Mechanisms and methods to combat biofilm tolerance

Z. Yan*[,1] and **B. Kjellerup**[1]

[1] University of Maryland, College Park

Biofilm form when bacteria adhere to an inert or animate surface and form a glue-like coat consisting of extracellular polymeric substances (EPS). The combined effect of tolerance factors (such as limiting penetration of antibiotics, altering metabolic pathways, changing gene expression when exposed to bacteria, and protecting persister cells) make biofilms difficult eradicate with antibiotics. Current methods of treatment with antibiotics are insufficient and this chapter will focus on novel treatment methods towards biofilm infections. Quorum sensing inhibitor (QSI) administration interferes with bacteria's ability to coordinate cell density. Curlicides are small-molecule inhibitors capable of preventing the amyloid biogenesis necessary for biofilm formation. A variety of agents from plants and marine sponges have also shown to have biofilm dispersal effects, which can be paired with antibiotics to eradicate biofilms.

1. Introduction

1.1 Biofilm tolerance

Bacteria typically grow as single (planktonic) cells or as aggregates (biofilms). Biofilms adhere to surfaces and typically produce a matrix of extracellular polymeric substance (EPS). In the 1980s, biofilms were identified as the source for slow-progressing infections, where patients experienced the clinical signs of infection but bacteria were not detected (culture negative) and the effects of antibiotics were diminished or nonexistent [1]. The tolerance to antibiotics that biofilms exhibit is different from the antibiotic resistance that can occur when bacteria adopt resistance genes that change an antibiotic's target, rendering the antibiotic ineffective. In the case of biofilm tolerance, there are many factors that can contribute to reduced efficacy of antibiotics.

The biofilm matrix plays a role in interfering with the delivery of antibiotics. The matrix generally does not inhibit diffusion, but it can bind to the antibiotic compound, thus preventing binding to bacteria. Extracellular DNA, a key component of the biofilm matrix, has been found to mediate antibiotic resistance [2]. Extracellular DNA can bind cations from the environment, which serves as a cue for pathogenic bacteria such as *Pseudomonas aeruginosa* to induce genes that modify cell surface lipopolysaccharides, changing the cell membrane target for antibiotics. These changes result in tolerance against aminoglycosides and membrane- active biocides [3]. It is also possible for enzymes in the matrix to deactivate antibiotics, as seen by beta-lactamases' inactivation of beta-lactam antibiotics [4].

Biofilms may also vary in metabolic activity or gene expression, leading to antibiotic tolerance. In biofilms oxygen, pH and redox gradients and thus varying levels of growth and metabolic activity have been found. Anaerobic conditions and slow growth rate in bacteria decrease the efficacy of antibiotics such as quinolones [5, 6] It has been suggested that a general stress response induced by high cell density in biofilms can protect bacterial cells from environmental changes in temperature, pH, and chemical agents present [6]. This stress response often leads to the upregulation of efflux pumps, which dispel antimicrobials from the cell [7]. In patients with cystic fibrosis with chronic *P. aeruginosa* biofilm infections, polymorphonuclear leukocytes can accumulate to create anaerobic conditions as well [8].

Persister cells, the small percentage of dormant, non-dividing cells in the biofilm, also play a role in antibiotic tolerance. By remaining dormant, antibiotics may bind to these cells but the cells have no cell-wall synthesis, translation, or topoisomerase activity, and therefore antibiotics cannot corrupt these critical cell functions [9]. Beta-lactam have no activity on non-dividing cells [10] while aminoglycosides have decreased activity on non-dividing cells [11].

1.2 Antibiotic Treatments

The two main strategies against biofilm infections using antibiotics alone are 1) high dosage topical treatments and 2) multiple sequential therapies. High dosage topical treatments allow for high levels of antibiotic exposure directly to the site of biofilm exposure, while maintaining low levels in the blood stream. This is beneficial as it can be used to target avascular areas and it decreases the risk of planktonic bacteria developing antibiotic resistance by limiting the number of bacteria exposed to antibiotics [12]. In patients with cystic fibrosis, nebulized antibiotics, such as aztreonam lysine [13], ciprofloxacin [14], tobramycin [15], colistimethate [16], are used daily to suppress exacerbations and have been shown to reduce bacterial load in sputum. However, inhaled anti-microbials have not been proven to be a better treatment for patients with ventilator-associated pneumonia. Studies have shown no difference in signs of respiratory infection in patients on intravenous antibiotics compared to those on intravenous and nebulized antibiotics [17]. Topical antibiotics are used to coat catheters to prevent biofilm formation that causes catheter-related bloodstream infections [12]. Topical forms of tobramycin and vancomycin have also been shown to reduce the incidence of biofilm infections

