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REVIEW

Antibiotic-induced biofilm formation

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ABSTRACT

Surface-attached colonies of bacteria known as biofilms play a major role in the pathogenesis of device-related infections. Biofilm colonies are notorious for their resistance to suprainhibitory concentrations of antibiotics. Numerous studies have shown that subminimal inhibitory concentrations of some antibiotics can act as agonists of bacterial biofilm formation in vitro, a process that may have clinical relevance. This article reviews studies demonstrating that low-dose antibiotics induce bacterial biofilm formation. These studies have provided important information about the regulation of biofilm formation and the signaling pathways involved in global gene regulation in response to cell stressors. It is still unclear whether antibiotic-induced biofilm formation contributes to the inconsistent success of antimicrobial therapy for device infections.

KEY WORDS: *c-di-GMP, Extracellular DNA, PNAG, ppGpp, Subinhibitory, Sub-MIC*

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INTRODUCTION

Matrix-encased bacterial biofilms that form on implanted medical devices often lead to life-threatening systemic infections and device failure. Biofilms are resistant to killing by antibiotics at concentrations that are 10-1000 times greater than concentrations needed to kill free-living or "planktonic" cells (1). This high level of biofilm resistance makes most device-related infections difficult or impossible to eradicate using conventional antimicrobial chemotherapy. Factors that contribute to biofilm-mediated antimicrobial resistance include inefficient diffusion or sequestering of the agent within the biofilm matrix (1, 2), the slow growth rate of biofilm cells (3), the presence of "persister" cells or antibiotic-resistant small-colony variants (4, 5), and other unknown phenotypic differences (6).

Numerous studies have shown that subminimal inhibitory (sub-MIC) concentrations of some antibiotics, although not able to kill bacteria, can inhibit biofilm formation. A good example is the macrolide antibiotic azithromycin, which efficiently inhibits *Pseudomonas aeruginosa* biofilm formation at concentrations as low as 1/128 of the MIC (7, 8). *P. aeruginosa* forms biofilms in the lungs of cystic fibrosis patients and contributes to progressive lung damage and respiratory failure (9). Although most *P. aeruginosa* clinical isolates are resistant to azithromycin (MIC >64 μg/ml), low-dose azithromycin therapy has been shown to improve lung function in cystic fibrosis patients (10, 11). Sub-MIC levels of azithromycin have been shown to inhibit quorum-sensing and production of the mucoid biofilm matrix polysaccharide alginate, both of which are required for *P. aeruginosa* biofilm formation (7, 12-14). Thus, part of the clinical effect of low-dose azithromycin chemotherapy may be due to its ability to inhibit *P. aeruginosa* biofilm formation.

In contrast to the effects of sub-MIC macrolides on *P. aeruginosa* biofilm formation, numerous studies have shown that some antibiotics, when present at concentrations below the MIC, can significantly induce biofilm formation in a variety of bacterial species *in vitro*. This process may have clinical relevance because bacteria are exposed to sub-MIC concentrations of antibiotics

at the beginning and end of a dosing regimen, between doses, or continuously during low-dose therapy (15). In addition, cells buried deep within a biofilm colony may be exposed to sub-MIC concentrations of antibiotics because of diffusion gradients (2). The widespread use of antibiotics as growth promoters in agriculture may further exposure of bacteria to low levels of the drugs (16).

This article reviews studies showing that subminimal inhibitory concentrations of antibiotics induce bacterial biofilm formation *in vitro*. The term "sub-MIC" will be used to refer to concentration of antibiotics below the MIC (<1 MIC). Many authors use the term "subinhibitory concentration" when referring to sub-MIC concentrations of antibiotics. However, this term is not accurate because it specifically refers to concentrations of antibiotics that do not affect the growth of the organism being tested (17).

OVERVIEW OF THE LITERATURE

The observation that sub-MIC concentrations of antibiotics can interfere with some bacterial functions was first reported in 1940 by Arthur Gardner, a Professor of Bacteriology under Howard Florey at Oxford University (18). Gardner showed that *Clostridium perfringens* formed elongated, unsegmented filaments in the presence of sub-MIC penicillin. Numerous studies subsequently showed that sub-MIC antibiotics readily alter the ultrastructure and antigenicity of bacteria, their adherence to epithelial cells, their synthesis and secretion of enzymes and toxins, their growth rate both *in vitro* and *in vivo*, and even the induction of prophages (19-23). In addition, sub-MIC antibiotics have been shown to provoke considerable transcription activation at low levels (17). Recent studies using transcriptomic approaches have shown that the expression of as many as 5% of bacterial promoters may be affected by sub-MIC antibiotics (24-31).

The first study demonstrating that sub-MIC antibiotics can induce bacterial biofilm formation *in vitro* was reported in 1988 by Gordon Christensen, a pioneer in the field of staphylococcal biofilms and their relevance to device infections (32). In this study, one out of three strains of *Staphylococcus epidermidis* showed a 65% increase in biofilm formation in the presence of 1/4 MIC rifampin as determined by crystal violet staining. None of the strains showed an increase in biofilm formation in response to 1/4 MIC concentrations of eight other antibiotics, and most showed a decrease. Only a handful of additional articles on the subject were published until 2005, when a classic paper by Hoffman et al appeared in the journal *Nature* (33). In this study, sub-MIC concentrations of tobramycin were shown to induce *P. aeruginosa* biofilm formation through a mechanism that involves the intracellular second messenger cyclic dimeric guanosine monophosphate (c-di-GMP). This study sparked interest in the field and resulted in the publication of about a dozen additional articles over the past six years. A complete list of studies demonstrating that sub-MIC antibiotics induce bacterial biofilm formation is presented in Table I.

