ACS Infectious Diseases

Viewpoint

Cite This: ACS Infect. Dis. XXXX, XXX, XXX−XXX

pubs.acs.org/journal/aidcbc

# Hijacking the Bacterial Circuitry of Biofilm Processes via Chemical "Hot-Wiring": An Under-explored Avenue for Therapeutic Development

Ingrid K. Wilt,[†] Taylor P. A. Hari,[†] and William M. Wuest*[,†,‡]

[†]Department of Chemistry, Emory University, 1515 Dickey Drive, Atlanta, Georgia 30322, United States

[‡]Emory Antibiotic Resistance Center, Emory University School of Medicine, 201 Dowman Drive, Atlanta, Georgia 30322, United States



**ABSTRACT:** Biofilm-associated infections are linked to chronic and recurring illnesses. These infections are often not susceptible to current antibiotic treatments because of the protective exocellular matrix and subpopulations of dormant or "persister" cells. Targeting bacterial circuitry involved in biofilm formation, including two-component systems, quorum sensing, polysaccharide structural integrity, and cyclic nucleotide signaling pathways, has the potential to expand the existing arsenal of therapeutics, thus catalyzing a second golden age of antibiotic development.

Bacteria grow and proliferate either as single, independent cells (planktonic phenotype) or in organized aggregates commonly referred to as biofilms.[1−3] Conventional antibiotic treatments target free-living planktonic bacterial populations through cell membrane disruption[4] or one of five essential biosynthetic processes: the generation of protein,[5] RNA,[6] DNA,[7] peptidoglycan,[8] or folic acid.[9] Unfortunately, these processes are downregulated in biofilms via complicated bacterial circuitry controlled through a number of conserved feedback loops, severely limiting the effectiveness of conventional treatments. Further exacerbating the issue is the facility that resistance traits are exchanged within a biofilm through horizontal gene transfer, which has led to the rapid evolution of antibacterial resistance, emphasizing the critical need for novel therapeutics.

Among the most successful at developing these ecological advantages are the "ESKAPE" pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter sp.*), which were identified by the CDC as posing the greatest threat to human health.[10] Most acute infections are often dominated by planktonic bacterial populations and can be cured within days if the proper treatment is initiated timely and appropriately.[11] Conversely, numerous chronic infections have revealed that the infecting bacteria aggregate in the biofilm phenotype, and are surrounded by a complicated matrix of extracellular polymeric substances (EPS). Biofilm-associated infections represent one of the major threats to modern medicine as they jeopardize surgical procedures, implants, and basic sterilization techniques in hospital settings. As a result, significant research has focused on identifying mechanical, physical, and chemical strategies for preventing biofilm formation and promoting biofilm dispersion.

The focus of this Viewpoint is to introduce the burgeoning, and somewhat understated, threat of chronic biofilms to accentuate historical approaches by synthetic chemists in the context of controlling the bacterial circuitry of biofilm communities and to address pressing areas of underexplored research avenues that require both significant investigation and investment.

**Relevance.** Over 80% of human bacterial infections involve biofilm-associated microorganism. Of the ESKAPE pathogens, *P. aeruginosa*, *S. aureus*, and *A. baumannii* are involved in biofilm infections. *P. aeruginosa* and *S. aureus* biofilms are found in over 50% of patients with cystic fibrosis (CF) lung infections,[12] chronic wound infection, catheter-associated UTI, chronic rhinosinusitis, chronic otitis media, and contact lens-related keratitis. *S. aureus* is associated with chronic osteomyelitis, chronic rhinosinusitis, endocarditis, chronic otitis media, and orthopedic implants.[10] More recently, *A. baumannii* infections (commonly referred to as "Iraqibacter") have become a critical medical concern in conflict zones and Veterans Affairs (VA) hospitals, particularly in biofilm-related combat wounds.[13]

Often, a combination of microorganisms leads to severe polymicrobial biofilm infections, thus increasing persistence and tolerance to antibiotic treatments because these organisms can trade resistance cassettes across species and even genus.[14] Furthermore, adherence of bacteria to biotic and abiotic surfaces plays a crucial role in the development of acute infection particularly in the case of indwelling devices.[15] Thus far, the impact of biofilm formation has likely been underestimated, and the investigation of antibiofilm agents is of critical importance and inadequately addressed by both industry and academia.

**Biofilm Characterization and Composition.** Biofilm formation initiates when planktonic cells attach to biotic or abiotic surfaces (Figure 1). Initial adhesion is reversible; however, the committed formation of a biofilm is associated with the production of an EPS matrix.[16] This matrix consists of microbial cells (2−5%), proteins (<1−2%, including enzymes), exopolysaccharides (1−2%), extracellular DNA (eDNA, <1−2%), and water (up to 97%).[17] Adhesion of cells occurs

