

Chemical Countermeasures for the Control of Bacterial Biofilms: Effective Compounds and Promising Targets

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Abstract: The pathogenic nature of many infectious bacteria is enhanced by their ability to form surface-associated, protected communities known as “biofilms.” Due to various factors, bacteria in biofilm communities display significantly greater resistance to traditional antimicrobial therapies than their planktonic brethren. This resistance complicates many common bacterial infections, resulting in recurrent ear infections, bacterial endocarditis, chronic lung infection in cystic fibrosis, infectious kidney stones, and surface infection of implanted medical devices. Owing to the serious nature of many biofilm-mediated infections and the near-complete dearth of effective strategies for treating them, efforts are underway to further understand the nature of bacterial infections involving biofilms and to discover and develop effective therapies to combat them. Particularly, several classes of chemical compounds have shown promise in combating biofilms when used in conjunction with traditional antimicrobials. The vast majority of these compounds exert their anti-biofilm properties through disruption of “quorum sensing,” a common means of intercellular communication in bacterial communities that allows coordinated expression of virulence factors and facilitates formation of the oft-complex architecture of mature bacterial biofilms. Other new chemical entities are effective against biofilms without necessarily affecting quorum sensing. This review summarizes salient research in the development of effective chemical countermeasures for Gram-negative and Gram-positive bacterial infections involving biofilms.

INTRODUCTION

In a public announcement by the U.S. National Institutes of Health, it was stated that over 80% of microbial

microorganisms are thought to be surface associated, the prevalence and significance of microbial biofilms have only recently captured the attention of the scientific community. Biofilms can be defined as a population of bacteria living in

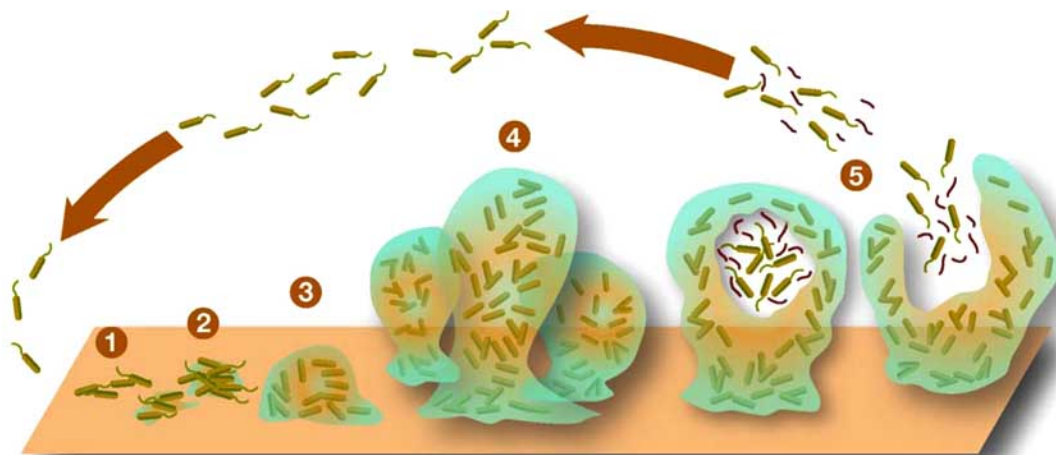


Fig. (1). A model of stagewise bacterial biofilm development general to many motile bacterial species. Stage 1 involves reversible attachment to a surface. In maturing to stage 2, the bacterial cells secrete exopolymers and attachment becomes irreversible. Stage 3 denotes early maturity, as three-dimensional architecture begins to appear. Complex architecture is found as growth continues to stage 4, generally considered as fully mature. Stage 5 is called the “dispersion” stage, where structures within the biofilm develop hollow cavities filled with hypermotile cells that are released upon opening of the channels to spread and begin the process anew. Adapted from Stoodley *et al. Annu. Rev. Microbiol.* **2002** pp. 187-209.

infections in the human body are mediated by biofilms [1]. Incredibly, though the vast majority of the world’s

organized structures attached at a solid-liquid interface. They differentiate in stages and possess remarkable structural complexity (Fig. 1).

Such surface-associated microorganisms are responsible for a legion of common ailments, such as lung infection in

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Table 1. Common Biofilm-Forming Pathogenic Bacteria and the Diseases They Cause

Organism	Gram +/-	Biofilm associated disease(s)
<i>Agrobacterium tumefaciens</i>	-	plant pathogen
<i>Burkholderia cepacia</i>	-	cystic fibrosis lung infection
<i>Erwinia carotovora</i>	-	plant pathogen, root infection
<i>Haemophilus influenzae</i>	-	ear infection
<i>Neisseria gonorrhoeae</i>	-	urethral infection
<i>Pseudomonas aeruginosa</i>	-	burn wound infection, cystic fibrosis lung infection etc.
<i>Staphylococcus aureus</i>	+	burn wounds, bacterial endocarditis, catheter infection etc.
<i>Staphylococcus epidermidis</i>	+	nosocomial sepsis, catheter infection
<i>Streptococcus mutans</i>	+	tooth decay

patients with cystic fibrosis (CF) [2], otitis media (commonly known as ear infection) [3, 4], periodontitis [5], wound infection in burn patients [6], infections caused by a variety of surgical implants [7], endocarditis [8], urinary tract infections [9], and many others. With such a range of clinically significant infections and infecting species involving biofilms (Table 1), it is little surprise that the number of publications involving biofilms has risen dramatically in recent years. Despite this proliferation of research and enhanced understanding of biofilms, clinically useful therapies have yet to emerge. Precious few chemical compounds have specific anti-biofilm properties (apart from

classical antimicrobial activity), and among these none are of current clinical utility. Indeed, an urgent need exists for the development of agents to effectively eradicate biofilms either alone or in tandem with traditional antibiotics. This review summarizes research toward this end and discusses future strategies to approach this multifaceted and currently intractable problem. A fitting introduction to biofilms will be presented in this review suitable to educate a newcomer to the field, though several superb and comprehensive reviews on biofilms [10, 11] and their recalcitrance toward traditional antimicrobial treatments [1, 12-14] have been assembled.

GLOSSARY OF ABBREVIATIONS/TERMS

agrABCD- The gene cluster coding for two-component quorum sensing system in *Staphylococcus aureus*.

AHL (N-acyl homoserine lactone)- The active signaling molecule in quorum sensing cascades in Gram-negative organisms.

AIP (autoinducing peptide)- The general term for an active signaling molecule in quorum sensing cascades in many Gram-positive organisms.

Biofilm- A population of microbes living in organized structures attached at a solid-liquid interface. Biofilms generally are composed of both microbes and an excreted matrix called "EPS".

CF (cystic fibrosis)- A common, fatal genetic disorder that affects approximately 30,000 individuals in the United States. Defects in an ion transport channel called CFTR cause the lungs of CF patients to produce thick mucus that is not easily cleared and is a fertile nidus for infection with many organisms, notably *Pseudomonas aeruginosa*

EPS (extracellular polymeric substance)- This substance, often dubbed "matrix", allows biofilm bacteria to adhere strongly to surfaces and is composed of excreted polysaccharides and other materials.

LasI/LasR- The primary quorum sensing cascade in *P. aeruginosa*, bearing considerable similarity to LuxI/LuxR, the archetypical gram-negative quorum sensing cascade found in *Vibrio fischeri*.

LuxI/LuxR- The first quorum sensing system to be extensively studied, found in the marine bacterium *Vibrio fischeri*.

PQS (pseudomonas quinolone signal)- An active signaling molecule in *P. aeruginosa* that mediates expression of numerous genes

Quorum sensing- The process in which bacterial communities take a real-time census of their immediate population density by secreting and sensing signal molecules and modulating gene expression in response to changing population density.

RAP/TRAP (RNAIII-activating protein/ Target of RNAIII-activating protein)- The two-component quorum sensing system in *S. aureus* that facilitates expression of agr and, consequently, RNAIII.

RhlI/RhlR- The secondary quorum sensing system in *P. aeruginosa*; its expression is regulated by LasR.

RIP (RNAIII-inhibiting peptide)- A peptide of sequence YSPWTNF-NH₂ that inhibits staphylococcal QS by binding to TRAP. This prevents RAP from activating TRAP-mediated phosphorylation cascades leading to expression of agr

RNAIII- As the crucial signaling molecule of the agr system, RNAIII acts directly as a transcription factor to effect genetic regulation afforded by the RAP/TRAP and agr QS systems in *S. aureus*.

TraI/TraR- The primary QS system in *Agrobacterium tumefaciens*. TraR is the only AHL receptor whose crystal structure has been solved; this structure has been used to model AHL receptors from other organisms.