DOSE-RESPONSE RELATIONSHIPS

The results of a typical experiment demonstrating that sub-MIC concentrations of antibiotics can induce bacterial biofilm formation are shown in Figure 1. In this experiment, three strains of *Staphylococcus aureus* were cultured in 96-well microtiter plates in the presence of increasing concentrations of methicillin. Strains 383 and 9897 are methicillin-resistant *S. aureus* (MRSA) with a MIC of >16 µg/ml, whereas strain Mu50 is methicillin-sensitive (MSSA) with a MIC of 8 µg/ml. After 18 hours of incubation at 37°C, the wells of the microtiter plate were rinsed with water and the biofilms were stained with crystal violet. Sub-MIC methicillin caused increased biofilm formation by all three strains in a dose-dependent manner as evidenced by the amount of bound crystal violet dye. Methicillin-induced biofilm formation by strain Mu50 was maximal at about 1/2 MIC (4-5 µg/ml). In addition to using the crystal violet binding assay, induction of biofilm formation by sub-MIC antibiotics has also been demonstrated using confocal and scanning electron microscopy (34-37), CFU enumeration (33, 36, 38), and measurements of exopolysaccharide production (38-40).

Examples of dose-response curves reported in the literature are shown in Figure 2. The fundamental nature of the dose response is biphasic and is characterized by low-dose stimulation of biofilm formation and high-dose inhibition. Some antibiotics can act as antagonists of biofilm formation at low levels, agonists at higher levels, and antagonists at still higher levels (e.g., Figs. 2F and O). These U-shaped and multiphasic dose response relationships, which are characteristic of many chemicals, drugs, hormones, biological molecules and physical

TABLE I - ANTIBIOTICS THAT INDUCE BACTERIAL BIOFILM FORMATION AT CONCENTRATIONS BELOW THE MIC

Class	Antibiotic	*Bacterium*	Ref.
Aminocoumarin	Novobiocin	*S. epidermidis*	[38]
Aminoglycoside	Gentamicin	*P. aeruginosa*	[33, 71]
	Various	*E. coli*	[35]
	Tobramycin	*E. coli*	[33]
	Tobramycin	*P. aeruginosa*	[31, 33]
b-lactam	Ampicillin	*S. intermedius*	[36]
	Imipenem	*P. aeruginosa*	[34]
	Imipenem	*A. baumannii*	[78]
	Nafcillin	*S. lugdunensis*	[66]
	Oxacillin	*S. aureus*	[61]
	Various	*E. coli*	[35]
Cefalosporin	Cefalexin	*S. aureus*	[43]
	Cefalotin	*S. aureus*	[64]
	Cefazolin	*S. lugdunensis*	[66]
	Cefamandole	*S. epidermidis*	[84]
	Cefotaxime	*S. typhimurium*	[40]
Chloramphenicol	Chloramphenicol	*E. coli*	[35]
Fluoroquinolone	Ciprofloxacin	*S. intermedius*	[36]
	Ciprofloxacin	*P. aeruginosa*	[31]
	Moxifloxacin	*S. lugdunensis*	[66]
	Various	*E. coli*	[35]
Glycylcycline	Tigecycline	*S. epidermidis*	[38]
Glycopeptide	Vancomycin	*S. epidermidis*	[38, 85, 86]
	Vancomycin	*S. aureus*	[61]
	Vancomycin	*S. lugdunensis*	[66]
Lipopeptide	Daptomycin	*S. lugdunensis*	[66]
Macrolide	Azithromycin	*S. epidermidis*	[37]
	Clarithromycin	*P. aeruginosa*	[76]
	Clarithromycin	*S. epidermidis*	[37]
	Erythromycin	*S. epidermidis*	[37]
	Various	*E. coli*	[35]
Oxazolidone	Linezolid	*S. aureus*	[66]
	Linezolid	*S. epidermidis*	[38, 66]
	Linezolid	*E. coli*	[35]
Rifamycin	Rifampin	*S. epidermidis*	[32]
	Rifampin	*S. lugdunensis*	[66]
	Rifampin	*E. coli*	[35]
Streptogramin	Quinupristin-dalfopristin	*S. epidermidis*	[54]
	Quinupristin-dalfopristin	*S. lugdunensis*	[66]
Sulfonamide	Trimethoprim/sulfamethoxazole	*S. lugdunensis*	[66]
Tetracycline	Tetracycline	*S. epidermidis*	[54]
	Tetracycline	*S. intermedius*	[36]
	Tetracycline	*S. lugdunensis*	[66]
	Tetracycline	*E. coli*	[35]
	Tetracycline	*P. aeruginosa*	[31]