Received: March 15, 2019




DOI: 10.1021/acsinfecdis.9b00104
ACS Infect. Dis. XXXX, XXX, XXX−XXX

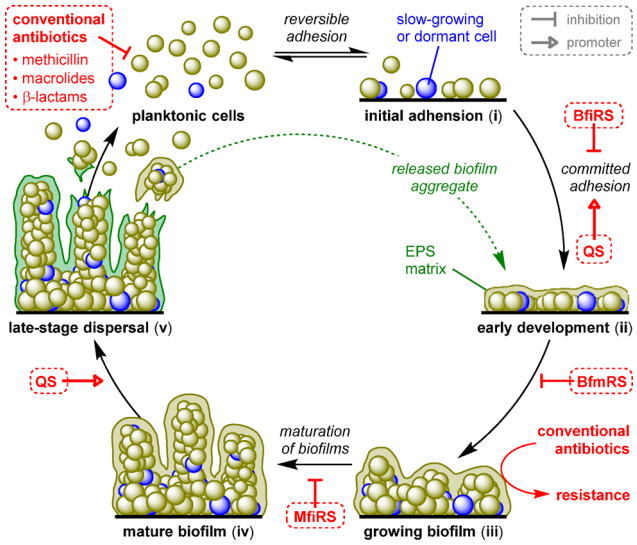


**Figure 1.** Biofilm life cycle. In the canonical view of the biofilm life-cycle, formation begins following the initial adhesion of free-moving planktonic cells to a surface (i). Early development of the EPS matrix correlates with committed adhesion of bacterial cells to a surface or aggregate regulated by quorum sensing and the TCS BfiRS (ii). The growing biofilm, regulated by the TCS BfmRS, is resilient to conventional antibiotic treatments and develops resistance rapidly through horizontal gene transfer (iii). Maturation of biofilms to stage (iv) is regulated by the TCS MfiRS. Biofilms begin to form three-dimensional fortresses with subpopulations of persister colonies. Late stage dispersal is controlled by quorum sensing to revert sessile cells to planktonic form (v).

through formation of microcolonies via cell division and EPS matrix production, leading to the formation of mature three-dimensional biofilm structures. At this stage, antibiotic resistance through horizontal gene transfer and existence of slow-growing or dormant (persister) cells is common and results in chronic infection.[18]

Although qualitative data on biofilms is plentiful, quantitative analyses, including specific chemical interactions within the EPS matrix, remain elusive due to both the complexity and insolubility of biofilms. Solid-state NMR techniques recently developed by Cegelski provided a complete account of the protein and polysaccharide components in the EPS matrix of an *E. coli* biofilm.[19] This technique could allow for the study of contacts existing between biofilm components and analysis of biofilm structures at the atomic level. Future investigations utilizing biosynthetic labeling strategies will provide more comprehensive data on biofilm development and assembly of the EPS matrix and are sorely needed.

**Challenges: Diagnosis and Infection Models.** Diagnosing biofilm-associated infections remains challenging as traditional methods are often unsuccessful at detecting the species responsible for infection. Multiple qualitative criteria were described by Parsek and Singh to facilitate improved identification of biofilm-associated infections:[20] (1) The existence of an aggregated bacteria, creating a localized infection, (2) resistance to conventional antibiotics, and (3) prolonged host-immune response.[10] Although these criteria allow for an initial assessment, it is critical to improve current methods for early diagnosis of biofilm infections to increase success of treatment options, specifically in patients at high risk for developing biofilm-associated infections. Further, if one were to develop narrow-spectrum therapies, then knowing the identity of the infecting pathogen would be critical for appropriate treatment.

In addition to diagnosis, investigations of biofilm formation have been hindered by inconsistencies between in vivo and in vitro biofilm models.[21] Historically, these assays are notoriously difficult to repeat due to very minute changes (oxygen concentration, media composition of growth surface) having dramatic effects on the robustness of the biofilm. Other complicating factors may arise from uncharacterized roles of biofilm in infections and insufficient data regarding the role of biofilms in health and disease settings. Furthermore, biofilms often consist of polymicrobial infections, thus traditional in vitro models may not account for the complexity of in vivo systems.[14] To improve in vitro models, continued investigation of the physiological environment surrounding the specific biofilm community is necessary. Recent advances from the Whiteley lab have demonstrated the potential of this approach as they have developed well-defined media recapitulating CF-sputum for the analysis of bacterial growth and activity of therapeutics under these conditions versus traditional media.[22]

## DISRUPTION OF BIOFILMS

Herein, we briefly highlight promising approaches to hijack chemical signaling that regulates biofilm processes. The examples presented below seek to "short-circuit" or "hot-wire" the bacterial circuitry instilled in these organisms. Bacteria have evolved to maximize the use of resources to not only multiply but also withstand assault from external stresses. Therefore, they have evolved sophisticated systems that rely on feedback loops, relay pathways, and threshold concentrations to both turn on and off biological responses akin to electrical circuits and light switches. The purpose of this viewpoint is not meant to be exhaustive and we encourage the reader to explore seminal reviews by Weinert[23] and Melander,[24] and any of the cited material for in-depth analyses of approaches to preventing biofilm formation and promoting biofilm dispersion.

**Two-Component Systems.** Two-component systems (TCSs) are an attractive target for antibacterial therapeutics because they are found primarily in prokaryotes, with few reported cases in lower eukaryotic organisms.[24] These regulatory systems are utilized by bacteria to control gene expression in response to chemical or physical changes in the environment (Figure 2). Typically, a TCS consists of a membrane-bound sensor histidine kinase (HK) and a DNA-binding response regulator (RR). In response to an extracellular change, the sensor HK is phosphorylated at a conserved histidine residue. The phosphoryl group is subsequently transferred to the RR at a conserved aspartate

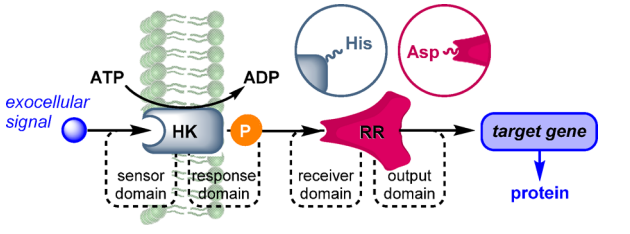


**Figure 2.** Two-component systems are composed of a histidine kinase sensor that responds to extracellular signals. Phosphorylation of the sensor triggers a response from the regulator receptor, resulting in gene expression.