Virulence factor- Any determinant that enhances pathogenicity of an invading microbe; biofilm formation is often considered a virulence factor in bacterial infections.

BIOFILM RESISTANCE TO ANTIMICROBIAL AGENTS

Biofilms are ubiquitous in nature and the clinic, and display amazing tenacity in the presence of biocides and antibiotics alike. For example, sodium hypochlorite, an oxidizing biocide of unparalleled efficacy, requires a 600-fold concentration increase to kill biofilms of wild-type *Pseudomonas aeruginosa* compared to planktonic organisms of the same strain [15]. Ampicillin has a 2 $\mu\text{g}/\text{mL}$ minimum inhibitory concentration (MIC) against a β -lactamase negative strain of *Klebsiella pneumoniae*. The same strain, when grown as a biofilm, survives quite handily (66% survival) when treated for 4 hours with 5000 $\mu\text{g}/\text{mL}$ ampicillin, a 2500-fold increase in MIC [16]. The amazing tenacity of bacterial biofilms in the presence of such compounds is a subject of intense study and debate.

Several hypotheses have been proposed and shown to account in varying degrees for biofilm resistance to antimicrobial agents. Without question, classical mechanisms of genetically encoded bacterial resistance can also operate in biofilms. Multi-drug efflux pumps, antibiotic-modifying enzymes, and bacterial target modifications are all still viable modes of resistance for biofilm bacteria. For example, biofilms of a β -lactamase positive strain of *K. pneumoniae* retard penetration of ampicillin into their interior regions, while β -lactamase negative strains do not [16]. This is most likely due to β -lactamase-mediated antibiotic hydrolysis being faster than penetration into the biofilm matrix. However, these classical resistance mechanisms do not explain the resistance observed even in strains of bacteria which do not possess known antibiotic resistance genes. To explain this phenomenon, several biofilm-specific alternative mechanisms of resistance

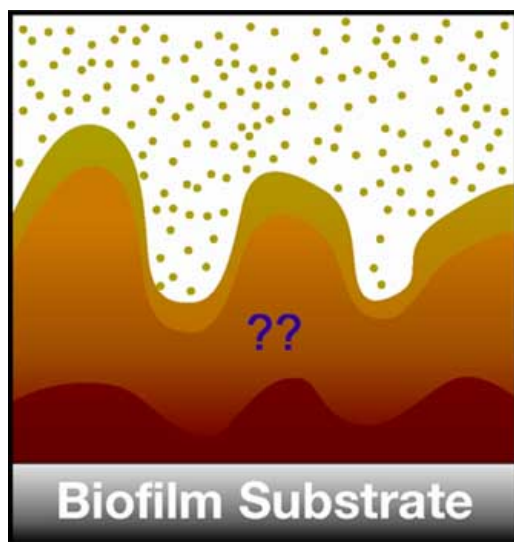


Fig. (2). A graphic summarizing various mechanisms of biofilm resistance to antimicrobial agents. An antimicrobial compound in solution is unable to penetrate all areas of the biofilm due to adhesion, as in the case of aminoglycosides. There are also areas of altered metabolic activity or reduced growth rate, rendering bacteriostatic drugs ineffective. Question marks remind us that differential gene expression allows biofilm bacteria to survive in ways we do not yet fully understand.

have been proposed. These mechanisms are summarized in (Fig. 2).

An oft-discussed factor in biofilms being refractory to antimicrobials is a failure of some of those compounds to penetrate all areas of the biofilm. This has been substantiated by a number of studies in which antibiotic concentration has been monitored at the base of bacterial biofilms. Examinations of real time concentrations of the antibiotic ciprofloxacin were performed to test its ability to pass through to the base of biofilms of *P. aeruginosa* using real time infrared spectroscopy. This study concluded that the biofilm was able to significantly reduce penetration of ciprofloxacin to the surface of the substrate; it was detected at the sterile surface in 40 seconds, while an otherwise similar surface with *P. aeruginosa* confluent biofilm required over 20 minutes to allow similar levels of ciprofloxacin to be detected [17]. This observation is not consistent across all classes of antibiotics, however, as a similar study utilizing a fluorescent analogue of tetracycline along with confocal laser scanning microscopy (CLSM) concluded that a strain of *Escherichia coli* grown as a biofilm shows tetracycline-mediated fluorescence throughout the entire three-dimensional space of the biofilm within seven to ten minutes after exposure of the biofilm to tetracycline [18]. The phenomenon of limited penetration of some antibiotics into biofilms is postulated to be due in part to the Extracellular Polymeric Substance (EPS) or “matrix” of the biofilm. This matrix is a strain-dependant, remarkably complex structure of polysaccharides and sometimes small peptides or DNA. Due to its highly polar nature, the EPS could act as an effective adsorbent for charged compounds or biocides like hypochlorite, and would in so doing limit penetration of these molecules into some bacterial biofilms. In fact, some aminoglycosides, which bear charged ammonium groups at physiological pH, have been shown to adsorb to and be retarded by the EPS produced by *P. aeruginosa* [19, 20]. The retardation of aminoglycosides has also been found reversible in the presence of alginate lyase, an enzyme proficient at catabolizing the EPS of Pseudomonads [21]. Thus, a considerable body of research suggests that the EPS functions as a chemical-retardant barrier to allow survival of biofilm bacteria in the presence of antibiotics that would be lethal to planktonic bacteria.

A second hypothesis to explain the resistance of biofilms to antibiotics is that the varied microenvironments within parts of the biofilm allow for several layers of defense against antimicrobial agents. It is well known that biofilms possess remarkable differentiation, having been compared to other differentiating bacteria with altered phenotypic states like “fruiting bodies” in *Myxococcus xanthus* or spores formed by most Bacillus species [22]. Biofilms are highly structured, often possessing water channels for shuttling nutrients and waste products to and from the interior portions of the biofilm [23]. In addition, oxygen is often consumed at the surface of such biofilms, providing for anaerobic pockets which can antagonize the action of some antimicrobials [24, 25]. Also, it is established that growth rates vary widely from location to location in bacterial biofilms. Since some antibiotics, such as the β -lactams, only inhibit the growth of actively dividing bacteria, non-proliferating regions of the biofilm would resist treatment with such antibiotics. Correspondingly, antibiotics with

bactericidal activities such as ciprofloxacin are more effective in treating *Escherichia coli* biofilms than β -lactams [26]. However, both classes still are less potent against biofilms of the bacteria than against their free-floating kin. Similarly, Evans and colleagues showed that cetrimide's effectiveness was directly proportional to the growth rates of Gram-negative organisms in biofilms [27]. Another set of microenvironmental niches in the biofilm which have received attention are localized areas of low pH due to accretion of acidic cellular waste products [28]. These acidic environments are postulated to aid in biofilm survival by antagonism of the activity of some antimicrobial compounds. Thus, by virtue of the varied microenvironments and growth rates present in biofilms, they are apt to survive treatment with antibiotics effective against growing, planktonic bacteria.

A third and more all-encompassing hypothesis is that differential gene expression in biofilm bacteria aids in bacterial survival through some heretofore unknown mechanism. Indeed, it has been shown that *P. aeruginosa* biofilms express more than 800 proteins at a difference at least six-fold from planktonic levels at some point in their maturation. This represents over 50% of the organism's proteome [29]. Similar studies in *E. coli* strains show 13-20% of the proteome expressed at significantly different levels in biofilms. Such pronounced physiological changes certainly contribute to the relative inefficacy of antimicrobials which are used to treat planktonic bacteria. Optimistically, they could unveil a number of antimicrobial targets not existent in free-floating bacteria as well. In pursuing these biofilm-specific antimicrobial targets, a key realization has been that differentiation to biofilm in many bacterial species requires concerted gene regulation brought about by the ability to sense population density.

BACTERIAL QUORUM SENSING

The ability to sense population density confers specific advantages to pathogenic bacteria: chances of successful attack on a host rise considerably when large numbers of bacteria invade together as opposed to individual cells attempting to brave immune system onslaught without the special protections conferred by overwhelming bacterial numbers. Accordingly, bacteria have evolved sophisticated methods by which to sense how many like bacteria are around them; this ability enables modulation of gene expression based on population density, and enhances viability in harsh environments (like the human body).

A number of bacterial species are now known to carry out a "real-time census" of their proximate population and, in response to increasing population density, to modulate their survival strategies through differential expression of many genes. This phenomenon, termed "quorum sensing" (QS), is a form of intercellular communication and allows bacteria to function effectively in a variety of uninviting environs.