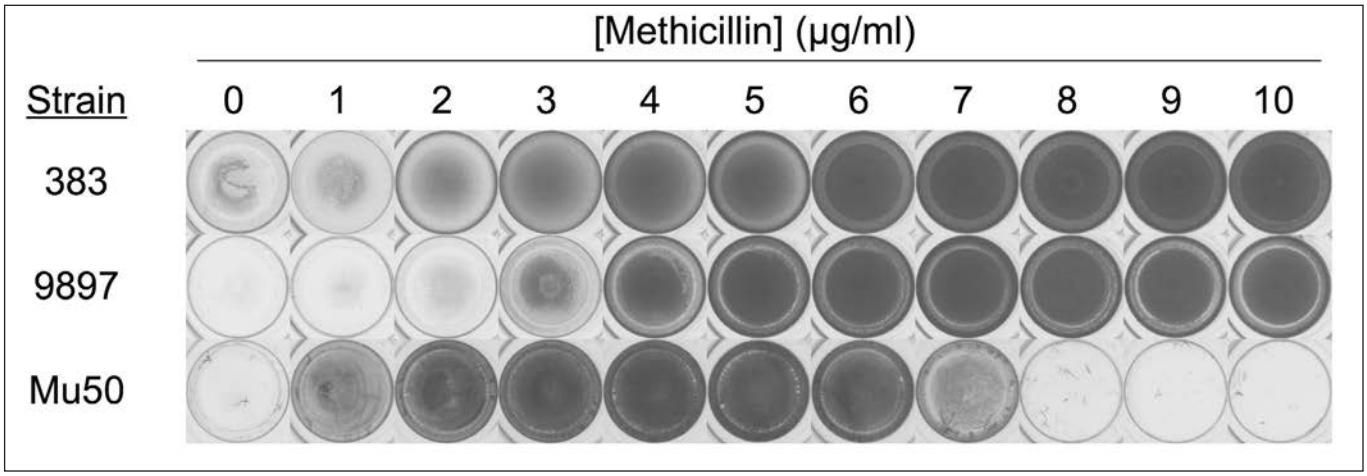

Fig. 1 - *Biofilm formation by* Staphylococcus aureus *in the presence of sub-MIC concentrations of methicillin. Three different* S. aureus *strains (383, 9897 and Mu50) were cultured in the wells of a 96-well polystyrene microtiter plate in the presence of increasing concentrations of methicillin (0-10 µg/ml). After 18 h, wells were rinsed with water and stained with crystal violet. Strains 383 and 9897 are methicillin-resistant* S. aureus *(MRSA; MIC > 16 µg/ml), whereas strain Mu50 is methicillin-sensitive (MIC = 8 µg/ml).*

stressors, are sometimes referred to as hormetic responses (17, 41, 42).

The maximum amplitude of the stimulus response and width of the stimulatory dose range varies for different antibiotics and different bacteria. For example, clarithromycin induces *P. aeruginosa* biofilm by more than 25-fold (Fig. 2D), whereas biofilm induction by *Streptococcus intermedius* in response to ampicillin and *Staphylococcus epidermidis* in response to vancomycin are less than two-fold (Figs. 2E and O). Similarly, *E. coli* exhibits biofilm induction over a narrow range of tobramycin concentrations (0.2 to 0.4 × MIC; Fig. 2J), but over a broad range of tetracycline concentrations (0.1 to 0.8 × MIC; Fig. 2N).

The concentration of antibiotic that induces maximum biofilm formation also differs for different antibiotics. For most antibiotics, maximum biofilm induction occurs at antibiotic concentrations corresponding to ≤1/2 MIC, but some antibiotics induce maximum biofilm formation at concentrations >1/2 MIC (Figs. 1C, F, and O). Among more than 40 studies testing the effects of sub-MIC antibiotics on biofilm formation, a majority tested antibiotic concentrations in serial two-fold dilutions starting at 1/2 or 1/4 MIC. This pattern of concentrations was chosen based on established methods for measuring antibiotic MIC values. Numerous studies utilized a single concentration of 1/4 or 1/2 MIC. Many of these studies showed no effect or an antagonistic effect of the drugs on biofilm formation. However, among those studies that tested concentration between 1/2 and 1 MIC, a majority (4 out of 5) demonstrated biofilm induction in this concentration range (33, 36, 38, 43). Thus, it may be necessary to test a wide range of sub-MIC antibiotic concentrations to detect biofilm induction, and not just concentrations at or below 1/2 MIC. For example, several studies failed to demonstrate biofilm induction by *S. epidermidis* in response to ≤1/2 MIC vancomycin (37, 44-48), probably because vancomycin exhibits maximal biofilm induction at a concentration of 3/4 MIC (38).

Several studies have examined antibiotic-induced biofilm formation in both antibiotic-sensitive and antibiotic-resistant strains. As can be seen in Figure 1, sub-MIC methicillin induces *S. aureus* biofilm formation in both MRSA and MSSA strains, although the concentration at which maximal induction occurs in higher for the MRSA strains than for the MSSA strain. Elliott et al (49) also showed that sub-MIC tobramycin induces biofilm formation in both tobramycin-sensitive and tobramycin-resistant *P. aeruginosa* strains, but maximum induction always occurs at the same drug concentration (0.2 to 0.3 µg/ml) regardless of the MIC of tobramycin for the strain. In contrast, Boehm et al (35) found that streptomycin induces biofilm formation in a streptomycin-sensitive strain but not in a strain with a point mutation in *rpsL* (encoding the S12 protein of the small ribosomal subunit) that confers resistance to high levels of streptomycin. Thus there is

