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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