QS, VIRULENCE, AND BIOFILM FORMATION

Many organisms rely heavily on quorum sensing for pathogenicity, and biofilm formation is one of a number of known determinants for effective virulence that are controlled

by quorum sensing [30]. Since adhesion to surfaces in the body is required for infection in many diseases, and since biofilm formation allows bacterial infections to persist stubbornly in the human body, biofilm formation itself is often considered a virulence factor. Because QS is thought to be critical to biofilm formation and virulence in a variety of pathogenic organisms, it has received much attention as a possible drug target, though exact QS contributions to biofilm formation are yet debated in some organisms [31]. To understand how this attention has been focused, it becomes necessary to examine quorum sensing more closely as it relates specifically to both Gram-negative and Gram-positive organisms.

QUORUM SENSING IN GRAM-NEGATIVE ORGANISMS

The Gram-negative quorum sensing (QS) systems have now been studied extensively, beginning with the marine organism *Vibrio fischeri* and continuing with the frequent human invaders *P. aeruginosa*, *Burkholderia cepacia*, *Vibrio cholera*, *Yersinia pestis* and others. In fact, at least 25 species of Gram-negative bacteria are now known to possess quorum sensing cascades with very similar features [32]. The most striking element of quorum sensing in Gram-negative organisms is the highly conserved set of three-component regulatory networks used by these organisms to efficiently regulate a wide variety of bacterial density-dependent activities such as metabolism, virulence, chemotaxis and, notably, biofilm production and maturation [30].

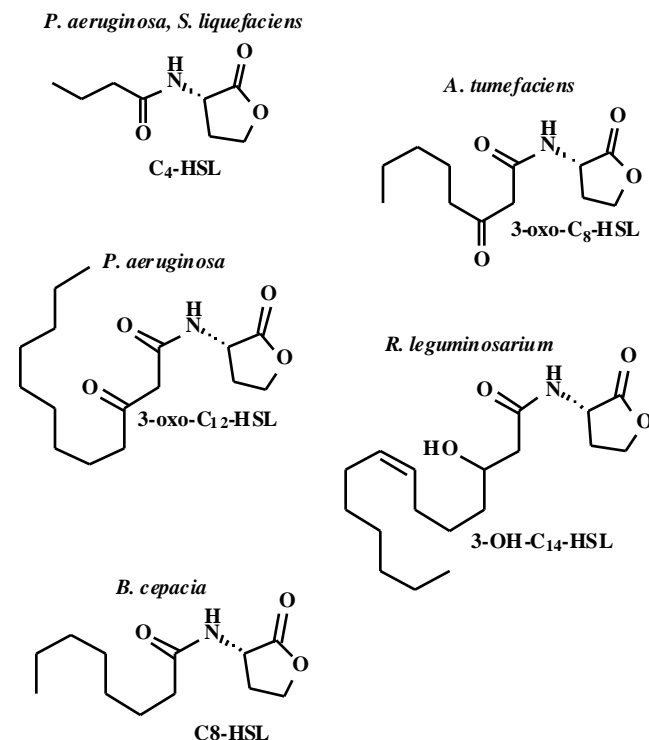


Fig. (3). Typical N-acyl homoserine lactone signals from Gram-negative bacteria. The common name of each signal compound appears below its structure and denotes both the number of carbons in its acyl chain and the position of functionality on that chain.

In keeping with the aforementioned “census” analogy, the bacteria have to send out some type of census form, or small molecule signal, to obtain information regarding how many neighbors surround them. The signal molecules operative in gram-negative organisms are nearly all derivatives of an N-acyl homoserine lactone (AHL) scaffold, differing only by virtue of their diverse acyl functionalities (Fig. 3) [33]. These molecules serve as sensitive secreted measures of local population density, and bind their cognate receptors at a threshold concentration (corresponding to a threshold bacterial population density). This binding is specifically reserved for AHLs of a given acyl side-chain, conferring a significant degree of species selectivity to a given signal [34, 35]. However, AHL-mediated crosstalk between various species does occur and can be symbiotic or antagonistic, depending on the given instance [36]. This binding incites a series of regulatory events that is still being deciphered, but is generally thought to include conformational change and multimerization of the receptor protein and its subsequent binding to DNA whereby transcriptional activation or repression of targeted genes occurs [30]. A number of superb reviews have been published on these systems in Gram-negative bacteria [35, 37, 38]. Fig. 4A shows a diagram of one such system: LasR/LasI from *P. aeruginosa*.

AHLs regulate a diverse variety of phenotypes in the organisms that rely on them, including extracellular protease production [39], alginate and rhamnolipid biosynthesis [40] and biofilm differentiation [41] in *P. aeruginosa*, production of extracellular enzymes and antibiotics in the plant pathogen *Erwinia carotovora* [42], plasmid copy number in *Agrobacterium tumefaciens* [43] and many others. In *P. aeruginosa* AHLs are synthesized by the LasI and RhlI synthases and “sensed” or bound by LasR and RhlR receptors. These proteins are close homologues of the LuxI/LuxR *Vibrio* enzymes that were discovered first.

QS PERTURBATION IN GRAM-NEGATIVE ORGANISMS AS AN ANTI-BIOFILM STRATEGY

It has been shown convincingly that QS and resulting biofilm formation are operative in *P. aeruginosa* infections in the CF lung [44], and this is thought to be among the reasons that conventional antimicrobial therapies are ineffective at eradicating lung infections of this organism in patients with CF. The importance of quorum sensing for effective biofilm formation and pathogenesis in CF [45] and other diseases has led to extensive research efforts toward blocking quorum sensing as a means to mitigate the virulence of *P. aeruginosa* and, ultimately, to eradicate these infectious bacteria.

Indeed, in several models of *P. aeruginosa* infection, genetic or small molecule inhibition of quorum sensing causes attenuation of virulence and enhanced clearance of infection. Wu and coworkers utilized a *lasI/rhlI* double-knockout mutant of PAO1 (wild-type) *P. aeruginosa* versus wild-type PAO1 itself in a rat model of lung infection. At 14 and 28 days post-inoculation, the rats infected with the mutant PAO1 had reduced lung damage and lower bacterial counts compared to wild-type infection. The wild-type bacteria were also able to delay effective immune response to infection, presumably due to production of biofilm matrix

exopolysaccharides under quorum sensing control [46]. A similar study confirmed this result in a rat model of chronic lung infection [47]. Pearson and coworkers demonstrated that *lasI* and *rhlI* single knockouts had attenuated virulence in a neonatal mouse model of pneumonia, while the double knockout was completely avirulent [48]. Other models studied include a mouse model of gastroenteritis [49], a burned mouse model of skin infection [50], and a novel mouse model of lung infection that closely mimics the disease state of CF [51]. Each of these models clearly demonstrates that *P. aeruginosa* uses quorum sensing to establish successful antimicrobial-resistant biofilm infections, and suggests that strategies to perturb quorum sensing are liable to produce tangible benefits in those infections.

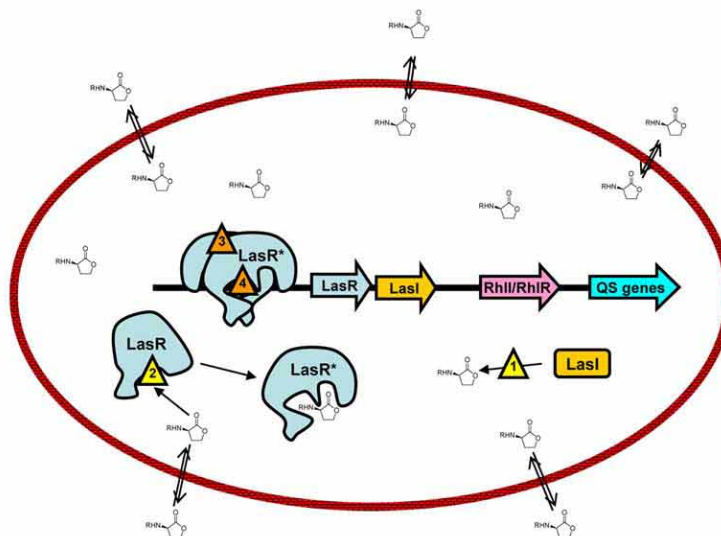
While these genetic knockouts provide proof-of-concept that perturbation of QS cascades can dramatically influence biofilm formation, disruption of QS with a drug-like small molecule is needed to move such strategies into the clinic. Elucidation of the active signals and receptors comprising common QS circuits has afforded a unique opportunity for the development of antimicrobial agents geared not toward killing the targeted organisms, but interfering with their ability to form biofilms or otherwise pathogenize humans as a result of an efficient quorum sensing-mediated attack [52, 53]. As these agents would diminish the ability of a pathogen to infect a human host but render the organism (and other beneficial organisms colonizing a human host) no inherent harm, it is postulated that these “antipathogenic” or anti-virulence drugs will be less likely to pressure the targeted organisms toward the quick development of resistance as is noted with classic antimicrobials. Analogous “anti-virulence” strategies are being pursued for *B. anthracis* [54] and *V. cholera* [55] infections.