DOI: 10.1021/acsinfecdis.9b00104
ACS Infect. Dis. XXXX, XXX, XXX−XXX

residue, resulting in a conformational change that leads to dimerization of the regulators and high-affinity DNA binding.[25]

TCSs have been linked to biofilm formation in three of the six ESKAPE pathogens, including *P. aeruginosa*, *A. baumannii*, and *S. aureus*. In *P. aeuriginosa*, the TCSs BfiRS, BfmRS, and MifRS have been demonstrated to control irreversible surface adhesion from stage i to ii, growth of biofilm from stage ii to iii, and maturation from stage iii to iv, respectively (Figure 1).[26] BfmRS has also been found to regulate surface adhesion via pili formation in *A. baumannii*.[27] In Gram-positive bacteria, LytRS regulates *S. aureus* production of eDNA, a key component of the EPS and biofilm formation.[24]

Although TCSs have been linked to biofilm formation, several challenges remain in designing an inhibitor of these regulatory systems to act as an antibiofilm agent. Agents could potentially act by blocking recognition of external stimulus by the HK, kinase autophosphorylation, kinase dimerization, phosphotransfer from kinase to regulator, RR dimerization, and binding of regulator to DNA.[24] Sensor domains of HK are typically unique to each kinase, making them challenging drug targets. Additionally, small molecules that bind to sensor domains have not been elucidated. More promising targets of TCSs include the catalytic domain of the HK and the receiver domain of the RR. Structural homology between the catalytic domain of the HK and receiver domain of the RR suggests a single agent could simultaneously disrupt the activity of sensor and regulator.[25] Additionally, these similarities reduce the probability that mutations will result in resistance that is associated with a lower binding affinity of the inhibitor to HK or RR.

The 2-aminoimidazole (2-AI) based agents developed by Melander and Blackwell represent first examples of a small molecule inhibitor of a TCS that is associated with biofilm growth.[28−30] Target identification of 2-AIs was completed by Melander and Cavanagh in 2012 through a pull-down assay, suggesting BfmR—a TCS involved in *P. aeruginosa* biofilm maturation—as a potential target. Further computational docking studies were completed with a BfmR model based on a high-resolution PhoP structure—a member of the OmpR response regulator family that has homology with the predicted BfmR structure. The computational data suggests 2-AI and its analogs bind to the interface between the N- and C-terminal regulatory and output domains.[31] With the success exhibited by 2-AIs, high-throughput screens to identify additional small molecular inhibitors of TCSs known to regulate biofilm formation in *A. baumannii* and *S. aureus* may prove promising avenues for drug discovery to inhibit nonessential life processes in the future. Additionally, investigating the cyclic di-GMP two-component signaling pathway may provide insight on its role in biofilm formation and maturation.

**Quorum-Sensing.** Quorum-sensing (QS) is the intercellular communication between bacteria and occurs when a critical density (or quorum) is reached (Figure 3). When the threshold is achieved, it triggers a switch in the bacterial circuitry that results in production of small molecules and elicits a phenotypic change. These small molecules, referred to as autoinducers, are released into the EPS matrix and recognized by others of the same species (or sometimes different species) to trigger a community response, such as biofilm formation or virulence.[32] Within the biofilm growth cycle, QS causes the committed adhesion (i−ii) and dispersal stages (v). Thus, investigating both antagonists and agonists or QS to prevent biofilm adhesion or to promote premature

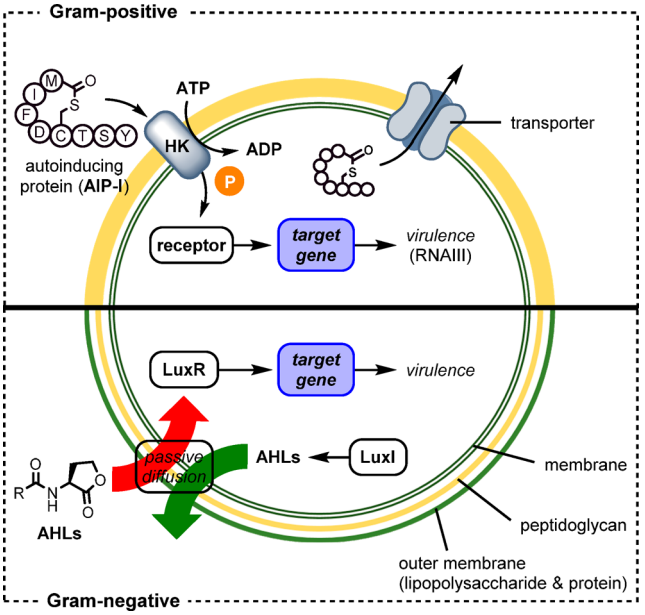


**Figure 3.** Quorum sensing in Gram-positive and Gram-negative bacteria relies on the production and recognition of small molecules to regulate virulence behavior.

dispersion of biofilms may prove advantageous when developing biofilm-controlling agents.[24] Additionally, investigating the differences between QS small molecules specific to Gram-negative and Gram-positive bacteria may facilitate the development of narrow-spectrum agents.