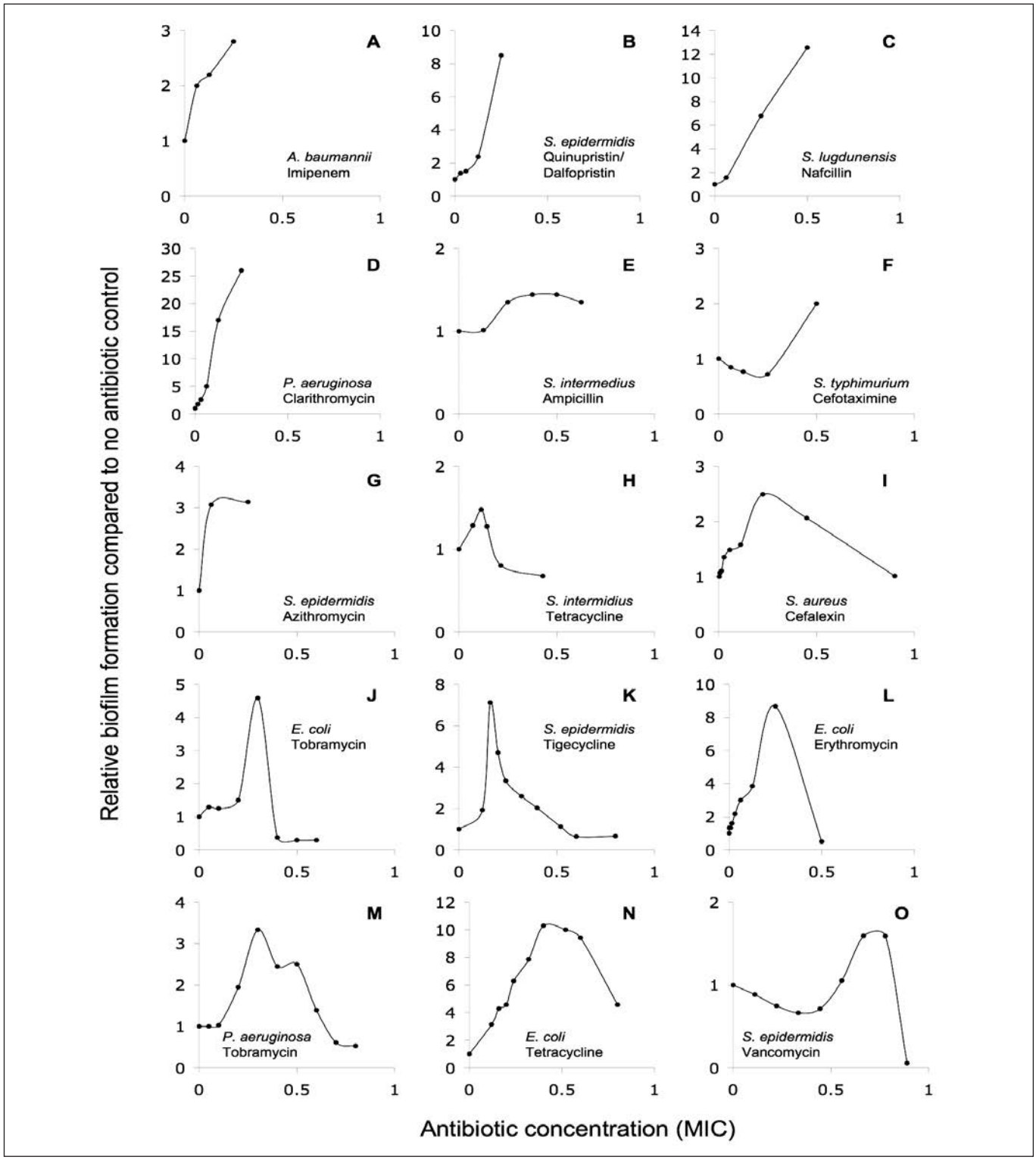

Fig. 2 - *Dose-response curves. The x-axis indicates the antibiotic concentration (relative to the MIC) and the y-axis indicates the amount of biofilm formation normalized to the amount of biofilm formation in the absence of antibiotic, which is given a value of 1. The bacterial species and antibiotic are indicated on each graph. References: A, (78); B, (54); C, (66); D, (76); E, (36); F, (40); G, (37); H, (36); I, (43); J, (33); K, (38); L, (35); M, (33); N, (35); O, (38).*

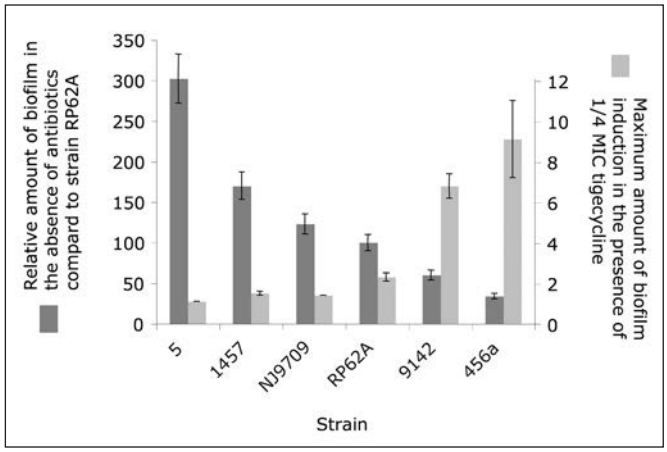

Fig. 3 - *Relationship between basal level of biofilm formation (dark gray bars) and biofilm inducibility (light gray bars) in six strains of* S. epidermidis*. The names of the strains are indicated on the bottom. The x-axis on the left indicates the relative amount of biofilm formation in the absence of antibiotics for each strain relative to the amount of biofilm formation exhibited by reference strain RP62A, which is given a value of 100. The x-axis on the right indicates the maximum amount of biofilm induction exhibited by each strain in the presence of 1/4 MIC tigecycline. Redrawn from (38), with permission of Elsevier Limited.*

no clear relationship between antibiotic susceptibility and biofilm induction.

MECHANISMS OF BIOFILM INDUCTION

Many studies investigating the mechanisms of antibiotic-induced biofilm formation have been carried out using the common device-associated pathogens *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus lugdunensis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Other studied have been carried out on the nosocomial pathogen *Acinetobacter baumannii*, the enteric pathogen *Salmonella enterica* serovar Typhimurium, and the oral bacterium *Streptococcus intermedius*, as outlined below.