Displayed in Fig. 4A is the current model of the AHL-mediated signaling cascade. This QS cascade reveals several targets for inhibition by small molecules; such compounds could disturb the production, reception, or resulting genetic manifestations of a quorum signal. In the four sections that follow, these potential opportunities for the perturbation of Gram-negative QS systems (and resultant biofilm disruption) are delineated. These four targets are 1) AHL production, 2) AHL binding to its receptor protein, 3) Dimerization of the AHL receptor, and 4) receptor-DNA binding. As described below, strategies 1) and 2) have been validated to a certain extent (represented in yellow in Fig. 4A), whereas 3) and 4) remain as unexploited targets (represented in orange in Fig. 4A).

1. Inhibition of AHL Production

The start of the quorum sensing cascade is the synthesis of AHL compounds by a LuxI homologue that is expressed at a basal level. As AHL signals are highly homologous, so are their synthases, generally utilizing S-adenosylmethionine (SAM) (Fig. 5A) as a critical intermediate to donate a nitrogen atom to what will become the homoserine lactone. Accordingly, traditional inhibitors of SAM-utilizing enzymes such as S-adenosylhomocysteine (Fig. 5B), sinefungin (Fig. 5C), and other intermediate mimics are potent inhibitors of AHL synthesis in RhlI, the synthase

A.



B.

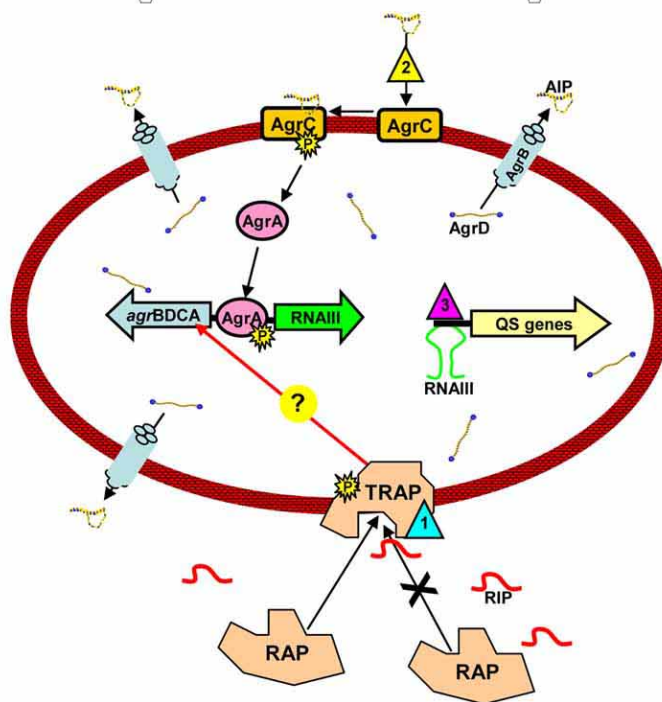


Fig. (4). (A.) A typical Gram-negative quorum sensing system, LasI/LasR from *Pseudomonas aeruginosa* in which triangles denote current (yellow) or potential (orange) points of quorum sensing perturbation as enumerated in the section of the text entitled “**QS PERTURBATION IN GRAM-NEGATIVE ORGANISMS AS AN ANTI-BIOFILM STRATEGY.**” The autoinducing compound, an N-acyl homoserine lactone, is synthesized by LasI (1) and binds its cognate LuxR-type receptor, called LasR in *P. aeruginosa*, at a threshold concentration (2). This causes conformational change in LasR which results in its dimerization (3), and binding to cognate DNA (4) as a positive regulator of QS-controlled genes, including secondary QS cascade RhlIR. (B.) Sequential staphylococcal two-component systems controlling biofilm formation and virulence factor production. (1). The protein RAP binds to its cell wall-associated receptor TRAP. Through uncertain mechanisms, this begins expression of the genes in the *agr* locus. These genes comprise a second two-component regulatory system whereby AgrD peptide is translated, processed by AgrB into autoinducing peptide (AIP), which binds its cell wall-associated receptor AgrC. AgrC phosphorylates AgrA, activating it as a promoter of RNAIII transcription. RNAIII is the transcription factor which then upregulates expression of virulence factors and determinants critical to biofilm formation

responsible for producing C_4 -HSL, the secondary messenger in *P. aeruginosa* [56]. Because SAM is a common intermediate in mammalian enzymes as well, pan-inhibition with a non-specific mimic would likely have undesirable manifestations in clinical applications. However, further

study and optimization could yield compounds with specific affinity for AHL synthases. Such compounds would be ideal QS inhibitors, as AHL synthases are produced in relatively low numbers before positive autoregulation begins as a “quorum” is attained.

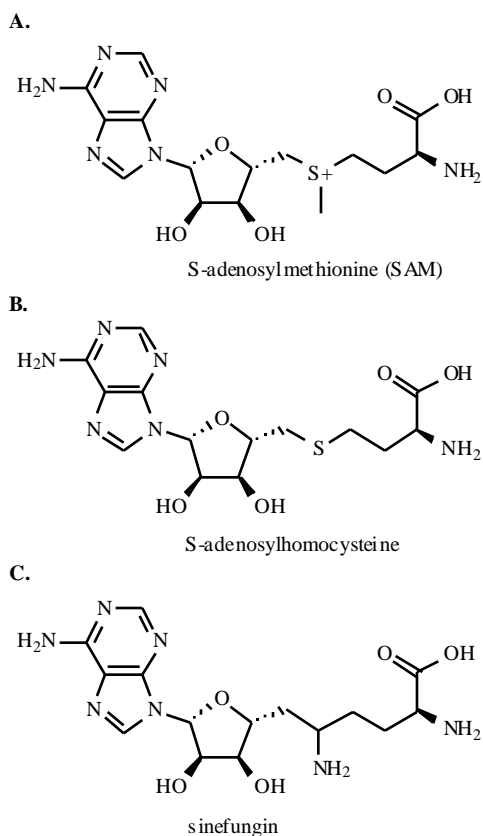


Fig. (5). S-adenosylmethionine and two known inhibitors, (B.) S-adenosylhomocysteine, and (C.) sinefungin. These compounds inhibit RhlI, the synthase of C4-HSL.

In addition, there are bacterial enzymes, generally from Gram-positive organisms, that are known to degrade homoserine lactone signals [57]. The benefit of degrading AHL signals is obvious: without a threshold concentration of AHL being reached, Gram-negative pathogens would be unaware of local population density and unable to form mature biofilms or secrete virulence factors. In particular, a *Bacillus* strain was found that expressed an enzyme, AiiA, which specifically hydrolyzed AHLs [58]. Transgenic plants expressing this enzyme were highly resistant to colonization and infection by *Erwinia carotovora*, a Gram-negative quorum sensing plant pathogen. In fact, Hentzer and Givskov have found many complex isolates of environmental organisms show AHL degradation activities [59].

Although enzymes are not common antimicrobial agents in the clinic, they may have specific applications in the fight against Gram-negative quorum sensing pathogens: in cystic fibrosis the unique accessibility of the lungs through nebulization of therapeutics allows for facile delivery of even proteins. In fact, recombinant human DNase I is currently inhaled by many CF patients under the pharmonym "Pulmozyme" [60, 61]. As DNA is a prevalent component of the mucus of CF patients, this enzyme reduces the viscoelasticity of that mucus and facilitates its clearance. Likewise, an optimized nebulized enzyme to hydrolyze AHLs which produced little or no immune response and had a reasonable half-life could possibly help to attenuate virulence and thwart biofilm production in early-stage *P. aeruginosa* colonization in CF patients and to aid them in

clearing infections before the lungs become chronically diseased.

2. Inhibition of AHL Binding to its Receptor

After the diffusible AHL signal is generated and its concentration grows high enough to facilitate binding by its cognate receptor, generally a LuxR homologue, the receptor is thought to change conformation upon signal binding, preparing it for the regulatory steps of the cascade which are to follow. Given that the structures of the AHL signals are known, and crystal structures of a LuxR homologue (TraR) are available (PDB entries 1H0M and 1L3L at 3 and 1.7 Å, respectively) [62], considerable possibilities exist for the inhibition of interactions between AHLs and LuxR-type receptors.