on surgical implants [18]. In this chapter several novel treatment strategies towards biofilm tolerance will be discussed (Figure 1).

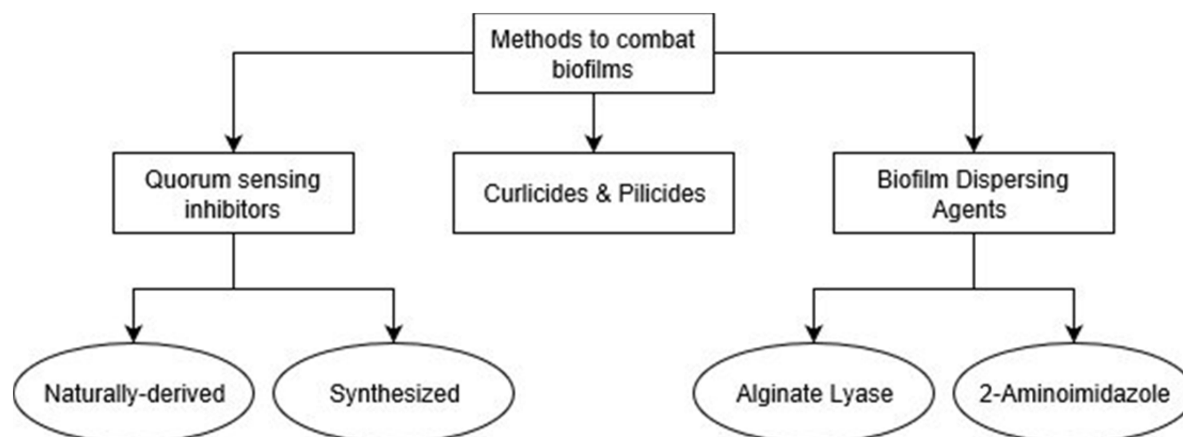

Figure 1. Overview of the novel treatments towards biofilm tolerance focused on in this chapter.

2. Quorum sensing inhibitors

2.1 Introduction to quorum sensing inhibitors

Quorum sensing refers to bacteria's ability to change gene expression in response to changes in population density. Bacteria produce and release autoinducers, which are signaling molecules that regulate processes such as virulence, antibiotic formation, and biofilm formation. They can be classified into three major groups, acylhomoserine lactones (AHLs), oligopeptides and the LuxS/autoinducer 2 [19, 24].

In the gram-negative bacteria quorum sensing pathway (Figure 2a), the two regulatory components are the R protein (transcriptional activator protein) and the autoinducer (AI) molecule that accumulates in a cell density dependent manner. Typically, gram-negative bacteria use AHLs as AI molecules. When the threshold AI level is met, the AI binds and activates the R protein, which induces gene expression of a target gene.

The quorum sensing pathway for gram positive bacteria (Figure 2b), such as in *Staphylococcus aureus* is more complicated. The two gene loci that control quorum sensing are *agr* (accessory gene regulator) and *sar* (staphylococcal accessory gene regulator) [43]. The *sar* locus encodes SarA, a DNA-binding protein that upregulates expression of *agr* operons. The *agr* operons *agrBDCA* is responsible for encoding the proteins that create and sense the peptide signal molecule AgrD and RNAIII. When AgrD reaches a critical level, it stimulates phosphorylation of AgrC, which then stimulates phosphorylation of AgrA. AgrA activates RNAIII, which leads to increased secretion of signal peptides, decreased expression of specific surface proteins, and induced expression of *agrBDCA*.