Staphylococcus epidermidis

Nearly 20 studies have investigated the effects of sub-MIC antibiotic on biofilm formation by *S. epidermidis*. Among these, seven studies showed enhanced biofilm formation in the presence of low levels of 11 different antibiotics with varied modes of action (Tab. I and Figs. 2B, G, K, and O).

In many strains of *S. epidermidis*, biofilm formation is dependent on production of poly-*N*-acetylglucosamine (PNAG), a cell surface polysaccharide that mediates various biofilm-related functions including intercellular adhesion (50) and resistance to killing by antimicrobial peptides and phagocytes (51, 52). PNAG synthesis is dependent on the proteins encoded by the *icaADBC* operon (53). Several studies have measured PNAG production and *ica* gene expression in response to sub-MIC antibiotics. Kaplan et al (38) observed a 2.6-fold increase in the amount of total hexosamine in the *S. epidermidis* biofilm matrix when cells were cultured in 1/4 MIC tigecycline, which is consistent with an increase in PNAG production. This increase was accompanied by a significant increase in biofilm CFUs and crystal violet binding (Fig. 2K). Using a reporter gene construct that linked the *ica* promoter to a β-galactosidase gene, Rachid et al (54) showed that sub-MIC concentrations of tetracycline, erythromycin and quinpristin-dalfopristin (1/70 to 1/2 MIC) increased expression of the *ica* promoter by 2.5 to 11-fold. Similarly, Wang et al (37) showed that *ica* gene expression was increased by 2.8-fold in response to 1/16 MIC erythromycin as determined by real-time RT-PCR. Taken together, these findings suggest that antibiotic-induced biofilm formation in *S. epidermidis* may depend on increased PNAG production. The expression of other biofilm-related genes including *atlE* (encoding a major autolysin), *fruA* (fructose-specific permease), *pyrR* (pyrimidine regulatory protein), *sarA* (global regulator) and *sigB* (sigma factor) were also increased by sub-MIC erythromycin (37).

Several studies examined antibiotic-induced biofilm formation in multiple strains of *S. epidermidis* that produce different amounts of biofilm. Pérez-Giraldo et al (39) showed that fluoroquinolone-induced biofilm formation by 12 *S. epidermidis* clinical isolates of unknown *ica* status was inversely proportional to the amount of biofilm formed in the absence of antibiotic. This same patterns was observed by Kaplan et al (38), who showed that the amount of biofilm induction caused by 1/4 MIC tigecycline in six *ica*-positive *S. epidermidis* strain was inversely proportional to the amount of biofilm formed in the absence of antibiotic (Fig. 3). These results suggest that expression of the *ica* genes may be suppressed in *S. epidermidis* strains that produce low amounts of biofilm, and that *ica* expression can be induced by sub-MIC antibiotics. In contrast, the inducibility of biofilm formation in *P. aeruginosa* by sub-MIC tobramycin was independent of the magnitude of biofilm formation in the absence of tobramycin (49).

Although PNAG is considered an important virulence factor in *S. epidermidis* device infections, not all *S. epidermidis* strains carry the *ica* locus. Biofilm formation in these *ica*-negative strains may depend on the production of extracellular DNA (ecDNA) (55, 56), proteinaceous adhesins (57), or teichoic acids (58, 59). Wang et al (37) showed that induction of biofilm formation by sub-MIC concentrations of macrolides was independent of the *ica* status of the strain. In their study, 25% of *ica*-positive strains (3 out of 12) and 50% of *ica*-negative strains (5 out of 10) exhibited increased biofilm formation in the presence of macrolides at 1/4 MIC. Thus, the precise role of PNAG in antibiotic-induced *S. epidermidis* biofilm formation is unclear, and PNAG-independent mechanisms of induction must exist. Consistent with this hypothesis, Kaplan et al (38) showed that biofilm formation by the *ica*-positive *S. epidermidis* reference strain RP62A in response to sub-MIC tigecycline and vancomycin was dependent on the production of ecDNA.

The 11 antibiotics that induce *S. epidermidis* biofilm formation at sub-MIC levels exhibit varied chemical structures and modes of action. They include cell wall biosynthesis inhibitors, translation inhibitors, and DNA gyrase inhibitors (Tab. I). In addition, other compounds can induce *S. epidermidis* biofilm formation when present at low levels including furanones (60) and sodium chloride (54). Since *S. epidermidis* biofilm formation is induced by diverse compounds with varied modes of action, these observations suggest that the biofilm induction response, whether through a PNAG-dependent or PNAG-independent mechanism, occurs as a result of a global response to cell stress rather than by the binding of antibiotics to specific cell receptors. The intracellular signals that mediate the *S. epidermidis* biofilm induction response are currently unknown, and aside from the *ica* locus, the genes involved in mediating the response are also unknown.

Staphylococcus aureus

In contrast to *S. epidermidis*, only a handful of studies have examined the effects of sub-MIC antibiotics on *S. aureus* biofilm formation. In addition to methicillin (Fig. 1), sub-MIC concentrations of the cell-wall-active antibiotics oxacillin, cefalexin, cefalotin and vancomycin, and of the translation inhibitor linezolid, have been shown to induce *S. aureus* biofilm formation (Tab. I). Mirani and Jamil (61) found that sub-MIC concentrations of vancomycin (0.9 × MIC) promoted *S. aureus* biofilm formation on nylon and silicon surfaces but not on glass surfaces, whereas sub-MIC oxacillin (1/16 MIC) promoted biofilm formation on glass surfaces but not on nylon or silicon surfaces. The amount of biofilm induction was about 3- to 4-fold as determined by using a crystal violet binding assay.