Many Gram-negative bacteria, including the ESKAPE pathogen *P. aeruginosa*, utilize acylated homoserine lactones (AHLs) as QS autoinducers.[33] Conversely, Gram-positive bacteria like *S. aureus* communicate via small cyclic autoinducing peptides (AIPs).[34] Several small molecules have been investigated as QS inhibitors. Bassler identified two *P. aeruginosa* QS receptors, LasR and RhlR (pyocyanin and biofilm formation), as targets of a library of meta-bromothiolactones (mBTLs), which is thought to mimic the native QS ligand. Bassler notes that LasR and RhlR reciprocally control critical virulence factors, thus tuning rather than total inhibition is crucial for blocking pathogenesis in vivo.[32] Although LasR leads the QS cascade and has been a traditional QS target, Bassler showed that the ΔLasR strain possesses virulence, thus concluding RhlR is pertinent for in vivo QS regulation and a promising target for QS-controlled biofilm formation.[35]

Flustramine-inspired analogs were also investigated as QS antagonists. Analogs were designed by Cavanaugh and Melander based on trends correlating amphipathic small molecules with increased biofilm inhibition noted in a previous study of the 2-AI scaffold TCS inhibitor (Figure 3). The pyrroloindole triazole amide flustramine C demonstrated *A. baumannii* biofilm inhibition by 30% at 100 $\mu$M. Additionally, two analogs (Figure 4, **10** and **24**) were deemed active against biofilm via a nontoxic mechanism.[36]

Although many QS antagonists are known, agonists remain a relatively unexplored field. In Gram-positive populations, specifically *S. aureus*, high concentrations of a small RNA (RNAIII) promote the productions of exotoxins, resulting in the dispersal of biofilms. Low concentrations of RNAIII promote the production of surface molecules that are critical


DOI: 10.1021/acsinfecdis.9b00104
ACS Infect. Dis. XXXX, XXX, XXX−XXX

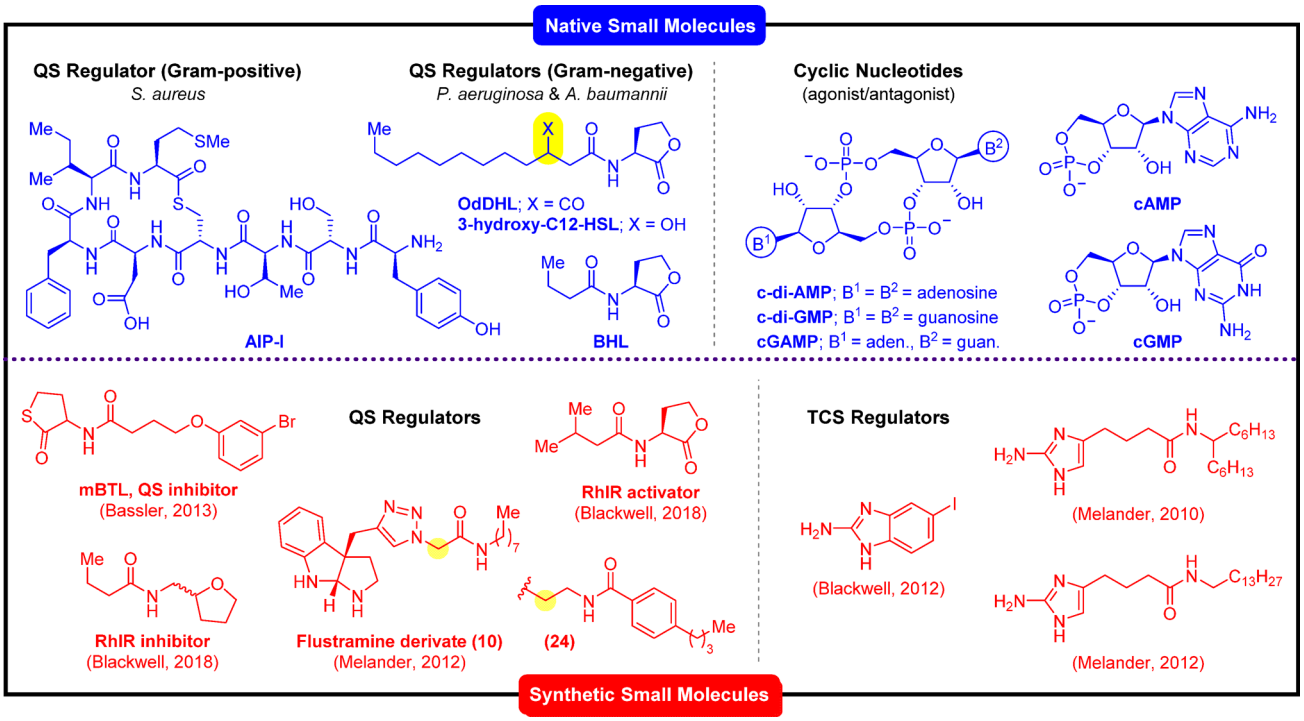


**Figure 4.** Native small molecule signals (top) and chemical modulators (bottom) highlighted in this viewpoint.

for initial biofilm adhesion. Uziel linked the initial production of RNAIII to RNAIII-activating protein (RAP) binding to TRAP (target of RAP), and high concentrations of RNAIII to the QS molecule AIP that activates RNAIII synthesis via *agr* signal transduction system. RNAIII-inhibiting peptide (RIP) was shown to block RAP binding to TRAP, but production of RNAIII still occurred via the AIP *agr* system.[37] Thus, further understanding of the complex RNAIII production system is necessary for improving either an inhibitor of RNAIII production to prevent biofilm adhesion, or to promote RNAIII synthesis that results in dispersal of biofilms. Additionally, Bassler demonstrated appending AIPs to abiotic surfaces can prevent biofilm formation of *S. aureus*.[34] Strategies to promote biofilm dispersal in Gram-negative bacteria, however, remain relatively unexplored.

