table 1) [12]. These increases were not seen when using drugs with other 'non-lysing' mechanisms but were also seen with membrane-disrupting detergents, leading to the hypothesis that antibiotic-induced biofilm formation may, in part, be due to eDNA release and cell lysis caused by antibiotic activity [12].

Listeria monocytogenes

Listeria monocytogenes is a cold-tolerant and salt-tolerant bacterium that can cause food poisoning associated with the consumption of contaminated ready-to-eat foods. It forms biofilms on food processing equipment and food surfaces and is shed from infected hosts as small biofilm-like aggregates [13]. Exposure of *Listeria* to subinhibitory levels of antimicrobials of various classes [4] or disinfectants [14] can increase biofilm formation, posing a problem in food plants where clean-in-place protocols (where processing equipment is not fully dismantled prior to being sanitized by application of disinfectants) could result in insufficient exposure of cells embedded in crevices or other hard-to-reach locations [15]. Exposure to subinhibitory antibiotics has been linked to changes in *L. monocytogenes* metabolism that result in increased tolerance to antimicrobials [16].

Salmonella enterica

S. enterica serovar Typhimurium is a common cause of acute gastroenteritis, a sometimes severe but generally self-limiting infection that is of major concern to food manufacturing and handling industries. Biofilms have been implicated in the transmission of food-borne pathogens like *Salmonella*, from their ability to tolerate harsh environments posed by desiccation and biocides. Sodium hypochlorite, a common biocide used in cleaning, induces biofilm formation in *S. enterica* when the bacteria is exposed to subinhibitory concentrations [17]. If used at suboptimal concentrations while cleaning food

preparation or manufacturing surfaces, sodium hypochlorite may cause *Salmonella* to more readily adopt a biofilm lifestyle.

Potential mechanisms of biofilm stimulation

The roles of antimicrobials in nature remain a topic of debate — are they signalling molecules that can kill, or lethal molecules that can act as social cues? The ability of subinhibitory concentrations of diverse antibiotics and disinfectants to provoke changes in bacterial physiology and behaviour is now well established, but the underlying mechanisms remain to be clarified. This phenomenon has been framed as a response to ecological competition, where detection of competitor strains by their ability to cause sublethal damage using toxic molecules leads to increased biofilm production [9••]. The most prevalent hypotheses about how antibiotics stimulate biofilm are the seeding of biofilm formation by dead cells and their products, or the induction of stress responses or other physiological changes by sublethal damage, leading to increased biofilm formation (Figure 1). Both these hypotheses are consistent with the observation that biofilm stimulation occurs in a dose-dependent manner, with maximal stimulation typically seen at approximately $^1/_4$ to $^1/_2$ the minimal inhibitory concentration (MIC). The following sections summarize various mechanisms proposed for the biofilm stimulation phenotype.