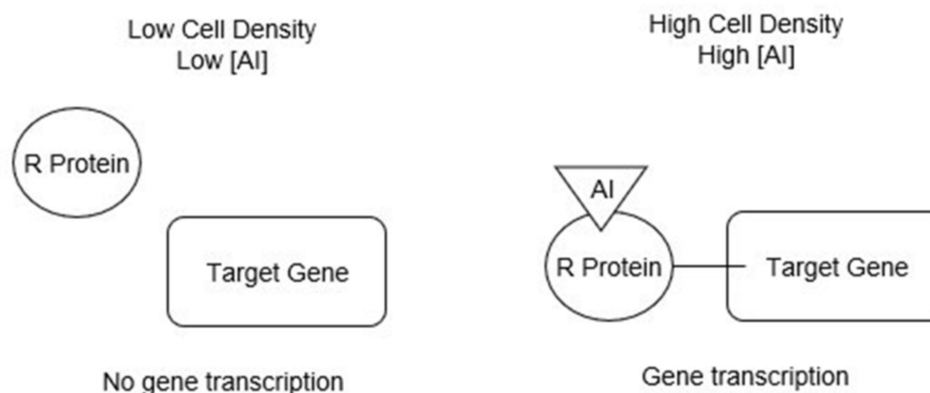

Figure 2a. Diagram of quorum sensing pathways in gram-negative bacteria

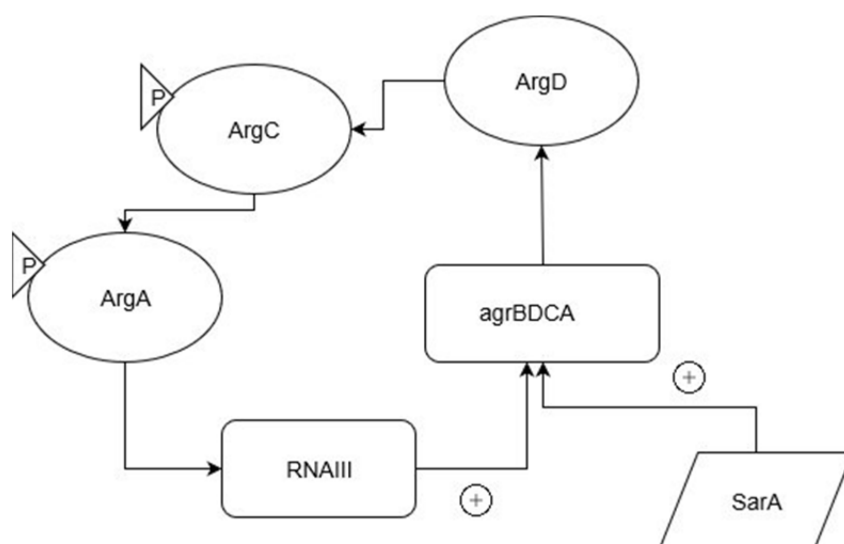

Figure 2b. Diagram of quorum sensing pathways in gram-positive bacteria

2.2 Naturally derived quorum sensing inhibitors

Inhibition of *P. aeruginosa* biofilms have been demonstrated by halogenated furanone compounds. The compound, Furanone 56, was derived from a secondary metabolite of the Australian macroalga, *Delisea pulchra*. It interferes with AHL-mediated quorum sensing by repression of LuxR-activated QSC gene transcription of the *lasB-gfp* (ASV) reporter fusion. Studies using green fluorescent reporters indicated transcription of the *lasB-gfp* genes in response to bacterial communication. Furanone 56 treated biofilms were found to be thicker with cells that had less green fluorescence expression. This indicated that the compounds were able to penetrate the biofilm as well as block cell signalling and quorum sensing in most biofilm cells. The treated biofilms also had decreased bacterial biomass. In addition, Furanone 56 was found to reduce the production of the virulence factors elastase and chitinase by *P. aeruginosa* biofilms [23].

The effects of Furanone 56 were also tested with *Escherichia coli* biofilms. At concentrations of 60 µg/mL, confocal scanning microscopy showed that this compound inhibited biofilm formation, decreased biofilm thickness by 55%, decreased the number of water channels, and decreased the percentage of living cells by 87% [25].