Very little is known about the mechanism of antibiotic-induced biofilm formation in *S. aureus*. Bisognano et al (62) showed that expression of the *S. aureus* fibronectin-binding proteins (FnBPs) was increased in some highly quinolone-resistant strains when cultured in medium supplemented with 1/4 MIC ciprofloxacin as determined by Western blot analysis. It has recently been shown that FnBPs promote biofilm formation in some *S. aureus* MRSA strains (63). Subrt et al (64) showed that sub-MIC concentrations of cefalotin (1/4 MIC) induced *S. aureus* biofilm formation but did not affect expression of agr, a quorum sensing system that modulates *S. aureus* biofilm formation and dispersal (65). Sub-MIC cefalotin also induced expression of the *S. aureus spa* (encoding protein A) and *lukE* (leukotoxin E) virulence genes (64). Although PNAG also plays a role in *S. aureus* biofilm formation (55), no studies on the expression of the *S. aureus ica* genes in response to sub-MIC antibiotics have been reported.

Staphylococcus lugdunensis

Frank et al (66) measured the effects of sub-MIC concentrations of 10 structurally diverse antibiotics on biofilm formation by 15 clinical isolates of *S. lugdunensis*. Nine of the ten antibiotics induced biofilm formation by at least 2-fold in at least one strain when tested at 1/32 to 1/2 MIC (Tab. I and Fig. 2C). The β-lactam nafcillin induced biofilm formation in 14 out of 15 strains (93%) to levels that were up to 14-fold greater than drug free controls as determined by crystal violet staining. None of the other antibiotics induced biofilm formation in more than 27% of the isolates. The mechanism of antibiotic-induced biofilm formation in *S. lugdunensis* has not been investigated.

Escherichia coli

At least five chemically diverse classes of translation inhibitors have been shown to induce biofilm formation in *E. coli* when present at sub-MIC levels (Tab. I and Figs. 2J, L, and N). As a result of a series of comprehensive studies reported by Boehm et al in 2009 (35), a detailed genetic and

biochemical model of *E. coli* biofilm induction in response to translation inhibitors has been proposed. In this model, a decrease in translational performance of the ribosome results in upregulation of PgaA and PgaD, two proteins required for the biosynthesis of PNAG in Gram-negative bacteria. Information about translation performance is relayed from the ribosome to the Pga machinery by means of two intracellular second messengers, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) and guanosine-bis 3',5'(diphosphate) (ppGpp). Ribosomal stress results in upregulation of YdeH, a diguanylate cyclase that increases intracellular c-di-GMP levels, and SpoT, a ppGpp 3'-pyrophosphohydrolase that degrades intracellular ppGpp. By unknown post-transcriptional mechanisms, increased c-di-GMP levels cause an increase in PgaD production, whereas decreased ppGpp levels cause an increase in PgaA production. Increased production of PgaA and PgaD results in increased PNAG production and biofilm formation.

In another study, Sailer et al (67) showed that sub-MIC concentrations of certain β-lactams, including carbenicillin, cefotetan, cephaloridine, cephalothin, and ticaricillin, induced expression of the *cps* gene, which is required for the synthesis of colanic acid capsular polysaccharide in *E. coli*. Expression of *cps* was not induced by other β-lactams (ampicillin, ceftazidime, ceftriaxone, penicillin) indicating that induction was not due to stresses accompanying general inhibition of peptidoglycan biosynthesis. Expression of *cps* was also not induced by antibiotics that inhibit protein synthesis (chloramphenicol, erythromycin, tetracycline) or DNA synthesis (ciprofloxacin, nalidixic acid, sulfisoxazole), indicating that *cps* induction was not caused by stresses accompanying cell death. Because colanic acid is important for maturation of *E. coli* biofilm architecture (68), these findings suggest that β-lactam-induced biofilm formation may also occur via upregulation of colanic acid production.

Boehm et al (35) measured the effect of more than 200 antimicrobials on *E. coli* biofilm formation by culturing biofilms in Biolog phenotype array microtiter plates (69). These plates contain four concentrations of each of a variety of test compound pre-aliquotted into the wells of a standard 96-well microtiter plate. The test compounds include antibiotics as well as a variety of non-antibiotic compounds such as chelators, detergents and metal ions. They found that low concentrations of a wide variety of compounds induced *E. coli* biofilm formation, including glycopeptide, cyclic peptide, fluoroquinolone, and β-lactam antibiotics, *p*-aminobenzoic acid analogs, and various membrane-active agents, chelators, salts, and toxic metal ions. Biofilm induction by *E. coli* in response to a broad range of antimicrobials and related compounds is consistent with the hypothesis that metabolic stress is the key signal that mediates the response.

Pseudomonas aeruginosa

More than a dozen studies have examined the effect of sub-MIC antibiotics on *P. aeruginosa* biofilm formation. Among these, five studies demonstrated biofilm induction in response to sub-MIC concentrations of various aminoglycosides, β-lactams and macrolides (Tab. I and Figs. 2D and M).