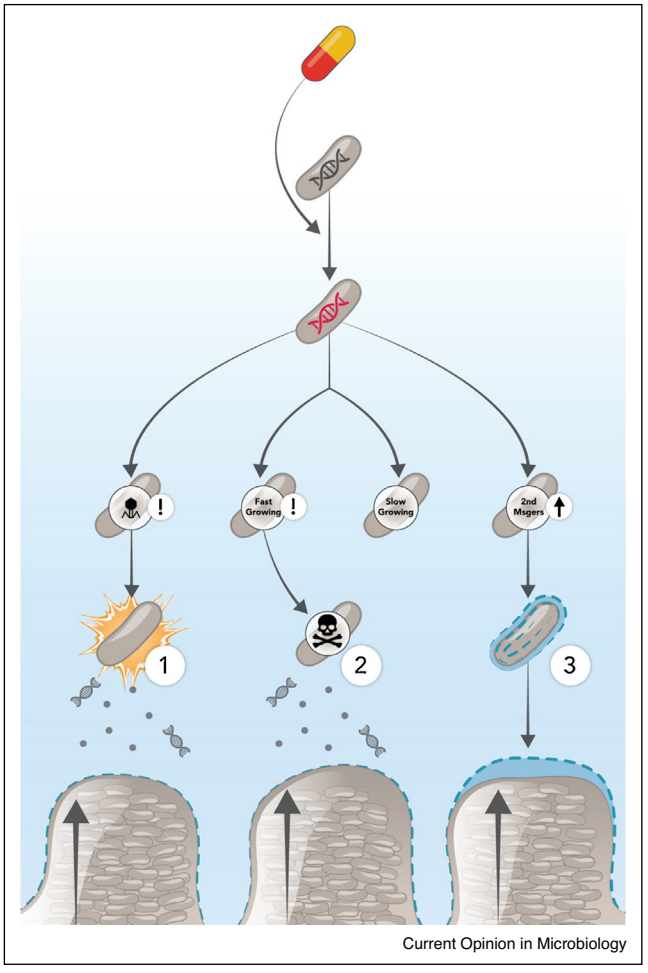
Increased adherence

Initial adherence to a surface is a critical early step in biofilm formation. Bacteria interact with surfaces using filamentous protein structures such as pili or flagella, as well as via cell surface proteins and polysaccharides. Biophysical studies showed that subinhibitory concentrations of norfloxacin or streptomycin induce physical changes in cell surface charge and hydrophobicity, resulting in more favourable interactions with surfaces that may contribute to increased biofilm formation [18,19]. Increased cell-to-surface interaction strength was shown using force spectroscopy, measured by single *S. aureus* cells coupled to an atomic force microscope tip [19]. Changes in surface protein content, as well as up-regulation of known adhesion-related proteins, have also been observed after treatment with subinhibitory antibiotics.

Stress responses

Biofilm stimulation has been documented for a broad range of bacterial and fungal species, suggesting that it may reflect general stress responses. However, there is evidence that only certain stimuli trigger biofilm development [20]. Heat or osmotic shock — which can also cause sublethal cellular damage and trigger protective stress responses — fail to stimulate biofilm formation. This finding suggests that only specific types of signals lead to biofilm induction. Along these lines, the toxic activity of thiocillins towards *Bacillus* could be decoupled from their biofilm stimulatory activity, suggesting that biofilm formation could be induced without overt damage [1••].

Figure 1



Potential mechanisms of biofilm stimulation by subinhibitory antibiotics. When bacteria are exposed to subinhibitory antibiotic concentrations (red-yellow capsule), one or more of the following events may be triggered. In scenario **1**, cell damage or stress leads to upregulation of prophages or phage holins expression, leading to explosive cell lysis and the release of eDNA and cellular contents that seed biofilm formation and contribute to matrix deposition. In scenario **2**, the natural physiological heterogeneity of cells in a population means that some will be more susceptible than others to the antibiotic at its subinhibitory concentration. These could be more rapidly growing cells, or cells expressing susceptible targets. The cell debris released by these dead cells could seed biofilm formation of the remaining, less susceptible subpopulation. Scenario **3** considers the potential ability of antibiotics to act as signalling molecules at subinhibitory concentrations, either directly or indirectly through the induction of cell stress responses or increase in production of secondary messengers (2nd Msgers) such as cyclic-di-GMP or ppGpp that modulate gene expression. These transcriptional changes could include changes in cell surface properties that increase adhesion to surfaces, and/or increased production of matrix components (blue dashed lines).

The response to subinhbitory antibiotics could be mediated in part through induction of oxidative stress pathways [21,22] although this mechanism of antibiotic action remains controversial [23,24]. The Hancock group proposed that biofilm formation by both Gram positive and Gram negative bacteria relies on activation of the stringent response, as antimicrobial peptides that specifically block this pathway prevent biofilm formation [25]. However, this hypothesis has been challenged by subsequent work [26].

Multiple studies reported that antibiotics such as beta-lactams that induce cell lysis can stimulate biofilm formation, whereas antibiotics that target the ribosome or DNA replication are less likely to do so [12,27]. Unique responses to specific antibiotics could initiate alterations in physiology that are predictive of a particular compound's mechanism of action, but ultimately these changes may feed into more general stress responses that include increased biofilm formation.