Recently, Blackwell disclosed one of the first examples of agonists to regulate QS in Gram-negative bacteria. Modification of the native ligand of *P. aeruginosa* QS, *N*-butanoyl L-homoserine (HSL) was performed to probe the effect of non-native ligands on the regulation of QS. Non-native ligands promoted QS via regulation of RhlR activity, further supporting Bassler's claim that RhlR is essential for in vivo virulence of *P. aeruginosa*.[33] Although applications of HSL have yet to be discussed, non-native ligands may have the potential to upregulate quorum sensing to promote premature dispersal of biofilms, rendering the population more susceptible to conventional antibiotics.

**Polysaccharide Mimics.** Competition between two or more bacterial species has been shown to reduce biofilm formation, possibly through the release of nonantibiotic molecules. Ghigo demonstrated that when grown in a polymicrobial community, *E. coli* produce group II capsular polysaccharides that inhibit biofilm formation in both Gram-negative and Gram-positive pathogens, including ESKAPE pathogens *P. aeruginosa* and *S. aureus*. The polysaccharides likely interfere with biofilm formation through repulsion of cell adherence to biotic or abiotic surfaces.[14]

Polysaccharides may also interfere with biofilm formation by improper incorporation into the mature biofilm three-dimensional matrix. Townsend demonstrated this effect with human milk oligosaccharides (HMOs) that possess antibiofilm activity in *S. aureus*. His findings suggest supplementing bacterial colonies with HMOs results in the incorporation of non-native polysaccharides into biofilm matrices, thus disrupting structural integrity and resulting in collapse of the three-dimensional biofilm structures. The main advantage of HMOs is their nontoxic profile. However, due to variability of the content of HMOs between samples tested, antibiofilm results are inconsistent.[38] Isolating the critical polysaccharide in HMOs for antibiofilm activity may provide more accurate and precise bioactivity data. Additionally, investigating complex microbiomes or cocultures of bacteria may reveal key information on utilizing bacteria competition to inhibit biofilm formation.

**Cyclic Dinucleotides.** Cyclic dinucleotide secondary messengers have been linked to biofilm formation and biofilm-related characteristics including antibiotic resistance and the development of persistence.[23] Cyclic dimeric guanosine monophosphate (c-di-GMP) plays a crucial role in the activation of biofilm formation, specifically the expression of EPS matrix components including eDNA, exopolysaccharides, adhesive pili, and surface-anchored adhesins necessary for three-dimensional biofilm structures. Furthermore, c-di-GMP is hypothesized to control the switch between free-swimming and sessile bacteria, and subsequently acute and chronic infections. Although commonly found in prokaryotes, c-di-GMP is not found in eukaryotes, rendering it an attractive target for antibacterial agents.[39]

Mechanistically, c-di-GMP binds to a protein or RNA receptor, which in turn regulates biofilm-associated processes at transcriptional, post-transcriptional, and post-translational levels. Regulation of c-di-GMP is controlled by a plethora of



DOI: 10.1021/acsinfecdis.9b00104
ACS Infect. Dis. XXXX, XXX, XXX−XXX

proteins (over 40 in *P. aeruginosa*), emphasizing the complexity of this signaling pathway.[40] Proteins containing c-di-GMP binding sites or involved in turnover often possess a sensory or signaling domain, suggesting that these enzymes likely respond to environmental cues and are akin to TCSs.[39] Additionally, elevated levels of c-di-GMP are associated with chronic *P. aeruginosa* infections.[40] Sequestering c-di-GMP with high affinity binders has also been linked to premature dispersal of biofilms.[39] This evidence suggests developing small molecule or protein signals to down-regulate production of or sequester c-di-GMP may be potential therapies for biofilm-associated infections.

**Current Research and Unidentified Targets.** Many processes involved in biofilm formation remain underexplored, such as nonself AIPs or QS small molecules, cyclic nucleotides (beyond c-di-GMP), and multispecies infections. Nonself AIPs inhibit RNAIII synthesis in *S. aureus* species, but these findings have not been applied to other systems.[37] Furthermore, anti-RAP antibodies have been proposed as a potential biofilm vaccine to prevent RNAIII synthesis and initial adhesion of cells to biotic or abiotic surfaces.[37] Although the role of c-di-GMP in biofilm formation is forefront, many nucleotide secondary messengers are involved in biofilm formation including cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), cyclic-diadenosine monophosphate (c-di-AMP), and cyclic guanosine monophosphate-adenosine monophosphate (cGAMP).[40] Both cAMP and c-di-AMP are common to bacterial systems;[41] however, cGMP and cGAMP remain more elusive. Signaling pathways relying on c-di-AMP likely play critical roles in Gram-positive pathogen virulence[42] and cGMP was shown to regulate virulence and biofilm formation in the phytopathogen *Xanthomonas campestris*,[43] but its role is unexplored in the ESKAPE pathogens. Investigations of cAMP, cGMP, c-di-AMP, and cGAMP are still in the early stages and further studies are necessary to fully understand the role they play in regulating biofilm formation.