This potential has not escaped the watchful eye of Nature, as numerous organisms in symbiosis or direct competition with Gram-negative bacteria produce AHL analogues that modulate QS, providing a wealth of lead compounds. In the absence of evolved immune systems, many marine plants are especially susceptible to bacterial colonization and resultant disease [63], but *Delisea pulchra*, a marine macroalga, produces a set of compounds called halogenated furanones that inhibit quorum sensing with moderate potencies [64]. This organism uses these compounds to effectively prevent colonization by *Serratia liquefaciens* and other Gram-negative organisms [65]. Structures of several of these compounds are shown in Fig. 6A. At least 30 such compounds are produced by this organism, and an inverse relationship has been shown between numbers of microbes at the algal surface and concentration of halogenated furanones at the same spot [66]. Investigation of its ecological niche reveals that these inhibitors allow *D. pulchra* to control the balance of microflora in its natural environment, allowing Gram-positive organisms that are naturally poor colonizers of marine surfaces to outcompete Gram-negative organisms that would dominate given the ability to sense a quorum.

Due to the roughly AHL-like scaffold of these molecules, their known displacement of AHL derivatives from LuxR-type proteins [67], and the fact that their effects are partially mitigated by overdosing with AHLs in some competition experiments [68], it had been reasonably estimated that they act as direct competitors to the AHL binding sites of LuxR-type receptors. However, direct competition of halogenated furanones with AHLs for the same binding pocket seems not to be the mechanism of inhibition for these compounds on all AHL receptors [69, 70]. Nonetheless, Hentzer and coworkers made synthetic derivatives (Fig. 6B) of the natural *Delisea* furanones and demonstrated that these compounds were able to inhibit *P. aeruginosa* QS circuits and markedly affect biofilm architecture in that organism [71]. In a followup study, production of other virulence factors was also stifled in the presence of these inhibitors [72]. Interestingly, the Givskov lab showed that, in *V. cholera*, the QS inhibition corresponded not to stable association of the furanone compounds with LuxR, but by accelerated turnover of LuxR in the presence of the compounds [69]. Full mechanistic insights into this fascinating data are not yet available.

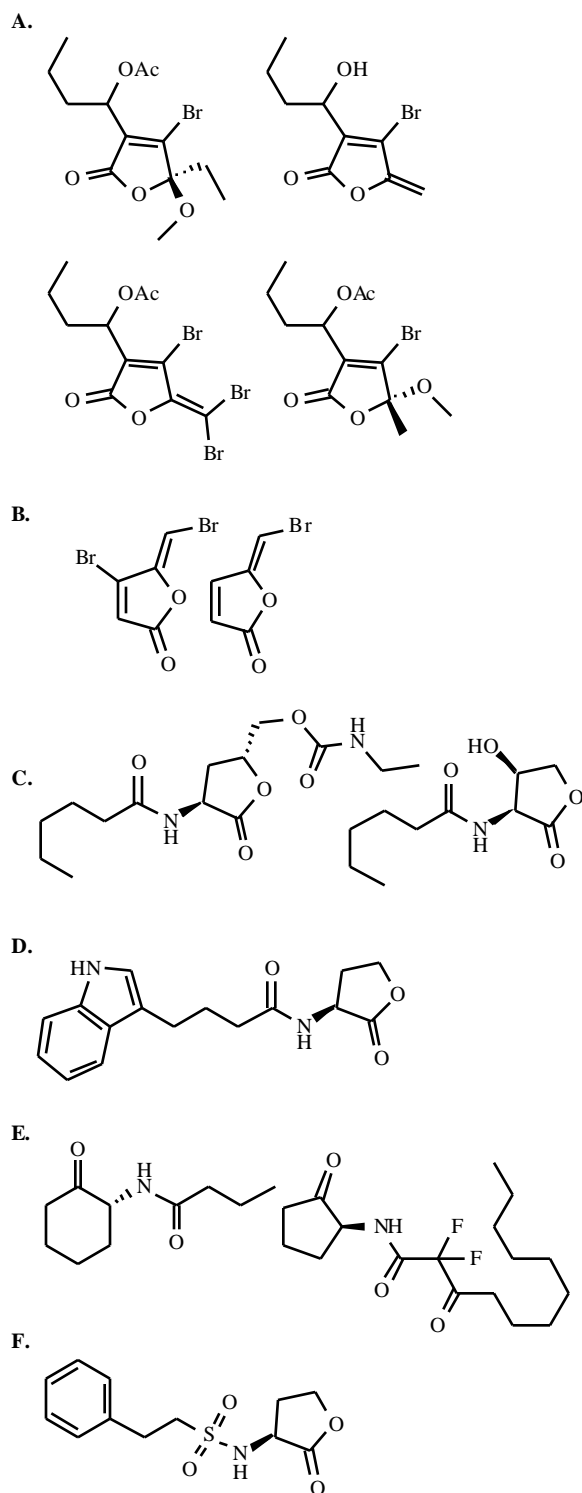


Fig. (6). Synthetic quorum sensing antagonists.

Additionally, some commonly cultivated plants such as pea, tomato, rice, and garlic secrete substances that affect QS regulation by AHLs in bacteria [73]. These inhibitors' structures have yet to be elucidated but seem to modulate QS in bacteria in a more specific fashion than the pan-inhibitory properties of the *Delisea* compounds. Obviously, structural insights and perhaps new optimizable lead compounds for inhibition of QS and biofilm formation could be gained by deciphering the source of these plants' inhibition of QS in neighboring bacteria.

Extensive biochemical studies have shed considerable light on the structures of a number of LuxR analogues, though to date only the TraR analogue of LuxR has a published crystal structure. These studies have revealed several structural trends that tend to hold across members of this enzyme family throughout diverse organisms. For instance, the N-terminal domains in LuxR-type proteins appear to be the active regions for binding to AHL signal molecules, while DNA binding is generally reserved for a sequence of amino acids close to the C-terminus of these receptors [62, 70, 74, 75].

A number of compounds designed to bind LuxR-type proteins have been synthesized. Generally these compounds are close structural homologues of the AHLs, and as a result some cause induction rather than inhibition of the QS cascades. Early efforts focused on altering the homoserine lactone moiety itself, utilizing a number of structures from pedestrian cycloalkanol and cycloalkanone analogues to more remotely related heterocyclic aliphatic and aromatic moieties [76, 77]. A number of these compounds show considerable promise as LuxR inhibitors. An interesting approach was undertaken in the Nielsen lab, where the 3- and 4- positions of the homoserine lactone ring were derivatized (Fig. 6C) to produce several attractive compounds. Some of these compounds were superb activators of the QS cascade in a LuxR-based screen, but none were potent inhibitors of LuxR-mediated quorum sensing [78].

On the topic of LuxR inhibitors, a particularly engaging report from the Blackwell laboratory detailed a higher-yielding microwave-driven synthetic scheme to produce a number of novel AHL derivatives. Evaluated in traditional reporter gene assays to assess QS inhibition, one of these derivatives was two orders of magnitude more potent than any previous AHL-based inhibitor and effectively stymied biofilm production in *P. aeruginosa* (Fig. 6D) [79]. Spring and coworkers recently synthesized and tested a number of derivatives which substituted 2-substituted cyclopentanone and cyclohexanone for the homoserine lactone moiety and found those compounds had enhanced stability in physiological solutions [80] (Fig. 6E). Castang *et al.* generated a focused library of N-sulfonyl homoserine lactones that also show antagonism of QS in the micromolar range, but these compounds may experience the same stability problems as other lactone-based inhibitors (Fig. 6F) [81]. Notably, their compounds were conceived utilizing a three-dimensional model of LuxR generated *via* sequence homology with TraR. Their modeling suggested that the inhibitors could function by preventing conformational change of LuxR into its active, dimeric, DNA binding conformation. This modeling strategy could prove useful for evaluating ligand docking into the putative active sites of LasR and RhlR from *P. aeruginosa* as well as other AHL receptors from organisms of particular ecological or medical importance. A similar homology model approach was recently used by Koch and coworkers to test a number of AHL receptor inhibitors comprising several classes against LuxR active site mutants (identified by biochemical studies in combination with examining the aforementioned TraR structure) [70]. Ideally homology models would not be needed, but until crystal structures for more of the receptor proteins are solved, these hybrid biochemical and computational strategies are likely the most attractive.

As a guideline for future research, it seems worthy of stating that the inherently labile functionality of the lactone ring itself [82] should be phased out in subsequent generations of AHL inhibitors, and certainly in those which aspire to clinical viability. Numerous scaffolds have been shown adequate as replacements for the natural L-homoserine lactone ring, and a “global” study incorporating all the structure-activity relationships (SAR) known for both the HSL ring and side chain could produce second generation inhibitors that are far more potent than any currently available.