Studies have also shown the abilities of common household food items to be effective as quorum sensing inhibitors that possess potential as biofilm therapies. Biofilms that were grown as continuous cultures and biofilms treated with garlic extract were more susceptible to polymorphonuclear leukocytes and tobramycin that without garlic extract treatment. When mice were given garlic extract or a placebo and then infected with left sided pulmonary infections, the test group showed increased inflmmation and increased clearing of infecting bacteria. *Cinnamomum zeylanicum*, commonly known as cinnamon, was similarly found to decrease biofilm formation. These results were confirmed with confocal laser scanning microscopy with quantitative analysis by COMSTAT and a direct microscopy assay that measured growth-normalizing adhesion [27]. Furocoumarins, naturally occurring compounds found in grapefruit juice, are also of interest for treatment of biofilms. The two types of furocoumarins isolated were DHB and Bergamottin. The inhibition of biofilm growth was measured via optical density at 590 nm. DHB resulted in 71.9% inhibition in *E. coli* O157:H7, 15.5% in *Salmonella typhimurium* and 18.1% in *P. aeruginosa* [28]. Tea extract contains catechin, which has been found to have bactericidal effects on *Streptococcus mutans* biofilms, which are responsible for oral infections like periodontitis. When nanoparticals of bioactive catechins were removed from tea, the bactericidal properties of green tea decreased [29]. PCR showed that epigallocatechin gallate suppressed the gtf B, C, D genes essential for the initial attachment involved in *S. mutans* biofilm formation [30].

Table 1. Naturally derived quorum sensing inhibitors' effects on various types of biofilms

Compound	Biofilm Type	Experimental Results
Furanone 56 [23, 25] (from *Delisea pulchra*)	*P. aeruginosa*	-Shown to block cell signaling and quorum sensing via GFP based analysis -Decreased production of virulence factors elastase and chitinase
	E. coli	- Decreased biofilm formation, thickness - Decreased percentage of living cells
Allium sativum (garlic) extract [26]	*P. aeruginosa*	- Biofilm cultures more susceptible to polymorphonuclear leukocytes and tobramycin -Mice prophylatically given garlic extract had increased bacterial clearing of pulmonary infection
Cinnamomum zeylanicum (cinnamon), oil component-cinnamaldehyde	*P. aeruginosa*	-Decreased quantity of cells in biofilm
Grapefruit juice (furocoumarins) [28]	*E. coli, S. typhimurium, and P. aeruginosa.*	- Furocoumarins inhibit N-acylhomoserine lactone (AI-1) and furanosyl borate diester molecule (AI-2) and decreased the optical density of biofilm samples
Catechin (tea extract) [29, 30]	*Streptococcus mutans*	-Responsible for bactericidal properties of green tea -Plays essential role in biofilm initial attachment

2.3 Synthetic quorum sensing inhibitors

In *P. aeruginosa*, the LasI synthase catalyzes the formation of autoinducers and bacteria with mutations in the *lasI* gene are incapable of forming mature biofilms [20]. One method to combat biofilm formation is to synthesize non-native (AHL) derivatives that block the natural AHL signals. By using a *P. aeruginosa* strain that produced GFP (Green Fluorescent Protein) to compare biofilm formation, the samples treated with non-native AHL were found to be less fluorescent, indicating decreased biofilm formation [21]. A specific non-native AHL derivative that was found to be effective against *P. aeruginosa* biofilms is meta-bromo-thiolactone (mBTL). This compound inhibited RhlR, a quorum-sensing receptor. Treatment with mBTL was found to inhibit the production of pyocyanin, a toxin typically produced by *P. aeruginosa,* and biofilm formation. Testing in the *Caenorhabditis elegans* nematode model revealed reduced nematode killing when treated with mBTL. When mBTL was tested in the human lung carcinoma cell line A549, it did not appear toxic and it reduced the killing by biofilms [22].

AI-1 activates the LasR protein, which initiates the quarum sensing cascade. To inhibit initiation of this cascade, a library of AI1 analogs were synthesized and 3-oxo-C12-(2-aminocyclohexanol) was found to be the most active agonist, with effect comparable to natural AI-1. Additionally, a variety of compounds were synthesized with small modifications to the structure of 3-oxo-C12-(2-aminocyclohexanol) and these were also confirmed as antagonists. Virulence factor assays revealed inhibitory effects on pyocyanin expression. Confocal laser microscopy of the *P. aeruginosa* PAO-JP2 showed decreased biofilm development compared to the control [30].