Perhaps the most clinically relevant, but still unexplored case is polymicrobial infections as most biofilm infections are a result of multiple bacterial species.[10] There is much ambiguity surrounding interspecies interactions involved in promoting or preventing biofilm formation. Group II capsular producing strains of *E. coli* were shown to inhibit production in *P. aeruginosa* and *S. aureus*, but other bacterial strains may promote biofilm formation in a multispecies setting. Additionally, over 50% of CF patients are often coinfected with *P. aeruginosa* and *S. aureus*. Coinfection is associated with decreased lung function and increased frequency of pulmonary exacerbations. Polymicrobial infections likely lead to more rapid pulmonary decline in CF patients, but an understanding of the dynamic relationship of coinfections as a predictor for patient outcome remains unclear.[12] Thus, there is critical need to study biofilm formation in complex polymicrobial communities to better predict and improve patient outcome.

## CONCLUSIONS AND FUTURE DIRECTIONS

The urgency of identifying new treatments for biofilm-associated infections cannot be overstated. The combined socioeconomic costs both in terms of dollars (prevention/treatment) and lives is significant and continues to increase.[15] From the literature discussed in this viewpoint, it is clear that although some progress has been made, more is needed. We would argue that there is high demand for additional support in this arena and from a personal perspective can articulate several reasons to enter the field. Foremost, biofilms effect virtually every aspect of life, ranging from ecological to health to combat, rendering a surplus of funding agencies and corporate entities interested in investing in this type of research. In addition, there are significant unmet needs that could easily become entire research careers for aspiring academics, for example:

(1) The engineering of methods for detecting small molecules produced both in single species and, more importantly, multispecies biofilm matrices are lacking. Novel scalable metabolic approaches, potentially using innovate nanotechnology, would be incredibly enabling, particularly as interest in the human microbiome increases.
(2) The development of species-specific, narrow-spectrum chemical tools, which can be used in a chemical genetic approach to deconvolute multispecies communities would provide critical information on the roles of certain commensal and pathogenic microbes in these ecological niches.
(3) Methods to better understand the biofilm architecture at the atomic level would allow one to better comprehend the dynamics between species and permit the development of new antimicrobial surfaces.
(4) Recent examples have demonstrated that agents specific to either biofilms or persister cells can potentiate existing antibiotics.[44] Further investigation of the synergy of biofilm agents and established drugs may lead to the development of antimicrobial cocktails that target a multitude of processes.
(5) Systematic analysis of biofilm processes should lead to the identification of new biological targets or critical protein−protein interactions for the development of new therapeutics. This may open the door for antivirulence compounds which could limit resistance development.

We are at the threshold of a second golden age of antimicrobial development. With the confluence of breakthroughs in genome sequencing, microscopy (both confocal and cryo-EM), microbiological techniques, and chemical synthesis, several new tools and skills are now at our fingertips. Akin to how our understanding of electrical circuitry has allowed us to move from a simple light bulb to intricate networks and renewable energy, so too has our abilities to better comprehend the chemical signaling and bacterial circuitry necessary for pathogen proliferation. It is our hope that the next generation of chemical biologists will also aspire to be chemical electricians and tackle these pressing health crises.

## AUTHOR INFORMATION

### Corresponding Author
*E-mail: wwuest@emory.edu.

### ORCID
William M. Wuest: 0000-0002-5198-7744

### Notes
The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We gratefully acknowledge funding from the National Institute of General Medical Sciences (GM119426) and the National Science Foundation (CHE1755698) for support. We would



DOI: 10.1021/acsinfecdis.9b00104
ACS Infect. Dis. XXXX, XXX, XXX−XXX

also like to thank members of the Wuest Lab for their helpful feedback.