3. Inhibition of Receptor Auto-Association

Several LuxR homologues have been studied extensively *via* biochemical methods. These studies have revealed a similar role for these receptors in many organisms. In general, the DNA-binding, active form of LuxR-type proteins is in a dimer or higher-order aggregate which is formed upon binding of an AHL signal [38]. Currently, no reports describe small molecule inhibitors of this multimerization step. This is unfortunate, as these receptor proteins only perform their intended functions when associated with one another; a compound that prevented association of AHL receptor monomers would likely abolish transcriptional regulation by the receptor, effectively freezing the quorum sensing cascade.

Examination of the high resolution crystal structure of TraR, a LuxR homologue, reveals strong association between the monomer units (Fig. 7). Even still, this dimerization does not occur until prompted by conformational adjustment upon binding an AHL signal. A molecule targeted to the face of the receptor where dimerization occurs could disturb this dimerization event, and as TraR only functions to bind DNA and regulate transcription as a dimer, the effect would be to abolish TraR's DNA-binding activity and transcriptional regulation, thereby thwarting quorum sensing. Some precedent exists

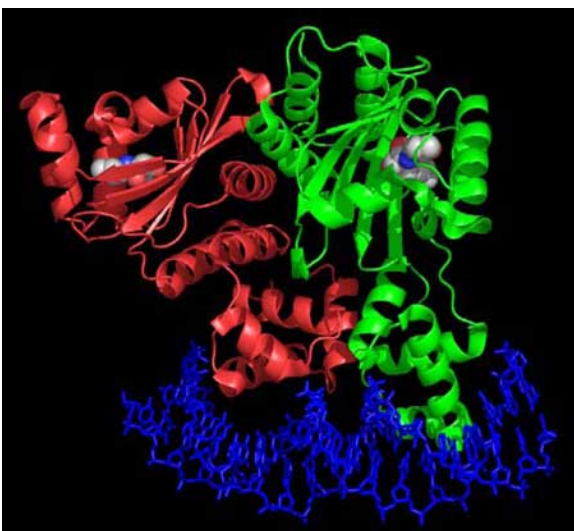


Fig. (7). Crystal structure of TraR homodimer complexed to cognate DNA. Identical TraR monomers bind the DNA substrate only as a homodimer. Spacefill models show the AHL signals bound to each TraR monomer.

for inhibition of protein homodimerization with small molecules [83], and the extensive homology between members of the LuxR protein family suggests that disruptors of one LuxR-type multimer could disrupt others of the family.

4. Inhibition of AHL Receptor-DNA Interaction

Another possible approach to the disruption of quorum sensing is the use of small molecule inhibitors designed to prevent the binding of LuxR-type receptors to their cognate DNA targets, often termed “Lux-type boxes.” Examples are present in the literature of small molecule inhibitors of transcription factor function [84, 85]. The C-terminal DNA-binding domains of the LuxR-type receptors have considerable homology with one another, as evidenced by their primary binding sequence changing little from species to species. However, some variation in binding specificity for given DNA sequences does exist, conferring a degree of speciation to different AHL receptors that is necessary when (as is often the case) more than one LuxR/LuxI type system is present in a single organism.

QS IN GRAM-POSITIVE ORGANISMS

Although the Gram-negative quorum sensing cascades have been studied more extensively, the importance of quorum sensing to biofilm formation and virulence in Gram-positive organisms has recently come to light. Though many parallels exist between Gram-negative and Gram-positive quorum sensing, there are important differences. In Gram-positive bacteria most QS signals are post-translationally modified peptides called autoinducing peptides (AIPs). Since AIPs do not diffuse readily across cell membranes like AHLs, they are secreted by ATP-binding cassette export systems [86, 87]. Correspondingly, the receptors for AIPs are transmembrane proteins rather than cytoplasmic. In addition, considerable heterogeneity exists in AIP structure as well as the mode by which a given AIP receptor induces or represses gene expression. Generally, activation of transcriptional changes (and subsequent differentiation, often including biofilm formation) occurs when the AIP signal is bound by the receptor kinase, activating complex phosphorylation events within the cell. These events include positive feedback and enhanced synthesis of the signaling peptide as well as synthesis of a battery of regulated genes often including virulence determinants [87]. Though many Gram-positive QS signals exist only as communication molecules, nisin and subtilin function dually as quorum signals and as potent secreted antimicrobials [88, 89].

In a particularly well-studied example, *S. aureus* possesses a two-component system called RAP/TRAP which is responsible for promoting transcription of RNAII, from whence *Agr*, another two component system, is translated. RAP/TRAP indirectly (through *agr*) promotes transcription of RNAIII, which acts to promote virulence factor formation and biofilm formation in *S. aureus* (Fig. 4B). RAP is a 30 kD protein excreted by the bacteria from early in their growth curve. As cells amass, RAP accumulates in the supernatant and eventually binds its transmembrane receptor TRAP, which activates *agr*, a secondary system with its own excreted peptidic, AIP.

AIP is translated from the mRNA of gene *agrD* and processed by AgrB that removes residues from N- and C-terminal sides of the peptide while shuttling it to the outside of the cell [90]. AgrC is the transmembrane receptor that binds the AIP made from AgrD peptide. This receptor, a histidine kinase, phosphorylates AgrA which initiates transcription of RNAIII. This regulatory RNA is the entity that ultimately effects regulation of many of the target genes under *Agr* control, including numerous toxins, other virulence factors, and determinants for effective biofilm formation. This complex cascade is represented in Fig. 4B.

INHIBITION OF GRAM-POSITIVE QUORUM SENSING AS AN ANTI-BIOFILM STRATEGY

Unfortunately, inhibitors of QS in Gram-positive organisms are rarer even than Gram-negative QS inhibitors. But that may be changing: study of an exoprotein-deficient mutant strain of *S. warneri* revealed a peptidic inhibitor, RNAIII-inhibiting peptide (RIP), that competes with RAP for binding to TRAP, thus preventing TRAP phosphorylation and stifling QS in *S. aureus* [91] (Fig. 4B-1). RIP has since been evidenced to be widely effective as a QS inhibitor against *S. aureus*, showing its utility *in vitro* and in several diverse *in vivo* models [92], though this and subsequent evidence of RIP's utility is still hotly debated [93]. RIP has been utilized to coat surfaces of implanted devices, preventing biofilm formation thereon [94]. RIP and a synthetic derivative [95] are the only widely utilized inhibitors of Gram-positive biofilm formation, and as peptides are thus suboptimal as drug candidates for biofilm-mediated Gram-positive infections like endocarditis [96].

As opposed to the high homology of RAP-TRAP (and RIP effectiveness) across strains of *Staphylococcus*, sequences of AIPs are highly strain dependent, and *agr* systems are divided into four classes based on these sequences [97-100]. Divergence in sequence among these peptides results in a considerable variation of activities for any given peptide toward other strains' AgrC receptors, with most completely inhibiting quorum sensing activities of other strains [100]. A notable exception is the *agrI* system, which has been shown to be strongly induced by AIP-IV (the AIP from *agrIV*) [101, 102], though another lab that attempted to synthesize AIP-IV and utilize it to activate cells of a group I strain saw inhibition of *agrI* [103]. These contrasting results have not been resolved, but further testing of both the synthetic AIP-IV and the isolated AIP-IV in classical organic identification methods would confirm structural homology between the samples. And though no universal inhibitor of AgrC variants exist, it is not impossible to think that AIPs from other AgrC classes might function similarly in this QS cascade as RIP does in the RAP/TRAP cascade. Given that approximately 90% of *S. aureus* strains possess a type I *Agr* system, AIP-2 which inhibits AgrC-1, could function as a *de facto* "universal" inhibitor of the *agr* cascade. A plethora of AIP derivatives have been prepared and evaluated for their proclivities in preventing quorum sensing; a review examining this work is available [104].

Activating-peptide analogues represent a primary class of AgrC inhibitors (Fig. 4B-2). Considering that mutations in

agr have shown both inhibitory and stimulatory effects on biofilm production in *S. aureus*, a greater understanding of these peptides' effects on particular strains need to be attained before widespread use, lest a potential therapy enhance biofilm formation in a Gram-positive invader and exacerbate rather than mitigate Gram-positive bacterial infections. And, just as in the Gram-negative cascades, no research documenting inhibitors of AgrA's DNA binding site (like LuxR binding to DNA in Gram-negatives) has been described, though screening for them would almost certainly be facile given that a fluorescently labeled oligonucleotide can serve as a sensitive binding detector.