The response of *P. aeruginosa* to sub-MIC aminoglycosides has been studied in detail. Hoffman et al (33) found that sub-MIC concentrations of tobramycin readily induced *P. aeruginosa* biofilm formation (Fig. 2M). Interestingly, several other antibiotics with varied modes of action including polymyxin B (a membrane-active peptide), chloramphenicol (a translation inhibitor) and carbenicillin (a cell wall synthesis inhibitor), had no effect on biofilm formation. These results suggest that biofilm induction by *P. aeruginosa* in response to tobramycin is a specific response to aminoglycoside antibiotics rather than a non-specific response to translation inhibition or other cell stressors. The authors screened a library of transposon insertion mutants and found three mutants that were defective in tobramycin-induced biofilm formation. All three mutants carried transposon insertions in a gene designated *arr*, for aminoglycoside response regulator. The *arr* gene encodes a c-di-GMP phosphodiesterase that degrades c-di-GMP and reduces intracellular c-di-GMP concentrations. The mechanism by which reduced c-di-GMP levels induce *P. aeruginosa* biofilm formation evidently did not involve the modulation of biofilm-associated cell surface appendages such as fimbria and pili. These results contradict those described above for *E. coli*, in which increased intracellular c-di-GMP levels promote biofilm formation. In fact, increased c-di-GMP levels generally result in an increase in exopolysaccharide and fimbria production, a decrease in motility, and increased biofilm formation, whereas decreased levels of c-di-GMP exert the opposite effects and induce biofilm dispersal in most bacteria (70). This contradiction may reflect the fact that

regulation of intracellular c-di-GMP levels is complex, with many bacteria possessing dozens of diguanylate cyclases and c-di-GMP phosphodiesterase homologues. In addition, biofilm formation by *P. aeruginosa* strains that lack the *arr* gene is still inducible by tobramycin (49). Thus, the biological effect of alterations in diguanylate cyclase levels is not easy to predict.

A recent study by Marr et al (71) also investigated the mechanism of aminoglycoside-induced biofilm formation in *P. aeruginosa*. These authors screened a mini-Tn*5-luxCDABE* fusion library for mutants that exhibit increased luminescence when grown in 1/4 MIC gentamicin. They found that luminescence of 33 of the 9408 *lux* fusions was up-regulated in the presence of sub-MIC gentamicin. Among these, 11 mutants were deficient in gentamicin-induced biofilm formation. These included known biofilm-related genes involved in fimbrial biogenesis, alginate production, and motility regulation as well as other genes involved in solute transport, antibiotic resistance, energy metabolism, transcriptional regulation, and genes of unknown function. These findings demonstrate that screening mutant libraries is likely to be useful approach for elucidating the mechanisms of antibiotic-induced biofilm formation.

Linares et al (31) showed that sub-MIC concentrations of tobramycin, ciprofloxacin and tetracycline induced *P. aeruginosa* biofilm formation by approximately 2-fold as determined by crystal violet staining. The antibiotic concentrations that caused maximum biofilm induction were at or just above the concentration that caused a decrease in the growth rate. Using microarray technology, these authors found that sub-MIC concentrations of all three antibiotics caused a significant increase or decrease in the expression of 5% to 7% of 555 genes in a subgenomic array selected as relevant for the development of chronic colonization and infection, antibiotic resistance, transcriptional regulation, and stress response, including some genes that had previously been shown to play a role in biofilm formation. Bagge et al (34) also used microarray technology to study the effects of sub-MIC concentrations of the β-lactam antibiotic imipenem on *P. aeruginosa* biofilm formation. They identified 34 genes that were induced or repressed in biofilms exposed to 1/2 MIC imipenem. Among these, five genes involved in alginate metabolism (*algD*, *algG*, *algJ*, *algF* and *algA*) were induced more than 10-fold by imipenem at 1/2 MIC. Alginate production and biofilm formation were also increased, indicating that *P. aeruginosa* biofilm induction in response to sub-MIC β-lactams evidently involves upregulation of alginate biosynthesis.

As mentioned above, sub-MIC concentrations of macrolide antibiotics have generally been shown to inhibit *P. aeruginosa* biofilm formation *in vitro* (12, 72-75), and long-term administration of low level azithromycin improves the clinical outcome in cystic fibrosis patients colonized with *P. aeruginosa* (10, 11). In one study, however, Garey et al (76) showed that sub-MIC concentrations of the macrolide antibiotic clarithromycin induced biofilm formation by up to 25-fold in 44 *P. aeruginosa* clinical isolates when tested at 1/32 to 1/2 MIC (Fig. 2D). One other study found that clarithromycin at 0.03 to 0.003 × MIC had no effect of *P. aeruginosa* biofilm formation (77), although only one strain of *P. aeruginosa* was tested in this study. These findings suggest that clarithromycin and azithromycin may exhibit different biofilm inducing activities against *P. aeruginosa.*

Acinetobacter baumannii

Nucleo et al (78) showed that imipenem induced biofilm formation in one out of three clinical strains of *A. baumannii*. The amount of biofilm induction was 3-fold when the antibiotic was present at 1/16 to 1/4 MIC (Fig. 2A). Low levels of $FeSO_4$ also stimulated biofilm formation in the imipenem-inducible strain, and both imipenem- and $FeSO_4$-induced biofilm formation occurred in the presence of cellulase, suggesting that induction is not dependent on increased cellulose production. Imipenem at 1/4 MIC was shown to induce expression of genes encoding a ferrichrome-iron receptor and a TonB-dependent siderophore receptor as determined by real-time RT-PCR, and cells exposed to sub-MIC imipenem contained increased levels of the corresponding proteins in their membranes as determined by SDS-PAGE and MALDI-TOF analysis. The mechanism by which imipenem induces expression of these *A. baumannii* iron uptake genes and proteins is unknown. The role played by iron uptake in biofilm induction is also unknown, but it evidently does not involve the upregulation of adhesive pili (78).