Based on the structure of naturally occuring furanone, a library of brominated furanones was synthesized and tested on *Salmonella enterica* serovar *Typhimurium* biofilm formation; these bacteria are the most common cause of bacterial food-borne illnesses. Several of the synthesized furanones were found to inhibit biofilm formation but not bacterial growth. Microarray studies of the most effective compounds, (Z)-4-bromo-5-(bromomethylene)-3-alkyl-2(5H)-furanones, showed genes that were controlling metabolism, stress response, and drug sensitivity were induced and genes controlling metabolism, the type III secretion system, and flagellar biosynthesis were repressed. Interestingly, there was no evidence that furanones interfered with known quorum sensing systems in *Salmonella* [32]. A different bromonated furanone, (Z)-5-bromomethylene-2(5H)-furanone, was shown to inhibit biofilm formation in *Streptococcus anginosus*, *Streptococcus intermedius*, and *S. mutans* (bacteria that cause oral infections). It was hypothesized that this compound interfered with AI-2 signaling because the furanone had no effect on biofilm formation ability of AI-2 *luxS* mutants of the three species [33].

Table 2. Synthetic quorum sensing inhibitor effects on various types of biofilms

Compound	Biofilm Type	Experimental Results
Meta-bromo-thiolactone (mBTL) [22]	*P. aeruginosa*	- Inhibit the production of virulence factor, pyocyanin - Reduce death rate of *C. elegans* exposed to biofilms - Reduce killing rate of human lung carcinoma cells
3-oxo-C12-(2-aminocyclohexanol) [30]	*P. aeruginosa*	- Antagonists synthesised from this compound decreased virulence factor, pyocyanin - Decreased biofilm noted via GFP analysis
Brominated furanone [32]	*Salmonella enterica* serovar *Typhimurium, Streptococcus anginosus, S. intermedius* and *S. mutans*	-Inhibit biofilm formation but does not seem to interfere with quorum sensing in *Salmonella enterica* serovar Typhimurium

3. Curlicides/Pilicides

Curli are amyloid fibers found on the cell surface of many Enterobacteriaceae that control cell-cell and cell-surface interactions responsible for adhesion to cell or inert surfaces. Curli also serve as an adhesive or structural scaffold needed for biofilm formation. Pili are extracellular adhesive fibers that mediate biofilm formation, binding, and host cell invasion. Type 1 pili contains FimH adhesion, which allows bacteria to bind to receptors on the surface of the lumen of bladder epithelia cells, causing UTI. Curlicides and pilicides prevent the formation of curli and pili respectively.

Uropathogenic *E. coli* (UPEC) biofilms cause chronic urinary tract infections (UTI). Recently, UPECs have evolved to develop resistance to many first-line antimicrobials such as nitrofurantoin, ampicillin, fluoroquinolones, and sulphamethoxazole/trimethoprim [34]. N-(4-chloro-phenyl)-2-{5-[4-(pyrrolidine-1-sulfonyl)-phenyl]-[1,3,4]oxadiazol-2-yl sulfanyl}-acetamide (AL1) was found to inhibit type 1 pili biogenesis, thus preventing bacteria from adhering to bladder epithelial cells with no disruption of bacterial growth [35]. Mannosides are another group of compounds that were designed to block the FimH adhesion tip. In mice infected with catheter associated UTI, mannosides reduced biofilm formation, biomass, biofilm-adherant cells [36].

FN075 and BibC6 are two curlicides that have similar chemical properties as ring-fused 2-pyridones pilicides. As a result, they also function as pilicides. Since curli and pili play independent roles in promoting UPEC biofilms, these two compounds possess qualities that make them uniquely suitable as potential therapies against UPEC biofilms. In cell cultures of UTI89, a UPEC strain, these compounds prevented curli formation by blocking the polymerization of a major curli subunit protein CsgA and amyloid biogenesis. UTI89 cultures also revealed that the ability to form pellicles, biofilm formations in the air-liquid interface requiring interbacterial adhesion, is dependent on the presence of curli. These curlicides also prevented the formation of biofilms on polyvinyl chloride (PVC) [37].