RNAIII itself might serve as a future drug target as well, should advances in RNA binding methodology continue. In such a strategy, a small molecule would bind RNAIII directly, preventing it from acting as a transcription factor and mediating biofilm formation or production of other virulence factors in Gram-positive bacteria (Fig. 4B-3). A small number of drugs currently target RNA, and active research continues in this field [105-108].

AI-2/luxS: A QS SIGNAL OPERATIVE IN BOTH GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

The AI-2 signal is noteworthy both in that it controls biofilm formation in a number of significant bacterial species (*Bacillus cereus* [109], *Escherichia coli* [110], *Haemophilus influenzae* [111], *Streptococcus mutans* [112], *Klebsiella pneumoniae* [113], etc.) and that those species comprise both Gram-positive and Gram-negative organisms. The term AI-2 is used to refer to a number of derivatives of a common precursor, 4,5-dihydroxy-2,3-pentanedione (DPD), that are synthesized by LuxS synthases (Fig. 8). AI-2 signaling has been reviewed elsewhere [114], but it is important to note that competitive inhibitors of AI-2 have been synthesized and shown effective in blocking the effects of AI-2 [115, 116].

OTHER TARGETS FOR BIOFILM DISRUPTION

Though AHL- and AIP-mediated signaling pathways have necessarily encompassed the majority of the discussion in this review, there are other targets for disturbing biofilm formation both quorum sensing related and unrelated. Often these targets become more organism-specific.

For instance, in *P. aeruginosa*, a third QS cascade exists with a non-AHL signal, 2-heptyl-3-hydroxy-4-quinolone. This compound, termed the *Pseudomonas* Quinolone Signal (PQS) (Fig. 9), is synthesized from a precursor called anthranilic acid. Its receptor, PqsR, controls expression of 143 genes [117], many implicated in quorum sensing processes. Direct links between PQS and biofilm formation/maturation have not been solidified. Calfee *et al.* showed that methyl anthranilate, an analogue of PQS, inhibited PQS production and mitigated production of several virulence factors, including elastase, in *P. aeruginosa* in a dose-dependent fashion [118]. Full characterization of the enzymatic pathways controlling PQS synthesis and monitoring might unearth other easily targetable enzymes to prevent PQS production or reception.

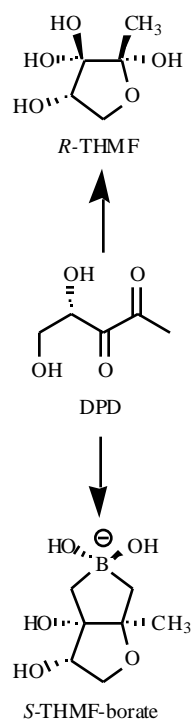


Fig. (8). Some common AI-2 molecules. Intermediate **DPD** is synthesized by LuxS and undergoes rearrangement, producing various AI-2 molecules including **R-THMF** and **S-THMF-borate**, the active AI-2 signals in *Vibrio harveyi* and *Salmonella enterica* serovar Typhimurium, respectively. Adapted from Camilli *et al. Science* **2006** pp. 1113-1116

Musk and coworkers discovered that iron salts are potent inhibitors of biofilm formation in *P. aeruginosa* both in static and flow-cell models (Fig. 10) of biofilm growth [119], and that excess iron can prevent biofilm-mediated resistance of *P. aeruginosa* to tobramycin by preventing biofilm formation (Fig. 11). Interestingly, production of other virulence factors, save production of iron chelators (pyoverdine and pyochelin), was unaffected by excess iron addition (Musk, D.J., unpublished observations); reduction in synthesis of these chelators seems expected in iron-laden growth conditions. It could be that the chelators themselves have a role in signaling for biofilm formation: a recent study by Ferreras and coworkers revealed that inhibitors of siderophore biosynthesis effectively prevented virulence

factor production in both *Yersinia pestis* and *M. tuberculosis* [120] (Fig. 12). A hypothetical role for iron in biofilm formation is thus postulated in (Fig. 12). A specific target for iron's inhibitory effects has yet to be deciphered, though complex regulatory events courtesy of the iron-mediated ferric uptake regulon (*fur*) likely are operative. The intimate connections between iron and biofilm formation in *P. aeruginosa* have been noted by other labs, as very low iron concentrations (due to iron sequestration by lactoferrin) also can prevent biofilm formation by *P. aeruginosa* [121]. The links between iron and virulence in cystic fibrosis patients, though still hotly debated, are indelible [122-125].

Since the discovery that iron inhibits biofilms in *P. aeruginosa*, excess iron has also been shown to inhibit biofilm formation in *S. aureus* and *Streptococcus mutans*, Gram-positive organisms [126, 127]. These interesting findings have important implications for the treatment of cystic fibrosis; direct correlation between iron deficiency in CF patients and severity of lung infection by *P. aeruginosa* has been noted [124]. Since iron is an essential nutrient, most *in vivo* settings are iron-starved, with iron being sequestered by host proteins [128]. If the frequent CF pathogens *S. aureus* and *P. aeruginosa* have evolved to thrive in this environment, it stands to reason that changing their environment by adding iron could prevent effective biofilm formation, pathogenesis, or chronic infection. Of course, questions of iron's pulmonary and systemic toxicity would need to be effectively addressed before such an approach could prove effective.

Another intriguing development in anti-biofilm efforts is the discovery that macrolide antibiotics, particularly azithromycin and clarithromycin (Fig. 13), delay or disrupt biofilm formation at subinhibitory concentrations. Ichimaya and coworkers first noted significant inhibition of exopolysaccharides at 1/16 the MIC for that strain, and confirmed inhibition of biofilm formation using scanning electron microscopy (SEM) [129]. Subsequent studies in more rigorous flow channel experiments showed sub-inhibitory azithromycin to delay but not inhibit biofilm formation in *P. aeruginosa*, with pre-formed biofilms not affected by azithromycin addition [130]. Clarithromycin, a closely related macrolide, was shown to have similar effects against biofilms of *Mycobacterium avium*, an opportunistic pulmonary pathogen. Like azithromycin, it was ineffective at

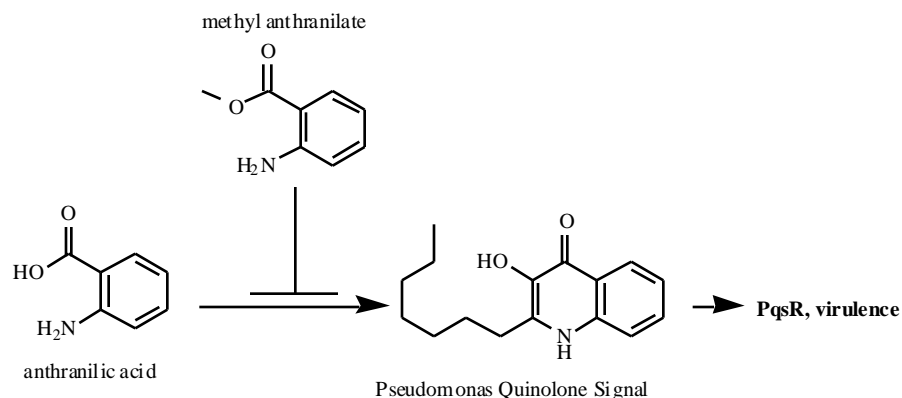


Fig. (9). Anthranilic acid is a crucial precursor in the synthesis of the *Pseudomonas* Quinolone Signal (PQS). Synthesis of this molecule is inhibited by the precursor analog methyl anthranilate

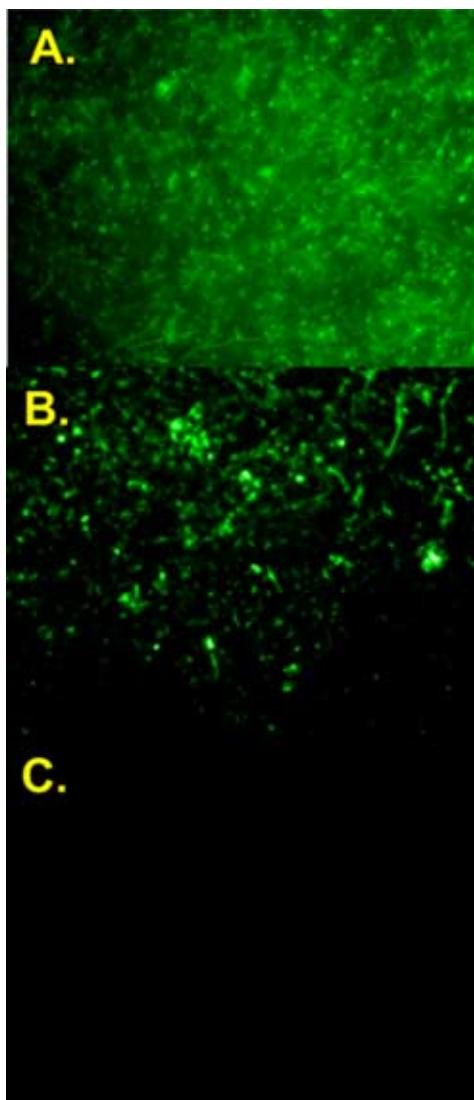


Fig. (10). Iron disrupts pre-formed biofilms of *P. aeruginosa* PA14. At 5 days (A.), biofilm had achieved confluent growth and was visualized by fluorescence microscopy. At this time point, 200 μM ferric ammonium citrate was added to the media and flow was continued through the chamber for 3 more days. At day 8 (B.), partial disruption of the biofilm was evident, and by day 10 (C.), the biofilm was completely vanquished.