Salmonella enterica serovar Typhimurium

Majtán et al (40) measured the effects of sub-MIC concentrations of gentamicin, ciprofloxacin and cefotaxime on biofilm formation by five human clinical isolates of *Salm*.

Typhimurium. They found that cefotaxime induced biofilm formation by about 2-fold in three strains when present at 1/2 MIC (Fig. 2F). Sub-MIC cefotaxime also caused a significant increase in exopolysaccharide production in two of the inducible strains as determined by chemical analysis of extracted exopolysaccharides. One potential limitation of this study was that fact that only the five highest biofilm-producing strains (from among 75 clinical isolates) were selected for analysis in the biofilm induction assay. As described above for *S. epidermidis*, strains that produce high levels of biofilm in the absence of antibiotics may not exhibit biofilm induction in the presence of sub-MIC antibiotics.

Streptococcus intermedius

Ahmed et al (36) found that sub-MIC concentrations of ampicillin, ciprofloxacin, and tetracycline all induced biofilm formation in a wild-type strain of *S. intermedius*. The amount of biofilm induction was less than 2-fold when antibiotics were present at 1/14 to 1/2 MIC (Figs. 2E and H). Biofilm induction did not occur in an isogenic *luxS* mutant strain exposed to sub-MIC antibiotics. The *luxS* gene encodes an enzyme (LuxS) responsible for the synthesis of autoinducer-2 (AI-2), a furanosyl borate diester that functions as an intra- and interspecific quorum-sensing signal in numerous Gram-positive and Gram-negative bacteria (79). LuxS is required for biofilm formation by *S. intermedius* (80), which suggests that the mechanism of antibiotic-induced biofilm formation in *S. intermedius* involves the AI-2/LuxS pathway. Induction of biofilm formation in staphylococci by sub-MIC concentrations of furanone is mediated by an increase in PNAG production that is also dependent on LuxS (60).

CLINICAL RELEVANCE

It is tempting to speculate that induction of biofilm formation by sub-MIC antibiotics contributes to the inconsistent success of antimicrobial therapy for biofilm-related device infections and other biofilm-related infections. As mentioned above, some bacterial cells are likely to be exposed to sub-MIC antibiotics during the course of antimicrobial chemotherapy, either because of varying gradients of systemic antibiotic concentrations over the course of the dosing regimen, or because their location deep within the biofilm colony exposes them to sub-MIC antibiotics due to diffusion gradients. So far, there is little evidence to support this hypothesis. Elliott et al (49) found that approximately half of *P. aeruginosa* strains isolated from cystic fibrosis patients exhibited biofilm induction upon treatment with sub-MIC tobramycin, indicating that the inducible phenotype is common among clinical isolates. However, more studies are needed to determine whether there is a relationship between biofilm inducibility and response to therapy.

ECOLOGICAL CONSIDERATIONS

It has been suggested that biofilm formation upon exposure to sub-MIC antibiotics may represent an inducible mechanism of antibiotic resistance (33, 49). According to this hypothesis, biofilm formation may have evolved, for example, as a strategy to counter antibiotic production by soil bacteria (33). This hypothesis is based on the assumption that the primary function of antibiotics in nature is to inhibit the growth of competitors. One of the most interesting ideas to emerge over the past few years is the notion that antibiotics are not only bacterial weapons for fighting competitors, but also signaling molecules that may regulate the homeostasis of microbial communities (17, 31, 81-83). Antibiotics constitute only a small portion of the millions of organic compounds produced by microbes. They have received the most attention only because of their therapeutic utility. It is possible that at the low concentrations likely to be found in the environment, the majority of these compounds play important roles in the modulation of metabolic function in natural microbial communities. Since sub-MIC antibiotics provoke considerable transcription activation, these observations suggest that antibiotics and antibiotic-resistance genes may have evolved not as weapons and defense systems, but possibly as interspecific signaling molecules analogous to other low molecular weight compounds such as peptides, quorum sensing signals, rhamnolipids, halogenated furanones, alkaloids, fatty acids and surfactants that have been shown to modulate bacterial physiology (84). Supporting this hypothesis, phylogenetic analyses have shown that antibiotic resistance genes such as β-lactamases probably originated millions of years before the modern antibiotic era, and diverse genes encoding β-lactamases have been identified in metagenomic analyses of microbiota from

remote habitats such as Alaskan soil where antibiotic concentrations are normally below detection limits and certainly not in the MIC range for most environmental bacteria (82).

CONCLUSIONS

Numerous studies have demonstrated that sub-MIC concentrations of a variety of chemically distinct antibiotics with different modes of action can significantly induce biofilm formation in phylogenetically diverse Gram-negative and Gram-positive bacteria *in vitro*. These studies have already identified two novel genes involved in c-di-GMP-mediated regulation of biofilm formation, *E. coli ydeH* (encoding diguanylate cyclase) and *P. aeruginosa arr* (encoding c-di-GMP phosphodiesterase). The results of these studies have also helped substantiate a role for the intracellular signals c-di-GMP and ppGpp and for AI-2/LuxS quorum-sensing in biofilm formation in a variety of bacteria. Evidently there is no single mechanism of antibiotic-induced biofilm formation, but a global response to cell stress seems to play a role in many bacteria. It appears that screening mutant libraries for mutants deficient in biofilm induction will be a valuable tool for elucidating the mechanisms of antibiotic-induced biofilm formation. More studies on antibiotic-induced biofilm formation are warranted. Clinical studies that identify a relationship between biofilm inducibility and response to therapy will help establish the clinical relevance of this phenomenon. A better understanding of this process may help guide antibiotic therapy and lead to the development of novel cotherapeutic agents that would suppress the biofilm induction response.

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