4. Biofilm Dispersing Agents

4.1 Alginate Lyase

Alginate lyase is an enzyme that degrades alginate, a major component of the biofilm matrix. By destroying this matrix, the biofilm should be dispersed to planktonic bacteria that can be treated with antibiotics or leukocytes. Biofilm cell viability assays used the Alamar Blue reagent to quantify *P. aeruginosa* biofilm viability. The enzyme alone reduced the biofilm signal by 25% and reduced the signal to nearly undetectable levels, when combined with tobramycin treatment. The enzyme and tobramycin were also shown to almost eliminate viable cells in the planktonic state [38]. However, due to the low catalytic activity of the wild type alginate lyase, there is a need to find mutants with higher catalytic efficiency. After developing a structural model, modifications to the enzyme were made to create the K197D/K321A mutant, with greatly improved catalytic efficiency. Ciprofloxacin-resistant *P. aeruginosa* (CRPA) was cultured to test the efficiency of the K197D/K321A mutant. SEM showed decreased biofilm formation of CRPA when treated with alginate lyase (both wild type and mutant strains) and the antibiotic Tozacin. The mutant required a lower dose of Tozacin to degrade the CRPA biofilm [39].

Helicobacter pylori, bacteria that can cause gastric ulcers, can form biofilms in human gastric mucosa. Analysis of the minimal inhibitory concentration in sessile cells (sMIC) showed a median sMIC of 30-15 µg/mL, when the samples were treated with clarithromycin or alginate lyase alone compared to a median sMIC of 0.5-2 µg/mL when the compounds were combined. This indicated a significant increase in susceptibility of *H. pylori* in the clarithromycin. This indicated synergistic activity in the combination of clarithromycin and alginate lyase [40].

4.2-Aminoimidazole- Derived Agents

The biofilm dispersing agent 2-aminoimidazole/triazole conjugate, referred to as “Compound 1”, was found to be the most active in its class of molecules. In studies “Compound 1” was capable of dispersing and inhibiting bacterial biofilms [41]. The effectiveness of biofilm dispersal was measured by EC_{50}, which is the concentration necessary to elicit a 50% dispersion of the biofilm. The EC_{50} of “Compound 1” alone against an *S. epidermidis* biofilm was 325 ± 26 nM but combined treatment with the antibiotic novobiocin reduced the EC_{50} to 0.67 ± 0.11 nM. Test of the EC_{50} of the compound alone against a *P. aeruginosa* PDO300 biofilm was 13.2 ± 1.1 µM, while in combination treatment with tobramycin the EC_{50} was reduced to 0.0077 ± 0.0015 µM. Similarly, in *S. aureus* biofilms, dispersal increased threefold with the addition of “Compound 1”. There was a significant synergistic relationship between the compound and antibiotics that was also shown by resensitizing multi-drug resistant bacteria to traditional antibiotics. This ability was evaluated for a multidrug-resistant strain of MRSA (BAA-44). The compound was successful in resensitizing MRSA to Gentamicin (86.3 ± 0.4 % reduction), Erythromycin (81.9 ± 2.3 % reduction), Penicillin (94.8 ± 1.0 % reduction), Methicillin (90.5 ± 1.7 % reduction), Tetracycline (69.2 ± 2.3 % reduction) [42].

5. Concluding remarks

Many of the quorum sense inhibitor compounds agents tested as potential biofilm therapies are derived from nature. From the naturally derived compounds, chemists can synthesize compounds with slight modifications for different species of bacterial biofilms. It is logical that many potential biofilm therapies are based on natural sources as these organisms had to evolve and generate defence mechanisms against biofilms for survival. There is a lot of promise for compounds derived from common food products as they are less likely to be toxic to human, since they are ingested daily. Novel therapies against biofilms often serve as adjuncts to either the immune system or antibiotic treatments. By eliminating the “film-like” characteristics that make biofilms difficult to eradicate, traditional methods will hopefully be sufficient to eliminate planktonic bacteria. Some bacteria, such as *E. coli* possess special characteristics, which allow tailored therapies like curlicides and pilicides.

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