treating established biofilms [131]. Important steps have been taken toward identifying new biofilm-compromising protein targets by using random transposon-mediated mutagenesis to silence genes followed by testing for impaired biofilm formation. It was in just such a study that the *pel* genes required for efficient biofilms in *P. aeruginosa* as well as in the Gram-negative plant pathogen *Ralstonia solanacearum* were identified as crucial [132, 133]. Other studies showed a similar cluster of genes, dubbed *psl*, to have similar effects on biofilm production in *P. aeruginosa* [134, 135]. These genes are known to code for proteins (PslA was shown to be especially critical) which serve to synthesize critical components of the EPS in Gram-negative bacteria. Inhibitors that stunted the synthesis of EPS could halt biofilm formation at its earliest stages, rendering treated bacteria susceptible to traditional antibiotics.

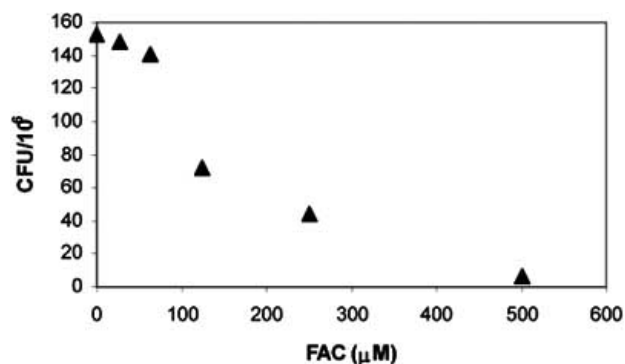


Fig. (11). Treatment with ferric ammonium citrate (FAC), an iron salt, increases susceptibility of *P. aeruginosa* to tobramycin by preventing biofilm formation. After allowing 48 h of stationary growth in the FAC concentrations indicated, cultures were treated with 64 $\mu\text{g}/\text{mL}$ tobramycin for 4 h, a treatment that would kill planktonic bacteria but not biofilm bacteria. Cultures were then plated to assess survival.

Another very interesting protein that has anti-biofilm target potential is GroEL1 in mycobacteria. The Hatfull lab recently characterized this protein as a non-essential entity that modulates the synthesis of mycolates, long chain fatty acids that both comprise a large portion of mycobacterial cell walls and are also essential to biofilm maturation. Knockout mutations *via* phage integration into *groEL1* produced mutant strains severely defective in biofilm formation in *Mycobacterium smegmatis* [136]. Since *Mycobacterium tuberculosis* also has this gene, it could represent a future target for biofilm perturbation in that important pathogen. It will be first important to determine with surety whether biofilm is important to the pathogenesis of *M. tuberculosis*, as this information is currently unknown [136].

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Bacterial communities that band together as biofilms are responsible for a variety of intractable infections. Biofilms display tremendous tenacity in rebuffing traditional antimicrobial therapies and surprising fluidity in regulating expression of their genes in concert with one another to efficiently deal with rapidly changing and actively hostile environs. As expounded upon in this review, efforts to date have produced leads, but no clinically useful agents capable of remediating bacterial infections involving biofilms.

And even if progress continues in this area and compounds capable of treating biofilm infections are found, questions will remain to be answered. Would one administer an anti-biofilm compound alone in some cases, relying on the immune system to finish clearing a planktonic infection that was once biofilm and thus protected? Would this approach be effective in immunocompromised individuals who often harbor biofilm-mediated chronic infections? Or would an antibiotic be given after, or in tandem with, treatment with an anti-biofilm compound? Will the clinical effectiveness of anti-biofilm agents be limited to use in prophylactic capacities? Would resistance develop to anti-biofilm therapies similar to its rapid evolution against antibiotics? Only time and trial will tell.

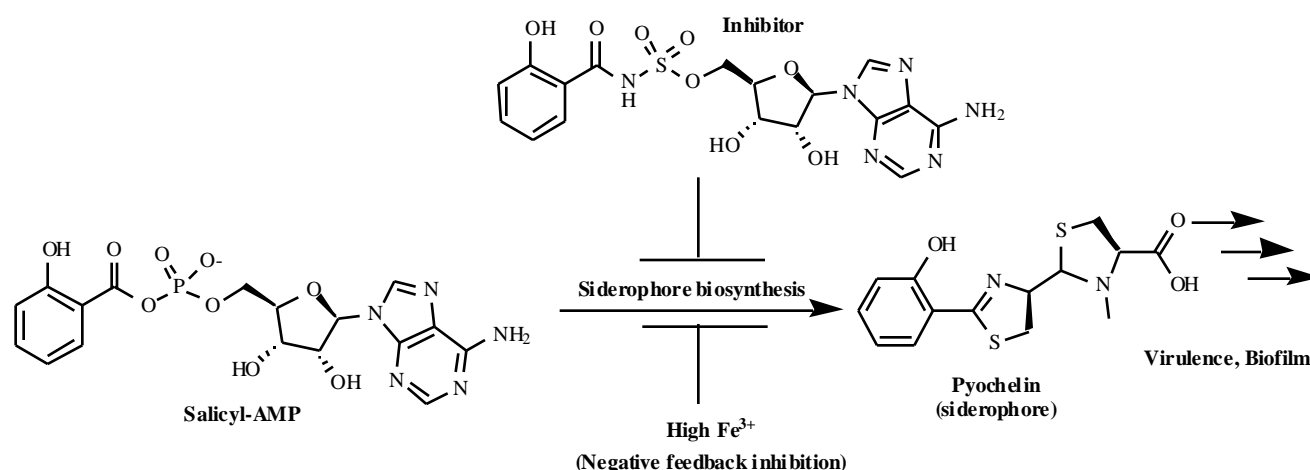


Fig. (12). Inhibition of siderophore biosynthesis as a putative anti-biofilm strategy. Small molecule inhibitors have proven to halt synthesis of iron chelators like pyochelin and mitigate virulence in bacteria. High iron may be functioning similarly in preventing biofilm formation.

Though some potential does exist for the discussed compounds to become of wide application clinically, it would behoove researchers to focus on expanding the range of biofilm-specific targets they probe in search of effective countermeasures to pernicious biofilm infections. As the list of new genes and protein products crucial to effective biofilm formation grows longer, opportunities will increase for the discovery of novel chemical entities that will inhibit crucial cellular components of biofilm formation in both Gram-positive and Gram-negative organisms.

to breathe easy and will lay biofilm-mediated *S. aureus* infections to rest in peace.

ACKNOWLEDGEMENTS

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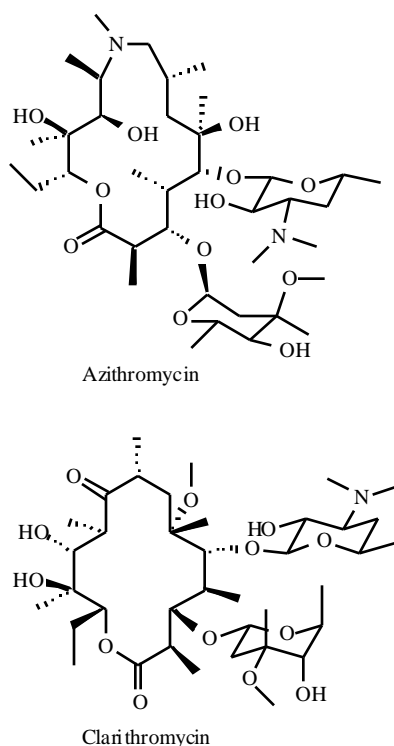


Fig. (13). Structures of azithromycin and clarithromycin, two macrolide antibiotics known to perturb biofilm formation at sub-inhibitory concentrations.

And perchance, in the near future, new classes of biofilm-inhibiting small molecules will allow cystic fibrosis patients

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