Release of eDNA

An alternative or ancillary mechanism for biofilm stimulation by sublethal antibiotic concentrations could be the death of a subpopulation of cells and release of their contents. Most bacterial populations will exhibit some heterogeneity in susceptibility due to differences in physiology, growth rate, or timing of the cell cycle, with some cells dying at concentrations below the minimal inhibitory concentration of the bulk population [28,29]. Multiple species of Gram positive and Gram negative bacteria, mycoplasmas, archaea, and fungi incorporate eDNA as a major component of the biofilm matrix [30–32]. The release of extracellular DNA (eDNA) by the most susceptible members of the population could seed increased biofilm formation by remaining cells (Figure 1).

In addition to cell death directly from antibiotic action, eDNA release can occur via activation of programmed cell death mechanisms that result in cell lysis. For example, *P. aeruginosa* releases eDNA through explosive lysis of a subset of cells through the activity of the Lys endolysin, encoded within a R-pyocin and F-pyocin gene cluster. Explosive cell lysis is induced by exposure to genotoxic antibiotics such as mitomycin C and ciprofloxacin via the SOS response regulator, RecA [33••]. Both staphylococci and enterococci require expression of peptidoglycan hydrolases for biofilm formation, which allow for lysis of a subpopulation and release of eDNA [34–37].

Exploitation of the biofilm stimulation phenotype for therapy and drug discovery

Due to the inexorable rise of resistance, many antibiotics are losing their utility. Efforts to preserve the effectiveness of remaining molecules include attempts to identify adjuvant molecules that can block resistance or inhibit biofilm formation to maintain microbes in a more susceptible state [38,39•,40,41]. As our understanding of the mechanisms of biofilm stimulation by subinhibitory antibiotics improves, we may be able to identify antibiotic adjuvants that block stress pathways or activate dispersal mechanisms, aiding in antibiotic action.

High throughput screening of whole cells against libraries of synthetic compounds or mixtures of natural products remains a critical tool for drug discovery. Using whole cell screens bypasses potential problems of poor permeability and efflux but has the disadvantage that the target(s) of the molecules must be subsequently identified [42]. For practical reasons, screening is typically performed at a single arbitrary concentration which could fall anywhere on the scale from ineffective to well above the MIC for a specific molecule. For new antimicrobials, a typical first pass seeks to identify molecules that inhibit growth of intact cells at the selected screening concentration.

Since biofilm stimulation occurs at concentrations below those that cause sterilization of a culture and is responsive to many chemically distinct molecules, this phenotype could potentially provide a wider screening window. Molecules that might otherwise be overlooked because they fail to kill the test organism at the concentration tested could still be identified as potentially toxic hits if they stimulate biofilm development compared to a vehicle control. Using this phenotype to report on potential toxicity of a particular molecule is also attractive in that it does not require prior purification. For example, Oliveira and colleagues [9••] used spent culture medium to demonstrate that soluble pyocins were responsible for the biofilm stimulation of one *P. aeruginosa* strain by another.

A potential disadvantage of using biofilm stimulation as a screening tool is the labour-intensive and time-intensive nature of current biofilm assays. However, a better, molecular-level understanding of the subinhibitory antibiotic-responsive pathways that lead to biofilm stimulation will provide an opportunity to build tools such as transcriptional reporters coupled to informative promoters that could be used for more rapid whole cell screening. Such reporters could indicate upregulation of secondary messengers such as cyclic-di-GMP [43] that are critical to biofilm formation, or of matrix components such as polysaccharides [44]. This type of reporter-based approach was used to identify novel thiocillins produced by *B. cereus* [1••]. As our current antibiotics continue to lose their effectiveness to widespread resistance, we will need to use creative approaches such as these to ensure that we do not miss potentially useful molecules.

Conflict of interest statement

Nothing declared.

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