## REFERENCES

(1) Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., Flemming, H.-C., and Kjelleberg, S. (2016) Biofilms: An emergent form of bacterial life. *Nat. Rev. Microbiol. 14* (9), 563−575.
(2) Koo, H., Allan, R. N., Howlin, R. P., Stoodley, P., and Hall-Stoodley, L. (2017) Targeting microbial biofilms: current and prospective therapeutic strategies. *Nat. Rev. Microbiol. 15* (12), 740−755.
(3) Satpathy, S., Sen, S. K., Pattanaik, S., and Raut, S. (2016) Review on bacterial biofilm: An universal cause of contamination. *Biocatal. Agric. Biotechnol. 7* (5), 56−66.
(4) Epand, R. M., Walker, C., Epand, R. F., and Magarvey, N. A. (2016) Molecular mechanisms of membrane targeting antibiotics. *Biochim. Biophys. Acta, Biomembr. 1858*, 980−987.
(5) Mccoy, L. S., Xie, Y., and Tor, Y. (2011) Antibiotics that target protein synthesis. *Wiley Interdiscip. Rev. RNA 2* (2), 209−232.
(6) Ma, C., Yang, X., and Lewis, P. J. (2016) Bacterial Trascription as a Target for Antibacterial Drug Development. *Microbiol. Mol. Biol. Rev. 80* (1), 139−160.
(7) van Eijk, E., Wittekoek, B., Kuijper, E. J., and Smits, W. K. (2017) DNA replication proteins as potential targets for antimicrobials in drug-resistant bacterial pathogens. *J. Antimicrob. Chemother. 72* (5), 1275−1284.
(8) Nikolaidis, I., Favini-Stabile, S., and Dessen, A. (2014) Resistance to antibiotics targeted to the bacterial cell wall. *Protein Sci. 23* (3), 243−259.
(9) Bourne, C. (2014) Utility of the Biosynthetic Folate Pathway for Targets in Antimicrobial Discovery. *Antibiotics 3* (1), 1−28.
(10) Römling, U., and Balsalobre, C. (2012) Biofilm infections, their resilience to therapy and innovative treatment strategies. *J. Intern. Med. 272* (6), 541−561.
(11) Murray, C. J. L., and Lopez, A. D. (1997) Alternative projections of mortality and disability by cause 1990−2020: Global Burden of Disease Study. *Lancet 349* (9064), 1498−1504.
(12) Limoli, D. H., Yang, J., Khansaheb, M. K., Helfman, B., Peng, L., Stecenko, A. A., and Goldberg, J. B. (2016) *Staphylococcus aureus* and *Pseudomonas aeruginosa* co-infection is associated with cystic fibrosis-related diabetes and poor clinical outcomes. *Eur. J. Clin. Microbiol. Infect. Dis. 35* (6), 947−953.
(13) Sebeny, P. J., Riddle, M. S., and Petersen, K. (2008) *Acinetobacter baumannii* Skin and Soft-Tissue Infection Associated with War Trauma. *Clin. Infect. Dis. 47* (4), 444−449.
(14) Rendueles, O., and Ghigo, J. M. (2012) Multi-species biofilms: how to avoid unfriendly neighbors. *FEMS Microbiol. Rev. 36* (5), 972−989.
(15) Shah, S. R., Tatara, A. M., D'Souza, R. N., Mikos, A. G., and Kasper, F. K. (2013) Evolving strategies for preventing biofilm on implantable materials. *Mater. Today 16* (5), 177−182.
(16) Mooney, J. A., Pridgen, E. M., Manasherob, R., Suh, G., Blackwell, H. E., Barron, A. E., Bollyky, P. L., Goodman, S. B., and Amanatullah, D. F. (2018) Periprosthetic Bacterial Biofilm and Quorum Sensing. *J. Orthop. Res. 36* (9), 2331−2339.
(17) Jamal, M., Tasneem, U., Hussain, T., and Andleeb, S. (2015) Bacterial Biofilm: Its Composition, Formation and Role in Human Infections. *Res. Rev. J. Microbiol. Biotechnol. 4* (3), 1−14.
(18) Chung, P. Y., and Toh, Y. S. (2014) Anti-biofilm agents: recent breakthrough against multi-drug resistant *Staphylococcus aureus*. *Pathog. Dis. 70* (3), 231−239.
(19) Reichhardt, C., and Cegelski, L. (2014) Solid-state NMR for bacterial biofilms. *Mol. Phys. 112* (7), 887−894.
(20) Parsek, M. R., and Singh, P. K. (2003) Bacterial Biofilms: An Emerging Link to Disease Pathogenesis. *Annu. Rev. Microbiol. 57* (1), 677−701.
(21) Bjarnsholt, T., Alhede, M., Alhede, M., Eickhardt-Sørensen, S. R., Moser, C., Kühl, M., Jensen, P. Ø., and Høiby, N. (2013) The *in vivo* biofilm. *Trends Microbiol. 21* (9), 466−474.
(22) Palmer, K. L., Mashburn, L. M., Singh, P. K., and Whiteley, M. (2005) Cystic Fibrosis Sputum Supports Growth and Cues Key Aspects of *Pseudomonas aeruginosa* Physiology. *J. Bacteriol. 187* (15), 5267−5277.
(23) Fontaine, B. M., Duggal, Y., and Weinert, E. E. (2018) Exploring the Links between Nucleotide Signaling and Quorum Sensing Pathways in Regulating Bacterial Virulence. *ACS Infect. Dis. 4* (12), 1645−1655.
(24) Worthington, R. J., Blackledge, M. S., and Melander, C. (2013) Small-molecule inhibition of bacterial two-component systems to combat antibiotic resistance and virulence. *Future Med. Chem. 5* (11), 1265−1284.
(25) Walthers, D., Tran, V. K., and Kenney, L. J. (2003) Interdomain Linkers of Homologous Response Regulators Determine Their Mechanism of Action. *J. Bacteriol. 185* (1), 317−324.
(26) Mikkelsen, H., Sivaneson, M., and Filloux, A. (2011) Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. *Environ. Microbiol. 13* (7), 1666−1681.
(27) Tomaras, A. P., Flagler, M. J., Dorsey, C. W., Gaddy, J. A., and Actis, L. A. (2008) Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology 154* (11), 3398−3409.
(28) Frei, R., Breitbach, A. S., and Blackwell, H. E. (2012) 2-Aminobenzimidazole Derivatives Strongly Inhibit and Disperse *Pseudomonas aeruginosa* Biofilms. *Angew. Chem., Int. Ed. 51* (21), 5226−5229.
(29) Ballard, T. B., Richards, J. J., Wolfe, A. L., and Melander, C. (2008) Synthesis and Antibiofilm Activity of a Second-Generation Reverse-Amide Oroidin Library: A Structure-Activity Relationship Study. *Chem. - Eur. J. 14* (34), 10745−10761.
(30) Bunders, C. A., Richards, J. J., and Melander, C. (2010) Identification of aryl 2-aminoimidazoles as biofilm inhibitors in Gram-negative bacteria. *Bioorg. Med. Chem. Lett. 20* (12), 3797−3800.
(31) Thompson, R. J., Bobay, B. G., Stowe, S. D., Olson, A. L., Peng, L., Su, Z., Actis, L. A., Melander, C., and Cavanagh, J. (2012) Identification of BfmR, a Response Regulator Involved in Biofilm Development, as a Target for a 2-Aminoimidazole-Based Antibiofilm Agent. *Biochemistry 51* (49), 9776−9778.
(32) O'Loughlin, C. T., Miller, L. C., Siryaporn, A., Drescher, K., Semmelhack, M. F., and Bassler, B. L. (2013) A quorum-sensing ihibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. *Proc. Natl. Acad. Sci. U. S. A. 110* (44), 17981−17986.
(33) Boursier, M. E., Moore, J. D., Heitman, K. M., Shepardson-Fungairino, S. P., Combs, J. B., Koenig, L. C., Shin, D., Brown, E. C., Nagarajan, R., and Blackwell, H. E. (2018) Structure-Function Analyses of the *N*-Butanoyl L-Homoserine Lactone Quorum-Sensing Signal Define Features Critical to Activity in RhlR. *ACS Chem. Biol. 13* (9), 2655−2662.
(34) Kim, M. K., Zhao, A., Wang, A., Brown, Z. Z., Muir, T. W., Stone, H. A., and Bassler, B. L. (2017) Surface-attached molecules control *Staphylococcus aureus* quorum sensing and biofilm development. *Nat. Microbiol. 2*, 17080.
(35) Mukherjee, S., Moustafa, D., Smith, C. D., Goldberg, J. B., and Bassler, B. L. (2017) The RhlR quorum-sensing receptor controls *Pseudomonas aeruginosa* pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer. *PLoS Pathog. 13* (7), e1006504.
(36) Bunders, C., Cavanagh, J., and Melander, C. (2011) Flustramine inspired synthesis and biological evaluation of pyrroloindoline triazole amides as novel inhibitors of bacterial biofilms. *Org. Biomol. Chem. 9* (15), 5476−5481.
(37) Balaban, N., Goldkorn, T., Gov, Y., Hirshberg, M., Koyfman, N., Matthews, H. R., Nhan, R. T., Singh, B., and Uziel, O. (2001) Regulation of *Staphylococcus aureus* Pathogenesis via Target of RNAIII-activating Protein (TRAP). *J. Biol. Chem. 276* (4), 2658−2667.
(38) Ackerman, D. L., Craft, K. M., Doster, R. S., Weitkamp, J. H., Aronoff, D. M., Gaddy, J. A., and Townsend, S. D. (2018) Antimicrobial and Antibiofilm Activity of Human Milk Oligosacchar-


DOI: 10.1021/acsinfecdis.9b00104
*ACS Infect. Dis.* XXXX, XXX, XXX−XXX

ides against *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Acinetobacter baumannii*. *ACS Infect. Dis. 4* (3), 315−324.
(39) Meissner, A., Wild, V., Simm, R., Rohde, M., Erck, C., Bredenbruch, F., Morr, M., Römling, U., and Häussler, S. (2007) *Pseudomonas aeruginosa cupA*-encoded fimbriae expression is regulated by a GGDEF and EAL domain-dependent modulation of the intracellular level of cyclic diguanylate. *Environ. Microbiol. 9* (10), 2475−2485.
(40) An, S. Q., and Ryan, R. P. (2016) Combating chronic bacterial infections by manipulating cyclic nucleotide-regulated biofilm formation. *Future Med. Chem. 8* (9), 949−961.
(41) Townsley, L., Huynh, T. N., Woodward, J. J., Shank, E. A., and Yannarell, S. M. (2018) Cyclic di-AMP Acts as an Extracellular Signal That Impacts *Bacillus subtilis* Biofilm Formation and Plant Attachment. *mBio 9* (2), e00341-18.
(42) Römling, U. (2012) Cyclic di-GMP, an established secondary messenger still speeding up. *Environ. Microbiol. 14* (8), 1817−1829.
(43) Chin, K. H., Lee, Y. C., Tu, Z. Le, Chen, C. H., Tseng, Y. H., Yang, J. M., Ryan, R. P., McCarthy, Y., Dow, J. M., Wang, A. H. J., and Chou, S. H. (2010) The cAMP Receptor-Like Protein CLP Is a Novel c-di-GMP Receptor Linking Cell-Cell Signaling to Virulence Gene Expression in *Xanthomonas campestris*. *J. Mol. Biol. 396* (3), 646−662.
(44) (a) Kim, W., Hendricks, G., Zhu, W., Van Tyne, D., Steele, A.D., Keohane, C., Fricke, N., Conery, A.L., Shen, S., Rahamuthiah, R., Pan, W., Lee, K., Rajamuthiah, R., Fuchs, B. B., Vlahovska, P. M., Wuest, W. M., Gilmore, M. S., Gao, H., Ausubel, F. M., and Mylonakis, E. (2018) A new class of synthetic retinoid antibiotics effective against bacterial persisters. *Nature 556*, 103. (b) Kim, W., Steele, A. D., Zhu, W., Csatary, E. E., Fricke, N., Dekarske, M. M., Jayamani, E., Pan, W., Kwon, B., Sinitsa, I., Rosen, J. L., Conery, A. L., Fuchs, B. B., Vlahovska, P. M., Ausubel, F. M., Gao, H., Wuest, W. M., and Mylonakis, E. (2018) Discovery and Optimization of nTZDpa as an Antibiotic Effective Against Bacterial Persisters. *ACS Infect. Dis. 4*, 1540.


DOI: 10.1021/acsinfecdis.9b00104
*ACS Infect. Dis.* XXXX, XXX, XXX−XXX